Transfer of blood containing primordial germ cells between chicken eggs development of embryonic reproductive tract

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Received: 11 May 2007 / Accepted: 19 September 2007 / Published online: 16 October 2007
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Abstract The present study was carried out to investigate development of recipient chicken embryonic reproductive tracts which are transferred chicken primordial germ cells (PGCs). It is thought that differentiation of PGCs is affected by the gonadal somatic cells. When female PGCs are transferred to male embryos, it is possible that they differentiate to W-spermatogonia. However, the relationship development between PGCs and gonads has not been investigated. At stage 12–15 of incubation of fertilized eggs, donor PGCs, which were taken from the blood vessels of donor embryos, were injected into the blood vessels of recipient embryos. The gonads were removed from embryos that died after 16 days of incubation and from newly hatched chickens and organs were examined for morphological and histological features. The survival rate of the treated embryos was 13.6% for homo-sexual transfer of PGCs (male PGCs to male embryo or female PGCs to female embryo) and 28.9% for hetero-sexual transfer PGCs (male PGCs to female embryo or female PGCs to male embryo) when determined at 15 days of incubation. The gonads of embryos arising from homo-sexual transfer appeared to develop normally.

In contrast, embryos derived from hetero-sexual transfer of PGCs had abnormal gonads as assessed by histological observation. These results suggest that hetero-sexual transfer of PGCs may influence gonadal development early-stage embryos.

Keywords Chicken · Embryo · Gonad · PGCs · Transfer

Introduction

Avian primordial germ cells (PGCs) are an important resource for production of transgenic offspring. PGCs have been reported to originate from the epiblast (Eyal-Giladi et al. 1981), and appear in hypoblast of the germinal crescent region. PGCs circulate in the vascular system of the developing embryo and finally migrate into the germinal ridge (Fujimoto et al. 1976; Kuwana 1993), where the cells differentiate into spermatogonia or oogonia. PGC transfer between embryonic blood vessels is a common technique for establishment of germ line chimeric chickens (Naito et al. 1994; Ono et al. 1996; Yamaguchi et al. 2000; Furuta et al. 1999; Furuta and Fujihara 1999; Furuta et al. 2001), and it has been reported that exogenous genes may be introduced into PGCs to produce transgenic chicken (Naito et al. 1998; Inada et al. 1997; Eguma et al. 1999; Furuta et al. 2000; Furuta and Fujihara 2000). For confirmation that transfer of PGCs is technically possible, quail PGCs have been

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observed in chicken gonads using immunohistochemistry (Ono et al. 1998), and the capability of producing chimeric chickens has been investigated by progeny testing (Naito et al. 1994; Furuta et al. 2001).

Female PGCs transferred to male embryos have been demonstrated to differentiate to spermatogonia in male gonads (Tagami et al. 1997). These results would suggest that PGCs might produce W-chromosome spermatogonia.

The progressive proliferation and differentiation from primordial germ cells to oogonia or spermatogonia is regulated by several factors during embryonic development. Pleiotropic cytokines, which are members of the transforming growth factor beta (TGFbeta) family, have key roles in tissue morphogenesis and growth. Potential roles for TGFbeta have been identified in gonad and secondary sex organ development, spermatogenesis, ovarian function, immunoregulation of pregnancy, embryo implantation and placental development. In the chicken embryo gonad, it has also been reported that the TGFbeta2 gene is expressed in the ovary and testis during early embryonic development. The level of TGFbeta2 was observed to be high during the day 7 embryo and gradually decreased until the beginning of day 14 or 17 of the embryonic period (Hattori et al. 2002). Avian estrogen is necessary to introduce feminization in normal females and cytochrome P450 aromatase is an important enzyme to convert testosterone to estrogen. In the male gonad, however, the absence of P450 aromatase results in the accumulation of testosterone (Woods and Erton 1978; Yoshida et al. 1996), which induces masculinization. However, the masculinizing effect of testosterone treatment in ovo is only temporary in the female, probably due to the effect of endogenous ovarian aromatase produced by the ovary. Anti-Mullerian hormone (AMH) is responsible for regression of the Mullerian ducts in males during embryonic development (Vigier et al. 1987). AMH also represses P450 aromatase biosynthesis indicating an indirect masculinizing effect of AMH (Vigier et al. 1989). Inhibition of aromatase activity can result in differentiation of the right and left testes or ovotestis in female chickens (Abinawanto et al. 1996; Burke and Henry 1999; Elbrecht and Smith 1992).

In this study, the development of embryos and sexual characteristics of chickens were determined

following the hetero-sexual transfer of the PGCs. PGCs play a critical role for determining the gonadal development. We examined the gonadal development of day 15 embryos and newly hatched chickens as well as examining the expression of P450 aromatase and AMH in day 7 embryos following hetero-sexual transfer of PGCs.

Materials and methods

Donor PGCs

Fertilized eggs were obtained from white leghorn maria line hens (GHEN Corporation, Gifu Japan). The eggs were incubated for 48–50 h and were then cracked to obtain PGCs. At this time, PGCs in blood vessels of embryos were at stage 12 to 15 (Hamberger and Hamilton 1951).

Recipient embryos

Fertilized eggs obtained from white leghorn laura line hens (GHEN Corporation, Gifu Japan) were employed as recipient embryos. The eggs were incubated until the same stage as the donor eggs.

Transfer of PGCs

The blood containing PGCs was collected from the blood vessels of embryos using fine glass pipettes preaspirated with a drop of 199 medium supplemented with 10% fetal calf serum (FCS). A window of approximately 10 mm in diameter was opened at the sharp end of the egg. About 4–7 μ L of blood was aspirated from the blood vessels of recipient embryos. The donor blood containing PGCs was injected into the same vessel from which the blood was removed in recipients. The small hole on the surface of the recipient embryo's vessel was closed with a drop of 199 medium with FCS. The windows on the recipient eggs were closed with tape and the eggs were allowed to continue to incubate. Statistical difference between homo-sexual and hetero-sexual transfer was tested by Chi-square method.

Histological examination of gonads

The gonads of newly hatched chickens and dead embryos after day 15 were removed, fixed with Bouin's solution and processed for routine histological examination. Paraffin sections were stained with HE to observe histological features under a light microscope.

Expression of AMH and P450 aromatase

Total RNA was extracted from the gonads of day 7 embryos following transfer of PGCs using Trizol Reagent (invitrogen, Carlsbad/Ca, USA). Reverse-transcription was carried out on total RNA using oligo (dT) 12–18 primers. The degree of AMH expression was determined by Real Time PCR. PCR was used to assess whether the P450 aromatase gene was to expressed or not.

Results

The total number of embryos manipulated was 221, including 96 embryos from homo-sexual PGC transfers (male to male or female to female) and 125 embryos from hetero-sexual PGC transfers (male to female or female to male). Survival rate of embryos after 7 days and 15 days incubation having transferred PGCs was 40.0% and 13.6% for male to male and female to female homo-sexual transfer, respectively, and 42.9% and 28.9% for male to female and female to male hetero-sexual transfer, respectively (Table 1, 2, 3). The number of surviving embryos of hetero-sexual

Table 1 Number of survival of recipient embryos after 15 days of incubation, following transfer of PGCs

Sexes of donor and recipient Donor → recipient	Number of embryos manipulated	Number of surviving embryos (%)
Male → male	33	5 (15.2)
Female → female	33	4 (12.1)
<i>Sub-total (homo-sexual)</i>	66	9 (13.6)
Male → female	37	9 (24.3)
Female → male	60	19 (31.7)
<i>Sub-total (hetero-sexual)</i>	97	28 (28.9)*

Note: Significance at * $p < 0.05$ versus ratio of surviving embryos in homo-sexual transfer

Table 2 Abnormity of recipient gonads after transfer of PGCs

Sexes of donor and recipient Donor → recipient	Number of gonads investigated	Morphological and histological abnormity of gonads
Male → male	5	0 (0.0)
Female → female	4	0 (0.0)
<i>Sub-total (homo-sexual)</i>	9	0 (0.0)
Male → female	9	3 (33.3)
Female → male	19	3 (15.8)
<i>Sub-total (hetero-sexual)</i>	28	6 (21.4)

Table 3 Number of survival recipient embryos after 7 days of incubation

Sexes of donor and recipient Donor → recipient	Number of embryos manipulated	Number of surviving embryos (%)
Male → male	11	4 (36.4)
Female → female	19	8 (42.1)
<i>Sub-total (homo-sexual)</i>	30	12 (40.0)
Male → female	13	6 (46.2)
Female → male	15	6 (40.0)
<i>Sub-total (hetero-sexual)</i>	28	12 (42.9)

transfer at 15 days of incubation was highly significantly greater than that of homo-sexual transfer.

It was judged more than normal gonad by morphological and histological examination as described, no abnormal gonads were observed in the embryos after 15 days of incubation following homo-sexual transfers. On the other hand, morphological and histological deformities were observed in the gonads of 3 out of 9 embryos arising from male to female transfer and 3 out of 19 embryos arising from female to male transfer (Table 2). The gonads in embryo arising from hetero-sexual transfer contained many flat sexual cords in the right gonad. Although right gonad of normal female degenerate in early embryo stage, no degeneration was observed when the gonad was transferred male PGCs (Fig. 1).

The level of expression of AMH mRNA in the gonads with and without (control) hetero-sexual transfer was examined by Real Time PCR (Fig. 2a, b). Expression AMH mRNA was much higher in the male than in female gonads, but no significant difference ($p > 0.05$) was observed between control gonads and gonads of embryos arising from

Fig. 1 (a) Female gonad (control). Note: Degeneration of the right gonad (arrow), (b) Histological features of female gonad. (c) Abnormal gonad following hetero-sexual (male to female) transfer of PGCs. Note: No degeneration of the right gonad (arrow) (d) Histological features of right gonad. Bar indicates 50µm. Note: Sexual cord in the tissue (arrows)

Fig. 2 The expression of Anti-Mullerian gene in the gonad following hetero-sexual transfer of PGCs. (a) female to male (b) male to female

hetero-sexual transfer in either sex. The P450 aromatase gene was not expressed in male gonads when PGCs of females had been transferred.

Discussion

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). Chimeric birds have also been produced by PGCs transfer from quail to chicken embryos (Ono et al. 1996; 1998).

PGCs are not thought to be involved in determining sex, and the cells usually differentiate to the germ cells of the sex which has already been determined according to the somatic sex. It has been reported when female PGCs transferred to male embryos was

differentiated to w-spermatogonia in recipient male gonads (Tagami et al. 1997). Abnormal development of female gonad was observed in the present study as shown in Fig. 1 (photo C). However, it is not yet clear whether PGCs of donor embryos involved in the sex determination of recipient embryos. In our present experiment, hetero-sexual transfer of PGCs developed donor-derived gonads. This result suggests the possibility of altering gonadal development of recipient embryos via hetero-sexual transfer of PGCs.

The expression of AMH showed no significant difference ($p > 0.05$) between gonads arising from control and hetero-sexually transferred PGCs. However, the individual embryo which showed the most abnormal degree of expression was 17% (2/12). PGCs migrated and established a germinal ridge, and development of a gonad and sex differentiation started in early stage embryos. P450 aromatase required for estrogen synthesis. AMH is expressed after stage 25, and the many genes of sex determination are expressed 3 days after incubation. The gonads of chicken embryos are “indifferent” or “bipotential” at days 3.5–4.5, being morphologically indistinguishable between the sexes (Smith and Sinclair 2004). PGCs may have affected these development. However, there were no significant changes in the level of AMH and P450 aromatase following hetero-sexual transfer in this experiment. The PGCs were transferred with blood, and both, blood cells and serum, influence development of gonads. The quantitative assessment of PGCs transfer caused gonad abnormality is the subject for future studies.

The results obtained from the present study suggest that hetero-sexual transfer of PGCs may induce development of gonads having the opposite sex to somatic cells. In the present study, using PGCs containing blood vessels of donor embryos with fetal calf serum, the survival of recipient embryos was significantly higher for hetero-sexual transfers than for homo-sexual transfers (Table 1). In addition, morphological and histological abnormalities were also observed in the recipient embryos (Fig. 1). It is considered that some causative agents inducing the above mentioned facts may be latently present in the blood vessels or calf serum. Future investigations will deal with these problems.

Acknowledgements This work was supported in part by the grant-in-aid for Scientific Research 17380176 from the Ministry of Education, Science, Sports and Culture of Japan.

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