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Regeneration of Muscular Dystrophy Chickens by Transplantation of Early Blastodermal Cells into Recipient Embryos

Akira Fujiwara[1,2], Tamao Ono[2] and Hiroshi Kagami[2]

[1] Nippon Institute for Biological Science, Laboratory Animal Research Station, Kobuchisawa, Yamanashi 408–0041, Japan
[2] Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399–4598, Japan

A novel strategy has been developed to generate muscular dystrophy chickens by means of germline chimeras. Donor embryos were obtained from the New Hampshire chicken; NH-413 strain which have genes responsible for Fukuyama type muscular dystrophy (Saito *et al.,* 2005). Donor cells were isolated from the center of area pellucida of the blastoderms. Recipient embryos were obtained from White Leghorn chicken; Line-M. The generated chimeric chickens had the donor derived brown plumage in the down in some extent, suggesting that the cells containing muscular dystrophy were introduced into the chimeras. These chimeric chickens have been raised until sexual maturity. The chimeric chickens were back-crossed to donor strain; the NH-413 strain. The phenotype of some of the offspring was very similar to that of the donor strain. The offspring showed some characters typical to the muscular dystrophy.

It was suggested that the donor derived NH-413 strain offspring was generated. The established system should be one of the powerful strategies for breeding and regeneration of the muscular dystrophy chickens.

Key words: blastoderm, chicken, germline chimera, muscular dystrophy, regeneration

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Introduction

Chickens manifesting muscular dystrophy symptoms have been reported since 1960's. Muscular dystrophy refers to a group of inherited diseases marked by progressive weakness and degeneration of the skeletal, or voluntary, muscle that prevents normal movements. The diseases are associated with mutation in the genes encoding several classes of muscle proteins, whose purpose maintaining the normal function of the membrane of muscle cells (Imamura *et al.,* 2000). The disease is one of the most important diseases to be effectively treated. However, there is little clinical treatment for complete recovery. Animal models for the etiological and pathological studies of human muscular dystrophies have been established in mice, dogs and cats (Nonaka, 1998). The diseases symptoms and conditions are not identical among these species because of different pathological manifestations of similar genetic defects. Therefore, establishing additional models will contribute to a better understanding of the mechanisms of muscular dystrophies and provide a basic and applied strategies for clinical therapy trials for such incurable diseases. A New Hampshire chicken; NH-413 strain which have candidate genes relating to Fukuyama type muscular dystrophy was found (Saito *et al.,* 2005).

The origin (Ginsburg and Eyal-Giladi, 1987; Urven *et al.,* 1988; Kagami *et al.,* 1997), migratory pathway (Fujimoto *et al.,* 1976; Ando and Fujimoto, 1983; Yasuda *et al.,* 1992), collection of pluripotent cell from blastoderm or PGCs has been studied extensively. Based on these knowledge, the technologies of transplanting donor avian pluripotent cells from the stage X blastoderms or PGCs into the recipient embryos was established to generate germline chimeras. These embryo manipulation systems have been considered as one of the most powerful tools for production of avian chimeras (Petitte *et al.,* 1990; Naito *et al.,* 1994a, b; Etches *et al.,* 1996) and transgenic birds (Brazolot *et al.,* 1991). Applied these embryo engineering techniques, it was challenged to generate germline chimera of muscular dystrophy NH-413 strain and a White Leghorn strain (L-M strain) in Nippon Institute for Biological Science (NIBS). By conducting the strategy, it was aimed to regenerate offspring of muscular dystrophy chicken.

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Correspondence: Prof. H. Kagami, Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399–4598, Japan. (E-mail: kagami@shinshu-u.ac.jp)

Materials and Methods

Experimental Animals

L-M strain (WL: white feather: Fig. 1)

The L-M strain was established for the research and

Fig. 1. **Recipient strain: White Leghorn (L-M: established in NIBS).** Original White Leghorn colony of this strain was introduced from the Komatsu farm in 1966, and the Line M strain was established in 1969.

Fig. 2. **Donor strain: New Hampshire (NH-413: established in NIBS).** Original colony was introduced from California University in 1976. The chickens have been onset of Muscluar dystrophy. Therefore, conventional breedings and maintenance are very difficult.

development as part of a laboratory animal model strain, and the SPF colony is established. The original colony was a white leghorn strain introduced from the Komatsu farm in 1966, and the Line M strain was established in 1969 in our laboratory.

NH-413 strain (New Hampshire: brown feather: Fig. 2)

The chickens were introduced to our laboratory from California University in 1976. The disorder is transmitted co dominantly by a single gene, whose phenotype is modified by other "background" genes (Asmundson and Julian, 1956; Wagner *et al.,* 1970). Fertility and hatchability of the NH-413 strain were very low as compared to that of the control strain. Conventional breeding and management of these chickens have been very difficult.

Production of Chimeric Chickens

For the production of chimeras, donor and recipient embryos were obtained from NH-413 and L-M (WL), respectively. The donor stage X blastoderm was obtained (Eyal-Giladi and Kochav, 1976; Eyal-Giladi *et al.,* 1981). Excess yolk and blood around the blastoderm was washed off by PBS. The blastoderms were dissociated and dispersed in DMEM containing 10% chicken serum (Carsience *et al.,* 1993). These cells were used for donor. The White Leghorn's fertilized eggs were used as recipients. A sharp needle was pushed through the yolk mass to reach the subgerminal cavity of the blastoderm to remove the cells from the center of the area pellucida. Of about 500 donor cells were microinjected into the subgerminal cavity of the recipient embryos (Kagami *et al.,* 1997).

Embryo Culture

The manipulated embryos were cultured *ex vivo* by the modified methods of Perry (1988) and Naito *et al.* (1990). As the initial incubation, the embryos were incubated for 3 days at 38.5 Celsius. Again, the manipulated embryo and egg yolk were transferred to a larger host egg shell. An additional 18 days of incubation was conducted until they hatched (Hamburger and Hamilton, 1951). In case, brown pigment was present in the feather, the chicks were judged as the somatic chimeras. Strategies of present studies are depicted in Fig. 3.

Progeny Test

To test the germline transmission ability of blastodermal cells to form germline chimeras, the mature birds were progeny-tested by mating with NH-413 strain, according the following procedure: male chimeras were crossed with NH-413 strain's females and a female chimera was crossed with a NH-413 strain's male. Phenotypes of hatched chicks were individually recorded.

Results

Total 60 chimeric embryos were produced (Table 1). Among them 17 chimeric embryos dead until 4 days, moreover 39 embryos dead until 20 days. Finally, 4 chicks were hatched from the 60 manipulated embryos.

The 4 chicks were raised until maturity (Table 2). Three out of 4 chicks were somatic chimeras (75%) and a chicken without feather chimerism was classified as putative chimeras (25%). In these 3 somatic chimeras, two were males; ID# 601, 602, and one was female; ID# 603. The chimeras were raised to sexual maturity and test mating was conducted with donor strain. Three somatic chimeras were raised until maturity and mated with NH-413 strain to investigate the contribution of donor cells to the germline. Collected 104 eggs were incubated and 92 chicks were hatched. A male chimera ID# 0601 was sterile. When a male chimera (ID# 602) and a female chimera (ID# 603) was test mated with the donor strain, 42 and 18 donor-derived offspring were hatched, respectively. Therefore, the proportions of donor-derived offspring in these chimeras were 87.5% (42/48) in chimera

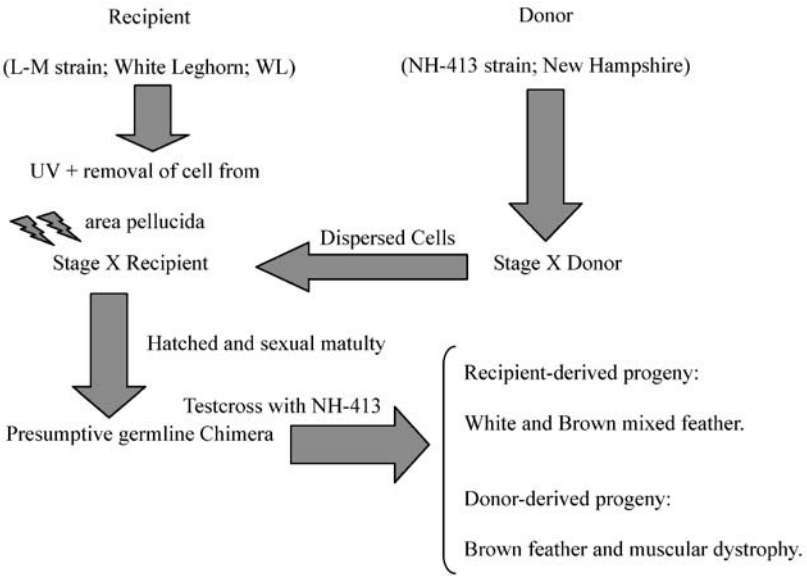

Fig. 3. **Strategy of present studies.** For the production of chimeras, donor and recipient embryos were obtained from NH-413 strain and L-M strain (WL), respectively. A sharp needle was pushed through the yolk mass to reach the subgerminal cavity of the blastoderm to remove the cells from the center of the area pellucida. Donor cells were microinjected into the subgerminal cavity of the recipient embryos. The manipulated embryos were cultured *ex vivo*. An additional 18 days of incubation was conducted until they hatched.

Table 1. **The number of embryonic mortality and survival to hatching following transfer of blastodermal cells from stage X NH-413 strain embryos into L-M strain at the same stage of development**

Number of treated embryos NH-413 to L-M	The number of mortality at days		Number of hatched chicks
	<4	<20	
15	5	9	1
15	4	10	1
30	8	20	2
Total: 60	17	39	4

Table 2. **The number of donor- and recipient-derived offspring obtained by crossing somatic chimeras with donor NH-413 strain***

Chimera ID (sex)	Percentage of donor-derived feather	Total number of offspring	Number of offspring		Percentage of donor-derived offspring
			donor-derived	recipient-derived	
0601 (male)	5	0	0	0	0
0602 (male)	20	48	42	6	87.5
0603 (female)	70	44	18	26	40.9
**0604 (male)	—	—	—	—	—

* Total 104 fertilized eggs were used.
** A chimera numbered as 0604 died before sexual maturity.

Fig. 4. **A generated chimera by transplantation of donor embryonic cells.** Brown feather derived from the donor cells were observed in the feather of the chimeric chickens.

Fig. 5. **Two types of progenies derived from the germline chimera.**
A: Type-I: Phenotype and symptom were very similar to the NH-413 strain.
B: Type-II: White and Brown feather were mixed. Phenotype and symptom were not aliked to that of the NH-413 strain.

ID# 0602 and 40.9% (18/44) in chimera ID# 0603, respectively (Table 2).

When the donor blastoderm of NH-413 strain was incorporated into the recipient embryo of L-M strain, somatic chimerism was observed by existence of the brown feather pigmentations from the NH-413 strain (Fig. 4). When a female somatic chimera (ID# 0603) was at 2 weeks of age, the proportion of the donor-derived feather was about 5%. The proportion of the donor derived feather increased about to 15% to 30%, at 1 and 3 months, respectively. The chimera showed frequent abnormalities in behavior as in the muscular dystrophy. In the other 2 chimeras, the proportion of the donor-derived feather has been decreased as they got older.

Two types of offspring were generated by back cross using these chimeras. The offspring was classified as the following two types. Type-I is offspring with very similar phenotype and the symptom to the NH-413 strain (Fig. 5 A). Type-II is offspring with mixed feather of brown (donor) and white (recipient) that is very similar to that of crossbreed between NH-413 strain and White Leghorn (Fig. 5B).

The fertility of usual NH-413 strain was 38.2% as indicated in Table 3. Only 18.3% could reach to sexual maturity in the conventional case. The fertility greatly improved to 90.2% in the chimeras. Also, 87.2% of the hatched chimeras could reach to sexual maturity.

Discussion

Germline chimeric chickens could be produced by using blastodermal cells from stage X embryos injected into the subgerminal cavity of recipient embryos. Also, chimeras could be produced by using PGC obtained from the germinal crescent region (Vick *et al.,* 1993) from embryonic blood (Naito *et al.,* 1998) or from embryonic gonads (Chang *et al.,* 1997), and transferred directly into bloodstream of embryos. Chicken chimeras between different strains based on plumage color marker (Petitte, 1990; Thoraval *et al.,* 1994) and interspecies chimeras (Ono *et al.,* 1996) have been produced by transferring blastodermal cells.

In the present study, we could successfully generate germline chimeras between the NH-413 and L-M strain as recipient. The localization and extent of donor-derived brown feathers seemed to be random. The generated chimera showed an abnormal symptom, suggested that the insertion of the muscular dystrophy gene. It was thought that the reproductive cell from L-M and NH-413 strain was committed into the chimeric gonads. Although, the proportion of the donor-derived brown feather in a chime-

Table 3. **Comparison of fertility and hatchability among strains L-M, NH-413 and generated chimeras***

	Fertility (%)	Hatchability (%)	Survival rate (%)
L-M strain	92.5±5.7[a]	90.5± 6.3[a]	90.0± 6.9[a]
NH-413 strain	38.2±7.5[b]	28.6±23.7[b]	18.3±13.5[b]
Generated chimera	90.2±6.3[a]	89.8± 5.4[a]	87.2± 5.7[a]

* Value represent the mean±SE in chickens.
[a,b]: Value without common superscripts are significantly different (p-test; a: $p>0.01$, b: $p<0.01$).

ra ID# 0602 (20%) was lower than in a chimera ID# 0603 (70%), the proportion of donor cell transmission to their offspring was higher in a chimera ID# 0602 (87.5%) than in a chimera ID# 0603 (40.9%). It was suggested that the chimeric proportion in feather and germline transmission were unrelated (Table 2).

Two types of offspring could be obtained by the test mating. It seemed that Type-I had an on set of muscular dystrophy since their phenotypes and the symptoms were very similar to that of the NH-413 strain. The low fertility, hatchability and survival rate of the conventional NH-413 strain were greatly improved by generating the germline chimeras (Table 3). Thus, the offspring from donor strain could be produced very efficiently in the established novel system. This is the first scientific report that the chickens with muscular dystrophy could be effectively regenerated by embryo engineering.

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