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Research Article
Activation of Migratory Ability in Male Mouse Primordial Germ Cells by *in vitro* Organ Culture

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Abstract

Background and Objective: Primordial Germ Cells (PGCs) migrate dynamically during the embryonic stage to the Genital Ridge (GR), the precursor of the immature gonad. After settling in the GR, PGCs differentiate to form germ cells and generally lose the ability to actively migrate. However, in the male mouse germline, germ cells can actively migrate in the testis after birth. The aim of the present study was to investigate PGCs that had completed their migration to the GR using an *in vitro* protocol to reproduce germ cell migration. **Materials and Methods:** Migration of mouse PGCs was investigated using *in vitro* organ culture in combination with serum supplementation of the culture medium. **Results:** By culture of male GR *in vitro* in supplemented mouse serum, PGCs were induced to migrate from the organ. But, PGCs did not migrate in Fetal Bovine Serum (FBS). Also, PGC migration was shown to induce in medium containing FBS supplemented with recombinant mouse Stromal Derived Factor-1 (SDF-1) which is known as a migration signal for PGCs. **Conclusion:** The results obtained here demonstrate that male PGCs in the GR potentially maintain their migratory ability and that SDF-1 supplementation induces their migration. A new method for analysis of germ cell migration has therefore been developed.

Key words: Primordial germ cell, migration, genital ridge, CXCR4, SDF-1

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Primordial Germ Cells (PGCs) are the progenitors of the germ cell lineage. The PGCs are established independently from somatic cell lineages early in embryogenesis and undergo sex-dependent differentiation and maturation. The PGCs form clusters in a specialized layer of epithelium in the urinary tract termed the urothelium and begin to express germline-specific genes while suppressing somatic cell transformation[1]. The PGCs are then released from the cluster and burrow into the hindgut epithelium to migrate toward the Genital Ridge (GR). During this period, PGCs show high levels of expression of transcription factors and carbohydrate antigens that play important roles in pluripotent cells, such as Oct4 and SSEA-1[2]. This characteristic suggested that the germline retains pluripotency and that this may serve as the basis for the reacquisition of pluripotency after the fertilization of gametes. Following migration to the hindgut epithelium, PGCs undergo cell cycle arrest and produce pseudopods to perform amoeba-like movements. They then pass through the dorsal mesentery and migrate to the position of the forming GR, cell cycle arrest is released and cell numbers increase[3]. At this time, germ-line cells express *Mouse vasa homologue* (Mvh), which is up-regulated in a germ cell-specific manner and enables them to be distinguished from somatic cells. *Vasa* is a maternal determinant 1st identified in the drosophila germline and has been shown to have a germ-cell specific expression pattern in most animal species[4]. It has been suggested that expression of Mvh is up-regulated in mouse germ cells by external signalling molecules[5]. Expression of Mvh can be detected in most PGCs by day 11.5 of embryogenesis (E11.5) as its expression is germ cell-specific, it can be used as a germ cell marker. At E12.5, when the migration of PGCs to the GR has finished, PGCs lose their migratory ability and differentiate into male or female germ cells[6].

The mechanism by which PGCs migrate to the GR is highly conserved among organisms and many of the molecules involved have been identified by cross-species comparisons[7]. The chemokine signalling SDF-1/CXCR4 axis is a pathway of interest concerning PGC migration, this axis has been studied as a regulatory mechanism for migration of leukocytes and cancer cells and found to involve a concentration gradient of SDF-1[8,9]. Mice with gene Knock-Out (KO) of Sdf-1 or CXCR4 have reduced numbers of PGCs, which is consistent with the presumed importance of this signal for migration of PGCs[10]. Furthermore, in the male germline, CXCR4 is expressed in spermatogonia and its transport to the stem cell niche region is regulated by SDF-1 signaling[11].

Although PGC migration has been studied by isolating individual PGCs and validating them with chemotaxis assay devices such as the transwell assay[12], there are little data on the post-migration PGCs. In addition, changes to the migration ability of PGCs during their differentiation into spermatogonia are not known. In this study, an organ culture method was used to demonstrate a mechanism for re-inducing germ cell migration in PGCs that had settled in the forming genital ridge.

MATERIALS AND METHODS

Study area: This study was conducted at Iwate University in Japan from April, 2017 to August, 2021.

Animals: E12.5 mouse embryos were recovered from female ICR mice (Charles River Laboratories Japan, Inc.). All mice were housed under a 12 hrs (7:00-19:00) light-dark cycle at 22-24°C in a barrier cage. The experimental protocols were approved by the animal ethics committee of Iwate University (No. A201626).

Isolation of E12.5 male GR: To isolate the male GR, the embryo was dissected from the uterus and extra embryonic tissues were removed. The gut tube and liver were removed from the body cavity. The GR is attached to the dorsal wall of the body cavity and adheres to the developing mesonephros. Male-specific testicular cords were identified and only male GRs were isolated. The GR and the mesonephros were gently teased apart with a needle. The GR was then washed with cold PBS and used for organ culture.

Preparation of mouse serum (MS): Samples of total blood were obtained from the hearts of 20 mice (8+weeks old) of both sexes. The mice were euthanized by cervical dislocation, a needle was inserted into the heart of each mouse and all the blood was collected. The collected blood was incubated at 4°C overnight. Then, it was centrifuged at 1500 rpm for 30 min at 4°C and the supernatant was stored in a fresh tube. The serum was immobilized for 30 min at 56°C and sterilized through a 0.25 µm syringe filter just before use.

Culture of mouse GR: The collected GRs were placed on poly-L-lysine coated glass adhesion slides (Matsunami Glass, Cat. No. C1110) in 4-well plates (Thermo Fisher Scientific, Cat No. 176740). The GRs were cultured in DMEM (Sigma, Cat. No. D4064) with 10% FBS (Gibco, Cat. No. 10270106) or 10% Mouse Serum (MS) and penicillin/streptomycin (Wako, Cat. No.

Table 1: Primer sequences designed and employed to amplify *Cxcr4* and *Gapdh* genes

Gene names	Primer sequences	Accession numbers
Cxcr4 (forward)	TCCTCCTGACTATACCTGACTTCATCT	NM_00911
Cxcr4 (reverse)	CCTGTCATCCCCCTGACTGAT	
Gapdh (forward)	AACTTTGGCATTGTGGAAGG	NM_001289726.1
Gapdh (reverse)	GGATGCAGGGATGATGTTCT	

168-23191), in a CO_2 incubator (37°C, 5% CO_2 in air). To analyze the effect of SDF-1 on germ cell migration, GRs were incubated in 10% FBS-DMEM with 100 μg mL^{-1} recombinant mouse SDF-1 (Wako, Cat. No. 196-12661). The concentration of SDF-1 was determined based on the report by Molyneaux *et al.*[10]. The GRs were cultured for 4 days and screened under a fluorescence microscope (OLYMPUS, BX-53), followed by immunostaining. Based on the report by Farini *et al.*[12], the germ cells that showed characteristics of motile cells were counted as migratory germ cells (n = 8). The growth areas of GR-derived cells were measured by ImageJ (Ver. 1.52 q) image analysis software (n = 10).

Immunofluorescence analysis: Cultured GRs were fixed in 4% paraformaldehyde for 30 min at room temperature and washed 3×5 min with cold PBS. Samples were then transferred to cold methanol for 10 min and washed 3×5 min with PBS. The samples were immersed in Blocking one (Nacalai Tesque, Cat. No. 03953-95) for 1 hr to block the non-specific binding of the antibodies. Subsequently, GRs were incubated at 4°C overnight with a rabbit anti-MVH polyclonal antibody (Abcam, Cat. No. ab13840) diluted 1:1000 and a rat anti-CXCR4 monoclonal antibody (R and D, Cat. No. MAB21651-100) diluted 1:50 in blocking one. After washing 3 times with PBS, GRs were incubated with donkey anti-rabbit IgG Alexa 594 (Abcam, Cat. No. ab150064) and DAPI solution (Dojindo, Cat. No. D523) for 1 hr at 37°C in the dark. After 3 washes with PBS, GRs were mounted on glass microscope slides in 90% glycerol/PBS and analyzed under an olympus fluorescence microscope.

qRT-PCR analysis: Total RNA was extracted from individual GRs using an RNeasy plus mini kit (Qiagen, Cat. No. 74134). A 500 ng aliquot of RNA was then reverse transcribed to cDNA using random primers and reverse transcriptase (Promega, Cat. No. A3500) according to the manufacturer's specifications. cDNA was amplified with TB Green Premix Ex Taq (Takara, Cat. No. RR420A) according to the manufacturer's protocol. Primer sequences are listed in Table 1. Amplification was conducted using a thermal cycler dice real-time system (Takara Bio, TP900). qRT-PCR data is shown as relative gene expression in cultures with FBS (n = 6), MS (n = 6), or E12.5 GR (n = 6) using the 2e-ΔCT calculation. To eliminate variations in the concentration of RNA used for the RT reaction, all the PCR data were normalized against an average of the housekeeping gene *Gapdh*.

Western blot analysis: Serum protein concentrations were measured using a qubit protein assay kit (Thermo Fisher Scientific, Cat. No. Q33211). Equal amounts of different proteins were then loaded onto a 10-20% gradient gel (Wako, Cat. No. 191-15031) and separated by SDS-PAGE. After electrophoresis, the gel was transferred to a PVDF membrane using the iBlot Dry Blotting System (Thermo Fisher Scientific, Cat. No. IB401002). After blocking with Blocking One for 1 hr, the membrane was incubated with an anti-SDF-1 antibody (protein tech, Cat. No. 17402-1-AP) diluted 1:500 in Blocking One at 4°C overnight, a secondary goat anti-rabbit IgG Alexa 488 (Invitrogen, Cat. No. A32790) diluted 1:1000 in Blocking one was then used for 1 hr at room temperature. Unbound antibodies in each step were removed by three washes with PBS-T. The membrane was analyzed using a fluorescence image analyzer (Biorad, Pharos FX).

Statistical analysis: The growth area of GR-derived cells, the numbers of MVH positive migration PGCs and the gene expression level between treated and control groups were analyzed with F-test followed by the student's t-test. A $p<0.01$ denoted a statistically significant difference.

RESULTS

Effect of FBS and MS on male GR culture: Isolated GRs were cultured with FBS or MS to determine if migration of male germ cells could be induced. GRs cultured with FBS showed adherence to and spreading along with the culture plate after 2 days of culture. By contrast, although GRs cultured in medium supplemented with MS showed similar growth and expansion as those cultured in FBS, they showed poorer growth of fibroblasts in (Fig. 1a). There was no significant difference in the tissue expansion area after 4 days between cultures using FBS or MS in (Fig. 1b). A gene expression analysis showed that *Cxcr4*, which is related to migration, had a significantly higher level of expression in GRs in culture than in E12.5 male GRs in Fig. 2.

Comparison of germ cell migration in cultures with FBS or MS: Cell proliferation and expansion did not differ significantly between cultures supplemented with FBS or MS. In the

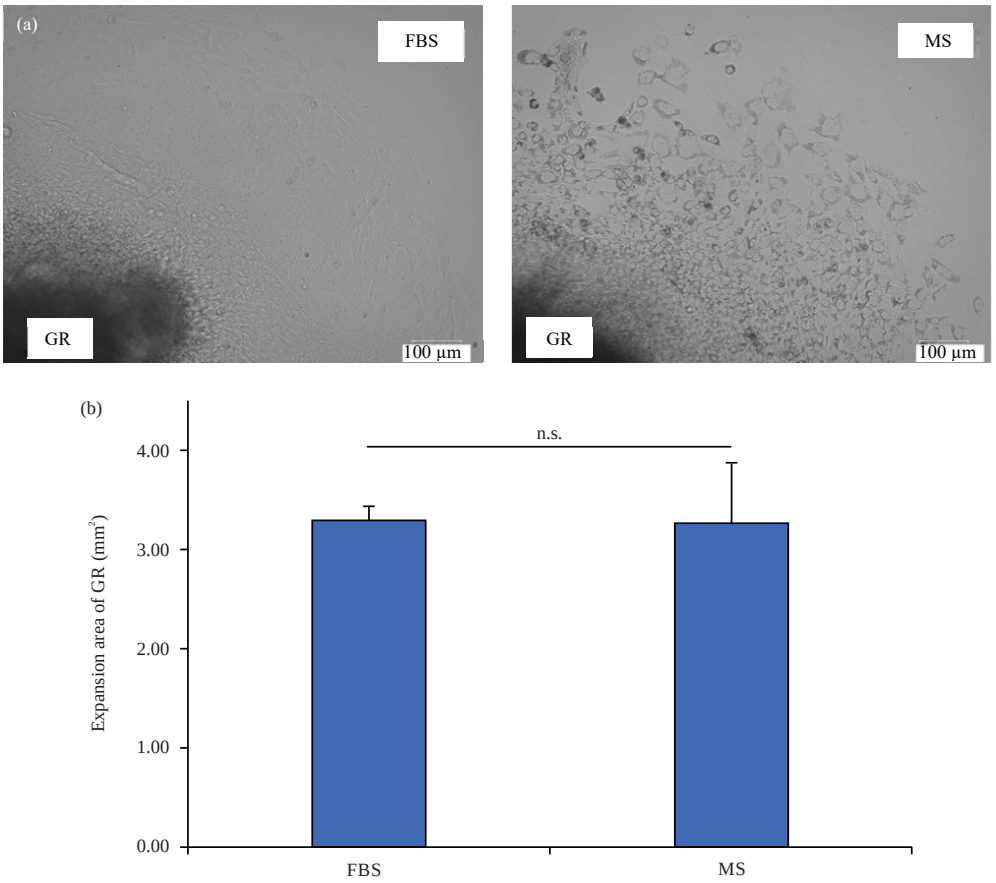

Fig. 1(a-b): Expansion and growth of GRs *in vitro* after supplementing the culture medium with FBS or MS, (a) Growth of fibroblasts in culture with FBS or MS and (b) Comparison of tissue expansion area in cultures with FBS or MS
Data are shown as Mean±SD and Scale bars = 100 µm

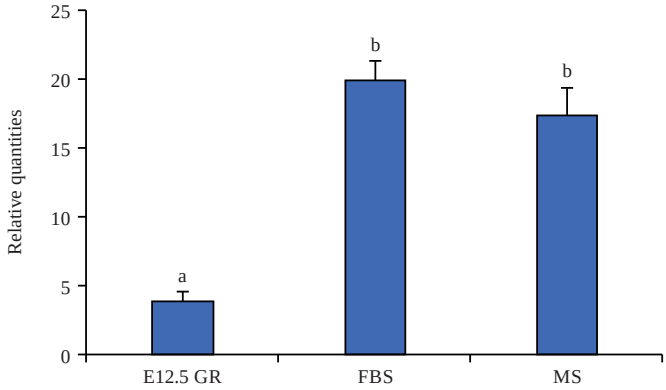

Fig. 2: Relative quantification of *Cxcr4* mRNA in the GR cultures
Gene expression was quantified concerning *Gapdh* and is shown as a Mean±SD, Significance was set at $p<0.01$ and different letters (a, b) Indicate significant differences between cultures

FBS-supplementation group, germ cells numbers increased slightly compared to the initial GR. The cells displayed a spherical morphology and did not undergo migration in (Fig. 3a). In the MS supplementation group, some germ cells were identified away from the GR and cells with elongation as seen during migration, were also observed in (Fig. 3b). The number of germ cells showing the characteristics of migrating cells increased significantly in MS-supplemented cultures

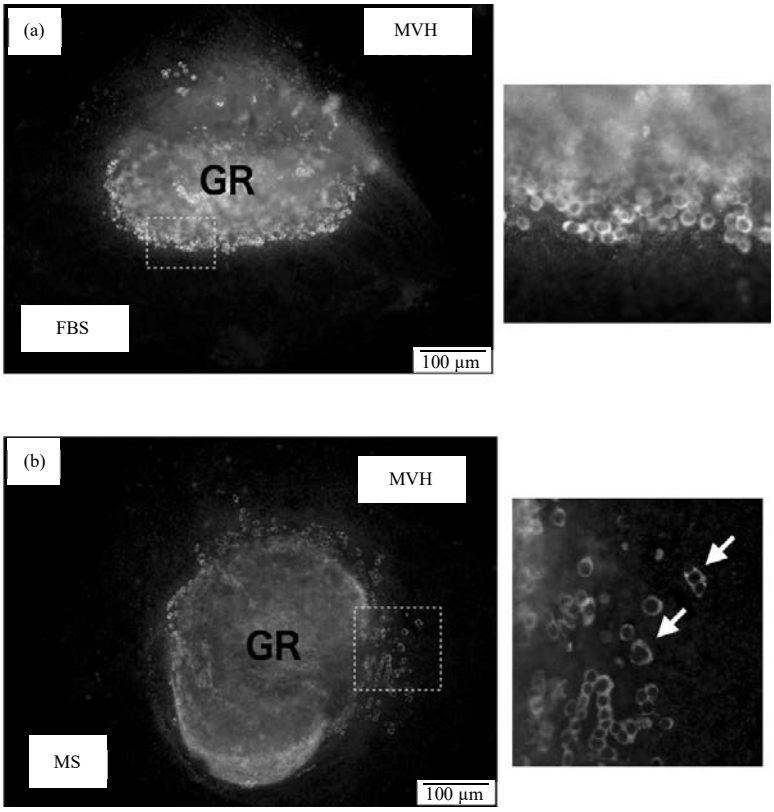

Fig. 3(a-b): Comparison of germ cell morphologies in cultures supplemented with FBS or MS, germ cells were immunostained for MVH, (a) 10% FBS-DMEM and (b) 10% MS-DMEM

Arrows indicate germ cells with morphological characteristics of migratory cells that are located away from the GR and scale bars = 100 µm

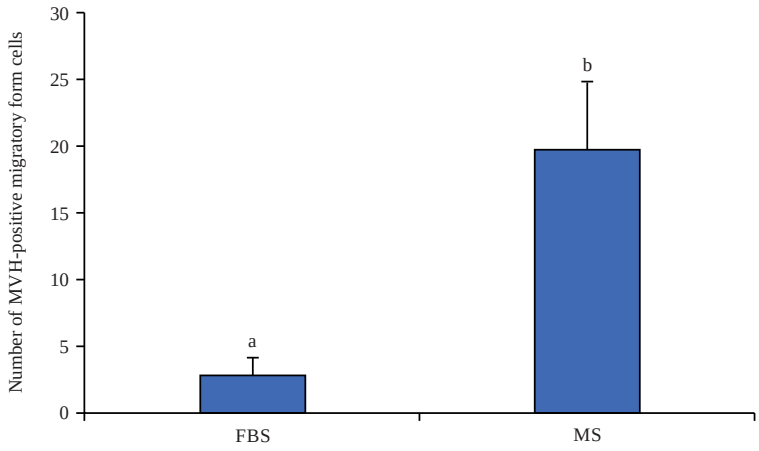

Fig. 4: Numbers of cells with migratory morphology in cultures with FBS or MS

Numbers of germ cells showing migratory morphologies are shown as Mean±SD, significance was set at $p<0.01$ and different letters (a, b) Indicate significant differences between cultures

compared to FBS-supplemented cultures ($p<0.01$) in Fig. 4 and the presumed migratory germ cells expressed CXCR4 in Fig. 5. CXCR4 (+)/MVH (-) lymphocytes were also observed in both MS-supplemented culture and FBS-supplemented culture.

Effect of SDF-1 supplementation on germ cell migration: Next, this study investigated whether the use of an SDF-1 supplement to the culture medium could induce germ cell migration. After the culture of GRs in a medium containing

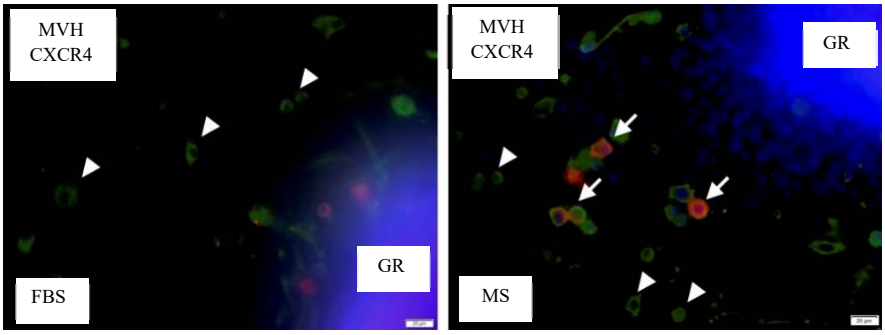

Fig. 5: Immunostaining for CXCR4 in germ cells cultured with FBS and MS

MVH and CXCR4 expression in presumptive migratory germ cells were identified by immunostaining: MVH (red), CXCR4 (green), DAPI (blue), arrows indicate CXCR4-expressing germ cells, arrowheads indicate CXCR4 (+)/MVH (-) lymphocytes and scale bars = 20 μm

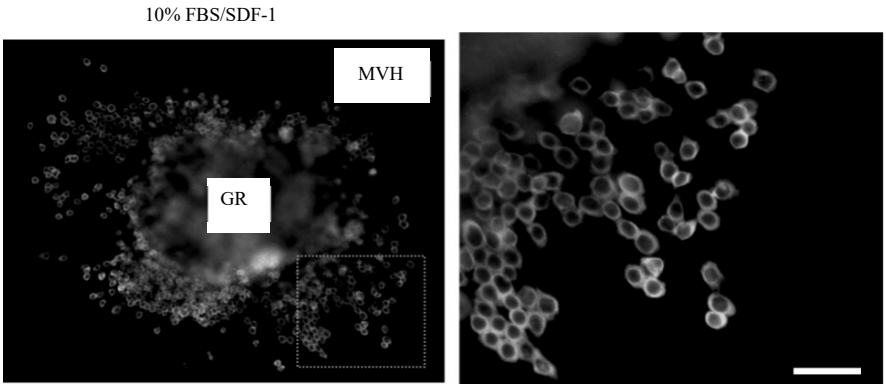

Fig. 6: Induction of germ cell migration by supplementation of SDF-1

Germ cells showing the morphological characteristics of migrating cells were identified by immunostaining for MVH and scale bar = 50 μm

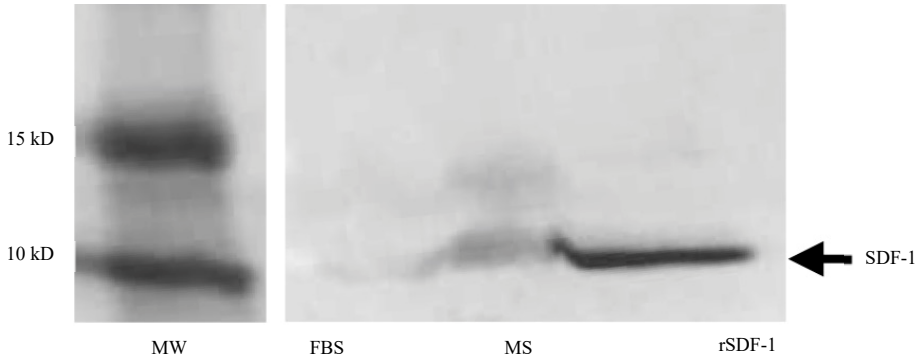

Fig. 7: Confirmation of SDF-1 in MS by western blotting

A band of the expected size for SDF-1 was observed after a western blot analysis of MS, no band was present in FBS, arrow indicates the molecular weight of SDF-1 and the response of recombinant mouse SDF-1 (rSDF-1) was used as a control

FBS and SDF-1, presumptive migratory germ cells were observed as was seen in the cultures using MS supplementation in Fig. 6. This suggests that MS may contain SDF-1. Western blot analysis of MS produced a band at the expected molecular weight position (10 kD) of SDF-1 in Fig. 7.

DISCUSSION

In this study, a method for inducing migration of male PGCs *in vitro* was developed, the main features of this system are the use of allogeneic serum and supplementation with SDF-1. Mouse PGCs are established at E7.0 and

migrate to their destination in an orderly fashion under the control of chemokine signals[13,14]. Once established in the GR, PGCs lose their migratory ability and undergo proliferation and differentiation[7]. However, in the male germline, gonocytes before birth and spermatogonial stem cells (SSCs) after birth are known to have the capacity to migrate[15]. The results of this study showed that this potential migratory capacity is maintained in male PGCs that have colonized the GR. This may be the basis for the retention of migratory ability in the male germ cell line.

Once settled in the GR, PGCs are located in testis cords (immature seminiferous tubules)[16]. The culture of the GRs may have broken down the 3-dimensional structure of testis cords and eliminated the boundaries that define the migratory range of PGCs, enabling them to exhibit motility. In addition, the disruption of the GR conformation may have resulted in a concentration gradient of chemokines required for migration[17]. CXCR4 expression was found here to be upregulated in the GR cultures.

The use of MVH staining to identify germ cells showed that PGCs could have morphological characteristics associated with migration and could move to the outside of the tissue when cultured with MS. Motile PGCs showed migratory germ cell morphology similar to that reported by Farini *et al*.[12] Fibroblasts were seen to expand outward in both FBS and MS. One possibility is that PGCs migrate in concert with fibroblast migration, however, the absence of a significant difference in the area of fibroblast expansion between FBS and MS cultures suggests this possible explanation is not correct. Migratory PGCs in culture expressed CXCR4. This suggests that SDF-1, which regulates PGC migration, might promote migration in organ culture, the analysis here showed that this was indeed the case and supplementation with SDF-1 gave a PGC migration pattern similar to that observed in the MS cultures. The germ cells induction by SDF-1 has been reported in several vertebrates and the results are reasonable[7]. A western blot analysis confirmed the presence of SDF-1 in MS. The results of this study showed that culture with FBS alone could not induce migration of PGCs. This might be due to the lack of SDF-1 in FBS or differences in the structure of mouse and bovine SDF-1 proteins. Support for the latter possibility comes from a report on the use of a Stem Cell Factor (SCF) supplement in cultures of chicken PGCs, it was found that mouse-derived SCF had little effect on the viability of chicken PGCs[18].

The migration of PGCs is crucial to the establishment of the gametogenic system. Interestingly, the ability to migrate has also been reported for male germ cells that have differentiated from PGCs. Gonocytes in the embryonic stage is located in the luminal side of seminiferous tubules. Gonocytes need to attach to the basal surface of the seminiferous tubule around birth for spermatogenesis to occur normally[19]. It has been reported that SDF-1/CXCR4 signalling is involved in the adhesion of gonocytes to the seminiferous tubules[20]. SSCs are lifelong stem cells that are required for spermatogenesis in the mature male mouse[21]. The SSCs are stimulated to migrate to the basement membrane of the seminiferous tubule by SDF-1/CXCR4 signaling[22]. The area near the basement membrane is called an "open niche" because of the presence of blood vessels that provide a rich supply of growth factors and other substances necessary for self-renewal. Thus, migratory ability plays an important role in the male germline. The results of this study indicate that PGCs settled in GR may potentially maintain their migratory ability. In birds and fishes, post migrated PGCs can migrate to GR when transplanted to a different location[23,24]. This suggests that the mechanism of maintaining the migratory ability of PGCs may be common among vertebrates. Furthermore, a report of single-cell analysis of mouse male germ cells shows that the expression of migratory markers changes before and after the mitotic arrest of PGCs[25]. However, the relationship between migration and the arrest of mitosis in male germ cells is not clear. It is important to study the migration ability of post-migration PGCs to understand the molecular mechanisms that maintain their migration ability during the differentiation process from PGCs to gonocytes to SSCs.

The use of SDF-1 or MS supplements in GR culture provides a simple method for investigating migration in PGCs and offers an effective alternative to other methods used to evaluate PGC migration ability, such as transwell analysis[12]. The new method may be of value in studies to elucidate the mechanisms responsible for the maintenance of migration ability in the male germline.

CONCLUSION

Organ culture of male GR activates the ability of germ cells to migrate. Germ cell migration can be easily achieved *in vitro* through the use of an SDF-1 supplement, which is required for germ cell migration, to the culture medium. This suggests that the migration of mammalian PGCs, which is difficult to observe *in utero*, can be reproduced *in vitro*.

SIGNIFICANCE STATEMENT

This study discovered that male mouse PGCs settled in GR migrated by supplementation of SDF-1. This fact indicates that

PGCs potentially maintain their migratory ability even after colonization of the GR. Therefore, it may be useful to elucidate the mechanism of maintaining the migratory ability of the male germline.

ACKNOWLEDGMENT

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