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Possible Production of Transgenic Chicken by Transferring Foreign Genes and Germ Cells

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외부유전자의 전이에 의한 배아세포와 트란스젠닉 가금 생산의 가능성

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ABSTRACT

In recent years, numerous researches have been carried out in author's laboratory to develop several kinds of methods for producing transgened chicken, leaving a lot of new findings. Some of them are very useful to search for new approaches necessary to improve the efficiency of hatchability and the survival rate of developing transgened embryos. The results obtained hitherto might be summarized as follows: (1) Foreign gene(*Lac* Z /Miw Z) introduced into blastodermal cells of developing embryos was successfully transferred to embryos, leading to the production of primordial germ cells(PGCs) carrying foreign DNA. However, hatched chickens failed to show the incorporation of introduced gene into the gonads. (2) When foreign gene was introduced into germinal crescent region(GCR), the gene was also efficiently incorporated into germ cells, resulting in the production of transgened chickens(offspring) which produced further offspring having foreign gene in the gonads. In this case, 2nd and 3rd generations of chickens were obtained through the reproduction of transgened birds. (3) In another way, the gene was injected into blood vessels of developing embryos at stage 13~15, creating PGCs having foreign gene, and produced some transgened chickens. In this work, the PGCs were transferred between embryos, resulting in the production of transgenic chickens. (4) In these experiments, PGCs were effectively employed for producing transgenic birds, developing some kinds of chimeric chickens from homo- or hetero-sexual transfer of the PGCs from embryos. This means that the gonads from donor PGCs developed in some degree to the stage of hatching. However, these gonads showed slightly abnormal tissues similar to ovotestis like organs through histological examination. (5) Avian Leukosis Virus(ALV) induced B cell line(DT40) successfully carried foreign genes into

chicken embryos, suggesting the possibility of the cells as a vector in this field of study in the future. (6) Inter-embryonic transfer of the PGCs also gave us some interesting results, suggesting the possibility of producing chickens carrying donor cells-derived gonads when hetero-sexual transfer of the PGCs was conducted. This also infers the possibility of creating the gonads having W-bearing spermatic cells. (7) The PGCs were cultured outside the body with great success, forming a sort of clumps having PGCs therein. In conclusion, this series of experiments in author's laboratory strongly suggest that transgenic chickens could be successfully produced in the very near future, particularly using the PGCs as a mediator for developing the gonads with homo- or hetero-sexual germ cells. This may also promise the possibility of producing W chromosome-bearing sperm cells even in the hetero-sexually developed gonadas.

(Key words: chicken, PGCs, transgene, embryos, germ cells, gonads)

INTRODUCTION

Lately, the researches on the production of transgenic chicken have been focused on mainly two kinds of tendency(Naito et al., 1998; Fujihara et al., 1998). One is to produce a sort of chimeric birds using PGCs and /or blastodermal cells and the other to introduce some kinds of foreign genes such as *lac* Z /Miw Z(Naito et al., 1991; Fujihara et al., 1998) and recently, green fluorescent protein(GFP) (Maruyama et al., 1998; Ebara and Fujihara, 1999a). Particularly, the latter gene, the GFP, has been successfully employed for studying transgenic animals including chickens (Maruyama et al., 1998; Ebara and Fujihara, 1999a). This special protein has some special features for using living cells such as developing embryos as research tools(Chalfie and Kain, 1998; Sullivan and Kay, 1999). This chemical may give us much more useful information to carry out the experiments on the production of transgened chickens in the near future. In this report, therefore, most recent results obtained from our laboratory will be demonstrated here in some detail, hoping that our final goal may be realized before long.

The goal of our study is to produce kind of transgenic White Leghorn hens, which may produce brown color-shelled eggs. In this respect, we have to search for genes regulating eggshell color in the brown chicken such strain as New Hampshire and Rhode Island Red using recent novel toosl for extracting DNA. In this connection, it seems very beneficial using germ cells including PGCs for producing transgenic chickens. This review, therefore, will extraordinarily focus special stress on recent works being done in author's laboratory, including some of the former researches which have already been summarized elsewhere(Fujihara et al., 1998: Fujihara, 1999a).

TRANSFECTION OF EXOGENOUS GENES INTOÅ@EMBRYOS

With regard to the method for introducing foreign genes into chicken embryos, a lot of researches have been carried out mainly empolying blastodermal cells of early developing chicken embryos(Naito et al., 1994). Most of the investigations for this purpose were to obtain fertilized ova from the oviducts of hens by surgical opeation or hormonal induction of prema-

ture oviposition. To overcome this troublesome task, the methods described below were exploited, bringing about desired outcomes, hoping future production of transgenic birds.

INJECTION OF FOREIGN GENES INTO BLASTODERMAL CELLS

Until recently, main approach for injecting foreign genes into chicken embryos was to use one-cell eggs, which were obtained, surgically from the oviduct(magnal region) of hens (Pettite et al., 1990; Naito et al., 1994; Maeda et al., 1997). This technique was confirmed to be time-consuming and hensacrifice which are also argumentative and controversial for those who keep the mind of animal welfare. Another way to introduce exogenous DNA into blastodermal cells without killing hens was waited for long time.

Author's laboratory, therefore, has developed a novel method to introduce foreign genes into blastodermal cells by using freshly oviposited fertilized eggs(Inada et al., 1996; 1997; 1998). In this study, the PGCs were also transgened, leading to the gonads carrying injected DNA, though next generation, transgenic offspring, was not successfully obtained(Inada et al., 1997). This technique will, however, provide a keen sense to those who work for this field of research in poultry sciences.

For transferring foreign genes into early developing chicken embryos, electroporation method was verified to be much mor effective as nonviral means of exogenous gene transfection to somatic cells of living chicken embryos *in ovo*(Muramatsu et al., 1997; 1998).

INRODUCTION OF EXOGENOUS DNA INTO GERMINAL CRESCENT

At first, germinal crescent region(GCR) was considered to be the site from where the PGCs originate, and migrate to the germinal ridges (GR) to settle and proliferate to be the gonads (Eyal-Giladi and Kochav, 1976; Eyal-Giladi et al., 1981; Han et al., 1994b; Hong et al., 1995; Ginsburg, 1997; Petitte et al., 1997; Guraya, 1998). However, recent works regarding PGCs origination confirmed that original site for PGCs in chickens is probably around the center of blastoderm of developing embryos(Kagami et al., 1997). The GCR might be the place to where the PGCs move after spending a given period of very short time in the central part of the blastoderm. Following moving to the GCR, the PGCs will migrate to the GR via blood circulation of developing embryos just at stage 13~15 of development(Fujimoto et al., 1976; Kuwana 1993).

In this connection, thus, several studies on PGCs development suggested that the incorporation of foreign genes into PGCs was demonstrated when the DNA was injected into both blastodermal cells and GCR almost in the same degree of incorporation rate(Han et al., 1994a; Fujihara, 1999a)., Even so, slightly higher integration rate of injected genes was observed in the case DNA was introduced into the GCR, suggesting that the PGCs residing in the GCR may possess a kind of physiological function peculiar to the developing stage in this site (Eguma et al., 1999).

To our surprise, the genes(*Lac* Z/Miw Z; GFP) introduced into the GCR of developing embryos was successfully transmitted to offspring (next generation), produng gametes, spermatozoa and ova(Ebara et al., 1998; Ebara and Fujihara 1999a,b,c). However, in this respect, lifespan of transgened chickens and

laying rate for hens, and semen production and sperm number for males were gradually decreased with the lapse of time after growing, suggesting the possibility of impared reproductive traits of transgened chickens in some extent(Ebara and Fujihara, 1999c). In the present study, transgenic chickens were characterised by producing unusula semen, including remarkably higher or lower number of spermatozoa in comparison with normal chickens(Ebara and Fujihara, unpublished data). These features peculiar to transgened chicken suggest that genetically treated chicken with exogenous DNA might have possessed introduced foreign genes in germ cells for long time, resulting in the production of offspring with transfected genes therein(Fujihara, 1999b). These interesting results strongly attract us to continue this series of experiments, hoping the possibility of creating new type of laying hens, which may produce a sort of eggs with desired shell color. Another desire of poultry men in the world is to develop the chickens with rapid growth rate via exogenously introduced gene like growth hormone obtained from other animals.

INTERJECTION OF FOREIGN GENES INTO EARLY EMBRYONIC GONADS

Following these works, foreign genes were introduced into the gonads of early developing embryos, resulting in unexpectedly low success rate(Hiyama and Fujihara, unpublished data). In this case, the gene(*lac* Z /GFP) was injected into the gonads of developing embryos at stage 18~20, the time when anlagen of the gonads of chicken reside between blood veseels and nephridial canal(Guraya, 1998). This trial led to the results that the level of introduced genes was decreased gradually with the lapse of time after the injection, though the incorporation of DNA into the gonads was obviously observed in the tissues following direct injection. This finding also suggests that most suitable time /period necessary to introduce foreign genes into the developing embryos. The injection of DNA into the GCR would be the best way for this purpose compared with other several ways during embryonic development. In this experiment, the injected GFP gene marker was clearly observed in the gonads following injection into the organs, though gradual decrease of the intensity of fluorescency of the protein was found as the time goes. This means that the introduction of foreign DNA into the developing gonads could be seriously limited to the stage at 26~28 (Hamburger and Hamilton, 1951). Direct injection of exogenous genes into the developing organs might not be successful way for transferring DNA to chickens, suggesting that higher integration rate of genes into the tissues or organs of developing chicken embryos might be obtained when the DNA was introduced at the early stage of embryonic development(Inada et al., 1997).

INTRODUCTION OF EXOGENOUS GENE INTO BLASTODERMAL CELLS VIA DT40 CELL LINE

The study of somatic diversification of chicken immunoglobulin genes was aided by the analysis of lymphomas induced by the avian leukosis virus (ALV)(Buerstedde et al., 1990). Neonatal ALV infection of susceptible strains of chickens caused B cell lymphomas with a latency of several months (Bezzubova et al., 1997)., The ALV-induced tumors and one cell line (DT40) derived therefrom continued to di-

versify their light chain genes outside of the bursal environment(Bezzubova et al., 1997). Thereafter, this ALV-induced chicken B cell line, DT40 cell line, has been used for increasing targeted integration of foreign genes(Buerstedde and Takeda, 1991). The DT40 cell line has recently been demonstrated to become possibly promising candidate for transferring exogenous genes into chicken embryos(Toba et al., 1999).

In our recent study, foreing gene (*Lac Z*/ Miw Z) incorporated in DT40 cells was injected directly into blastodermal cells of freshly oviposited unincubated fertilized eggs (stage X: Ginsburg, 1997). The DT 40 cells injected into the blastodermal cells were clearly detected in embryonic and extra-embryonic tissues of developing eggs. However, the degree of detection of DNA decreased gradually with the lapse of time following injection, suggesting that the possibility of excludeing the foreign cells from the intrinsic somatic cell groups of the embryos. The DT40 cells are, therefore, probably considered to be eliminated from inheritant somatic cells during their stay in the developing embryonic tissues. This special cell line has been thought to proliferate in the embryonic tissues to make clumps or cell colonies in the sites they reside(Taba et al., 1999).

However, the DT 40 cells are expected to be used for transferring foreign genes into animal cells without any heavy task. Thus, further investigation for improving the efficiency of transgenesis of the treated animals including chickens.

INTER-EMBRYONIC TRANSFER OF PRIMORDIAL GERM CELLS

The above mentioned several findings infer that the PGCs may have some sorts of gonad-determining factors, though the cells have so far been considered not to be involved in the determination of sex of birds(Niewkoop and Takatsuya, 1979). Even if this is certain, our experimental results showed that hetero-sexual transfer of the PGCs between developing embryos produced hetero-sexually treated gonads, showing ovotestis like gonads(Furuta et al., 1998; 1999a, b; Eguma et al., 1999). However, some of the hetero-sexually developed gonads possessed donor cell-derived organs, showing entirely reversed testis and ovary (Yamaguchi et al., 1966; 1998).

In this connection, several findings similar to ours demonstrated that sperm cells have been observed in the ovary like gonads in which PGCs were hetero-sexually transferred between embryos(Tagami et al., 1998; Kagami et al., 1995; 1998; Kagami and Hanada, 1997). These experiments suggest that future researches on hetero-sexual transfer of the PGCs may produce the male chicken with the testis carrying W chromosome-bearing sperm cells therein.

With respect to this matter, inter-species transfer of the PGCs has also been reported using chickens and quails(Ono et al., 1998). This finding suggests the possibility of creating chimeric birds between chckens and quails, which is indicative of new type of chickens for producing a sort of desired eggs suitable to home tables in general.

A series of these studies presume that inter-embryonic transfer of the PGCs would also be effective for producing transgened chickens as well as the introduction of exogenous genes into chicken embryos. In addition, the PGCs incorporated with foreign DNA may be much more productive for creating chimeric birds having many useful traits.

IN VITRO CULTURE OF PRIMORDIAL GERM CELLS

Until recently, the proliferation of PGCs outside the body has been considered to be perseverance task due to some kinds of unknown factors responsible for development of the cells (Niewkoop and Takatsuya, 1979). However, in our experiments, incredibly proliferated PGCs was found in the case the cells were cultured in combination with several growth factors and with specially prepared cells from the GR of early chicken embryos(Yang and Fujihara, 1999a,b). These experimental products also provide a lot of information to those who works with the PGCs.

Our previous researches confirmed that the PGCs have been successfully cultured *in vitro* when treated with some kinds of growth factors(Yang and Fujihara, 1998; 1999a, b). In this case, however, a sort of growth factors obtained from avian tissues were not always effective for *in vitro* proliferation of chicken PGCs. In the present study, a special attention was paid to utilize feeder cells from the GR of developing chicken embryos for stimulating PGC growth *in vitro*. As a result, GR-originated feeder cells were exclusively prominent for improving PGC propagation outside the body (Yang and Fujihara, 1998; 1999a,b).

Most recently, some kinds of modification of the feeder cells induced incredibly proliferated PGCs *in vitro*, when the PGCs were cultured for around 30 days together with a sort of growth factors, resulting in the rigid formation of cellular clump in which PGCs were completely incorporated and increased their number beyond our expectation(Fukushima et al., 1999). In this connection, pre-treatment of the feeder cells with a sort of antibiotics such as mitomycin led to non-proliferation of the PGCs. This result may indicate the possibility of producting gametic cells, spermatogonia or oogonia, outside the body in the future. Regarding this matter, several experiments are now in progress in author's laboratory.

With respect to *in vitro* culture of avian tissues, ther is a report demonstrating that modified pH was regulating factor for proliferation and longevity of cultured cells(Kuwana et al., 1996). Even in this study, *in vitro* culturing of PGCs was not successful compared with other avian tissues, suggesting that further researches will be needed to establish the culture methods peculiar to the PGCs.

POSSIBLE TRANSGENESIS USING SPERM CELLS AS VECTORS

A doubtful report shocked the world several years ago, originating from Italy, saying that most easiest method for producing transgenic animals was found by using sperm cells as vectors(Lavitrano et al., 1989). Since that time, many researchers having interest in transgenic animal production using sperm cells have tried to do the same study without obtaining successful results. As a conclusion, this experiment was recognized to be fallen into oblivion forever, However, in spite of such miserable results, a few scientists are still in expectation of possible use of sperm cells as vectors for transferring exogenous genes into animal cells.

In this connection, a wonderful success has been demonstrated in mammals, reporting that foreign genes injected directly into the testis were perfectly transferred to next generations of animals(Ogawa et al., 1995). The success in this experiment has been considered to be from

successive injection of DNA into the testis, because most of mammalian species possess their testis outside the body. This will make the injection easy compared with the case of birds. This report also caused a great sensation in the world of reproductive physiology, resulting in the production of many transgenic animals in the world.

On the other hand, a few researches have been done using chicken spermatozoa as vectors, though the results obtained hitherto are still far from perfect success(Nakanishi and Iritani, 1993; Tanigawa et al., 1996; Hasebe et al., 1996; 1998). In the case of our researches, an exogenous gene(*lac* Z/Miw Z) treated with ejaculated chicken spermatozoa was transferred successfully to fertilized eggs after artificial insemination(Hasebe et al., 1996; 1998). In this study, transfection reagent(Boehringer Mannheim, Germany) was found to be a useful tool for making transgened sperm cells, showing that no successful products were obtained without this chemical for chicken spermatozoa (Hasebe et al., 1996; 1998). In the case of chicken, testis-mediated transgene was not successful probably due to the difficulty of successive injeciton of DNA into the testis unlike the case of mammalian species(Hasebe et al., 1998). Because chicken testis were located inside the body, making the treatment difficult. Even in these difficult conditions, some trials are going on in order to develop a method for direct injection of the genes into the testis like mammals in our laboratory at present. Some successful reports may appear elsewhere in the world in the near future.

CONCLUSIONS

This series of experiments regarding transgenic chickens have been carrying out in our laboratory for several years, suggesting the possibility of producing these birds in the near future, though much more tough workd will be required to do these kinds of researches.

As described in the section of introduction, the main purpose of this study is to produce a strain of White Leghorn hens, which will lay brown-shelled eggs. If this become true, poultry industry in the world will change dramatically by producing most economical eggs. To do this, another task will be needed to search for new genes regulating egg shell color in the chicken, though several polygenes may be involved in the production of egg shell color. Even in these tough conditions, recently developed high technology regarding gene analysis will be surely supportive to make this kind of works success in the future.

Collectively, the experiments mentioned above are sure to be indicative to produce some kinds of transgenic chickens using exogenous DNA and/or intrinsic genes obtained from chicken tissues. Future heavy and tough works are needed to carry out these things.

적 요

최근까지 본 연구실에서는 형질전환 닭 생산을 위해 다양한 방법들을 이용한 연구들이 수행하고 있다. 이러한 방법들은 형질전환 개체의 생산 및 배자 생존율과 부화율을 증진시키기 위하여 유용하게 이용될 수 있을 것이다. 본 연구실의 결과를 요약하면 다음과 같다. (1) 외래 유전자 (*Lac* Z/Miw Z)의 배반엽 세포층(Blastodermal layer)내 미세주입에 의한 초기 배자 및 배자 원시생식세포(Primordial Germ Cells; PGCs)내 외래 유전자 전이를 확인하였다. 그러나 부화한 개체에서의 생식기내 외래 유전자 확인은 되지 않았다. (2) 외래 유전자를 생식선 반원(Germinal Crescent Region; GCR)내 미세주입에 의해 형질전

환 개체 생산 및 자손의 생식기내에서도 외래 유전자를 확인하였다. 이러한 생산선 반월내 미세주입의 경우에는 2세대, 3세대 까지의 자손에서도 형질전환 개체를 확인하였다. (3) 다른 방법으로 외래 유전자를 배발달 단계 13~15의 배자 혈관내로 미세주입하여 유전자가 전이된 원시생식세포와 형질전환 개체를 생산하였다. (4) 또한, 형질전환 개체 생산을 위하여 외래 유전자가 전이된 원시생식세포를 이용하였다. 이렇게 원시생식세포를 이용한 경우에는 일부 수용체 배자내에 공여체로 이용한 암·수 원시생식세포가 혼재되어 있다. 이러한 결과는 원시생식내 서로 반대 성의 원시생식세포가 배발달 과정에 있어 일정단계까지는 발달함을 제시하고 있다. 그러나 암·수 원시생식세포가 혼재되어 있는 경우에는 조직학적 검증결과, 생식기내 조직이 난소고환(Ovotestis)과 같이 비정상적으로 발달하였다. (5) Avian Leukosis Virus(ALV)를 이용하여 확립된 B cell line (DT40)에 외래 유전자를 전이한 후, 닭 초기 배자의 배반엽 세포층내 미세주입하여 형질전환가능성을 검증하였다. 이러한 세포주는 앞으로 형질전환 닭 생산을 위하여 이용 가능할 것으로 사료된다. (6) 암컷의 원시생식세포를 수컷의 배자에 미세주입하여 생식기 카이메라를 생산함으로써 W 염색체를 가진 정자생산을 위한 연구도 진행중에 있다. (7) 마지막으로, 원시생식세포를 체외 배양 실험도 성공적인 결과를 제시하고 있다. 결론적으로, 본 연구실에서 수행된 일련의 실험은 머지 않은 시일 내에 형질전환 개체 생산에 효율적으로 이용될 수 있을 것이며, 특히 원시생식세포를 이용하여 암·수 생식세포가 혼재된 카이메라를 생산함으로써 W 염색체를 가진 정자 생산에 이용될 수 있을 것으로 생각된다.

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