Chicken stem cell factor enhances primordial germ cell proliferation cooperatively with fibroblast growth factor 2

Daichi MIYAHARA1), Isao OISHI2), Ryuichi MAKINO1), Nozomi KURUMISAWA1), Ryuma NAKAYA1), Tamao ONO1), Hiroshi KAGAMI1, 3) and Takahiro TAGAMI4)

1) Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan
2) Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Osaka 563-8577, Japan
3) Institute for Biomedical Sciences (IBS), Interdisciplinary Cluster for Cutting Edge Research (ICCER), Shinshu University, Nagano 399-4598, Japan
4) Animal Breeding and Reproduction Research Division, National Agriculture and Food Research Organization Institute of Livestock and Grassland Science (NILGS), Ibaraki 305-0901, Japan

Abstract. An in vitro culture system of chicken primordial germ cells (PGCs) has been recently developed, but the growth factor involved in the proliferation of PGCs is largely unknown. In the present study, we investigated the growth effects of chicken stem cell factor (chSCF) on the in vitro proliferation of chicken PGCs. We established two feeder cell lines (buffalo rat liver cells; BRL cells) that stably express the putative secreted form of chSCF (chSCF1-BRL) and membrane bound form of chSCF (chSCF2-BRL). Cultured PGC lines were incubated on chSCF1 or chSCF2-BRL feeder cells with fibroblast growth factor 2 (FGF2), and growth effects of each chSCF isoform were investigated. The in vitro proliferation rate of the PGCs cultured on chSCF2-BRL at 20 days of culture was more than threefold higher than those cultured on chSCF1-BRL cells and more than fivefold higher than those cultured on normal BRL cells. Thus, use of chSCF2-BRL feeder layer was effective for in vitro proliferation of chicken PGCs. However, the acceleration of PGC proliferation on chSCF2-BRL was not observed without FGF2, suggesting that chSCF2 would act as a proliferation co-factor of FGF2. We transferred the PGCs cultured on chSCF2-BRL cells to recipient embryos, generated germline chimeric chickens and assessed the germline competency without FGF2, confirming by several studies [3–6]. However, the growth factors involved in vitro proliferation of chicken PGCs other than FGF2 are largely unknown. Thus, identification of another growth factor that support the in vitro proliferation of chicken PGCs would contribute to the optimization of culture conditions.

In mice, c-KIT, a receptor of SCF, is expressed on the surface of PGCs. SCF is produced by gonadal somatic cells, and the interaction between c-KIT and SCF is required for germ cell proliferation, anti-apoptosis and migration [7–12]. Based on these data, SCF has been used as the supporting factor for the in vitro proliferation and survival of murine PGCs [7–10]. In chickens, SCF has also been used for the culture of PGCs in various studies [1–5, 13, 14], but several studies insist that SCF has no apparent function in PGC proliferation. However, those studies used mouse or human recombinant SCF proteins, and mammalian SCF may not support the proliferation of chicken PGCs in vitro, because the amino acid identity between mammalian SCF and chicken SCF (chSCF) is less than 60% [15, 16]. Previous reports suggest that administration of chSCF might have a positive impact on in vitro proliferation of chicken PGCs [17, 18]. Thus, we elucidated whether chSCF is required for in vitro culture of chicken PGCs.

Mammalian SCF has two isoforms: a secreted form (longer form, SCF1) and a membrane-bound form (shorter form, SCF2) [19–23].
Fertilized eggs and animal care

Establishment of feeder cells stably expressing chSCF

RNA extraction

Establishment of feeder cells stably expressing chSCF

RT-PCR

Culture and maintenance of PGCs

Materials and Methods

Fertilized eggs and animal care

Fertilized eggs were obtained from White Leghorn (WL) and Barred Plymouth Rock (BPR) chickens, and were provided by the National Agriculture and Food Research Organization Institute of Livestock and Grassland Science (NILGS). All experiments in this study were performed in accordance with the Committee for the Care and Use of Experimental Animals at the NILGS.

RNA extraction

Total RNA was extracted from day 19 embryonic ovaries or cultured cells using an RNaseasy Plus Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Bio, Otsu, Shiga, Japan). The synthesized cDNAs were used for the subsequent assays.

In vitro growth assay

Culture and maintenance of PGCs

Circulating PGCs derived from HH14 embryonic blood or cultured PGCs were adhered onto a MAS-GP Type A coated glass slide
Matsunami Glass, Osaka, Japan) and fixed with 4% paraformaldehyde for 5 min at room temperature (RT). After several washes, cells were blocked with PBS containing 5% normal goat serum or Image-iT signal enhancer (Life Technologies) and incubated overnight at 4ºC with primary antibodies. Then, cells were incubated for 30 min or 1 h at RT with secondary antibodies. Subsequently, cells were counterstained with 1 μg/ml Hoechst 33342 (Dojindo, Kumamoto, Japan). Fluorescent images were captured using an Eclipse E1000 fluorescence upright microscope (Nikon, Tokyo, Japan), and these images were processed using Photoshop Elements (Adobe Systems, San Jose, CA, USA) for trimming and overlaying. Sources and dilution of used antibodies were as follows: rat anti-chicken vasa homolog (CVH) raised in our laboratory (1:10000) [27], mouse anti-stage specific embryonic antigen-1 (SSEA-1; 1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse anti-stage specific embryonic antigen-1 (SSEA-1; 1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse anti-stage specific embryonic antigen-1 (SSEA-1; 1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse anti-stage specific embryonic antigen-1 (SSEA-1; 1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), goat anti-rat IgG conjugated with Alexa Fluor 488 (1:1000, Life Technologies) and goat anti-mouse IgG or IgM conjugated with Alexa Fluor 594 (1:1000, Life Technologies).

Production of germline chimeric chickens and progeny test
PGC lines derived from WL chickens were cultured on chSCF2-BRL cells to produce germline chimeric chickens. PGCs (1.0 × 10^3 cells) were injected into the dorsal aorta of HH14-16 BPR embryos. Manipulated embryos were incubated until hatching, and the chicks were grown until sexual maturity. Male putative germline chimeric chickens were crossed with female BPR chickens (I/I) by artificial insemination to test the donor WL chicken (I/I)-derived spermatogenesis. White offspring (I/i) indicated the progeny of cultured PGC (WL)-derived chicks, whereas black offspring (i/i) indicated the progeny of recipient (BPR)-derived chicks.

Statistical analysis
Statistical differences in the proliferation rate of PGCs were calculated by the Tukey-Kramer method or Welch’s t-test. Data were regarded as significant at P < 0.05.

Results
Establishment and characterization of chSCF-expressing feeder cells
To analyze the possible function of SCF in chicken PGCs, we attempted to isolate chicken orthologs of SCFs. Among the four chSCF alternative splice variants, chSCF1 and chSCF2 are putative orthologs of the mammalian SCF secreted form and membrane-bound form, respectively (Fig. 1). The cDNAs encoding chSCF1 and chSCF2 were first isolated from a day 19 embryonic ovary as 864 bp and 762 bp genes. The chSCF1 or chSCF2 expression vectors were then transfected into BRL cells (Fig. 2A), and these cells were selected...
with G-418. After selection, we examined chSCF1 and chSCF2 expression in chSCF1-BRL cells and chSCF2-BRL cells by RT-PCR. Amplification products derived from chSCF1 (604 bp) and chSCF2 (502 bp) were detected in chSCF1-BRL cells and chSCF2-BRL cells, respectively (Fig. 2B). By contrast, chSCF1 and chSCF2 were not expressed in normal BRL cells.

c-KIT expression in circulating and cultured PGCs
To examine the expression of the chSCF receptor c-KIT in chicken PGCs, we performed immunocytochemistry on circulating PGCs derived from embryonic blood at HH14 and cultured PGCs. Endogenous c-KIT proteins could be detected in the PGC-like large spheres with CVH, a pan-germ cell marker, but not in CVH-negative blood cells (Fig. 3A). Furthermore, cultured PGCs also co-expressed CVH and c-KIT (Fig. 3B). CVH protein was localized to the cytoplasm, and c-KIT protein was localized to the cytomembrane and cytoplasm.

Growth effects of chSCF isoforms
To elucidate the growth effects of chSCF1 and chSCF2 on PGCs, WL-derived PGC lines (obtained from male embryos) were seeded onto chSCF1-BRL or chSCF2-BRL feeder layers using a PGC culture medium containing FGF2 (5 ng/ml). In vitro growth assays were started from 1.0 × 10^4 cells/ml of PGCs, and the cells were cultured for 20 days. Cultured PGCs were maintained as floating or weakly adhesive cells under every culture condition. The cultured PGCs were shaped like large spheres and had large nuclei and many lipids in their cytoplasm, and their morphological characteristics were not different from those of normal chicken PGCs (Fig. 4A) [5]. In addition, various diameters of cultured PGCs were observed under every culture conditions (6–20 μm). Cultured PGCs co-expressed CVH and undifferentiated cell marker SSEA-1 under each condition (Fig. 4B). CVH protein was localized in the cytoplasm, and SSEA-1 was localized on the cytomembrane, respectively. Thus, the CVH and SSEA-1 expression indicated that the cultured PGCs were...
maintained as germ cells in undifferentiated state. PGCs showed significant proliferation on the chSCF2-BRL feeder layer compared with the normal BRL and chSCF1-BRL feeder layers (P < 0.05). The cell numbers at 20 days after culture start were 1.81 ± 0.83 × 10^5 cells/ml (control), 3.38 ± 0.75 × 10^5 cells/ml (chSCF1-BRL) and 1.06 ± 0.35 × 10^6 cells/ml (chSCF2-BRL) (Fig. 5A). These data indicate that use of chSCF2-BRL cells can induce a more than fivefold increase in proliferation of PGCs compared with conventional culture conditions. To investigate whether chSCF1 and chSCF2 are sufficient for PGC proliferation, we performed a proliferation assay using a culture medium without FGF2. The results showed that PGCs did not sufficiently proliferate on feeder layers compared with the proliferation observed under culture conditions with FGF2 (Fig. 5B).

Germline transmission of cultured PGCs on chSCF2-BRL cells

To further assess whether the PGCs that hyperproliferated on chSCF2-BRL cells could differentiate into functional gametes, we produced germline chimeric chickens following transplantation of these cells and performed progeny tests. We analyzed male chimeric chickens, because cultured chicken PGCs could not complete normal gametogenesis in the gonads of the opposite sex [3, 28, 29]. Three male chimeric chickens were produced, and two chimeras were grown to sexual maturity. Two donor-derived white progenies (I/i) were generated from one germline chimeric chicken (ID 121), and the frequency of germline transmission was 3.39% (Fig. 6, Table 1). Thus, these data demonstrated that expanded PGCs following enhanced proliferation with chSCF2-BRL cells could differentiate into functional spermatozoa.

| ID  | Eggs set | No. of hatched chicks | No. of recipient PGC-derived chicks (I/i) | No. of cultured PGC-derived chicks (I/i) | % of cultured PGC-derived chicks |
|-----|----------|-----------------------|-------------------------------------------|------------------------------------------|---------------------------------|
| 103 | 32       | 29                    | 29                                        | 0                                        | 0                               |
| 121 | 65       | 32                    | 30                                        | 2                                        | 6.25                            |
| Total | 97       | 61                    | 59                                        | 2                                        | 3.39                            |
Discussion

In the present study, we first demonstrated that chSCF2 positively regulates PGC proliferation in vitro and that the hyperproliferated PGCs retained germline competency. We also clarified that the proliferative effect of chSCF2 requires FGF2, indicating that these two cytokines functionally interact and enhance chicken PGC proliferation.

SCF is an essential cytokine for the proliferation, survival, and migration of mice PGCs, and the SCF signal is also required for in vitro culture [7–12]. In mice, SCF1 has a limited effect on survival of PGCs, and thus SCF1 does not enhance the in vitro proliferation of PGCs [7]. Meanwhile, use of SCF2-expressing feeder cells is effective for long-term proliferation of mouse PGCs compared with use of medium supplemented with SCF1 [10]. In chickens, several studies reported that chSCF1 was an important factor for proliferation or differentiation in various cells including embryonic stem cells, normal erythroid progenitor cells and osteoblasts [30–32]. However, how SCF functions in chicken PGCs has been unknown. This study revealed that c-KIT, a receptor of chSCF, was expressed in proliferative PGCs in vivo, suggesting that chSCF functions as a growth factor that enables expansion of endogenous PGCs in chickens. Our data showed that chSCF2-BRL feeder cells improved the in vitro proliferation rate of chicken PGCs by fivefold compared with normal BRL feeder cells. Meanwhile, our data also demonstrated that chSCF1-BRL feeder cells did not sufficiently support the in vitro proliferation of chicken PGCs, and this result was consistent with the previous studies [17, 33]. Thus, chSCF2 was considered one of the essential factor for the in vitro proliferation of chicken PGCs.

On the other hand, another group showed that SCF2 sustained the migration of mice PGCs, and the SCF signal is also required for proliferative effect of chSCF2 requires FGF2, indicating that these two factors are required for PGC proliferation and that the hyperproliferated PGCs retain germline competency. chSCF2 has a high amino acid identity among various birds such as the quail (98%, Coturnix japonica, AAC59934), Japanese ibis (95%, Nipponia nippon, XP_009462259), and duck (94%, Anas platyrhynchos, XP_012960092). Thus, this PGC culture system combined with chSCF2 and FGF2 would be useful for the in vitro culture of PGCs derived from these birds. Furthermore, the present culture system would be extremely valuable for the cell based systems for cryopreservation of avian genetic resources and the generation of transgenic chickens.

Acknowledgments

This study was supported by a Research Fellowship for Young Scientists from the Japan Society for the Promotion Science (JSPS) to DM (14J10343). This work was also supported in part by the Industrial Technology Research Grant Program from of NEDO, Japan (to IO), JSPS KAKENHI Grant Number 25450481 (to IO) and Takeda Science Foundation (to IO). The authors thank the staff of the Poultry Management Section of the NILGS for taking care of the birds and providing the fertilized eggs. We thank Takafumi Mori for technical assistance in establishing the cultured PGC lines. We also thank Yoshiaki Nakamura, Kumiko Takeda, Eiji Kobayashi and Keihiro Nirasawa for kind support and discussion.

References

1. van de Lavoix MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ. Germline transmission of genetically modified primordial germ cells. Nature 2006; 441: 766–769. [Medline] [CrossRef]
2. Choi JW, Kim S, Kim TM, Kim YM, Seo HW, Park TS, Jeong JW, Song G, Han JY. Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells. PLoS ONE 2010; 5: e12968. [Medline] [CrossRef]
3. Macdonald J, Glover JD, Taylor L, Sang HM, McGrew MJ. Characterisation and germline transmission of cultured avian primordial germ cells. PLoS ONE 2009; 5: e15518. [Medline] [CrossRef]
4. Song Y, Duraisamy S, Ali J, Kühkkäytä J, Jacob VD, Mohammed MA, Eltigani MA, Anmeretty S, Shukla MK, Etches RJ, de Lavoix MC. Characteristics of long-term cultures of avian primordial germ cells and gonocytes. Biol Reprod 2014; 90: 15. [Medline] [CrossRef]
5. Miyahara D, Mori T, Makano R, Nakamura Y, Oishi T, Ono T, Nirasawa K, Tagami T, Kagami H. Culture conditions for mainain propagation, long-term survival and germline transmission of chicken primordial germ cell-like cells. Jpn Poult Sci 2014; 51: 87–95. [CrossRef]
6. Naito M, Harumi T, Kawanuma T. Long-term culture of chicken primordial germ cells isolated from embryonic blood and production of germline chimaeric chickens. Anim Reprod Sci 2015; 153: 50–61. [Medline] [CrossRef]
7. Dolci S, Williams DE, Ernst MK, Resnick JL, Brannan CL, Lock LF, Lyman SD, Boswell HS, Donovan PJ. Requirement for mast cell growth factor for primordial germ cell survival in culture. Nature 1991; 352: 809–811. [Medline] [CrossRef]
8. De Felici M, Dolci S, Pesce M. Cellular and molecular aspects of mouse primordial germ cell migration and proliferation in culture. Int J Dev Biol 1992; 36: 205–213. [Medline] [CrossRef]
9. Matsui Y, Tokuoe D, Nishikawa S, Nishikawa S, Williams D, Zebo K, Hogan BL. Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. Nature 1991; 353: 750–752. [Medline] [CrossRef]
10. Matsui Y, Zebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 1992; 70: 841–847. [Medline] [CrossRef]
11. Runyan C, Schaible K, Molyneaux K, Wang Z, Levin L, Wylie C. Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. Development 2006; 133: 4861–4869. [Medline] [CrossRef]
12. Gu Y, Runyan C, Shoemaker A, Surani A, Wylie C. Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantios, and provides a continuous niche throughout their migration. Development 2009; 136: 1295–1303. [Medline] [CrossRef]
GROWTH EFFECTS OF chSCF ON CHICKEN PGCs

13. Naito M, Harumi T, Kawana T. Expression of GFP gene in cultured PGCs isolated from embryonic blood and incorporation into gonads of recipient embryos. *Jpn Poult Sci* 2012; 49: 116–123. [CrossRef]

14. Tonou C, Cloquette K, Ectors F, Piret J, Gillet L, Antoine N, Desmecht D, Vanderplaschen A, Waroux O, Grobet L. Long term cultured and cryopreserved primordial germ cells from various chicken breeds retain high proliferative potential and gonadal colonisation competency. *Reprod Fertil Dev* 2014; DOI 10.1071/RD14194 [CrossRef]. [Medline]

15. Zhou JH, Obraki M, Sakurai M. Sequence of a cDNA encoding chicken stem cell factor. *Gene* 1993; 127: 269–270. [Medline] [CrossRef]

16. Petitte JN, Kulik MJ. Cloning and characterization of cDNAs encoding two forms of avian stem cell factor. *Biochim Biophys Acta* 1996; 1307: 149–151. [Medline] [CrossRef]

17. Karagenç L, Petitte JN. Soluble factors and the emergence of chick primordial germ cells in vitro. *Poult Sci* 2000; 79: 80–85. [Medline] [CrossRef]

18. Glover MJ, McGrew MJ. Primordial germ cell technologies for avian germplasm cryopreservation and investigating germ cell development. *Jpn Poult Sci* 2012; 49: 155–162. [CrossRef]

19. Zseo KM, Wyppych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA, Mendiaza EA, Langley KE. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver—conditioned medium. *Cell* 1990; 63: 195–201. [Medline] [CrossRef]

20. Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, March CJ, Boswell HS, Gimpel SD, Cosman D, Williams DE. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* 1990; 63: 235–243. [Medline] [CrossRef]

21. Flanagan JG, Chan DC, Leder P. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. *Cell* 1991; 64: 1025–1035. [Medline] [CrossRef]

22. Tsokas D, Zseo KM, Smith KA, Hu S, Brankow D, Sagg SV, Martin FH, Williams DA. Support of hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *Proc Natl Acad Sci USA* 1992; 89: 7350–7354. [Medline] [CrossRef]

23. McNiece IK, Briddell RA. Stem cell factor. *J Leukoc Biol* 1995; 58: 14–22. [Medline]

24. Wang Y, Li J, Ying Wang C, Yan Kwok AH, Leung FC. Epidermal growth factor (EGF) receptor ligands in the chicken ovary: 1. Evidence for hepattin-binding EGF-like growth factor (HB-EGF) as a potential oxytocin-derived signal to control granulosa cell proliferation and IGF-1 and IGF-2 expression. *Endocrinology* 2007; 148: 3426–3440. [Medline] [CrossRef]

25. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol* 1951; 88: 49–92. [Medline] [CrossRef]

26. Clinton M, Haines L, Bellior B, McBride D. Sexing chick embryos: a rapid and simple protocol. *Br Poult Sci* 2001; 42: 134–138. [Medline] [CrossRef]

27. Nakamura Y, Yamamoto Y, Usui F, Mushika T, Ono T, Saito AR, Takeda K, Nirasawa K, Kagami H, Tagami T. Migration and proliferation of primordial germ cells in the early chicken embryo. *Poult Sci* 2007; 86: 2182–2193. [Medline] [CrossRef]

28. Tagami T, Matsubara Y, Hanada H, Naito M. Differentiation of female chicken primordial germ cells into spermatogonia in male gonads. *Dev Growth Differ* 1997; 39: 267–271. [Medline] [CrossRef]

29. Tagami T, Kagami H, Matsubara Y, Harumi T, Naito M, Takeda K, Hanada H, Nirasawa K. Differentiation of female primordial germ cells in the male testes of chicken (Gallus gallus domesticus). *Mol Reprod Dev* 2007; 74: 68–75. [Medline] [CrossRef]

30. Pain B, Clark ME, Sheu M, Nakazawa H, Sakurai M, Samarut J, Etches RD. Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. *Development* 1996; 122: 2339–2348. [Medline]

31. Hayman MJ, Meyer S, Martin F, Steinlein P, Benc H. Self-renewal and differentiation of normal avian erythroid progenitor cells: regulatory roles of the TGF alpha/c-ErbB and SFc/c-kit receptors. *Cell* 1993; 74: 157–169. [Medline] [CrossRef]

32. van’t Hof RA, von Lindem M, Nijweide PJ, Benc H. Stem cell factor stimulates chicken osteoclast activity in vitro. *FASEB J* 1997; 11: 287–293. [Medline]

33. Yang G, Fajiwara N. Survival and proliferation of refined circulating primordial germ cells cultured in vitro. *J Reprod Dev* 1999; 45: 177–181. [CrossRef]

34. Miyazawa K, Williams DA, Gotah A, Nishimaki J, Broxmeyer HE, Toyama K. Membrane-bound Steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. *Blood* 1995; 85: 641–649. [Medline] [CrossRef]