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Developmental Genetic Analysis of Avian Primordial Germ Cells and the Application to Poultry Biotechnology

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ABSTRACT : A novel sterategy has been established to determine the origin of the Primordial Germ Cells (PGCs) in avian embryos directly and the developmental fate of the PGCs for the application to Poultry biotechnology. Cells were removed from 1) the centre of area pellucida, 2) the outer of area pellucida and 3) the area opaca of the stage X blastoderm (Eyal-Giladi & Kochav, 1976). When the cells were removed from the centre of area pellucida, the mean number of circulating PGCs in blood was significantly decreased in the embryo at stage 15 (Hamburger & Hamilton, 1951) as compared to intact embryos. When the cells were replenished with donor cells, no reduction in the PGCs number was observed. The removal of cells at the outer of area pellucida or at the area opaca had no effect on the number of PGCs. In case, another set of the manipulated embryos were cultured ex vivo to the hatching and reared to the sexual maturity, the absence of germ cells and degeneration of seminiferous tubules was observed in resulting chickens derived from the blastoderm in which the cells were removed from the centre of the area pellucida. It was concluded that the avian Primordial Germ cells are originated at the center of area pellucida. Developmental ability of the cells to differentiate into somatic cells and germ cells in chimeras were analyzed. Somatic chimerism was detected as black feather attributed from donor cells. Molecular identification by use of female- specific DNA was performed. It was confirmed that the donor cells could be differentiated into chimeric body and erythrocytes. Donor cells retained the ability to differentiate into germline in chimeric gonads. More than 70% of the generated chimeras transmitted donor derived gametes to their offspring indicating that the cells at the center of area pellucida had the high ability to differentiate into germ cells. A molecular technique to identify germline chimerism has been developed by use of gene scan analysis. Strain specific DNA fragments were amplified by the method. It would be greatly contributed for the detection of germline chimerism. Mixed-sex chimeras which contained both male and female cells were produced to investigate the developmental fate of male and female cells in ovary and testes. The sex combinations of donor and recipient of the resulting chimeras were following 4 pairs; (1) chimeras (ZZ/ZZ) produced by a male donor (ZZ) and a male recipient (ZZ), (2) chimeras (ZW/ZW) produced by a female donor (ZW) and a female recipient (ZW), (3) chimeras (ZZ/ZW) produce by a male donor (ZZ) and a female recipient (ZW), (4) chimeras (ZW/ZZ) produced by a female donor (ZW) and a male recipient (ZZ). It was found that genetically male avian germ cells could differentiate into functional ova and that genetically female germ cells can differentiate into functional spermatozoa in the gonad of the mixed-sex chimeras. An ability for introduction of exogenous DNA into the PGCs from stage X blastoderms were analyzed. Two reporter genes, SV-βgal and RSV-GFP, were introduced into the PGCs. Expression of bacterialβgal was imporved by complexing DNA with liposome detectedcc in 75% of embryos at 3 days embryos. At the embryos incubated for 1day, expression of the GFP was observed all the embryos. At day 3 of incubation, GFP was detected in about 70% of the manipulated embryos. In case of GFP, expression of the transgene was detected in 30 % of the manipulated embryos. These results suggested that the cells is one of the most promising vectors for transgenesis. The established strategy should be very powerfull for application to poultry biotechnology.

(Kew words : PGCs, germ line, sex chimera, poultry)

DETERMINATION OF ORIGIN OF PGCS

To produce germline chimeras, manipulation of the cells that retain the ability to differentiate into germ cells are essential. The origin of germline in vetebrates has been one of the most fundamental subjects in developmental biology. In avian species, primordial germ cells (PGCs) were localized in the epiblast of stage X blastoderm (Eyal-Giladi and Kochav, 1976). Majority of the PGCs at stage X was detected at the central disc of area pellucida by the PAS staining technique (Ginsburg & Eyal-Giladi, 1987; Muniesa & Dominguez, 1990). Primary tool to detect the presence of PGCs had been the PAS staining technique and the immuno-staining technique in recent years. Epitopes for PGCs are EMA-1 and SSEA-1 in chickens (Urven et al., 1988; Karagenc et al., 1996) and QH-1 in quail (Pardanaud et al., 1987). Despite the advancement in research techniques, the localization of PGCs within blastoderm has not been determined precisely (Eyal-Giladi, 1993; Etches et al., 1996). Furthermore, there was little evidence to support that putative PGCs which are detected by the PAS- and immuno-staining could be differentiated into the gametes; spermatozoa in males and ova in females.

A lack of the definitive evidence regarding the developmental origin of PGCs in the blastoderm has been mainly due to the limitation of experimental techniques for avian embryo manipulation. Recently, we have established a novel system to determine the origin of the Primordial Germ Cells (PGCs) in avian embryos directly and the developmental fate of the PGCs (Kagami et al., 1997; Kagami et al., 2001a; Kagami et al., 2001b). Approximately 700 cells were removed from 1) the centre of area pellucida, 2) the outer of area pellucida and 3) the area opaca of the stage X blastoderm (Eyal-Giladi & Kochav, 1976). When the cells were removed from the centre of area pellucida, the mean number of circulating PGCs per 1μl of blood was significantly decreased to 13 ($P<0.05$) in the embryo at stage 15 (Hamburger & Hamilton, 1951) as compared to intact embryos of 51. When the cells were replenished with 500 donor cells, no reduction in the PGCs number was observed. The removal of cells at the outer of area pellucida or at the area opaca had no effect on the number of PGCs. When another set of the manipulated embryos were cultured ex vivo to the hatching and reared to the sexual maturity, the absence of germ cells and degeneration of seminiferous tubules was observed in resulting chickens derived from the blastoderm in which the cells were removed from the centre of the area pellucida.

Together with these results it was first determined both *in vivo* and *in vitro* that the avian Primordial Germ Cells are originated at the center of area pellucida (Kagami et al., 1997; Kagami et al., 2000b).

DIFFERENTIATION TO SOMATIC CELLS

Blastodermal chimeras were produced with donor Barred Plymouth Rock (BPR) and recipient White Leghorn (WL). Manipulated embryos were ex vivo cultured until hatching (Perry, 1988; Naito et al., 1990; Ono et al., 1994). The donor contribution to somatic cells in chimeras were identified by the presence of the black feather derived from the donor cells. More than 60% of the generated chimeras contained the black pigment in the feather.

Chimeras were also produced for molecular identification of somatic chimerism. Erythrocyte and whole mount in situ hybridization was performed using a female-specific DNA probe. A W-specific DNA, Xho I family, was used as the probe (Tone et al., 1982; Kagami & Tomita, 1990; Kagami & Tomita, 1992). The probe was labeled with digoxigenin, hybridized to chimeric erythrocyte or embryos and visualized using sheep anti-digoxigenin antibody conjugated to alkaline phosphatase by immunological reaction (Conlon & Herrman, 1993). Distribution of the female-derived cells in the chimeras was observed. Embryonic tissues and the erythrocyte nuclei derived from the female were stained as dark violet. When the in situ hybridization was performed on chimeras at stages 3

to10 days of the ex vivo culture, the entire embryos were stained dark violet and the all erythrocytes contained nuclei stained dark violet, indicating the presence of the W-specific DNA fragment, in chimeric embryos which were produced by a female donor cells and a female recipient embryos. When chimeric embryos were produced by a male donor cells and a male recipient embryos, no staining was observed either in the embryonic body or erythrocytes. When chimeric embryos were produced by a female donor cells and a male recipient embryo, the fraction of the female derived tissue in the embryonic body was 7.3% at 3 days and it was increased to 11.0% at 10 days of the ex vivo culture. When the chimeric embryos were produced by a male donor cells and a female recipient embryo, almost all (more than 90%) of the embryonic bodies were derived from female. These results suggested that the male-derived donor cells could masuculinize the female recipient but female-derived donor cells could not feminize the male recipients.

It was concluded that the donor cells from stage X blastoderm could differentiated into somatic cells (feather, erythrocyte and embryonic body etc.) in resulting chimeras.

DIFFERENTIATION TO GERM CELLS

Experiments using chicken germline chimeras were conducted to confirm if the cells from center of area pellucida at the stage X blastoderm retained the ability to differentiation into germ cells or not (Kagami et al., 1995a; Kagami et al., 1997; Tagami & Kagami, 1998). Avian germline chimeras are also one of the most useful tools for the production of transgenic birds (Petitte et al., 1990; Naito et al., 1990; Brazolot et al., 1991; Watanabe et al., 1992; Carsience et al., 1993; Tajima et al., 1993; Ono et al., 1994; Etches et al., 1996). In the production of the germline chimeras, sterilization or elimination of recipients' PGCs is the critical step. At present, the g-ray irradiation is one of the most reliable methods for sterilizing the recipients' PGCs (Carsience et al., 1993). However, the use of g-ray irradiation is limited due to the cost and the equipment. Should the recipients' PGCs be eliminated from the blastoderm effectively by any other methods, the production of transgenic birds via germline chimeras would be greatly enhanced.

We have established a novel system to analyze the donor ability to differentiate into germ cells in chimeric gonads and to produce germline chimeras effectively for future application of transgenic poultry and to conserved endangered poultry. Donor cells from center of area pellucida at stage X blastoderm were microinjected into recipient blastoderms from which cells at the center of the area pellucida were removed. The distribution of donor BPR-derived black offspring (i/i) and recipient WL-derived white offspring (I/i) was recorded to estimate the contribution of the donor (i) and the recipient (I) to the germline, respectively. More than 70% of the chimera transmitted donor derived gametes to their offspring. However, chimeras transmitted donor-derived gametes to their offspring in very low frequencies in case donor cells were obtained from the outer of area pellucida (less than 10%) or area pellucida (less than 5%) of the stage X blastoderm.

These results indicated that the cells at the center of area pellucida retained the ability to differentiate into germ cells and that the established system could produce germline chimeras very effectively. A simple method to reduce recipients' PGCs was developed (Li et al., 2001). X-ray was irradiated to Japanese quail embryos. Using this method, number of circulating PGCs could be significantly reduced.

The cDNAs encoding two forms of quail stem cell factor (qSCF) were obtained using RT-PCR with nested primers (Petitte & Kulik, 1996). The deduced amino acid sequence of the long form of qSCF showed a high degree of homology with chicken (98%) and relatively low homology (approximately 53%) with various mammalian SCFs. Northern blot analysis with the qSCF cDNA revealed the expression of a 5.9 and a 2.7 kb transcript in several quail tissues. These findings should be important to understand the mechanism of PCCs' differentiation.

Recently, embryonic germ (EG) cell lines were established from primordial germ cells (Park & Han, 2000). PGCs from 5.5-day-old (stage 28) chicken embryonic gonads were isolated and cultureed in medium supplemented with stem cell factor (SCF), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), interleukin-11 (IL-11), and insulin-like growth factor-I (IGF-I). Use of these cells should be one of the most useful tools for the production of transgenic chickens and for studies of germ cell differentiation and genomic imprinting.

MOLECULAR IDENTIFICATION OF GERMLINE CHIMERISM

To identify the germline chimerism, only the established method was progeny test using feather color of offspring such as white (e. g. WL; *I/I*) or black (e. g. BPR; i/i) as the genetic marker. The chimeras were raised until sexual maturity and were mated with BPR. The distribution of black (fertilized with BPR-derived gametes (*i*)) and white offspring (fertilized with WL-derived gametes (*I*)) was recorded to assess the respective contribution of donor and recipient lineages to the germline (Pettite et al., 1990; Carsience et al., 1993; Pain et al., 1996; Kagami et al., 1997; Kagami & Hanada, 1997; Naito et al., 1999). The progeny test is useful to identify germline chimeris in chickens accurately. However, it takes long time to test the germline chimerism since several months is needed to reach sexual maturity in chickens. Thus, development of molecular technique to identify germline chimerism has been longed for. Recently, our research group has developed a molecular technique to identify cell derivation of donor and recipient of somatic and germline chimeras in chickens by use of gene scan analysis (Iwata et al., 2000). Strain specific DNA fragments were amplified by the method. By use of the gene scan analysis, cell derivation (e.g. WL or BPR) of germ cells or somatic cells could be identified. It was concluded that the established system would be greatly contributed for the detection of germline chimerism at any stage of development.

PRODUCTION OF MIXED-SEX CHIMERAS

Experiments in which the sex of donor and recipient embryos could be determined were conducted to investigate the developmental fate of male and female cells in ovary and testes (Kagami et al., 1995a; Kagami et al., 1995b). Donor embryos were obtained from BPR and the recipient embryos were obtained from WL, respectively. Chimeras were produced according to the method of Carsience et al., (1993) except that suspensions of blastodermal cells from individual donor embryos were prepared for injection. Approximately 50 cells obtained from the donor and the recipient were digested in 50 μl of buffer composed of 10 mM Tris, 1mM EDTA, 5% SDS and 10 μg/ml Proteinase K (pH 7.5) for 2 hours at 38.5℃. After the digestion, samples were centrifuged at 15,000 g for 5 minutes. A sample containing 5 μl of the supernatant was used for PCR reaction. The reaction condition and female-specific DNA primers for the PCR were as reported by Clinton (1994). A dense band of 315 bp was detected in females but not in males by the PCR. The sex combinations of donor and recipient of the resulting chimeras were following 4 pairs; (1) chimeras (ZZ/ZZ) produced by a male donor (ZZ) and a male recipient (ZZ), (2) chimeras (ZW/ZW) produced by a female donor (ZW) and a female recipient (ZW), (3) chimeras (ZZ/ZW) produce by a male donor (ZZ) and a female recipient (ZW), (4) chimeras (ZW/ZZ) produced by a female donor (ZW) and a male recipient (ZZ). As above, (1) and (2) are the same-sex chimeras and (3) and (4) are mixed-sex chimeras. These chimeras were raised until their maturity and were mated with BPR (*i/i*) and the distribution of black (fertilized with donor-BPR derived gametes (*i*)) and white offspring (fertilized with recipient-WL derived gametes (*I*)) was recorded to assess the contribution of the donor and recipient lineage to the germline, respectively (Kagami et al., 1995a; Kagami et al., 1995b; Kagami & Hanada,

1997).

Mixed-sex chimeras were also produced using PGCs from circulating embryonic blood in chickens (Furuta et al., 1999; Fujihara, 2000; Yamaguchi et al., 2000) and quail (Ono et al., 1996).

PRODUCTION OF FEMALE PGC-DERIVED SPERM

Phenotypic males (ZW/ZZ) combined by a female donor (ZW) and a male recipient (ZZ) produced black offspring and the phenotypic females (ZZ/ZW) combined by a male donor (ZZ) and a female recipient (ZW) produced black offspring from the mating, respectively. These data suggests that when cells from a male blastoderms (ZZ) were incorporated into a female chimera (ZZ/ZW), ZZ "oogonia" were included within the ovarian follicles and the chromosome complement of genetically male oogonia was processed normally during meiosis. Following ovulation, the male-derived ova were fertilized and produced normal offspring. In case, cells from female embryos (ZW) were incorporated into a male chimera (ZW/ZZ), ZW "spermatogonia" entered meiosis I and produced functional ZZ spermatocytes. The ZZ spermatocytes further differentiated into functional Z-bearing spermatozoa and produced offspring. By contrast, very small number of W-bearing spermatozoa was produced from the ZW spermatogonia, however, the W-bearing spermatozoa differentiated from the ZW spermatogonia was not functional. The production of W-bearing sperm was originally confirmed by southern hybridization using W-specific DNA probe (Kagami et al., 1995a; Etches & Kagami, 1997).

These findings as above is the first scientific evidence that genetically male avian germ cells can differentiate into functional ova and that genetically female germ cells can differentiate into functional spermatozoa in the gonad of the mixed-sex chimeras (Kagami et al., 1995a, Kagami and Hanada, 1997). The production of W-bearing sperm from female-derived PGCs were later reconfirmed by PCR (Simkiss et al., 1996; Naito et al., 1999) and FISH (Tagami et al., 1997; Tagami and Kagami, 1998).

STRAIN PREFERENCE FOR PRODUCTION OF FEMALE PGC-DERIVED SPERM

To elucidate the strain preference in donor and recipient for the production of W-bearing sperm, mixed-sex germline chimeric chickens were produced. The combination of donor and recipient was WL and BPR, and vice versa. Four sets of mixed-sex chimeras that had the male phenotype at sexual maturity were subjected to analysis: group 1, a female WL donor and a male BPR recipient; group 2, a male WL donor and a female BPR recipient; group 3, a female BPR donor and a male WL recipient; group 4, a male BPR donor and a female WL recipient. The mean number of W-bearing sperm detected by in situ hybridization among 10,000 sperm observed was 135, 158, 26 and 71 in groups 1, 2, 3 and 4, respectively. The numbers in groups 1 and 2 were both significantly higher than those of groups 3 and 4 ($p<0.05$). It is suggested that the combination of a WL donor and a BPR recipient produced W-bearing sperm more efficiently than the reverse combination (Kagami et al., 2001c).

VECTOR CELLS FOR TRANSGENESIS

In avian species, successful methods to generate transgenic birds has been the use of retorovirus (Salter et al., 1987; Bosselman et al., 1989) and the microinjection into zygote recovered from oviduct (Love et al., 1994). In chickens, transient expression of exogenous DNA was obtained when a reported gene construct was microinjected into the germinal disc of the fertilized embryos. However, there was no proof that the DNA had been incorporated into the genome.

Thus, an ability for introduction of exogenous DNA into the PGCs from stage X blastoderms were ana-

lyzed (Maruyama et al., 2000). Two reporter genes, SV-βgal and RSV-GFP, were introduced into the PGCs. Expression of bacterialβgal was imporved by complexing DNA with liposome detected in 75% of embryos at 3 days embryos. PGcs from the blastoderms (Kagami et al., 1997; Tagami and Kagami, 1998) were efficiently transfected *in vito* by lipofection. The rates of expression of the GFP were gradually decreased over the time for the incubation. At day 1 of incubation, GFP was detected in about 70% of the manipulated embryos. At day 3 of incubation, expression of the transgene was detected in 30 % of the manipulated embryos. These results suggested that the blastodermal cells from the center of the area pellucida at stage X was one of the most promising vectors for transgenesis.

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