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Development of Complete Culture System for Quail Embryos and Its Application for Embryo Manipulation

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Abstract : Gene and cell transfer technique will serve as a powerful tool for the genetic improvement of the poultry and to yield useful products. For avian transgenesis, Japanese quail may serve as an excellent animal model because of its small body size and fast growth rate. Recent progress was described on the manipulation of quail embryos such as the introduction of foreign genes and cells, and the subsequent culturing of the manipulated embryos yielding hatchlings. Intraspecific donor-derived offspring have been available in quail; however, further investigation will be required to obtain interspecific offspring with the aim of rescuing endangered species. Transgenesis will also be useful for improving the profitability and quality of poultry stocks and for developing stocks with novel uses. Considerable progress should soon be made toward the production of transgenic poultry. The key feature of the procedure described here is that embryos are initially taken out from the shell for ease of manipulation and then placed back in culture in addition to various operations midway during culture.

(Key words : chicken, culture, embryo, manipulation, quail)

INTRODUCTION

Poultry embryos are subjected to various environmental conditions in the course of normal development. For example, in the chicken embryo, on the first day, development takes place in the oviduct, where egg formation is completed by deposition of thick and thin albumen, uterine fluid, chalaza, inner and outer shell membranes, and the shell around the yolk; for the next 21 days, the enveloping layers act as a buffer between the embryo and the egg's environment. Chicken embryos have widely used in poultry biotechnology, however, Japanese quail may serve as an excellent alternative animal model because of its smaller body size and faster growth rate. Quail require 16 days to hatch and 6 weeks to mature to an adult body weight of less than 150 g, while chickens require 21 days to hatch and usually 5-6 months to mature, and their body weight exceeds 1 kg. Virtually all developmental information about the chicken embryo is applicable to the quail embryo, and much of it is applicable to embryos of other species.

Long-term culturing of embryos outside of their own shells and shell membranes was required for embryo manipulation. How to access the embryo while allowing it to develop normally has been the subject of many studies. Successful hatching of cultured poultry embryos was obtained for quail cultured from 2.5-day embryos (Ono and Wakasugi, 1984) and for chickens from 3-day embryos (Rowlett and Simkiss,1987). To manipulate embryos at earlier stages, such as before cell division in the hen's oviduct, a method which allows the culturing of such earlier developmental stages and which results in hatchlings, is required. Perry (1988) has reported such a complete culture method for chickens, which has proved to be applicable for gene transfer studies.

Emphasis here will be placed on the manipulation of quail embryos such as the introduction of foreign genes and cells, and the subsequent culturing of the manipulated embryos yielding hatchlings.

EMBRYO MANIPULATION

A number of attempts have been made to produce transgenic and germline chimeric birds. For example, DNA was introduced into zygotes by microinjection and the embryos were cultured *in vitro* thereafter (Sang and Perry, 1989). At the blastoderm stage, dispersed blastoderm cells were injected into a recipient embryo, resulting in germline chimeras (Petitte *et al.*, 1990; Kagami *et al.*, 1997), cells with gene lipofection were injected into blastoderm-stage embryos (Brazolot et al., 1989), DNA was transfected with a retroviral vector (Souza et al., 1984; Bosselman et al., 1989), and DNA was transfected with liposome and/or electroporation (Hong et al., 1998; Muramatsu et al., 1998; Naito et al., 2000). When the production of transgenic offspring is the target, DNA transfection into primordial germ cells (PGCs) with a retroviral vector (Allioli et al., 1994; Han et al., 1994a), ballistic DNA transfection into the germinal crescent (Li et al., 1995), transfusion of liposome-mediated DNA into PGCs (Watanabe et al., 1994) and use of recently established chicken embryonic germ cell lines (Park and Han, 2000) are the alternative strategies.

1. Microinjection of DNA into Zygotes

Transgenesis provides a useful tool for studying the expression and function of genes. Development of quail embryos from fertilization to blastoderm formation takes place in the oviduct. In the past, a major obstacle for the microinjection of DNA into the zygote was the culturing of embryos after the operation, but an appropriate culture system has now been developed.

When trying to introduce foreign genes into the cytoplasm of zygotes, the ovum covered with a thick albumen capsule was first used; however, we found that it was difficult to recognize the germinal disk through the capsular thick albumen. When this albumen was removed, the germinal disk at this stage was recognized more clearly with the aid of an image-processor system (Image Sigma-III, Nippon Avionics, Tokyo) and the introduction of genes was achieved into the center of the germinal disk. We introduced the plasmid construct pMiwZ into the naked (albumen capsule-removed) ova, and then cultured them *in vitro* and observed the gene expression at later embryonic stages (Ono et al., 1994a). The frequency and level of the gene expression were clearly higher than those in embryos with plasmids introduced through the albumen capsule (Yazawa et al., 1997). It should also be noted that the gene expression observed here was limited to subpopulations of cells in the embryo, indicating that the need for further improvement of the method of gene introduction, especially for germline incorporation.

2. Chimeras Obtained by Transfer of Blastoderm Cells and Their Use for Transgenesis

Dispersed blastoderm cells were microinjected into the blastoderms of unincubated quail eggs (Ono et al., 1994b; Jeon et al., 1977b). The newly laid, unincubated egg is accessible to the population of cells that differentiate into the cell lineage of gametes and this process can be performed without sacrificing the mother. The resulting animals are chimeras that carry the donor cell line and may produce donor-derived progeny if the donor cells differentiate into gametes. We transferred blastoderm cells of quail of the dominant black strain (D) to the wild-type plumage strain (WP). The embryos thus treated were cultured *in vitro* and a germline chimera was obtained (Ono et al., 1995a). For transgenesis, dispersed blastodermal cells were co-cultured *in vitro* with pMiwZ and a cationic liposome (Lipofectin) for gene transfection, and then injected into the host blastoderm. Gene expression was observed at later stages in embryos, but it was found to be transient (Ono et al., 1994b).

3. Introduction of PGCs and Genes into the Vascularized Embryo

PGCs of poultry display a unique migration pathway toward their target organ, the gonadal anlage, during early development. PGCs are the first identifiable precursor cells for gametes. These cells first arise from

the epiblast in the central zone of the area pellucida and gradually translocate to the hypoblast during the early stages of primitive streak formation (Eyal-Giladi et al., 1981; Ginsburg and Eyal-Giladi, 1987). During gastrulation, the PGCs migrate anteriorly via the hypoblast and come to reside in the extraembryonic germinal crescent (Eyal-Giladi et al., 1981; Urven et al., 1988; Han et al., 1994b; Hong et al., 1995). As soon as the blood vessels form at stage 10 (Hamburger and Hamilton, 1951), PGCs from the germinal crescent start to circulate temporarily through the bloodstream, and by stages 20~24 these PGCs have migrated into the gonadal anlage, where they rapidly proliferate and differentiate into either spermatogonia in the testis or oogonia in the ovary (Kuwana, 1993). These migration characteristics of PGCs facilitate their isolation and transfer in early developing avian embryos (Fujimoto et al., 1976; Chang et al., 1992; Yasuda et al., 1992).

A number of attempts have been made to produce germline chimeras and donor-derived offspring by the transfer of PGCs in chickens (Naito et al., 1994a; Tajima et al., 1993; Vick et al., 1993). Offspring were produced from frozen/thawed donor-derived PGCs (Naito et al., 1994b; Chang et al., 1998). It is also possible for PGCs to serve as vectors for introducing and expressing exogenous genes to produce transgenic birds (Allioli et al., 1994; Naito et al., 1998; Ebara amd Fujihara, 2000).

4. Enrichment of Donor PGC Concentration and Restriction of Recipient PGC Proliferation

To produce germline chimeras by the transfer of PGCs, it is critical to incorporate donor PGCs into the endogenous gonadal tissues of recipient embryos. The proportion of donor-derived gametes, however, will be determined by the ratio of the two populations, i.e., the donor- vs. the recipient-derived gametes in the chimeric gonad. Donor PGCs were enriched in concentration by means of Ficoll density gradient centrifugation (Yasuda et al., 1992). Alternatively, when donor PGCs were removed from quail embryos, they could be enriched by means of immunomagnetic cell sorting with the monoclonal antibody (mAb) QCR1 (Aoyama et al., 1992; Ono and Machida, 1999).

In addition, a higher proportion of donor-derived gametes is expected if recipient's PGCs can be removed, reduced in number or inactivated. The ideal recipient, therefore, would be a healthy animal which has normal reproductive organs but is sterile due to the absence only of the germ cells themselves. A number of attempts have been made to decrease the number of the recipient's PGCs; for example, for this purpose, 3~6 μl of blood was removed from the marginal vein (sinus terminalis) of the recipient chicken embryo and the blood-removed embryos exhibited about 30% fewer PGCs at stage 29 (Ono et al., 1998a). Recently, we have developed a simple method

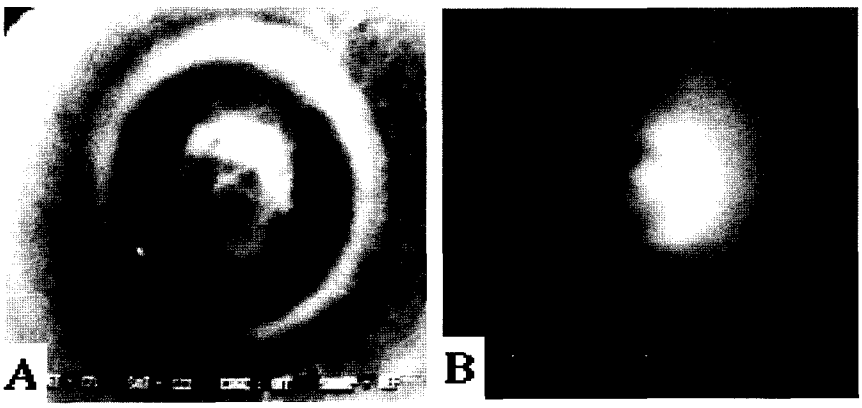

Fig. 1. Germinal disk of quail ova observed with the aid of an image-processor system (Image Sigma-III, Nippon Avionics, Tokyo). The Ovum was obtained from the oviduct 2.5 hr after the preceding oviposition. A, Albumen capsule removed ovum; B, Ovum encapsulated with thick albumen.

using soft X-ray irradiation (18 KV power, 20 cm distance, B-4, Softex, Tokyo) to reduce the number of quail PGCs, which should be useful for preparing recipient embryos for PGC-transfer studies (Li et al., 2001a). When embryos were exposed to the soft X-rays for 40 sec before incubation, the concentration of circulating PGCs was less than one-fifth that in controls after 2 days of incubation. Embryos at day 6 of incubation contained approximately half the number of PGCs compared to controls when they had been exposed before or at day 2 of incubation.

5. Identification of Interspecifically Transferred Donor Cells

In order to identify the transferred PGCs in the recipient animals, reliable markers that can distinguish between cells of exogenous and endogenous origin are required. We evaluated three mAbs, QCR1, QB2 and 2C9, for their ability to distinguish between chicken and quail PGCs in chimeric embryos (Ono et al., 1998ac). QCR1 and QB2 were raised against optic nerves of day 13 quail embryos (Aoyama et al., 1992). 2C9 was raised against gonads of day 6 chicken embryos (Maeda et al., 1994). All of the three mAbs represent direct markers that distinguish between quail and chicken germ cells, although the immunolabelings were limited to certain periods of development (Ono et al., 1998a).

A polymerase chain reaction (PCR) primer set that distinguished between the DNA of donor-derived germ cells and that of recipient cells would be useful as well as convenient for obtaining direct evidence that interspecifically transferred germ cells are present in the recipient animals. We have designed a primer set that amplifies fragments of both quail and chicken genomic DNA of different sizes at 458bp and 923bp, respectively. The primer set for microsatelitte locus LEI0171 on the chicken Z chromosome (GenBank Accession No. X85538; Schmid et al., 2000) amplified a fragment in chicken genomic DNA but not in quail genomic DNA (Li et al., 2001b), and in addition, a number of species-specific primer sets in poultry are candidates for our purposes (Kayang et al., 2000).

6. Interspecific Transfer of PGCs between Quail and Chickens

PGCs of quail embryos were transfused into chicken embryos (Ono et al., 1998a). Transfused quail PGCs settled in the gonads and constituted 14.2% of total PGCs in the gonad at stage 29. A transfused male hatchling contained two populations of spermatogonia derived from exogeneous quail and endogenous chicken PGCs.

Conversely, PGCs of chicken embryos were transfused into quail embryos (Ono et al., 1998c). In this case, transfused chicken PGCs settled in the gonads and constituted 5.6% of total PGCs in the gonad at stage 29. Seminal DNA samples taken from mature putative male chimeras were examined by PCR and the chicken DNA-specific band was observed in the semina of chimeras (Li et al., 2001b).

7. Intraspecific Transfer of PGCs in Quail

PGCs taken from D strain quail were transfused into the embryos of WP strain quail and cultured with System Q3 (hatchability, 31%; Ono et al., 1998b). The putative germline chimeras were raised and donor gamete-derived offspring were born from them. The transmission rates of the donor-derived gametes in the chimeric females and males were 1.8~8.3% and 2.6~63.0%, respectively.

CULTURE OF QUAIL EMBRYOS

Poultry embryos can be cultured from the zygote through hatching. The choice of culture method depends on the age of the embryo at the start of the experiment. In contrast to shell windowing, the culture allows easy access to the developing embryos, and thus is useful for analyzing the developmental process of embryos and embryo manipulation. Injections and microsurgical operations can be made into a specific portion of the embryo. These operations include transplantation of undifferentiated tissues and primordia, microsurgery of limb buds, etc.

Quail development is divided into three periods for

the purpose of culture: fertilization to blastoderm formation lasts for 1 day, embryogenesis for 2.5 days and embryonic growth for 13.5 days (Zacchei, 1961). Cultures are divided into three steps corresponding to the above three periods, respectively. Fertilization takes place in the anterior oviduct, after which the yolk-laden ovum is encapsulated in albumen secreted by the magnum. Around the time of the first division of the zygote, about 4.5 hr after ovulation, the shell membrane is deposited in the isthmus and the albumen is doubled in volume by the absorption of uterine fluid. In the final 18 hr of the oviductal phase, the shell is calcified. The second and third phases take place in the shelled egg. The three discrete culture steps meet the changing demands at successive stages of development, and the embryos are transferred from step to step at appropriate times.

1. Culture from Zygote to Blastoderm Formation

This step deals with the culture from the single-cell stage ovum before the attachment of the thick albumen capsule (or else after the capsule is removed) to the blastoderm stage (System Q1a; Ono et al., 1996). The thick albumen of a hen's egg (pH 7.2~7.4) is used as the culture medium. A fertilized ovum is obtained from the infundibulum or the upper magnum of a laying quail 60-90 min after the preceding oviposition. The culture set (Fig. 2A) is incubated without agitation for 24 hr in a cell culture incubator at 41.5°C with saturated humidity under 20% CO_2. The viability of embryos after 3 days in culture, i.e., System Q1a and successive System Q2 culture, is 50% (Ono et al., 1996).

There is an alternative protocol for the culture from the single-cell stage ovum after the attachment of the thick albumen capsule to the blastoderm stage (System Q1; Ono et al., 1994a). The thin albumen of a hen's egg is used as the culture medium. A fertilized ovum is obtained from a laying quail 2 hr and 30-45 min after the preceding oviposition. The culture set (Fig. 2B) is incubated without agitation for 24 hr in a cell culture incubator at 41.5°C with saturated humidity under 100% air. The viability of embryos after 3 days in culture, i.e., System Q1a and successive System Q2 culture, is 72% (Ono et al., 1994a).

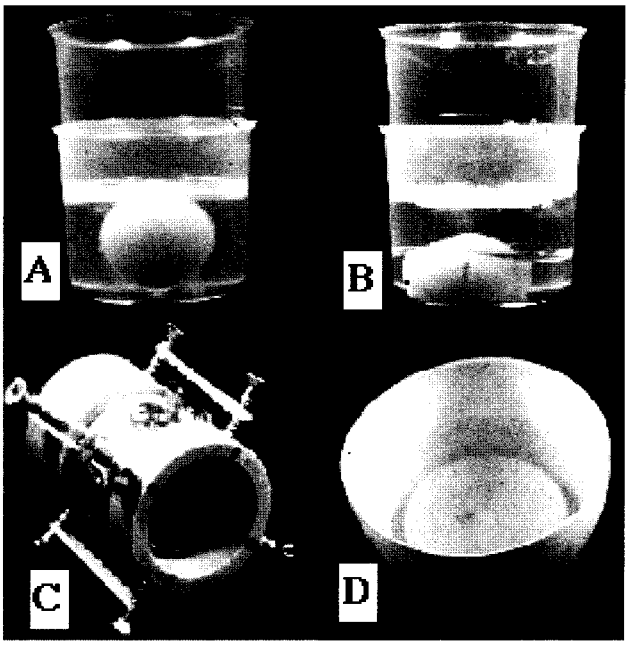

Fig. 2. Culture of quail embryos from single-cell stage to hatching.

A, System Q1a; quail ovum without thick albumen capsule is placed in a 20-ml cup and the cup is filled with chicken thick albumen (pH 7.2~7.4). The open surface of the cup is tightly sealed by putting another cup upon it with elimination of the inside air space. The ovum is submerged just below the surface.

B, System Q1; quail ovum with thick albumen capsule is placed in a 20-ml cup and chicken thin albumen is added up to the ovum's equatorial level. The germinal disk is set upward with an air space above it. The open surface of the cup is sealed by placing another cup inside it.

C, System Q2; Emptied quail shell which is cut horizontally at the position with 18-mm in diameter at its narrow end is used as the surrogate shell. The embryo from System Q1a or Q1 is transferred into it after removal of the thick albumen (if present). The shell is filled with chicken thin albumen and sealed tightly with cling film, a pair of plastic rings and elastic bands.

D, System Q3; A surrogate shell is prepared from a small chicken egg by the removal of its blunt-end half. The embryo from System Q2 is transferred into it and the open surface sealed with cling film.

2. Culture for Embryogenesis

After culturing for 24 hr from the single-cell stage, the embryos will have developped to the blastoderm

stage, which is equivalent to that in a freshly laid egg. The fertilized ovum (germinal disk with yolk) in the natural condition forms a shelled-egg in the oviduct, which includes albumen, chalaza, shell membranes and shell. During the culture in Systems Q1a or Q1, however, only the development of the embryo occurs without the addition of these associated substances. In this culture step, System Q2, the embryo is placed into a surrogate shell where embryogenesis is able to proceed (Fig. 2C, Ono et al., 1994a; Jeon et al., 1997a). Alternatively, the culture can be started at this point from the blastoderm stage. The culture set is placed in an incubator with the long axis of the shell held horizontally, and the embryo is then cultured for 48~60 hr at 37.5°C and 70% relative humidity in an atmosphere of 100% air while being rocked around the long axis at a 90° angle at 30 min intervals. At the end of System Q2, about 90% of embryos are surviving (Ono et al., 1994a).

3. Culture for Embryonic Growth

After culturing 48~60 hr in System Q2, the embryos are transferred to System Q3 for the embryonic growth and hatching (Fig. 2D, Ono et al., 1994a). If the embryonic development in System Q2 is continued, all the embryos die within several days in the absence of an air space. Therefore, the embryo is transferred into an extra-large shell with an artificial air space. Alternatively, the culture can be started newly from this point.

Hatchability was 19.4% and 25.0% when albumen capsule removed and non-removed embryos were cultured from the single-cell stage, respectively (Ono et al., 1994a, 1996). Embryos cultured from the blastoderm stage using Systems Q2 and Q3 showed 48.4% hatchability (Ono et al., 1994a). In contrast, when blastoderm-stage embryos were cultured from the beginning in System Q3 only, hatchability was 20.0%. Hatching of cultured quail embryos was first obtained by Ono and Wakasugi (1984) with only 3% hatchability. This low hatchability may have been due to culturing without rocking the eggs and to the use of autoclaved hens' eggshells. Nirasawa et al. (1992) cultured quail embryos in hens' eggshells from the blastoderm stage and obtained a hatchability of 17.6%. These results indicate that System Q1/Q1a is suboptimal for embryonic development from fertilization to blastoderm formation. System Q2, on the other hand, is more favorable than System Q3 for embryogenesis and morphogenesis, i.e., for about 2 days after blastoderm stage. Complete elimination of the air space above the embryo surface and the rocking angle of 90 degrees available in the former system probably contribute to its success (Ono et al., 1994a).

CONCLUSION

Culture technologies now permit the culture of poultry embryos from the single-cell stage through hatching. This technique is widely applied to maintain and yield manipulated birds. The use of embryonic chimeras which are produced using blastoderm cells and PGCs, has received considerable attention in the field of the conservation biology. Intraspecific donor-derived offspring have been routinely available in chickens and quail; however, further investigation will be required to obtain interspecific offspring with the aim of rescuing endangered species. Transgenesis will also be useful for improving the profitability and quality of poultry stocks and for developing stocks with novel uses. Considerable progress should soon be made toward the production of transgenic poultry.

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