Study on germline transmission by transplantation of spermatogonial stem cells in chicken

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ABSTRACT

As a bioreactor, bird has proved to be most efficient system for producing useful therapeutic proteins. More than half of the egg white protein content derives from the ovalbumin gene with four other proteins (lysozyme, ovomucoid, ovomucin and conalbumin) present at levels of 50 milligrams or greater. And the naturally sterile egg also contains egg white protein at high concentration allowing for a long shelf life of recombinant protein without loss in activity. In spite of these advantages, transgenic procedures for the bird have lagged far behind because of its complex process of fertilized egg and developmental differences.

Recently, a system to transplant mouse testis cells from a fertile donor male to the seminiferous tubules of an infertile recipient male has been developed. Spermatogenesis is generated from transplanted cells, and recipients are capable of transmitting the donor haplotype to progeny. After transplantation, primitive donor spermatogonia migrate to the basement membrane of recipient seminiferous tubules and begin proliferating. Eventually, these cells establish stable colonies with a characteristic appearance, which expands and produces differentiating germ cells, including mature spermatozoa. Thus, the transplanted cells self-renew and produce progeny that differentiate into fully functional spermatozoa.

In this study, to develop an alternative system of germline chimera production that operates via the testes rather than through developing embryos, the spermatogonial stem cell techniques were applied. This system consisted of isolation and in vitro-culture of chicken testicular cells, transfer of in vitro-maintained cells into heterologous testes, production of germline chimeras and confirmation of germline transmission for evaluating production of heterologous, functional spermatozoa.

▶ **Key words**: chicken, testicular cells, germline chimera, spermatogenesis, testis

I. Introduction

Because of their unique physiological characteristics and genetic attributes, birds are considered to be one of the most suitable organisms for developing transgenic bioreactors and experimental models (Sang H., 1994; 2000; McGrew MJ et al.,2004). To exploit this potential of birds, we endeavored to develop an effective chicken transgenic system for establishing embryo-mediated germline transmission methods (Han et al., 2002; Park et al., 2000, 2003a, 2003b) as the basis for transgenic manipulations. Germline chimeras yielding high rate of germline transmission (up to 49.7%)(Park et al., 2003b) were produced by transfer of in vitro-cultured primordial germ cells (PGCs) retrieved 5.5-day-old embryos into 2.5-day-old recipient embryos. However, these procedures are time-consuming and laborious. In addition, it has been difficult to retrieve sufficient numbers of PGCs that have retained pluripotency from developing embryos. The development of an alternative system of germline chimera production is therefore necessary, which will subsequently optimize the efficiency and feasibility of the chicken transgenic system.

In this study, we attempted to develop an alternative system of germline chimera production that operates via the testes rather than through developing embryos. This system consisted of isolation and in vitro-culture of chicken testicular cells, sterilization of recipient testes, transfer of in vitro-maintained cells into heterologous testes, production of germline chimeras and confirmation of germline transmission for evaluating production of heterologous, functional spermatozoa. To ensure efficient production of the progenies with different genotypes, this system creates a local chimeric state in the testes by the direct transfer of heterologous testicular cells, a mixture of cells containing pluripotent cells, into the seminiferous tubules of recipients. Accordingly, the time-consuming germline chimera production, which is central to the embryo-mediated method, is not necessary.

II. The origin and characteristics of spermatogonial stem cells

Primordial germ cells (PGCs), the first known origin of the germ cell lineage in both males and females, are derived from a small population of epiblast (embryonal ectoderm) cells and migrate from the base of the allantois along the hindgut to the genital ridges. In male genital ridges, the PGCs become enclosed by somatic supporting cells, the precursor Sertoli cells. PGCs and Sertoli cells then, together, form solid strands of cells which are called seminiferous cords. Later during development these cords form a lumen and become seminiferous tubules. When the PGCs are enclosed in the seminiferous cords they change morphologically and are then called gonocytes. The gonocytes proliferate for a few days and then arrest in the G0/G1 phase of the cell cycle. In rats and mice the gonocytes resume proliferation within a few days after birth to give rise to adult type spermatogonia (De Rooij et al., 1997).

A spermatogonia have traditionally been subdivided into A spermatogonia, which do not have heterochromatin in their nuclei, and B spermatogonia, which have abundant heterochromatin. In rats and mice Intermediate type spermatogonia can also be observed. In the spermatogonial compartment, Asingle (As), Apaired (Apr), and Aaligned (Aal) spermatogonia can be distinguished, according to their topographical arrangement

on the basal membrane of the seminiferous tubules.

III. Development of spermatogonial stem cell transplantation technique

The spermatogonial transplantation was developed by Brinster and his colleagues in 1994 (Brinster et al., 1994a; 1994b). And only simple injection of spermatogenic cells into the seminiferous tubules gave rise to donor cell derived spermatogenesis in the recipient testis. Space in the seminiferous tubule is divided into two compartments, basal and intraluminal, by Sertoli cells. Tight junctions between Sertoli cells demarcate the basal compartment from the intraluminal compartment. Therefore it was unlikely to expect that spermatogonial stem cells introduced into the lumen of the seminiferous tubule would pass through the tight junction of Sertoli cells to reach the basal compartment where spermatogenesis starts. For the success of this experiment several deliberate preparations were set up in advance. First, a transgenic mouse carrying an lacZ marker gene was used as a donor. Spermatogenic cells of the donor mouse stain blue when incubated with X-gal, whereby they are easily discernible from any type of recipient cells. The marker carrying donor cells made the results of spermatogonial transplantation unequivocal. Second, two different kinds of mice were prepared as recipient mice. Wild-type mice with the same genetic background as the donor mouse were treated with busulfan 4~8 weeks prior to the transplantation. Busulfan is an anti-cancer reagent which preferentially destroys spermatogonia in the mouse testis. Busulfan treatment eliminates germ cells in the testis, thus making space for donor cell colonization. The other recipient mouse was the W mouse that has a mutation of the c-kit gene. Because c-kit is essential for germ cell development, W mouse testis has few spermagonia and no spermatgenesis. Therefore no competitive endogenous germ cells are present in the seminiferous tubules of those mice, making the testes appropriate host testes. Testis cells are collected from donor mice by a twostep enzymatic digestion with collagenase followed by trypsin and EDTA. They are suspended in regular culture medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum) then injected into the recipient testis. Details of the transplantation procedure were described previously (Ogawa et al., 1997). Briefly, three different routes can be used. The method developed first is injection directly into the seminiferous tubules. The cell suspension infused in a seminiferous tubule flows through to the rete testis and then spreads into other seminiferous tubules, whereby most seminiferous tubules can be filled. Injection into rete cavity or through efferent ducts were developed later both of which proved to be feasible ways to introduce donor cells into the seminiferous tubules of the recipient testis. The process of colonization can be divided into three phases (Nagano et al., 1999). First, during the initial week, transplanted cells are randomly distributed throughout the seminiferous tubules, and a small number of stem spermatogonia reach the basement membrane. This initial step of colonization, namely settlement of stem spermatogonia on the basement membrane, is an event unique to spermatogonial transplantation. In the second phase, donor cells on the basement membrane divide and form a monolayer network of spermatogonia. This phase corresponds to the spermatogonial proliferation phase of regular spermatogenesis. Then, in

the third phase, after expanding along the basement membrane, the cells in the center of the network differentiate toward the lumen of the seminiferous tubules and establish a colony of spermatogenesis. The third phase usually starts around 1 month after transplantation. Spermatogenesis continues throughout the resipient's life and keeps expanding along seminiferous tubules, which confirms the presence of stem cells. In some successful cases recipient mice have become fertile and given rise to progeny of donor cell genotype when mated.

After this methodological breakthrough, various modifications and many new approaches were described. The time course of testicular repopulation after germ cell transfer is described (Parreira et al., 1998). The first meiotic germ cells arising from transplanted stem cells appeared after 1 month and increased permanently thereafter, indicating that the restoration of donor spermatogenesis is a slow and extended process. Xenologous transfer of germ cells from a number of species into mouse testis showed that in closely related species a reininitiation of spermatogenesis is achieved whereas in less related species a repopulation of stem cells, but no differentiation of germ cells can be observed (Clouthier et al., 1996; Ogawa et al., 1999a,b; Dobrinski et al., 1999, 2000). Although Reis et al. (2000) described complete absence of human spermatogonia after germ cell transfer into the mouse testis, Nagano et al. (2001) reported that baboon spermatogonial stem cells are able to repopulate the mouse testis indicating that even primate testicular stem cells survive and expand but do not differentiate in the rodent seminiferous epithelium. Rat germ cells differentiate according to the kinetics and topography typical for rats showing that the germ cell genotype and not the Sertoli cell dictates the developmental program (Franca et al., 1998). Spermatogonial transplantation offers interesting strategies for research on germ cells. It has become an assay to estimate the potential of germ cell development and a tool to determine whether the somatic environment or the germ cells are responsible for disturbed fertility of some transgenic animals. For example, the importance of c-kit expression of spermatogonia and stem cell factor expression of Sertoli cells was shown through transplantation experiments (Ogawa et al., 2000). Mahato et al. (2000) transplanted estrogen receptor-α (ERα) deficient germ cells into wild type testes, showing that the mutated germ cells induced qualitatively normal spermatogenesis. In contrast, after transplantation of germ cells from mice carrying the jsd mutation, no donor derived spermatogenesis can be established (Boettger-Tong et al., 2000). The use of GFP-positive spermatogonia allowed the real time observation of transplanted cell clones. These studies opened new pathways for the detailed study of stem cell colonization and germ cell development. The results indicated the presence of stem cell niches in the seminiferous epithelium (Ohta et al., 2000a,b). A clinically relevant approach for autologous germ cell transplantation in primates has been reported. The method is intratubular transfer of germ cell suspensions via the rete testis under guidance by ultrasonography. The detection of transplanted germ cells in the seminiferous epithelium of a macaque 4 weeks after autologous germ cell transfer revealed the success of the germ cell transplantation attempt (Schlatt et al., 1999a). These germ cell transfer technique was also applicable to surgically removed human testes. Follow up studies using macaque monkeys as animal models and

mimicking the gonado-toxic treatment of oncological patients by testicular irradiation show that germ cell retrieval and cryopreservation of testicular tissue before and transfer of germ cells after irradiation evoke an earlier and better recovery of spermatogenesis. Cryopreservation of testicular cell preparations seems to be the most promising approach for the storage of germ cells before transplantation. Cryopreservation of germ cell preparations prior to germ cell transfer did not interfere with repopulation of the testis and reinitiation of spermatogenesis (Avarbock et al., 1996). A recent study described various strategies for testicular cell preparations from murine and human testes and did not report major differences in cell survival comparing four commonly used cryoprotective agents (Brook et al., 2001).

Cross-species transplantation was conducted by in 1996. Production of rat sperm in mouse testes was achieved following cross-species spermatogonial transplantation fromrats tomice (Clouthier et al., 1996) and transplantation was subsequently also successful from mice to rats (Ogawa et al., 1999a; Zhang et al., 2003). Crossspecies transplantation of germ cells between rats and mice established that the cell cycle during spermatogenesis is controlled by the germ cell and not the Sertoli cell (Franca et al., 1998). Hamster spermatogenesis could also occur in the mouse (Ogawa et al., 1999b); however, with increasing phylogenetic distance between donor and recipient species, complete spermatogenesis could no longer be achieved in the mouse testis. Transplantation of germ cells from nonrodent donors ranging from rabbits, dogs, pigs, bulls, horses and ultimately non-human primates and humans, resulted in colonization of the mouse testis but spermatogenesis became arrested at the stage of spermatogonial expansion (Dobrinski et al., 1999, 2000; Nagano et al., 2001,2002). Therefore, the initial steps of germ cell recognition by the Sertoli cells, localization to the basement membrane and initiation of cell proliferation are conserved between evolutionary divergent species. However, when donor and recipient species are phylogenetically more distant than rodents, the recipient testicular environment appears to become unable to support spermatogenic differentiation and meiosis. This incompatibility of donor germ cells and recipient testicular environment could be overcome by co-transplantation of germ cells and Sertoli cells (Shinohara et al., 2003) or by testis tissue transplantation as described below (Honaramooz et al., 2002a). Although cross-species spermatogonial transplantation did not have the envisioned immediate practical application, it nonetheless provides a bioassay for stem cell potential of germ cells isolated from other species (Dobrinski et al., 1999, 2000; Izadyar et al., 2002).

Application of germ cell transplantation technology to non-rodent species, so far reported in pigs, goats, cattle and monkeys (Honaramooz et al., 2002b; Honaramooz et al., 2003a, 2003b; Izadyar et al., 2004; Schlatt et al., 1999), required the development of a new technical approach. While direct injection of donor cells into rodent seminiferous tubules is possible via the efferent ducts, this is not feasible in larger species. Instead, using a combination of ultrasound-guided cannulation of the centrally located rete testis with delivery of germ cells by gravity flow (Honaramooz et al., 2002b, 2003b), we succeeded in transplanting donor cells from transgenic donor goats into the testes of immunocompetent, prepubertal recipient animals. Once these goats became sexually mature, they produced sperm

carrying the donor haplotype and transmitted the donor genetic makeup to the offspring. This provided proof-of-principle that germ cell transplantation results in donor-derived sperm production and fertility in a non-rodent species (Honaramooz et al., 2003a). Importantly, donors and recipients were unrelated and immunocompetent while in rodents syngeneic, immunocompromised or immunosuppressed animals (Kanatsu-Shinohara et al., 2003; Zhang et al., 2003) have to be used as recipient animals. The testis is considered to be an immune privileged site, but it is unclear why transplantation between unrelated, immunocompetent animals is possible in domestic animal species but not in rodents. Nonetheless, this makes the technique infinitely more applicable in non-rodent species.

IV. Production of transgenic animals using spermatogonial stem cells

Since genetically engineered transgenic mice were produced by microinjecting foreign DNA into the pronucleus of a zygote, and then subsequently transferring the zygote into a recipient female (Palmiter et al., 1982; Gordon et al., 1983; Palmiter et al., 1986; Brinster et al., 1993). Several researchers tried to produce transgenic animals by number of methods, such as retroviral vectors (Kim et al., 1993) and embryonic stem cells (Anderson, 1992; Stewart, 1991). Recently, Brinster et al. (1994ab) reported a technique with which they transplanted testis-derived cells into mouse seminiferous tubules of infertile recipients and produced progeny derived from donor male germ cells. That technique was essential for the ultimately successful production of transgenic mice by genetic modification of the male germline. Combined with several transgenic method spermatogonial stem cell mediated technologies give some important adventages. First, introduction of the transgene into spermatogonial stem cells virtually assures germline integration. Second, a single experimental recipient can generate several germline transgenics because each donor stem cell-derived spermatogenic colony is likely to represent a unique chromosomal integration. Third, concatamerization is not observed with retroviral transduction, which will facilitate the use of inducible expression vectors (Kyle et al., 2002). Because the spermatogenic process is well conserved in mammals (Fritz et al., 1986), this method might be adapted to other species where transgenic methods are inefficient or not available. When combined with the rapidly expanding repertoire of somatic cell gene therapy vectors, this technology will facilitate the generation of transgenic animal models for the functional characterization of genes.

Kyele EO et al. reported that rat spermatogonial stem cells can survive and proliferate in short-term culture, although a net loss of stem cells was observed. Rat spermatogonial stem cells also were susceptible to transduction with a retroviral vector carrying a lacZ reporter transgene. Using a 3-day periodic infection protocol, 0.5 % of stem cells originally cultured were transduced and produced transgenic colonies of spermatogenesis in recipient mouse testes. De Miguel MP et al. reported that they developed an improved cell culture system in which spermatogonia survive and proliferate for several days. And they used this system to test the ability of a variety of murine and avian retroviruses to infect spermatogonia. We investigated the factors influencing retroviral transduction of spermatogonia, including the

proliferative status of the infected cell, the type of viral envelope, the type of retroviral long terminal repeat, and the method of viral delivery. In this study, many of the widely used retroviral vector systems can be used to successfully transducer spermatogonia at high efficiency. Moreover, retroviral delivery of MDM2, the major down regulator of p53, promotes spermatogonial survival in culture, suggesting that p53 plays a role in regulating spermatogonial apoptosis induced by growth factor deprivation. Nagano M et al. reported that male GSCs (Germline Stem Cells) of mice can be transduced in vitro by a lentiviral vector and generate complete spermatogenesis when transplanted into infertile host testes. Transduction effciencies were comparable to those for MMLV transduction using similar experimental conditions. The results suggest that both lentiviral and MMLV vectors could be effective in transducing GSCs of other species. Hamra FK reported that primary cultures of rat spermatogenic cells that did not bind to collagen matrix were able to colonize and form mature spermatozoa when transferred to testes of recipient males. Up to 73% of the progeny from matings with recipient males were derived from the transferred spermatogenic cells. Subsequently, two populations of germ cells were obtained by selection on laminin matrices. Both populations expressed the spermatogenic cell marker, DAZL, but not the somatic cell marker, vimentin. The cells that bound to laminin represented approximately 5% of the total population and were greatly enriched in ability to colonize a recipient testis, suggesting an enrichment in germ-line stem cells. The colonization potential was maintained for at least 7 days in culture. These cells were subsequently transduced with a lentiviral enhanced GFP reporter vector and then transferred to WT recipient males. After mating, 26 of 44 pups were derived from the cultured donor germ cells, and 13 pups carried the lentiviral transgene. Based on Southern analysis, the transgene was integrated at a different genetic locus in each animal and was transmitted to approximately 50% of pups in the F(2) generation. Thus, by using these procedures, approximately 30% of pups in the F(1) generation inherited and stably transmitted a lentiviral transgene that integrated at various genomic sites.

V. Stratages for germline chimera production in aves

Use of blastodermal cells

Efforts to isolate the chick equivalent of mouse ES cells have focussed on manipulation of cells from the embryos from new laid eggs. The chick embryo in the new laid egg is at a stage of development approximately equivalent to the stage in mouse embryogenesis when ES cells can be isolated from the inner cell mass. At this stage of development, the stage X blastoderm (Eyal-Giladi et al., 1976), is a single layer of cells that consists of two visibly distinct regions: the area pellucida or central disc, from which the embryo will develop, and the surrounding area opaca that gives rise to the extraembryonic membranes. Identification of cells equivalent to the inner cell mass of the mouse blastodermal embryo has not been possible as the organisation of the early chick embryo differs significantly from that of the mouse. Stern (1990) demonstrated that cells that will go on to form the hypoblast are scattered under the epiblast of the area pellucida at stage X. The chick homologue of the Drosophila gene vasa, that has an essential function in germ cell formation, has been identified (Tsunekawa et al., 2000). The cvh protein product is present in approximately 30 cells of the central zone

of the area pellucida. It is thought that these cells are the precursors of the chick primordial germ cells and therefore, that PGCs are determined before the egg is laid. Petitte et al. (1990) described the first experiments that suggested that stage X embryo cells could be transferred from one embryo to another and result in development of chimeric birds. Cells from stage X embryos of Barred Plymouth Rock chickens, which have distinctive black feathering, were injected into the subgerminal cavity of stage X embryos of an inbred line of Dwarf White Leghorns, which have white feathers. Feather-colour chimeric embryos were detected in 11.3 % of the manipulated eggs and one male chimera hatched that on breeding proved to be a low level germ line chimera. A significant improvement in the proportion of chimeric birds produced by this method, and the level of both somatic and germ line chimerism, was achieved by development of a method that compromised the recipient embryos. Exposure of intact eggs to 500~700 rads of g-irradiation from a 60Co source resulted in delayed development of the recipient embryos and over 60 % production of somatic chimeras. A high proportion of the birds were also germ line chimeras and in some cases apparently 100 % of the offspring of the chimeras were derived from the injected blastodermal cells (Carsience et al., 1993). These experiments have established the method for introduction of blastodermal cells and efficient production of high level chimeras. In parallel with the development of the method for production of chimeras, methods to culture and transfect the blastodermal cells have been developed. Chick blastodermal cells were initially maintained in culture for a short period, transfected with a lacZ reporter construct and injected into recipient embryos (Brazolot et al., 1991). Chimeric embryos were analysed and expression of the reporter gene detected. These experiments were extended to development of culture conditions that supported long term growth, for at least 35 passages, in culture of stage IX-XI blastodermal cells (Pain et al., 1996). The cells were cultured on inactivated mouse STO feeder cells, in the presence of cytokines and growth factors including bFGF, LIF and SCF, plus anti-retinoic acid monoclonal antibody (ARMA). The cells are very similar to murine ES cells, in morphology, cytokine-dependent proliferation, telomerase activity and expression of epitopes specific to murine ES cells. Germ line chimeric birds can be generated using cells maintained in culture for short periods of time (Speksnijder et al., 1999). Although efforts to improve the culture methods and recover genetically modified cells have been continued in several laboratories, no results describing germline transmission from gametes derived from genetically modified cells have been described.

Use of primordial germ cells

Primordial germ cells (PGCs) are the first identifiable precursor cell of sperm in male or egg in female. In birds, PGCs first arise from the epiblast and migrate to the region of gonadal anlagen via the embryonic blood vessel (Swift, 1914; Hamburger et al., 1951). And primordial germ cells in the chick are morphologically distinct and can be easily identified by staining using the periodic-acid Shiff method just after primitive streak formation. By stage 10 (Hamburger et al., 1951, approximately 18 h of incubation) they are concentrated in the germinal crescent at the anterior of the embryo. As the vascular system develops the PGCs enter the circulation and begin to circulate through the embryo. The number of PGCs in the blood

peaks between stages 15 - 16 of development. By stage 20 the PGCs actively migrate into the developing gonads. The literature describing the origin and development of primordial germ cells in the chick is reviewed by D'Costa et al. (2001). This easy access to the precursors of the gametes was recognized as a possible route to transgenesis: development of this method requires optimization of the PGC isolation and transfer process, genetic modification of the PGCs before transfer and manipulation of the recipient to allow a greater contribution to the germ line of donor-derived PGCs. PGCs have been isolated from the germinal crescent (Vick et al., 1993a), from the circulatory phase, when they were concentrated on a Ficoll density gradient by centrifugation (Tajima et al., 1993) and from the gonads after colonization (Han et al., 2002). Han et al. (2002) have developed a method for long term culture of gonadal PGCs that may form the basis of a method for transfection and selection of stably modified PGCs. Park et al. (2003) reported more than 45% of germline transmission rate when 10-day-cultured gPGCs were injected to recipient embryo.

VI. Application of spermatogonial stem cells in aves

- **Experiment 1 : Generation of local chimeric status by testicular cell transfer into heterogeneous testes.**

In the first experiment, a significant (P=0.0096) treatment effect was detected among different retrieval methods. As shown in Figure 2, greater viability was detected in testicular cells retrieved by the one-step method (method 3) than in cells retrieved by other methods. Accordingly, the method 3 was employed for subsequent experiments. In the second experiment, the Trypan blue staining of testicular cells showed that the transplanted cells were successfully injected into the seminiferous tubules within 24 h

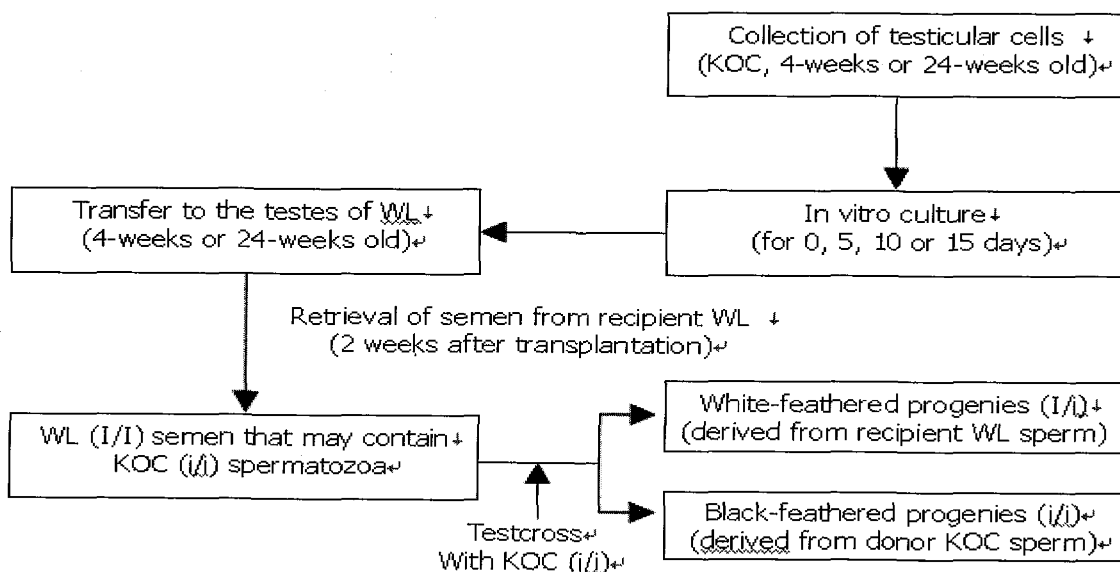

Figure1. General procedure for inducing germline transmission by the transfer of chicken testicular cells.

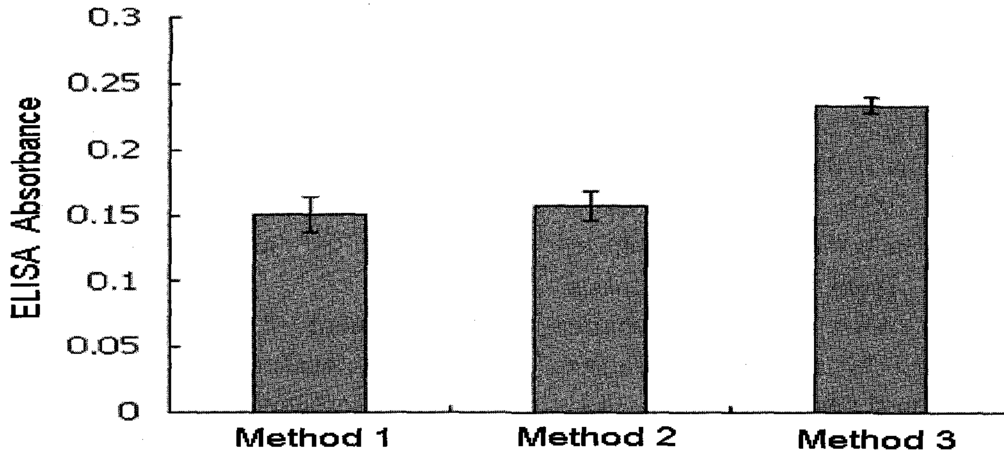

Figure2. Comparison of cell viability of testicular cells isolated from the testes separated by different dissociation methods.

after the transfer in all cases.

Subsequently, 64 recipients were used for testicular cell transplantation; among the 64 recipients, the production of heterogenic progenies was detected in five cases (7.8 %) based on testcross analysis (Table1). Three cases involved the injection of adult cells into adult recipients, one case involved the injection of cells from adult into juvenile, and one case involved the injection of cells from juvenile into juvenile. This pattern of transmission was observed after the transfer of spermatogonia what were injected immediately or cultured for 5 or 10 days. The

Table1. Induction of germline transmission by transfer of testicular cells collected from the testes of Korean Ogol chickens

Age of		culture of donor cells for (days)	No of recipients	No.(%) of recipients that produced progenies with black feathers	Length from cell transfer to the first hatching of progenies with black feathers	No. of hatched progenies	No.(%) of black feathered progenies
Donor KOC	Recipient WL						
24 weeks	24 weeks	0	4	2(50)	38 and 45 days	318	3(0.9)
		5	4	1(25)	45days	254	2(0.8)
		10	4	0(0)	N/A	188	0(0)
		15	4	0(0)	N/A	280	0(0)
4 weeks	24 weeks	0	4	0(0)	N/A	97	0(0)
		5	4	0(0)	N/A	161	0(0)
		10	4	0(0)	N/A	125	0(0)
		15	4	0(0)	N/A	48	0(0)
24 weeks	4 weeks	0	4	0(0)	N/A	257	0(0)
		5	4	0(0)	N/A	407	0(0)
		10	4	1(25)	188 days	487	2(0.4)
		15	4	0(0)	N/A	155	0(0)
4 weeks	4 weeks	0	4	0(0)	N/A	107	0(0)
		5	4	1(25)	137 days	185	1(0.5)
		10	4	0(0)	N/A	201	0(0)
		15	4	0(0)	N/A	166	0(0)

periods of time from transfer to hatching of the first progeny with black feathers were 38 and 45 days in the case of adult into adult, 188 days in the case of adult into juvenile, and 137 days in the case of juvenile into juvenile. The percentage of germline transmission (spermatogenesis of transplanted cells), which was calculated as the proportion of black-feathered progeny to total progeny, was 0.4~0.9 %.

- **Experiment 2 : In vitro culture and characterization of testicular cells**

Because both WLs and KOCs were employed as sources of pluripotent spermatogonial stem cells in our conventional embryo-mediated system, we attempted to develop a culture system for the testicular cells of these two strains. The testicular cells retrieved from juvenile or adult male KOCs were cultured in modified DMEM, which was supplemented with LIF, FGF2, and IGF1. Regardless of donor age, testicular stroma cells proliferated rapidly and formed a confluent monolayer 5-6 days after seeding. Both colony-forming (probably spermatogonia or germline cells) and monolayer-forming (probably stroma cells) cells were derived from the culture of the testicular cells. The colonies adhered firmly to the monolayer, and no signs of degeneration that was determined by both morphological criteria and cell viability assay were detected. Two types of colonies developed after the culture. Under microscopic observation, some of the colonies formed well-delineated cell masses, while others consisted of isolated cells that were linked by cell-to-cell bridges. The bridge formation within colonized cells became eminent during subculture of colony-forming cells and the isolated colonies formed predominantly in subcultures of the KOC testicular cells. Similar to KOC, the culture of WL testicular cells also yielded both colonies and monolayer during primary culture. Retrospective comparison showed rapid proliferation of WL testicular cells relative to KOC testicular cells.

In the second experiment, several cells at seeding and colony-forming testicular cells on day 15 of culture (at the end of primary culture) were positive for PAS, anti-SSEA-1, anti-SSEA-3, and anti-SSEA-4 antibody staining. However, both cells did not react to AP staining. The age of donor cells did not affect staining affinity patterns.

We conclude from our results that heterologous, fertile spermatozoa could be produced by transfer of testicular cells into juvenile or adult testes. Others have demonstrated the capability of intact or in vitro-cultured spermatogonia to induce germline transmission after transfer into recipients (Brinster et al., 1994; Nagano et al., 2003). Transplanted cells (presumptively spermatogonia, dedifferentiated spermatogenic cells or germline stem cells) may become established in the spermatogonial cell layer of the seminiferous tubules of the recipient testes. The testicular tissue of the recipients supported spermatogenesis of the injected spermatogonia that subsequently yielded functional spermatozoa.

The testicular cells retrieved by the optimized methods of this study consisted of various types of germline cells, sertoli cells and stroma cells. Presumably, spermatogonia, germline stem cells and/or dedifferentiated spermatogenic cells of the transplanted testicular cells might participate in the spermatogenesis of the recipient testes. It has generally been presumed that spermatogonial (germline) stem cells continuously proliferate to finally become undifferentiated type A

spermatogonia (De Rooij et al., 1998; Dym, 1994; Russell et al., 1990; Brinster et al., 1998). Brinster and Zimmerman (1994) and Ogawa et al. (2001) demonstrated that the transplanted donor spermatogonial stem cells can participate in spermatogenesis in the seminiferous tubules of recipient mice and generate donor-derived progenies. Live births have been achieved after transplantation of spermatogonia into adult testes, confirming the 'stemness' of the spermatogonia in the mouse (Brinster et al., 2003). Considering short-term culture of the testicular cells before transfer, however, it might be very little case that dedifferentiate spermatogenic cells participated in producing heterogenic progenies in this study.

Mature spermatozoa were not observed among the transferred cells of the cell suspension. It is possible that hatched progeny with black feathers were derived from primary or secondary spermatocytes, or from round spermatids, which could have been transplanted. However, it might be very little in this case. In general, spermatogenic cells in the early stages of spermatogenesis do not stay in the testicular tissues for longer than 17 days (Bellve et al., 1977). Considering the fact that the hatch of the progeny with black feathers was detected at more than 38 days after transfer in this experiment, this possibility may almost certainly be excluded. To date, there have been no reports on the potential feasibility, pluripotency, and gene targeting of spermatogenic cells in domestic animals.

The testis-mediated system involves less time and labor for creating chimeric status than does the embryo-mediated system (Nagano et al., 2003), i.e., the procedures for pluripotent cell transfer to the developing embryo, embryo development and hatching (21 days), and sexual maturation of the chimera (5-6 months) are omitted in the new system (Han et al., 2002). Our study suggests the possibility of developing this alternative, testis-mediated system that allows the use of testes as a reservoir for new stem cells. Sufficient numbers of the cells that give rise to functional spermatozoa are simply retrieved from this newly discovered reservoir in young and adult chickens, which may be provide for transgenesis for bioreactor production. We are now undertaking the experiment on in vitro-maintenance of colony-forming cells, and subsequent characterization and differentiation of the established cells. Nevertheless, our results are preliminary as a total of only 5 cockerels had chimeric testes as shown by a low frequency of donor-derived sperm shown by breeding. More detailed information on the population of pluripotent cells in the testicular cells and accessibility of transgenic technology for testicular cell-derived, chicken pluripotent cells is necessary, as well as the improvement of the transmission efficiency by modifying various sub-protocols.

국문 적요

생체반응기로서 조류는 유용단백질 생산에 있어서 가장 효율적인 시스템으로 알려져 있다. 계란 난백의 반이상을 차지하는 단백질은 다른 네가지의 단백질 (라이소자임, 오보뮤코이드, 오보뮤신, 콘알부민)과 함께 오브알부민 유전자로 부터 만들어진다. 그리고 자연적으로 멸균된 상태인 계란은 이러한 높은 농도의 난백과 함께 재조합 단백질의 안정적인 보관을 유지할 수 있다. 그러나 이러한 여러 장점에도 불구하고, 조류의 형질전환 방법은 복잡한 수정과정과 발생학적인 차이점으로 인하여 다른 동물에 비하여 매우 뒤쳐져 있다.

최근 정상인 수컷 마우스 정소로부터 분리된 세포를 불임인 수컷 마우스의 정소에 이식을 통한 생식선카이메라 생산 시스템이 개발되었다. 이식된 세포로부터

정자형성이 이루어졌으며, 수용체에서는 정자 및 자손의 생산이 가능하였다. 이식과정을 살펴보면, 정소세포 내에 포함된 정원세포는 이식 후에 정소세관의 기저막 부분으로 이동하여 분열을 시작한다. 그리고 이 세포는 생식세포로 분화하여 성숙된 정자생산을 하게 된다. 이러한 과정으로 이식된 정원세포는 자가 분열에 의한 증식, 생식세포로의 분화 및 정자 생산이 이루어지게 된다.

따라서 이 연구에서는 기존에 행하여져 왔던 배아를 이용한 방법과는 다른 닭에서의 생식선 카이메라 생산 방법으로서 정원세포를 응용한다. 이 시스템은 정소세포의 분리, 체외배양, 체외배양된 정소세포의 이식 그리고 검정교배를 통한 생식선 카이메라의 생산 확인으로 구성된다.

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