Expression of mRNA for 3HADH in manipulated embryos to produce germline chimeric chickens

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Abstract Germline chimeric chickens were produced by the transfer of primordial germ cells (PGCs) or blastoderm cells. The hatchability of eggs produced by transfer of exogenous PGCs is usually low. The purpose of the present study was investigated to express (3-hydroxyacyl CoA dehydrogenase) 3HADH which is a limiting enzyme in the beta-oxidation of fatty acids for hatching energy. Manipulations of both donor and recipient eggshells were as follows. A window approximately 10 mm in diameter was opened at the pointed end of the eggs at stage 12–15 days incubation. Donor PGCs, taken from the blood vessels of donor embryos from fertilized eggs at the same stage of development, were injected into the blood vessels of recipient embryos. The muscles of chicks in the eggs with transferred PGCs were removed after 20 days of incubation. A cDNA was prepared from the total RNA. The expression of 3HADH in the manipulated embryos was investigated using real-time PCR analysis. Real-time PCR analysis showed that expression of 3HADH was reduced in the muscles of manipulated embryos.

Keywords Manipulated embryo · PGCs · 3HADH · Hatch

Introduction

Avian primordial germ cells (PGCs) are an important resource for producing transgenic offspring (Fujihara 1999). PGCs originate from the epiblast and circulate in the vascular system the developing embryo (Eyal-Giladi et al. 1981; Fujimoto et al. 1976; Kuwana 1993). The cells eventually differentiate into spermatogonia or oogonia. It has been reported that exogenous genes could be introduced into the PGCs to produce a transgenic chicken (Naito et al. 1998; Inada et al. 1997; Eguma et al. 1999; Furuta et al. 2000; Furuta and Fujihara 2000, van de Lavoie et al. 2006). The transfer of PGCs from donor to recipient embryos is a commonly used technique to establish germline chimeric chickens (Naito et al. 1994; Ono et al. 1996; Yamaguchi et al. 2000; Furuta et al. 1999, 2001, 2007, 2008). The chickens produced by the transfer of PGCs were shown to be chimeric by using a progeny test (Naito et al. 1994; Furuta et al. 2001). The production of germlines of chimeric chicken is difficult because of the low hatchability of eggs with manipulated embryos. Windowed eggs prepared for the injection of exogenous PGCs showed an increase in abnormally developed embryos. However, when the eggs were completely filled with albumin or saline, the percentage of abnormal embryos was reduced (Fisher and Schoenwolf 1983).

It has been reported that lipid, as energy substrate, is mainly available for embryonic development at 12–18 days of incubation (Sato et al. 2006). Although

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glucose is important for development of the embryos, fatty acids are the main energy source for hatching (Ohta et al. 2007). The growth of chicks after hatching is influenced by the nutrients in the yolk remaining in the peritoneal cavity. There was no significant relationship between the egg's component weight and the egg weight prior to incubation. There was a significant correlation between moisture and fat before hatching (Nir et al. 1990). It is suggested that water in the eggs is produced by fat metabolism when hatching humidity is low.

In this study, expression of the 3-hydroxyacyl CoA dehydrogenase (3HADH) gene related to fatty acid oxidation as an energy source for hatching was examined in manipulated embryos that had low hatchability.

Materials and methods

Donor PGCs

Fertilised eggs were obtained from White Leghorn hens. The eggs were incubated at stage 12–15 (Hamburger and Hamilton 1951) and were then cracked to obtain PGCs. At this stage the PGCs appear in blood of embryos.

Recipient embryos

Fertilised eggs obtained from the same strain of White Leghorn hens were used as recipient embryos. The eggs were incubated for the same length of time as the donor eggs.

Transfer of PGCs

A aliquots of 7–10 µl of blood containing PGCs were collected from the blood vessels of donor embryos using fine glass pipettes preaspirated with a drop of 199 medium supplemented with 10% foetal calf serum (FCS). A window approximately 10 mm in diameter was opened in the pointed end of the recipient eggs. Blood was aspirated from the blood vessels of the recipient embryos. The donor blood containing PGCs was injected into the same vessel. The small hole in the vessel in the recipient embryos was covered with a drop of 199 medium with FCS and the eggshell windows in recipient eggs were

closed using Scotch Tape (3 M Co., Tokyo, Japan). Incubation of these eggs was continued.

Control

Fertilised eggs obtained from the same strain of White Leghorn hens were used. The untreated eggs served as a control and were incubated for 20 days.

Measurement of expression of 3HADH

Total RNA was extracted from the neck, breast and thigh muscles of hatching embryos at 20 days incubation. The RNA was extracted from a total 22 embryos (windowed 5, manipulated 8 and control 9) using Trizol Reagent (Invitrogen, CA, USA). The RNA samples were subjected to electrophoresis in 1% agar gel containing 2.2 M of formaldehyde. Ribosomal RNAs were verified using ethidium bromide staining of the gel. Poly A⁺ RNA was prepared from the total RNA using Oligotex-dT 30 Super (Takara Co., Shiga, Japan). cDNA was prepared from the Poly A⁺ RNA using Superscript (Invitrogen). The cDNA in 1 µl served as template in 25 µl PCR reaction buffer containing 1× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.2 µM of each primers that has been reported for 3HADH by Real Time PCR (Abe et al. 2006).

Statistical analysis

An ANOVA was used to determine the overall statistical significance of effects due to treatment. The results are presented as means ± SEM.

Results and discussion

The number of embryos surviving at 20 days incubation was 8 out of 29 manipulated (27.6%) and 10 out of 30 of windowed eggs (33.3%) though 92.3% of the control eggs had surviving embryos, as shown in Table 1. The number of embryos surviving in the control was significantly greater ($p < 0.01$) than in manipulated and windowed eggs.

In the neck muscle, expression of 3HADH in embryos from manipulated embryo and from windowed eggs was 0.25 ± 0.09 and 0.49 ± 0.13 , respectively (Fig. 1a). This was approximately 65.6 and

Table 1 Number of surviving embryos after 20 days incubation

	Control	Manipulated embryos	Windowed eggs
Number of surviving embryos (%)	12/13 (92.3) ^a	8/29 (27.6) ^b	10/30 (33.3) ^b

Different superscripts show significant difference ($p < 0.01$)

33.0% of the control level, but no significant difference ($p > 0.05$) was observed between control eggs and windowed eggs. Expression of 3HADH in the breast muscle of embryos from manipulated eggs was 0.27 ± 0.16 and from windowed eggs was 0.09 ± 0.04 (Fig. 1b). This was 67.6 and 88.8% of the baseline level, respectively. The results were significantly lower ($p < 0.05$) than that in the control. In the thigh muscle, expression in manipulated egg embryos was 0.19 ± 0.04 and in windowed egg embryos was 0.36 ± 0.09

(Fig. 1c). This was 86.1 and 61.2% of the baseline level, respectively, but no significant difference ($p > 0.05$) was observed between control eggs and windowed eggs. In the liver, expression in manipulated egg embryos was 0.06 ± 0.03 and in windowed egg embryos was 0.17 ± 0.08 (Fig. 1d).

Chimeric chickens have previously been produced by the transfer of PGCs at the embryonic stage, and donor-derived offspring were obtained (Naito et al. 1994; Furuta et al. 2001).

In general, hatchability of the eggs that were manipulated embryos was reduced. Mortality of manipulated embryos and windowed egg embryos was higher than the control in this study. Fisher and Schoenwolf (1983) showed that a higher number of abnormal embryos develop in manipulated eggs than non-treated ones and the development of abnormal embryos results in low hatchability. However, when the eggs were filled with albumin or saline, abnormal development was reduced.

Fig. 1 The expression of 3HADH gene in embryos at 20 days of incubation Mean $\pm$ SEM. Means with different letters show significant differences ($p < 0.05$). **a** Neck muscle (hatching muscle), **b** Breast muscle, **c** Thigh muscle, and **d** Liver

It has been reported that lipids are main energy source in the later period of embryonic development (Sato et al. 2006) and the energy from glucose metabolism and hexokinase activity was not used for hatching (Ohta et al. 2007). Fatty acids are the main energy source for hatching. The expression of 3HADH as a fatty acid metabolism enzyme reduced hatchability of manipulated and windowed eggs. The 3HADH gene is related to fatty acid oxidation as an energy source for hatching. In the snapping turtle, embryos developing in wet environments hatched at a larger size than those in dry condition. This shows an effect of moisture on embryonic metabolism and growth. Moisture also affected patterns of incorporation of lipids and proteins into developing embryos and thereby affected patterns of growth. When water was decreased in the egg, fatty acid synthesis is reduced in the embryos (Janzen et al. 1990).

There have been no studies on the relationship between 3HADH and hatchability of embryos. In this study, the manipulated embryos showed a reduction of 3HADH which is the enzyme related to fatty acid oxidation as an energy source for hatching. It is considered that one factor of low hatchability of the manipulated embryos is caused by the opening in the egg shell.

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