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# Identification of a Technique Optimized for the Isolation of Spermatogonial Stem Cells from Mouse Testes

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## Abstract

To date, there are no protocols optimized to the effective separation of spermatogonial stem cells (SSCs) from testicular cells derived from mouse testes, thus hindering studies based on mouse SSCs. In this study, we aimed to determine the most efficient purification method for the isolation of SSCs from mouse testes among previously described techniques. Isolation of SSCs from testicular cells derived from mouse testes was conducted using four different techniques: differential plating (DP), magnetic-activated cell sorting (MACS) post-DP, MACS, and positive and negative selection double MACS. DP was performed for 1, 2, 4, 8, or 16 h, and MACS was performed using EpCAM ($MACS^{EpCAM}$), Thy1 ($MACS^{Thy1}$), or GFR α1 ($MACS^{GFRα1}$) antibodies. The purification efficiency of each method was analyzed by measuring the percentage of cells that stained positively for alkaline phosphatase. DP for 8 h, $MACS^{Thy1}$ post-DP for 8 h, $MACS^{GFRα1}$, positive selection double $MACS^{GFRα1/EpCAM}$, and negative selection double $MACS^{GFRα1/α\text{-}SMA}$ were identified as the optimal protocols for isolation of SSCs from mouse testicular cells. Comparison of the purification efficiencies of the optimized isolation protocols showed that, numerically, the highest purification efficiency was obtained using $MACS^{GFRα1}$. Overall, our results indicate that $MACS^{GFRα1}$ is an appropriate purification technique for the isolation of SSCs from mouse testicular cells.

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## INTRODUCTION

Spermatogonial stem cells (SSCs) can self-renew indefinitely and differentiate into mature spermatozoa via spermatogenesis (de Rooij 2017; Sakai et al. 2018; Takashima and Shinohara 2018). Therefore, efficient transmission of genetic information to the next generation is mediated by SSCs in the processes of mating, *in vitro* fertilization and artificial insemination (Goossens et al. 2003; Park et al. 2014; Aponte 2015; Kawasaki et al. 2016), permanent preservation of male reproduction through cryopreservation (Lee et al. 2013; Yango et al. 2014; Aliakbari et al. 2016), overcoming male infertility via transplantation, generation of transgenic sperm via the introduction of target genes into the cytoplasm (Kanatsu-Shinohara et al. 2008; Wyns et al. 2010; Kim et al. 2014; Forbes et al. 2017), production of transgenic animals by generation of transgenic sperm (García-Vázquez et al. 2010; Wang et al. 2017), and performance of patient-specific cell therapy by acquisition of pluripotency (Kanatsu-Shinohara et al. 2004; Dym et al. 2009), indicating the usefulness of SSCs.

SSCs are present in a very small population (0.02 – 0.03%) of total testicular germ cells located in the seminiferous tubules (Kanatsu-Shinohara et al. 2010; Ishii et al. 2012; Rastegar et al. 2013). Accordingly, an efficient system for the isolation of SSCs from the seminiferous tubules of testes is required to successfully investigate the maintenance, differentiation, and cryopreservation of SSCs. To date, SSCs have been isolated from various species, including the mouse, rat, pig, cow, and human, using a variety of techniques, including velocity gravitational sedimentation, discontinuous Percoll gradients, differential plating (DP), magnetic-activated cell sorting (MACS), and fluorescent- activated cell sorting (Hamra et al. 2008; Izadyar et al. 2011; Liu et al. 2011; Zheng et al. 2014; Giassetti et al. 2016; Zhang et al. 2016). However, direct comparison of the efficiencies of the different SSC isolation techniques has not been reported, preventing the development of an isolation system customized to SSCs derived from specific species.

Thus, to identify a technique optimized for the isolation of SSCs from the testes of mice, we isolated SSCs from mouse testes using a combination of DP and MACS or MACS based on antibodies not or detecting proteins expressed specifically on the surface of mouse SSCs, and the isolation efficiencies of each method were compared.

## MATERIALS AND METHODS

### 1. Animals

Three-week-old male ICR mice purchased from DBL (Eumseong, Korea) were used as spermatogonial stem cells (SSCs) donors. All of the animal housing, handling and experimental procedures were performed according to the Animal Care and Use Guidelines of Kangwon National University and approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval no. KW-130307-1).

### 2. Isolation of testicular cells from mouse testes

Testes were obtained from mice sacrificed by cervical dislocation and washed with Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea). Next, the tunica albuginea and epididymis were removed from the testes, and seminiferous tubules were dispersed by forceps and digested by incubation for 20 min in 0.5 mg/ml type IV collagenase (Worthington Biochemical, Lakewood, CA)-supplemented Dulbecco's modified Eagle's medium (DMEM; Welgene) at 37°C. The fragmented seminiferous tubules were washed with DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) and digested with 0.125% trypsin-EDTA (Welgene) for 5 min at 37°C. Finally, the undigested testicular cells were discarded through a 70-μm nylon mesh (SPL, Pocheon, Korea) and the filtered testicular cells were allocated to the following experiments.

### 3. Differential plating (DP) method

The 100-mm Petri dishes (SPL) were coated with 0.1% (w/v) gelatin (Sigma-Aldrich, St. Louis, MO). The testicular cells retrieved from mouse testes were plated on gelatin-coated Petri dishes and incubated in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic solution (Welgene) at 37°C for periods described in experimental design, and the floating cells were collected (Figure 1).

### 4. Magnetic-activating cell sorting (MACS)

The MACS was performed using CELLection™ Biotin Binder Kit (Invitrogen, Carlsbad, CA) in accordance with the instruction manual. Briefly, Dynabeads-antibody complexes were formed by incubating Dynabeads provided in the kit with biotin-conjugated antibodies for 1 h at room temperature. Then, the retrieved testicular cells from mouse testes were incubated with Dynabeads-

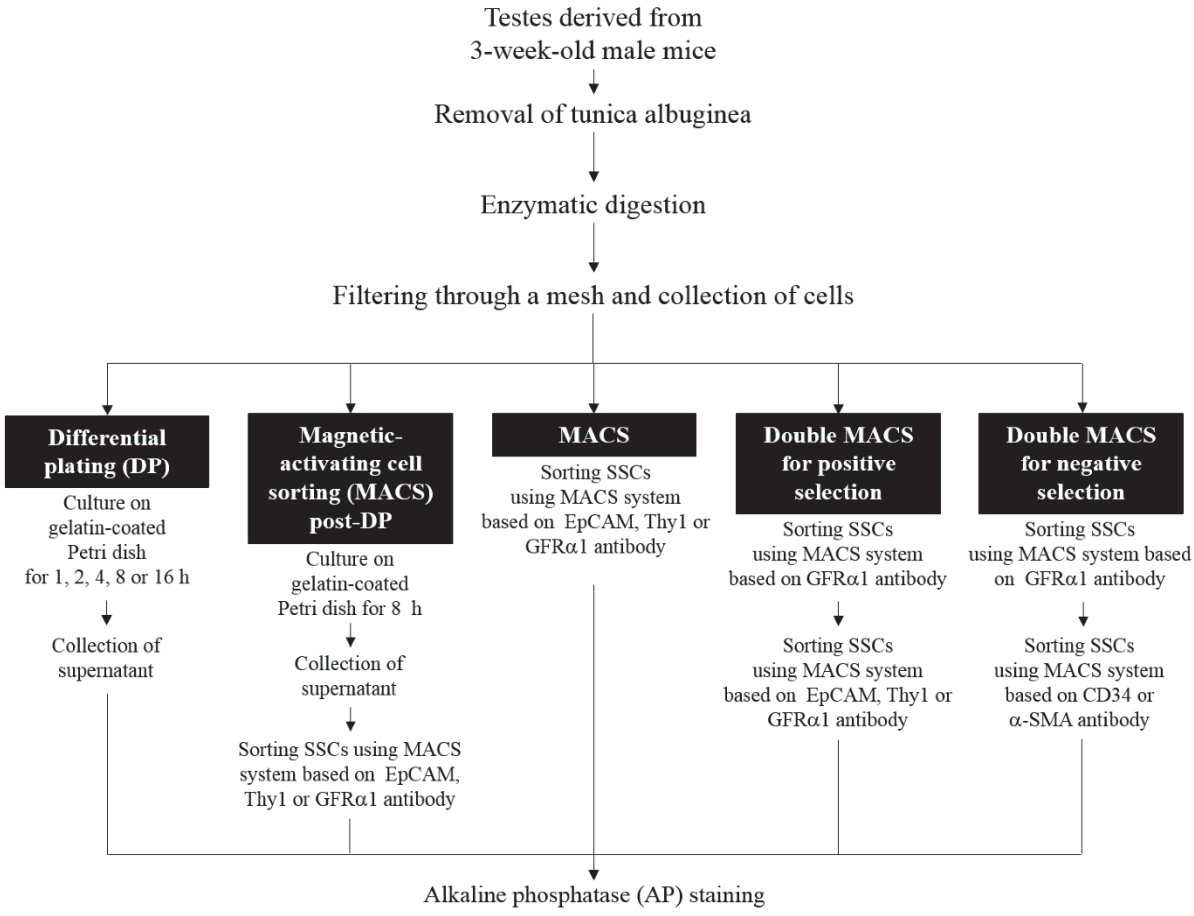


Figure 1. Experimental design

antibody complexes in BSA solution consisting of DPBS supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) for 30 min at 4°C. Separation of cells attached to Dynabeads-antibody complexes (Cell-antibody-Dynabeads) and cells not attached to Dynabeads-antibody complexes was conducted for 2 min in a magnet. In case of positive selection using antibodies detecting mouse SSC markers, the buoyant cells not attached to Dynabeads-antibody complexes were discarded and the Cell-antibody-Dynabeads washed twice with BSA solution in a magnet were incubated in releasing solution mixing 196 µl RPMI 1640 (Welgene) supplemented with 1% (v/v) FBS, 1 mM $CaCl_2$ (Sigma-Aldrich) and 4 mM $MgCl_2$ (Sigma-Aldrich) with 4 µl releasing buffer provided in the kit for 20 min at room temperature. Subsequently, Dynabeads isolated from the Cell-antibody-Dynabeads was removed by incubating for 2 min in a magnet and the remaining buoyant cells were collected. In case of negative selection using antibodies not detecting mouse SSC markers, the buoyant cells not attached to Dynabeads-antibody complexes were collected by discarding the Cell-antibody-Dynabeads in a magnet.

## 5. Experimental design

How to isolate SSCs from testicular cells derived from mouse testes more efficiently was determined by measuring AP activity of SSCs isolated in the following way; differential plating (DP), magnetic-activating cell sorting (MACS) post-DP, MACS, double MACS for positive selection, and double MACS for negative selection. In the DP method, the putative mouse SSCs were collected by incubating testicular cells on gelatin-coated Petri dishes for 1, 2, 4, 8 or 16 h. Retrieval of putative mouse SSCs through MACS post-DP was conducted by exposing cells isolated by DP to the MACS system based on EpCAM (a mouse SSC marker), Thy1 (a mouse SSC marker) or GFRα1 (a mouse SSC marker) antibody. MACS was performed by adjusting testicular cells to the MACS system based on EpCAM, Thy1 or GFRα1 antibody. Double MACS consisted of MACS repeated twice. In case of double MACS for positive selection, putative mouse SSCs included in the testicular cells were isolated through the MACS system based on antibody showing the best isolation efficiency in MACS, followed by re-exposing them to

Table 1. Antibodies used for magnetic-activating cell sorting (MACS)

| Antibody Name | Catalog Number | Company | Dilutions |
|---|---|---|---|
| Biotin-conjugated rat anti-EpCAM IgG2 | 118204 | BioLegend | 1:250 |
| Biotin-conjugated rat anti-Thy1 IgG2 | NBP1-28033 | Novus Biologicals | 1:250 |
| Rabbit anti-GFRα1 IgG | sc-10716 | Santa cruz biotechnology | 1:100 |
| Biotin-conjugated goat anti-rabbit IgG | sc-2040 | Santa cruz biotechnology | 1:200 |
| Biotin-conjugated rat anti-CD34 IgG2 | 119304 | BioLegend | 1:250 |
| Rabbit anti-α-smooth muscle actin IgG | ab5694 | Abcam | 1:100 |

the MACS system based on mouse SSC marker (EpCAM, Thy1 or GFRα1) antibody. Double MACS for negative selection was conducted by re-adjusting putative mouse SSCs isolated from the testicular cells by MSCS system based on antibody showing the best isolation efficiency in MACS to the MACS system based on CD34 (a testicular stromal cells marker) or α-smooth muscle actin (α-SMA; a peritubular myoid cells marker). Subsequently, the putative mouse SSCs purified by each protocol were stained through AP staining and the efficiency of each protocol was evaluated and compared by measuring the percentage of mouse SSCs with AP activity. The experimental design and the detailed information regarding the used antibodies are shown in Figure 1 and Table 1, respectively.

### 6. Alkaline phosphatase (AP) staining

The cells purified through each isolation protocol were fixed with 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo-ku, Japan) and washed with DPBS. Then, the fixed cells were stained with AP solution consisting of 0.1 M Tris buffer (pH 8.2) supplemented with 0.2 mg/ml naphthol AS-MX phosphate, 2% (v/v) dimethyl formamide, and 1 mg/ml Fast Red TR salt (all from Sigma-Aldrich) for 30 min at room temperature. Subsequently, the stained SSCs were rinsed with DPBS and the percentage of the positively stained cells was enumerated using a hemocytomer under an inverted microscope (CKX-41; Olympus, Tokyo, Japan).

### 7. Statistical analysis

The Statistical Analysis System (SAS) software (SAS Institute Inc, Cary, NY) was used for statistical analysis of the numerical data shown in each experiment. Moreover, the percentage of cells stained positively for AP was compared among all experimental groups using a generalized linear model (PROC-GLM) in the SAS package. The less than 0.05 of $p$ value was regarded as a significant difference.

## RESULTS

### Effect of incubation time on the purification efficiency of SSCs from mouse testicular cells using DP

To determine the incubation time resulting in optimal purification of SSCs isolated from mouse testicular cells using DP, mouse testicular cells were incubated for 1, 2, 4, 8, or 16 h on gelatin-coated Petri dishes. As shown in Figure 2, extension of the incubation time did not induce a significant difference in the purification efficiency (as determined by the percentage of alkaline phosphatase-positive cells). The highest purification efficiency was detected after 8 h of incubation.

### Effect of antibodies on the purification efficiency of SSCs from mouse testicular cells using MACS post-DP, MACS, positive selection double MACS, and negative selection MACS

Next, to determine the antibody resulting in optimal purification of SSCs isolated from mouse testicular cells using MACS post-DP, the cell population sorted by DP for 8 h was further purified by MACS using EpCAM, Thy1, or GFRα1 antibodies. Even though MACS using the Thy1 antibody ($MACS^{Thy1}$) showed the highest purification efficiency (Figure 3), there was no significant difference in purification efficiency among the antibodies. Next, to determine the optimal antibody for purification of SSCs from mouse testicular cells using MACS, SSCs isolated from mouse testicular cells were sorted by MACS using EpCAM, Thy1, or GFRα1 antibodies. As shown in Figure 4, no significant difference in purification efficiency was observed among the different antibodies, but the highest purification efficiency, numerically, was achieved using GFRα1 antibody- based MACS ($MACS^{GFRα1}$).

To enhance the purification efficiency using $MACS^{GFRα1}$, the cell population sorted by $MACS^{GFRα1}$ was further purified by positive selection MACS, using antibodies targeting proteins expressed on the surface of mouse SSCs (EpCAM, Thy1, or GFR α1), or negative selection MACS, using antibodies targeting

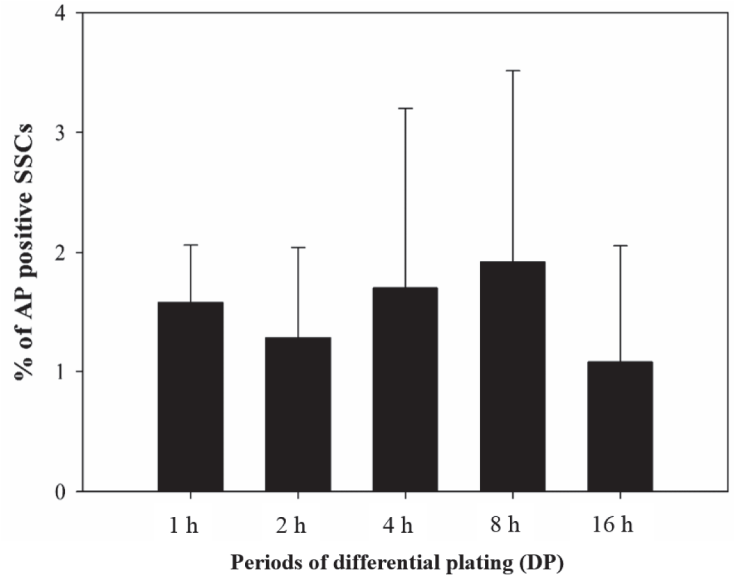


Figure 2. Determination of incubation times enhancing purification efficiency of SSCs from mouse testicular cells in the DP method. Testicular cells retrieved from mouse testes were incubated on 0.1% (wt/v) gelatin-coated Petri dish for 1, 2, 4, 8 or 16 hours (h) at 37°C and AP activity as an indicative of SSCs was analyzed in the floating cells not attached to gelatin-coated Petri dish through AP staining. Subsequently, the purification efficiency was determined by dividing the number of cells stained positively by AP staining by the number of total cells experiencing AP staining. Although there was no significant difference among periods of DP, numerically the highest percentage of AP-positive SSCs was induced by conducting a DP method for 8 h. Data represent the means ± standard deviation of three independent experiments.

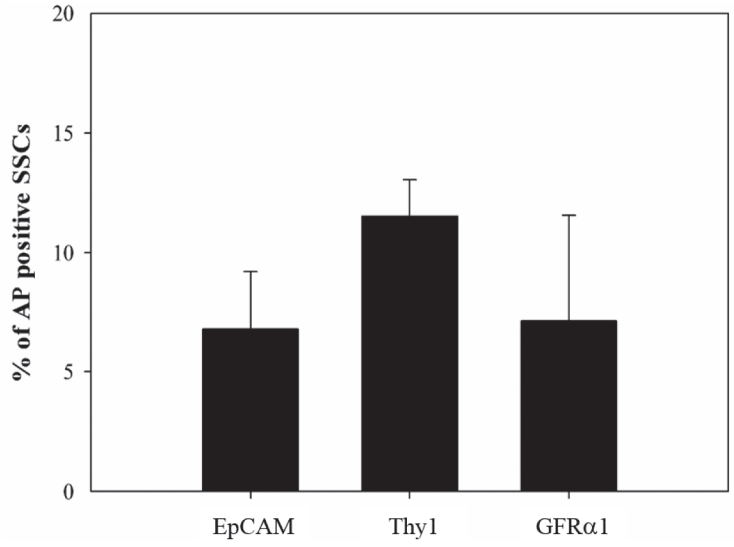


Figure 3. Determination of antibodies enhancing purification efficiency of SSCs from mouse testicular cells in the MACS post-DP method. Testicular cells isolated from mouse testes were incubated on 0.1% (wt/v) gelatin-coated Petri dish at 37°C. After 8 h of incubation, the floating cells not attached to gelatin-coated Petri dish were additionally sorted by MACS system based on EpCAM (a SSC marker), Thy1 (a SSC marker) or GFRα1 (a SSC marker) antibody. Subsequently, AP activity as an indicative of SSCs was analyzed in the finally sorted cells through AP staining and the purification efficiency was determined by dividing the number of cells stained positively by AP staining by the number of total cells experiencing AP staining. As the results, putative SSC populations isolated from mouse testicular cells through Thy1 antibody-based MACS post-DP ($MACS^{Thy1}$ post-DP) method showed numerically the highest percentage of AP-positive SSCs without a significant difference. Data represent the means ± standard deviation of three independent experiments.

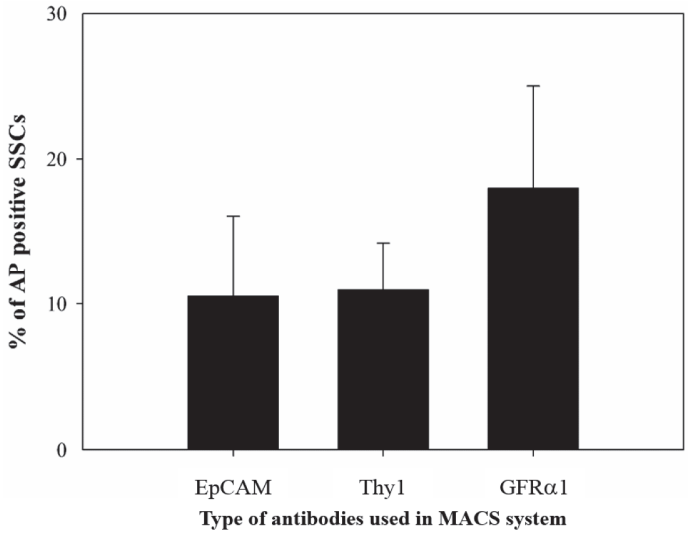


Figure 4. Determination of antibodies enhancing purification efficiency of SSCs from mouse testicular cells in the MACS method. Testicular cells isolated from mouse testes were sorted using a MACS system based on EpCAM (a SSC marker), Thy1 (a SSC marker) or GFRα1 (a SSC marker) antibody and AP activity as an indicative of SSCs was analyzed in the finally sorted cells through AP staining. Subsequently, purification efficiency was determined by dividing the number of cells stained positively by AP staining by the number of total cells experiencing AP staining. Putative SSC populations isolated from mouse testicular cells through GFRα1 antibody-based MACS (MACS$^{GFR\alpha1}$) method showed numerically the highest percentage of AP-positive SSCs. However, there was no significant difference among types of antibodies. Data represent the means ± standard deviation of three independent experiments.

non-cell-surface proteins expressed in mouse SSCs (i.e., CD34, a testicular stromal cell marker or α-SMA, a peritubular myoid cell marker). Following isolation of mouse testicular cells by MACS$^{GFR\alpha1}$, use of the EpCAM antibody in double MACS$^{GFR\alpha1/EpCAM}$ resulted in greater purification efficiency compared with the Thy1 and GFRα1 antibodies (Figure 5), although the differences were not significant. Moreover, in negative selection double MACS, use of the α-SMA antibody in double MACS$^{GFR\alpha1/\alpha\text{-}SMA}$ increased the purification efficiency compared with the CD34 antibody (double MACS$^{GFR\alpha1/CD34}$), although this increase was not significant (Figure 6).

### Determination of an optimized technique for the isolation of SSCs from mouse testicular cells

The most optimal purification method for the isolation of SSCs from mouse testicular cells was determined by comparison of the purification efficiencies derived from DP for 8 h, MACS$^{Thy1}$ post-DP for 8 h, MACS$^{GFR\alpha1}$, double MACS$^{GFR\alpha1/EpCAM}$, and double MACS$^{GFR\alpha1/\alpha\text{-}SMA}$. As shown in Figure 7, the purification efficiency of SSCs from mouse testicular cells was significantly higher using MACS$^{GFR\alpha1}$ and double MACS$^{GFR\alpha1/EpCAM}$ compared with the other methods. Moreover, higher purification efficiency was achieved using MACS$^{GFR\alpha1}$ compared with double MACS$^{GFR\alpha1/EpCAM}$. These results demonstrate that MACS$^{GFR\alpha1}$ is the optimal purification method for the isolation of SSCs from mouse testicular cells, compared with the other techniques evaluated.

## DICUSSION

Here, we report that the MACS$^{GFR\alpha1}$ protocol exhibited the best purification efficiency for SSC isolation from testicular cells derived from mouse testes. Numerically, the highest purification efficiency was observed using DP for 8 h, MACS$^{Thy1}$ post-DP for 8 h, MACS$^{GFR\alpha1}$, positive selection double MACS$^{GFR\alpha1/EpCAM}$, and negative selection double MACS$^{GFR\alpha1/\alpha\text{-}SMA}$. Comparison of these protocols revealed that MACS$^{GFR\alpha1}$ showed the highest purification efficiency numerically of mouse SSCs. These results will help reduce the inaccurate data derived from experiments using SSCs isolated directly from mouse testes.

DP, which is based on differential adherence speed, is a physical methodology used to purify a specific cell type from a mixed cell population (He et al. 2015). The significantly lowest purification efficiency of the tested methods was observed with gelatin-based DP (Figure 7). This may be a result of weak binding of testicular cells to the gelatin or strong binding of SSCs to

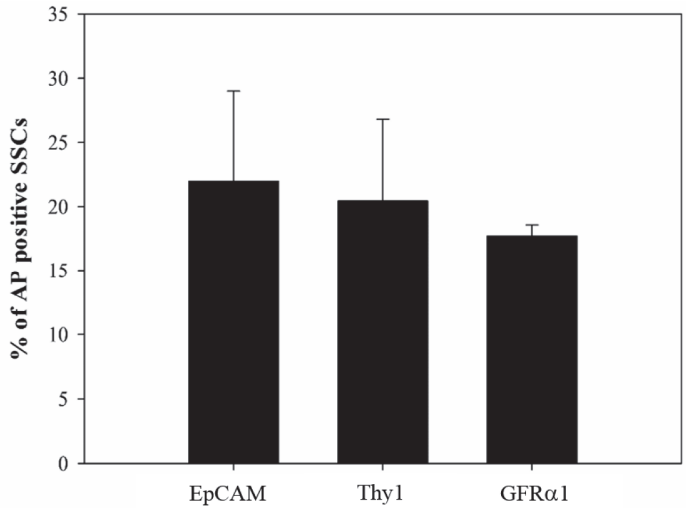


Figure 5. Determination of antibodies enhancing purification efficiency of SSCs from mouse testicular cells after GFRα1 positive (GFRα1$^+$) sorting in the double MACS for positive selection method. Putative SSC populations were retrieved from mouse testicular cells using MACS system based on GFRα1 (a SSC marker) antibody (MACS$^{GFR\alpha1}$) and purified once more for positive selection using MACS system based on EpCAM (a SSC marker), Thy1 (a SSC marker) or GFRα1 (a SSC marker) antibody. Subsequently, AP activity as an indicative of SSCs was analyzed in the finally sorted cells through AP staining and purification efficiency was determined by dividing the number of cells stained positively by AP staining by the number of total cells experiencing AP staining. Although there was no significant difference among types of antibodies for positive selection, numerically the highest percentage of AP-positive SSCs was detected in the usage of EpCAM antibody in the second MACS post-GFRα1 antibody-based MACS (double MACS$^{GFR\alpha1/EpCAM}$). Data represent the means ± standard deviation of three independent experiments.

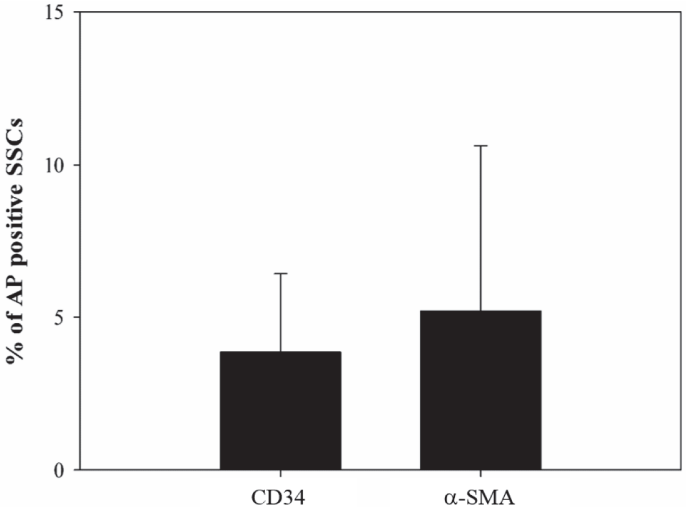


Figure 6. Determination of antibodies enhancing purification efficiency of SSCs from mouse testicular cells after GFRα1 positive (GFRα1$^+$) sorting in the double MACS for negative selection method. Putative SSC populations were retrieved from mouse testicular cells using MACS system based on GFRα1 (a SSC marker) antibody (MACS$^{GFR\alpha1}$) and purified once more for negative selection using MACS system based on CD34 (a testicular stromal cell marker) or α-SMA (a peritubular myoid cell marker) antibody. Subsequently, AP activity as an indicative of SSCs was analyzed in the finally sorted cells through AP staining and purification efficiency was determined by dividing the number of cells stained positively by AP staining by the number of total cells experiencing AP staining. Although there was no significant difference between types of antibodies for negative selection, the usage of α-SMA antibody in the second MACS post-GFRα1 antibody-based MACS (double MACS$^{GFR\alpha1/\alpha\text{-}SMA}$) revealed numerically higher percentage of AP-positive SSCs than those of CD34 antibody. Data represent the means ± standard deviation of three independent experiments.

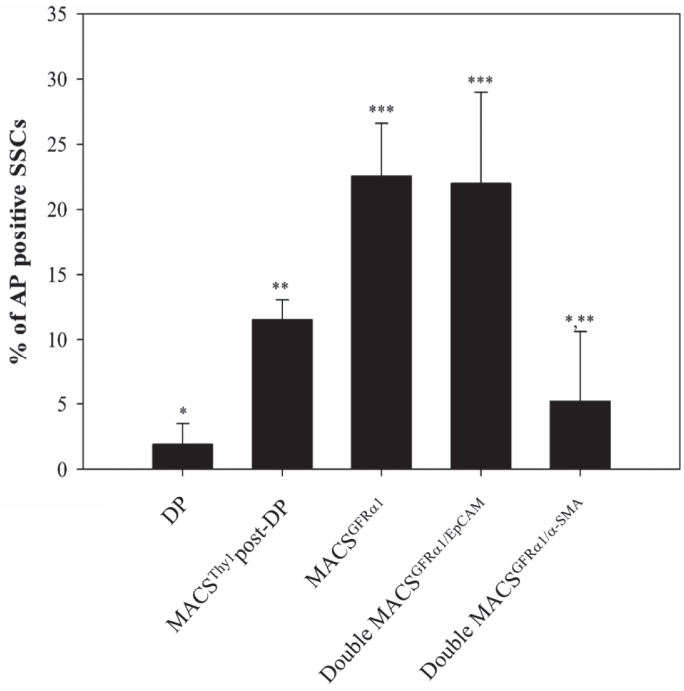


Figure 7. Comparison of purification efficiencies induced by diverse SSC isolation methods showing maximum efficiency in the isolation of SSCs from mouse testicular cells. Putative SSC populations were isolated from testicular cells derived from mouse testes by DP, MACS$^{Thy1}$ post-DP, MACS$^{GFR\alpha1}$, double MACS$^{GFR\alpha1/EpCAM}$, or double MACS$^{GFR\alpha1/\alpha\text{-SMA}}$. AP activity as an indicative of SSCs was analyzed in the cells finally sorted by each isolation method through AP staining. Subsequently, purification efficiency was determined by dividing the number of cells stained positively by AP staining by the number of total cells experiencing AP staining. Putative SSC populations retrieved by MACS$^{GFR\alpha1}$ and double MACS$^{GFR\alpha1/EpCAM}$ showed significantly the highest percentage of SSCs stained positively by AP staining. Data represent the means ± standard deviation of three independent experiments. $^{*\text{-}***}p<0.05$.

the gelatin, suggesting gelatin to be an inappropriate substrate in this case. Accordingly, the use of extracellular matrix proteins that specifically interact with integrin heterodimers expressed on the surface of undifferentiated mouse SSCs can help increase the purification efficiency of DP.

EpCAM, Thy1, and GFRα1 are membrane proteins expressed on the surface of mouse SSCs (Phillips et al. 2010; Guo et al. 2014). Therefore, antibodies detecting these surface marker proteins can be actively applied in MACS (He et al. 2015). However, to date, the surface marker proteins with the best efficacy in MACS-based isolation of SSCs from mouse testicular cells have not been identified, rendering it difficult to obtain mouse SSC populations with high purity. In this study, we determined that use of an antibody targeting GFRα1, which is expressed on the surface of mouse SSCs, resulted in the best purification of SSCs isolated from mouse testicular cells (Figure 4), indicating the usefulness of the GFRα1 surface protein in MACS-based SSC purification from mouse testicular cells.

Despite that MACS$^{GFR\alpha1}$ showed the best purification efficiency among the methods tested, the efficiency (< 25%) was not very high. This may be due to the enzymes used for isolation of SSCs from testicular tissues. Generally, a variety of digestive enzymes such as trypsin, pronase, dispase, and collagenase are used, which can induce minor or major damage to cellular junctions, membranes, surface receptors, antigens, and cytosolic contents (Miersch et al. 2018; Schmidt et al. 2018). Therefore, cell surface antigens impaired by enzymatic digestion may reduce antigen - antibody affinity, resulting in low purification efficiency with MACS. Accordingly, the development of novel digestive methods that induce less cell surface damage during cell harvest from testicular tissues will be necessary to improve purification efficiency.

Theoretically, double MACS, which is conducted sequentially using antibodies detecting different cell surface proteins, is expected to result in higher purification efficiency compared with single MACS. However, this study did not detect greater purification efficiency using double MACS (Figure 7). Generally, the purification efficiency of MACS is determined by antigen - antibody affinity,

and the antigen - antibody complex is not completely dissociated after positive sorting by MACS (He et al. 2015). Therefore, this incomplete dissociation may interfere with the attachment of other antibodies, which later bind to the antigens targeted by the antibodies in the previous step; this may explain the lack of increased purification efficiency using double MACS$^{GFR\alpha1/EpCAM}$ and double MACS$^{GFR\alpha1/\alpha\text{-SMA}}$ compared with MACS$^{GFR\alpha1}$.

In conclusion, this study showed that MACS$^{GFR\alpha1}$ was an appropriate purification technique for the isolation of SSCs from mouse testicular cells. This finding will be useful for future studies on the maintenance, differentiation, and cryopreservation of mouse SSCs. However, the development of purification techniques with greater efficiency will be necessary for conducting mouse SSC-based researches in the future.

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