REVIEW ARTICLE

Perspectives on avian stem cells for poultry breeding

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ABSTRACT

Stem cells have prulipotency to differentiate into many types of cell lineages. Recent progress of avian biotechnology enabled us to analyze the developmental fate of the stem cells: embryonic stem cells / primordial germ cells (PGCs). The stem cells were identified in the central area of the area pellucida of the stage X blastoderms. These cells could be applied for production of germline chimeras and organ regeneration. Generation of medical substrate in transgenic chickens has considerable interests in pharmaceuticals. Sex alteration of the offspring should be enormously beneficial to the poultry industry. Fertilization of the sex-reversed sperm could lead to sexual alteration of the offspring. These strategies using stem cells / PGCs should be one of the most powerful tools for future poultry breeding.

Key words: chicken, primordial germ cells, poultry breeding, stem cells.

INTRODUCTION

Stem cells should maintain self-renewal ability and pluripotency both *in vivo* and *in vitro*. The embryonic stem cells (ES cells) were isolated from the inner cell mass of mouse blastocysts (Martin & Evans 1975). Human embryonic stem cells were derived from the blastocysts (Thomson *et al.* 1998). It was shown that retrovirus-mediated transfection with four transcription factors, Oct-3/4, Sox 2, KLF 4 and c-Myc, into mouse fibroblasts has generated induced pluripotent stem (iPS) cells (Takahashi & Yamanaka 2006). These advancements could open up new frontiers for regenerative medicine.

Avian stem cells have also been considered as one of the most useful tools for embryo engineering. The stem cell technology could be applied for generation of chimeras, generation of pharmaceuticals in eggs, and genetic conservation of endangered birds. The cells were obtained from the area pellucida of the blastoderms. The obtained stem cell clusters were dissociated to single cells in the appropriate medium. The cells were used for production of chimeric chickens (Fig. 1). The stem cells could also be used for a vector of gene transfer. Furthermore, regeneration of a functional leg was achieved in chimeric chickens. A novel strategy to isolate primordial germ cells (PGCs) has been developed (Yamamoto *et al.* 2007b). Effective generation of germline chimeras has been achieved (Kagami 2002, 2003a; Kagami *et al.* 2006). Sex-reversed gametes from the stem cells were generated in mixed-sex chimeras. Fertilization of the W-sperm could lead to production of female offspring, alone. Stable production of transgenic poultry should be one of the most important subjects. Recent progress in animal genome editing (Hai *et al.* 2014; Hsu *et al.* 2014; Park *et al.* 2015) could lead to genome modification. These strategies could open up frontiers for novel poultry breeding.

Development of stem cells

Clarification of the developmental mechanism of stem cells or PGCs has been one of the most basic subjects in avian reproductive biology. The stem cells were detected in the center of area pellucida of stage x blastoderm by periodic acid Schiff (Eyal-Giladi & Kochav 1976; Ginsburg & Eyal-Giladi 1987), equi merozoite antgen 1 / stage-specific embryonic antigen 1 (EMA-1/SSEA-1) (Karagenc *et al.* 1996; Urven *et al.* 1988) and by chicken homolog to vasa (CVH) protein (Nakamura *et al.* 2007). As the embryonic development proceeds, the stem cells were destined to differentiate as PGCs. The PGCs were detected in the ventral surface of epiblasts and subsequently translocated to hypoblasts (Petitte *et al.* 1997). An

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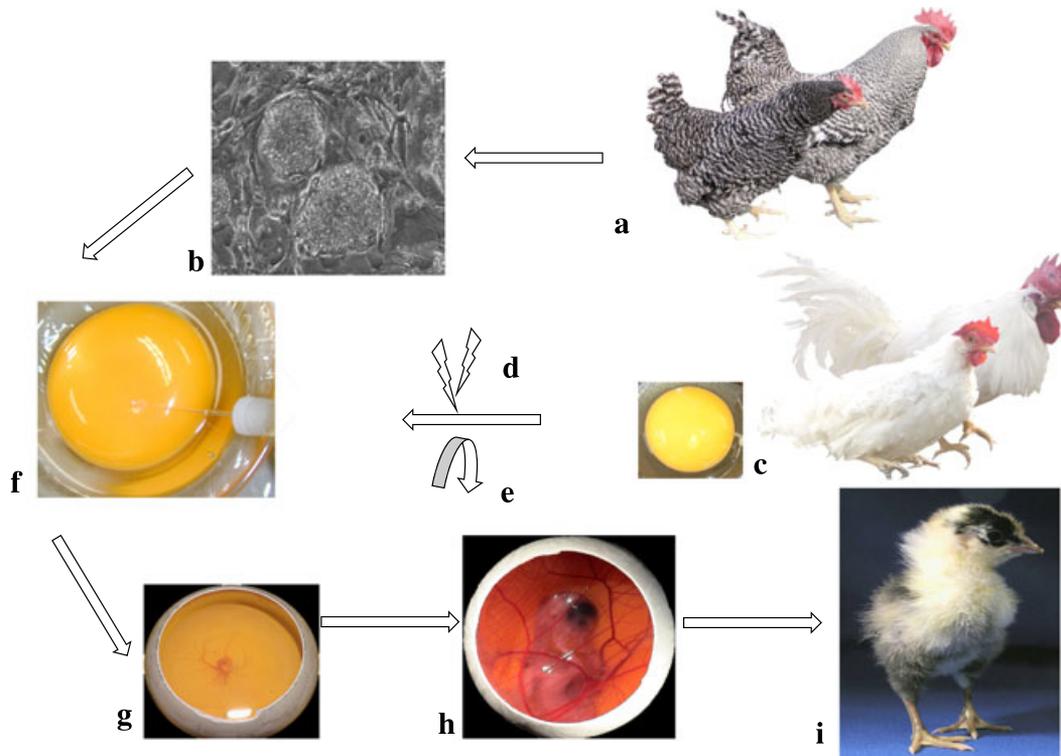
Figure 1 Production of chimeric chicken by stem cells. (a) Donor embryos were obtained from Barred Plymouth Rock. (b) Stem cells were isolated from the center of the area pellucida in the blastoderm. (c) Recipient embryos were obtained from White Leghorn. (d, e) The recipient blastoderm was irradiated (d) and the stem cells were surgically removed (e). (f) Donor stem cells were injected into the recipient. The manipulated embryo was cultured. (g) The embryos proceeded to development. (i) The donor contribution in chimeras could be identified by the feather pigmentation at hatching.

experimental strategy to determine the origin and development of PGCs has been established by embryo engineering. Donor stem cells fluorescently labeled by PKH26 were analyzed in chimeric embryos (Yamamoto *et al.* 2007a). The cells were injected into the center of the area pellucida of the recipient. The dynamic states of the donor cells were tracked during development of the chimeric embryos, thereby allowing cell fate analysis of the area pellucida. The manipulated embryos that were injected with marked donor cells from the center of the area pellucida were cultured. The presence of the marked donor cells was confirmed in many embryos, through observation of embryos at each developmental stage under a fluorescence microscope. The results suggest that the migration system and developmental fate of cells are almost identical to those of PGCs. It was concluded that many PGCs or their precursor cells are localized in the blastodermal center of the area pellucida (Kagami *et al.* 1995).

Elucidation of the stem cell development has also been conducted by molecular analysis. A predominant gene in germline formation was isolated and used to trace the germline development in chickens (Tsunekawa *et al.* 2000). The germline-specific expression of CVH protein was detected throughout all stages of development. Strong gene expression of chicken PouV (cPouV) which is an avian homologue of Oct4, was detected (Lavial *et al.* 2007). It was shown that the cPouV and the chicken Nanog gene are required for the maintenance of pluripotency and self-renewal in chickens. Due to these observations, it was suggested that the mechanisms by which Oct4 and Nanog regulate pluripotency and self-renewal are not exclusive to mammals. Full length chicken dead end homologue (CDH) was isolated with an open reading frame of 329 amino acids (Aramaki *et al.* 2007). RNA-binding motif in the chicken CDH was highly homologous to those in humans, mice and *Xenopus*. The temporal and spatial distribution of PGCs was also investigated by in situ hybridization (ISH) on whole-mount embryos with CDH complementary RNA (cRNA) as a probe. Chicken embryos from stage X to stage 20 were subjected to ISH. The hybridized samples were then sectioned to analyze the translocation of PGCs. CDH-positive cells could be counted from stage X to stage 4, with minimally 30 cells at the blastoderm and approximately 260 cells at the germinal crescent. Thus, specific expression of CDH messenger RNA (mRNA) has been established in chicken PGCs. It was concluded that isolated CDH is specifically expressed in chicken PGCs during embryogenesis.

Stem cell differentiation into germline or somatic tissues

Avian stem cell chimeras should be one of the most promising tools to induce germline development (Kagami *et al.* 1995, 1997; Petitte *et al.* 1990). The manipulated embryos were cultured by an ex vivo system (Naito *et al.* 1990; Perry 1988). The stem cells injected into recipient translocated into the hypoblast (Watanabe *et al.* 1992). Some of the stem cells were destined to differentiate into PGCs. The cells entered the embryonic circulation associated with blood vascular formation. The cell left capillary vessels to the germinal epithelium and migrated to the primitive gonad. In the gonad, the cells differentiated into either spermatozoa or ovum. Generated germline chimeras could be applied for transgenic birds, conservation of endangered birds and sexual manipulations (Brazolot *et al.* 1991; Kagami 2003a,b,

2006; Naito *et al.* 1990; Tagami & Kagami 1998; Tajima *et al.* 1993). The circulating PGCs in embryonic blood were isolated conventionally by ammonium chloride potassium buffer (Yamamoto *et al.* 2007a), and the germline chimeras were generated effectively (Figs 2–5).

To generate the germaline chimeras, sterilization or elimination of recipient's PGCs should be one of the most critical subjects. Irradiation of gamma rays has been evaluated as a useful method. But gamma rays are not used due to the cost and equipments needed. The recipient's PGCs were surgically removed from the blastoderm and the donor stem cells were microinjected into the recipients (Kagami & Hanada 1997; Kagami *et al.* 2000). Elimination of the recipient's PGCs improved the production efficiencies of germline chimeras. Busulfan was used for the enhanced production of chicken germline chimeras (Song *et al.* 2005). The treatments resulted in a
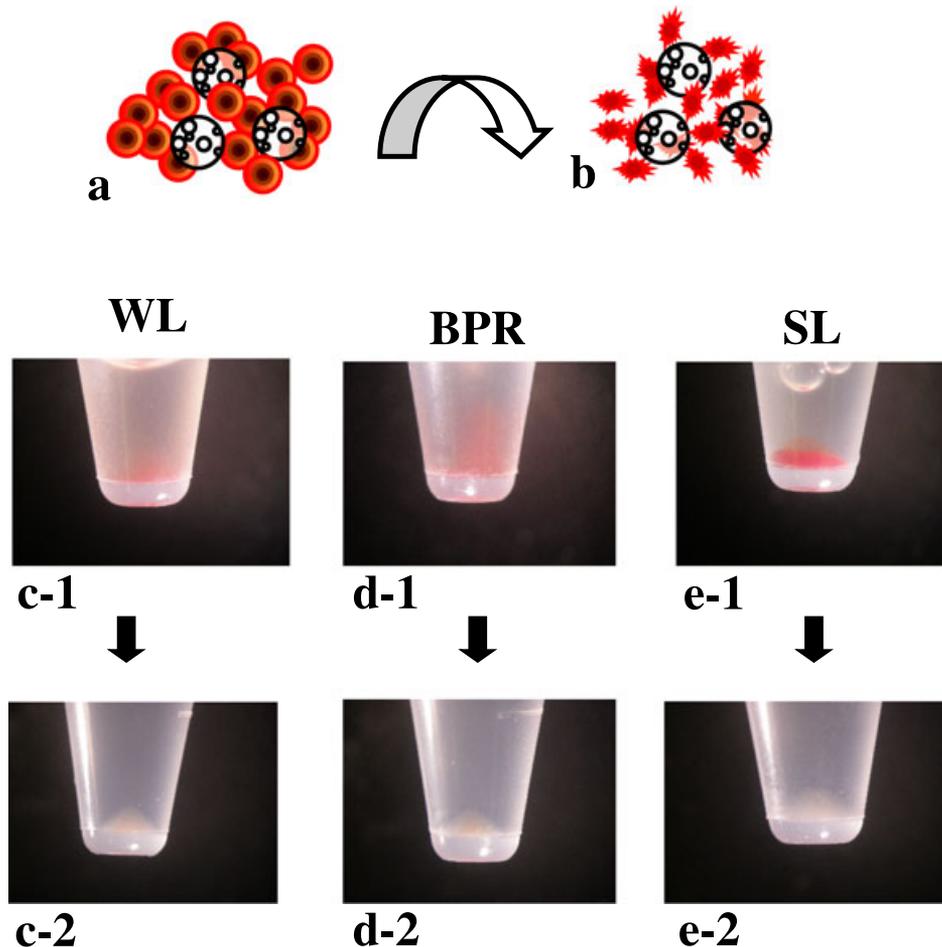

Figure 2 A novel isolation system of the circulating primordial germ cells (PGCs). (a) Circulating blood in the chick embryos contained blood cells and PGCs. (b) Most of the blood cells were destroyed by mixing with ammonium chloride potassium (ACK) buffer and the PGCs alone remained. c-e: The obtained circulating blood samples (c-1: White Leghorn, d-1: Barred Plymouth Rock, e-1: Silky) were mixed with the ACK buffer and the original red color changed to white by destroying the blood cells with ACK (c-2: White Leghorn, d-2: Barred Plymouth Rock, e-2: Silky).
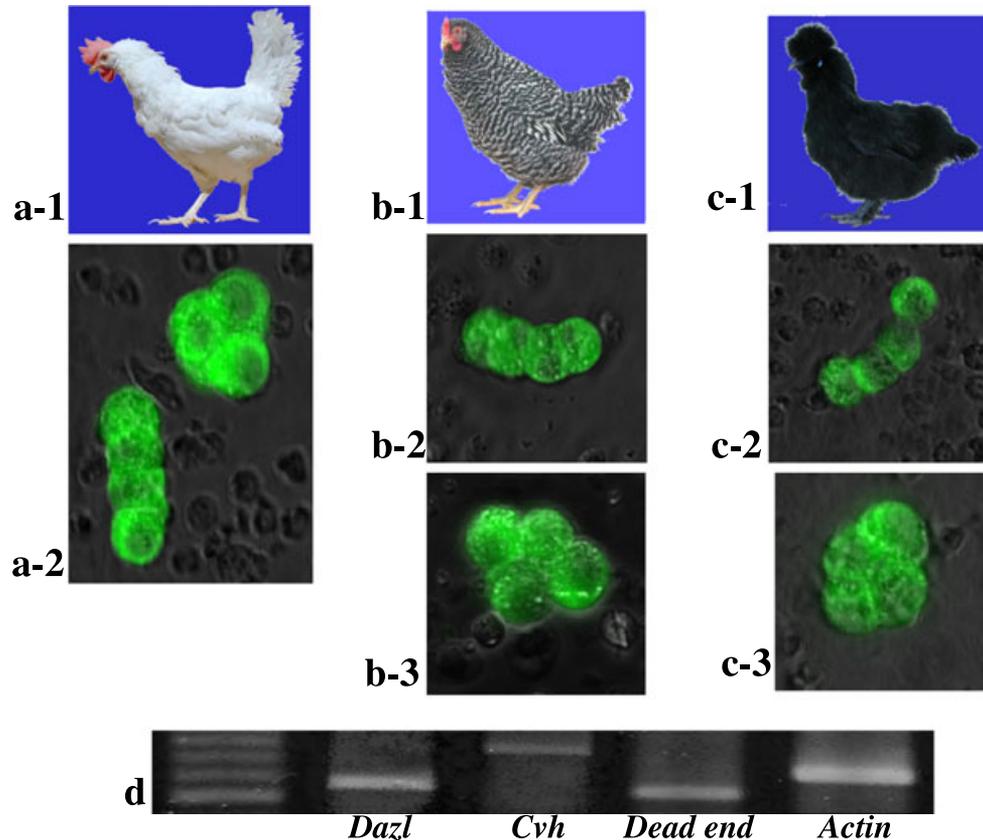
Figure 3 Detection of the isolated primordial germ cells (PGCs) by immunological staining and the gene expression. a–c; Blood samples were obtained embryos of White Leghorn (a-1), Barred Plymouth Rock (b-1) and Silky (c-1). After these blood samples were treated by ammonium chloride potassium (ACK) buffer, immuno-histochemical analysis with chicken homolog to vasa (CVH) antibody was performed. The PGCs could be detected by obvious staining with the antibody in White Leghorn (a-2), Barred Plymouth Rock (b-2, 3) and Silky (c-2, 3). (d) Strong gene expression of *Dazl*, *Cvh* and *Dead end* was detected in the isolated PGCs.

significant reduction in PGCs when compared to controls. It was suggested that the administration of a busulfan into the recipient eggs improved the depletion of endogenous PGCs and increased the efficiency of germline chimera production (Nakamura *et al.* 2010a,b).

Chimeras have been generated between Barred Plymouth Rock (black feather) and White Leghorn (white feather). Somatic differentiation of the stem cells was identified by the presence of black feathers. Therefore, feather color was found to be one of the most useful markers to judge somatic differentiation. Using a chicken female-specific DNA probe, the XhoI family (Tone *et al.* 1982), whole mount in situ hybridization to the chimeric erythrocytes was performed. Differentiation of stem cells to erythrocyte was identified. Regeneration of the functional organ was challenged by the stem cells. When the stem cells from Silky as donor was injected into the recipient blastoderm of White Leghorn, a functional leg was regenerated in chimeras (Figs 6 and 7). Inter-specific chimeras were generated between chicken and mice.

When the mice ES cells as donors were injected into recipient chicken blastoderms, chimeras could be generated. These inter-specific chimeras should be useful for mammalian small organ regeneration in chick embryos (Fig. 8).

Sex determination and reversal

Avian sex is determined by the pairs of sex-chromosomes, ZZ as male/ ZW as female. Development of the gonad has provided insights into the molecular genetics of vertebrate sex determination (Smith & Sinclair 2004). The pathways were found to be comprised of both conserved and divergent factors. During embryonic development, estrogens play an important role in gonadal sex differentiation. Some of the critical genes for mammalian sex determination are also expressed in the chicken, but their expression patterns were different. Although the molecular regulation for sex determination in birds remains unknown, some genes have recently emerged. Expression of DMRT1 gene, which is linked
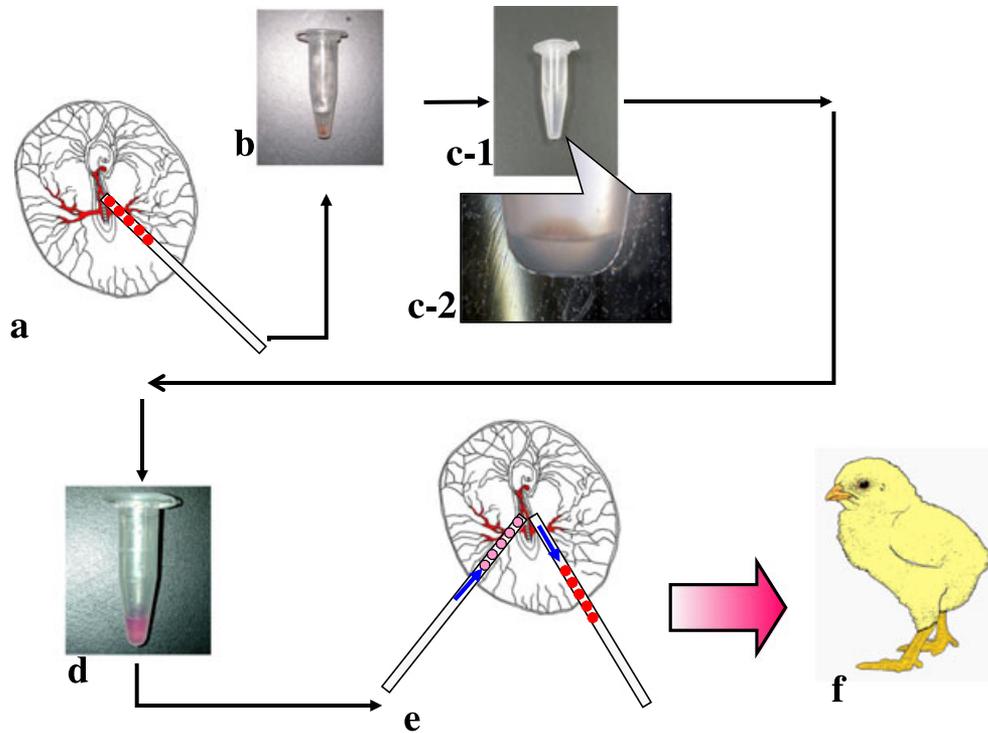
Figure 4 Production of chimeric chicken by primordial germ cells (PGCs) isolated by ammonium chloride potassium (ACK) buffer. (a) Circulating blood isolated from embryo. (b) The blood sample was transferred into a tube. (c) The sample was mixed to destroy the blood cells (c-1 and the higher magnification, c-2). (d) The remaining PGCs were resuspended in a medium. (e) Some of the endogenous blood was removed and the isolated PGCs were injected. (f) Germline chimeras were produced.
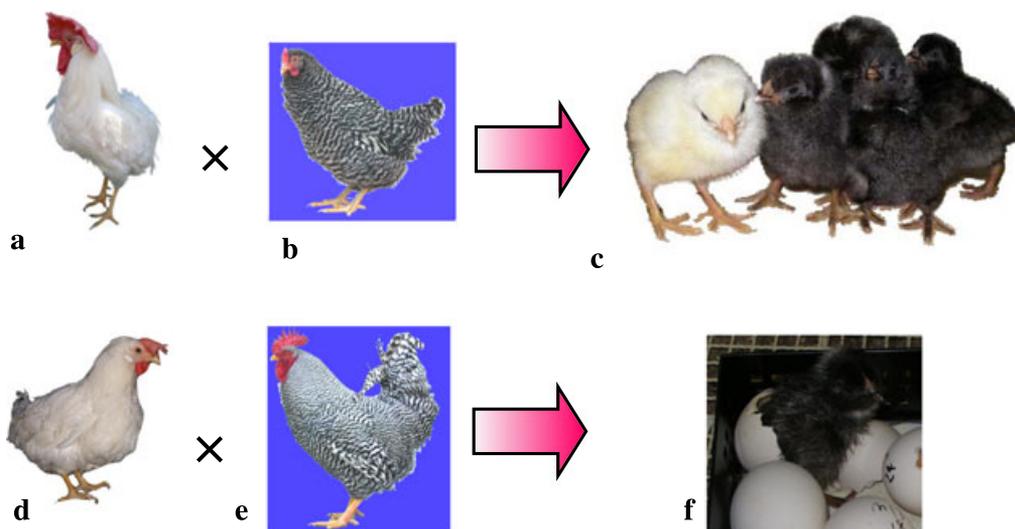

Figure 5 Generation of donor-derived offspring by the germline chimeras. By using primordial germ cells (PGCs) from Barred Plymouth Rock (black feather) as donor and recipient embryos from White Leghorn (white feather), a germline chimera was generated. (a-c) Male germline chimera (a) was test-mated with a female of the donor strain (b) and the donor-derived offspring (black chicks) was produced. (d-f) Female germline chimera (d) was test-mated with a male of the donor strain (e) and the donor-derived offspring (black chicks) was produced.

into the Z-chromosome, suggested a Z-dosage model of avian sex determination. Gene expression of HINTW, which is linked into the W-chromosome, suggested as a candidate female determinants. As above, avian sex determination involves genes acting in the gonads, but the exact nature of the sex
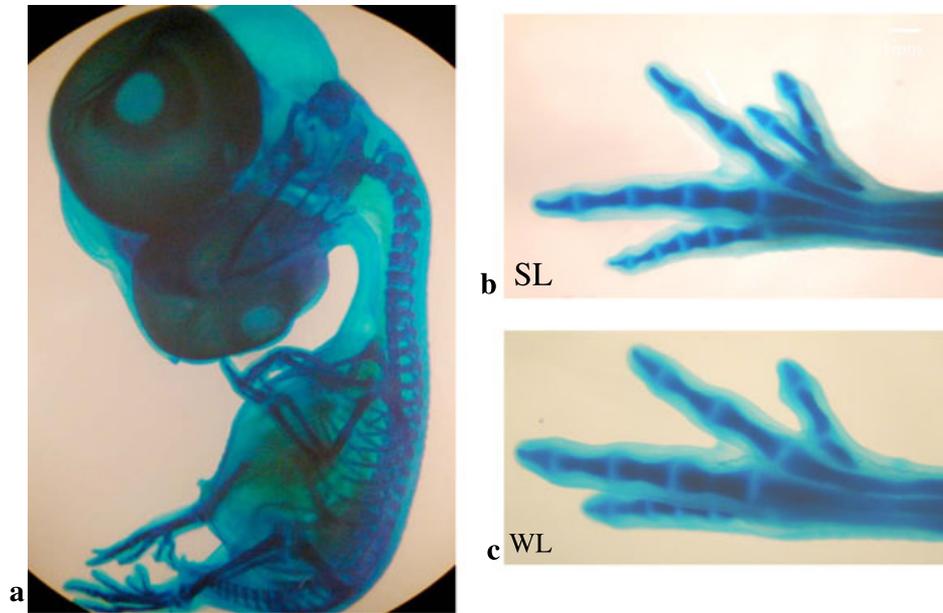
Figure 6 Comparison of the bone structure between Silky (SL) and White Leghorn (WL). (a) Bone structure of a WL embryo. (b, c) Five fingers in SL (b) and four fingers in WL (c) were observed in the legs as their strain-specific characteristics.
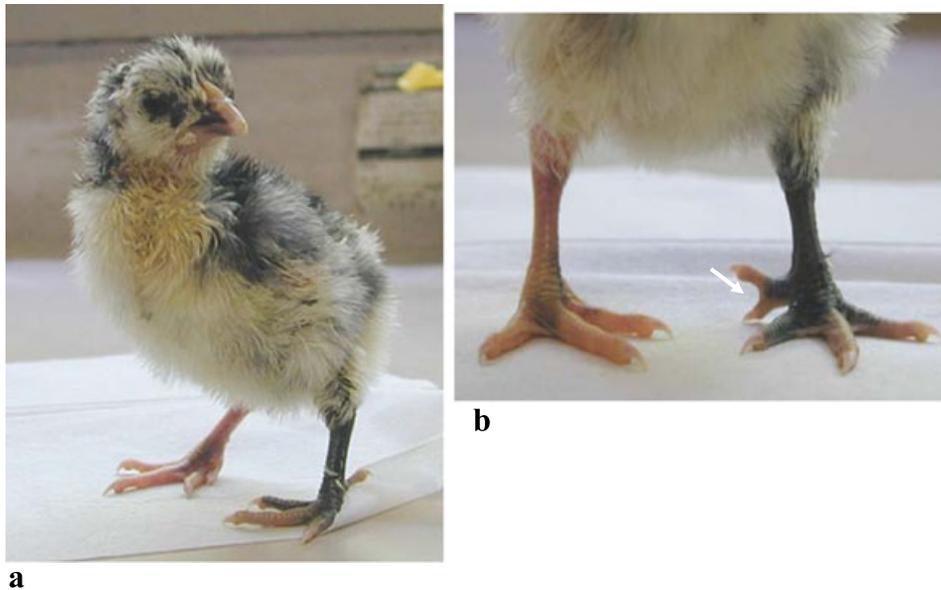

Figure 7 Regeneration of functional leg in chimeras. (a) Chimeras were generated by donor stem cells of Silky (SL) and White Leghorn (WL) embryos as recipients. (b) Functional leg of SL (black leg in right with five fingers (arrow)) was regenerated in the chimera.

determinant(s) and its expression systems remain uncertain (Smith *et al.* 2007).

Induction of sex reversal should be beneficial for better understanding of avian sex determination. Phenotypic sex reversals were induced by several methods. Surgical ovariectomy was conducted (Reyss-Brion & Scheib 1980). Many of these treated bird showed phenotypic sex reversal with retarded testicular gonads. Masculinization by ovariectomy was enhanced by pretreating the female embryos with tamoxifen. Testis from the male embryos were implanted to female embryos (Stoll *et al.* 1982). Joint use of testis grafting and ovariectomy improved the masculinization. Transient sex reversal was induced by embryonic estradiol treatment (Van Krey 1990). Embryonic treatment of females
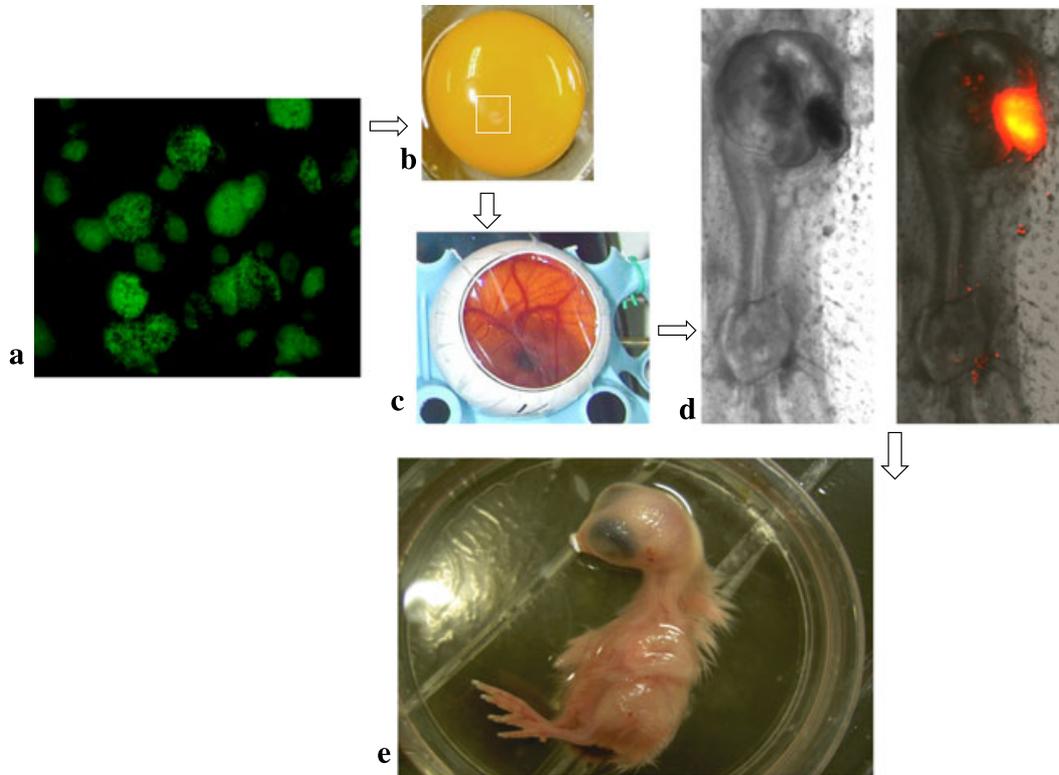
Figure 8 Production of inter-specific chimeras between mice and chicken. (a) Mice embryonic stem (ES) cells expressing green fluorescence protein was used as donor. (b) Chicken blastoderm was subjected as a recipient. (c) Chimeric embryo was cultured. (d) Mice ES cells were committed to the embryo and differentiation in chimeras was observed. (e) The inter-specific chimeras were further developed.

by aromatase inhibitor resulted in sex reversal with testicular gonads (Elbrecht & Smith 1992). When the sex-reversed hens reached sexual maturity, they were subjected to mating with normal roosters. However, any offspring by the mating could not be obtained.

Generation of sex-reversed gametes

Using the mixed-sex chimeras, Kagami *et al.* (1995) first suggested possible generation of W-bearing spermatozoa from female stem cells. Female stem cells as donors were injected into male recipients. These chimeras showed male phenotypes with functional testes. Generation of the W-bearing sperm in mixed-sex chimeras were confirmed by Simkiss *et al.* (1996) by PCR. The W-spermatozoa was confirmed by ISH (Tagami *et al.* 1997, 2007). Strain preference was confirmed in donor and recipient for the production efficiency of the W-bearing sperm in mixed-sex germline chimeras (Kagami *et al.* 2002a,b). It is suggested that the combination of a White Leghorn donor and a Barred Plymouth Rock recipient produced W-bearing sperm more efficiently than the reverse combination.

Sexual differentiation in the female was also reversed to the male by injection of an aromatase inhibitor into the embryo. Intracytoplasmic sperm ingection (ICSI) of W-sperm into quail oocyte revealed that a single spermatid and sperm directly collected from the testes is capable of fertilizing an oocyte (Hrabia *et al.* 2003). Using the ICSI, single sperm of a sex-reversed hen was injected into mouse oocyte to evaluate the fertilizability of the sperm (Takagi *et al.* 2007). Pronuclei derived from the sperm were observed.

Generation of offspring with sex-reversed gametes

Chicken male and female stem cells in the ovary and testis was evaluated by injecting the stem cells into recipients to form same-sex and mixed-sex chimeras (Kagami & Hanada 1997). Male stem cells were incorporated into a female chimera. The ZZ 'oogonia' were included within the ovarian follicles and the chromosome complement of genetically male oogonia was processed normally during meiosis. Following ovulation, the male-derived ova were fertilized and produced normal offspring. In

the testes, ZW spermatogonia enter meiosis I and produce functional ZZ spermatocytes. The production of Z-derived offspring from ZW spermatogonia indicates that female primordial germ cells can become functional Z-bearing sperm in the testes. Based on knowledge of gamete's sex-reversal, a novel strategy for the production of clone-like chickens has been challenged. Stem cells from one blastoderm were injected into several recipients. Male and female chimeras were generated by the donor cells derived from the one blastoderm. If the donor-derived offspring could be generated by mating with the pair of male and female chimeras, the genome component of the offspring should be similar to that of the original one blastderm, a clone-like chicken (Figs 9 and 10). Germline chimeras were also produced by transfer of blastodermal cells into recipient embryos of the opposite sex (Naito *et al.* 2001). The donor cells could also give rise to functional gametes in an opposite-sex recipient, although the frequency was low. In fish, testicular germ cells can colonize sexually undifferentiated embryonic gonads and produce functional eggs (Okutsu *et al.* 2006). Spermatogonia isolated from the adult testes contained a cell population that could differentiate into functional eggs. The spermatogonia, probably spermatogonial stem cells, seemed to be sexually bipotent and sexual differentiation of germ cells is controlled solely by the somatic microenvironment, rather than being cell autonomous in fish. Artificial sex reversal of the gametes and the application for sexual alteration of offspring is greatly beneficial for poultry production (Etches & Kagami 1997).
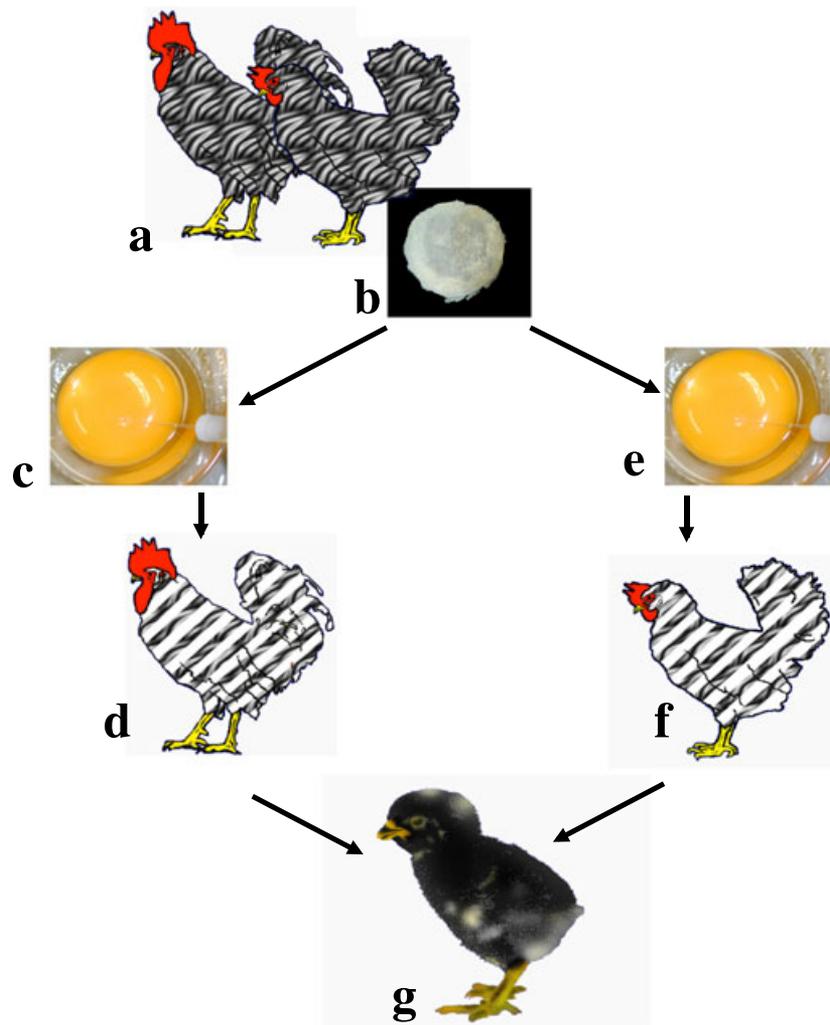

Figure 9 A challenge for the production of clone-like chickens. (a-b) Donor stem cells were obtained from one blastoderm (b) of the Barred Plymouth Rock (a). (c-f) The donor stem cells were injected into a male (c) or a female recipient (e) of Whilte Leghorn to generated a male (d) or a female (f) chimeras. (g) If the black chicks were generated by mating the male and female chimeras, the genome component should be very similar to that of the original donor blastoderm clone-like chicken.
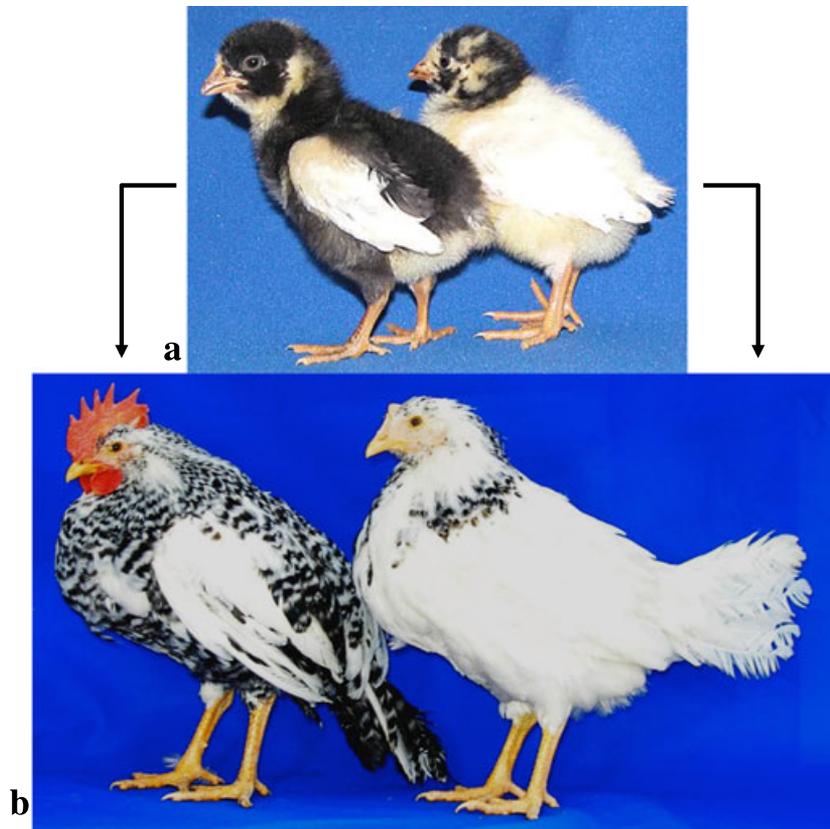
Figure 10 Chimeras generated by donor stem cells derived from one embryo. (a) A pair of male (left) and female (right) chimeric chicks generated by donor stem cells from one embryo. (b) The male and female chimeras reached sexual maturity. These male and female chimeras should be mated to test if clone-like offspring could be generated.

CONCLUSION

Advancement of stem cell engineering and molecular biology could contribute to avian biology and the poultry industry. Poultry regeneration using embryonic and somatic stem cells (Usui *et al.* 2009), poultry cloning and generation of transgenic poultry (Leighton *et al.* 2008; van de Lavoir *et al.* 2006; Zhu *et al.* 2005) should be one of the most important subjects (Park *et al.* 2014). Improvement of the cell culture should also be critical (Miyahara *et al.* 2014). Scientific knowledge (Nakamura *et al.* 2007) and technological refinement (Barrangou *et al.* 2015; Yamamoto *et al.* 2007a) should open up new frontiers for poultry breeding.

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