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Enrichment of Testicular Gonocytes and Genetic Modification Using Lentiviral Transduction in Pigs[1]

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ABSTRACT

Gonocytes are long-lived primary germ cells that reside in the center of seminiferous cords until differentiation into spermatogonia that drive spermatogenesis. In pigs, gonocytes have research value in the production of transgenic offspring through germline modification and transplantation. However, the rarity of pig gonocytes has raised the need for an efficient isolation method. Therefore, in this study we use components of extracellular matrix, laminin, fibronectin, and collagen type IV and their derivative, gelatin, to establish a negative selection system for functionally viable gonocytes in neonatal pig. We then demonstrate functional analysis with genetic modification using lentiviral transduction and successfully transplant the donor gonocytes, which colonized the seminiferous tubules of the recipient mouse. The most effective selection method was established by sequential use of laminin and gelatin, in which the purity of gonocytes was 80% and the recovery rate of gonocytes was 78%. The selected gonocytes were labeled with fluorescent dye PKH26 and transplanted into busulfan-treated immunodeficient mouse testes. The fluorescent gonocytes colonized the recipient testes, and the resultant germ cell colonies were visible up to 4 mo after transplantation. When gonocytes were transplanted after transduction with an enhanced green fluorescent protein marker gene using lentiviral vectors, the transduced germ cell colonies were visible up to 6 mo and displayed an estimated transduction efficiency of 11.1%. These results can be applied and extended to isolate and enrich gonocytes of other species for in vitro and in vivo studies and to assist in genetic modification of male germline stem cells of livestock species.

extracellular matrix, gonocyte, lentiviral transduction, lentivirus, pig, spermatogonial stem cell, transplantation

INTRODUCTION

In many vertebrates, including mammals, germ cells are the vehicle through which genetic information is transmitted from generation to generation. In an embryo, primitive germ cells called primordial germ cells (PGCs) journey from the base of allantois to the hindgut and then migrate to colonize the genital ridges, where the germ cells become gonocytes in the developing gonads [1]. Gonocytes in males, also called prospermatogonia, are the long-lived primary germ cells that reside in the center of seminiferous cords of neonatal testes of mammals such as rodents and livestock species [2, 3]. Gonocytes can be distinguished visually from the adjacent Sertoli cells based on their large circular morphology and prominent nuclei [2, 4]. After birth, gonocytes resume proliferation and migrate to the basement membrane of the seminiferous cords, initiating spermatogenesis by differentiation into spermatogonial stem cells (SSCs) that drive the lifelong and unlimited production of spermatozoa [5, 6]. From this point, compared with gonocytes, the proliferative SSCs are morphologically smaller and difficult to identify.

Theoretically, in rats a single SSC has the capability to produce 4096 mature spermatozoa through spermatogenesis, as is true for other species, including pigs [7]. Therefore, male germline stem cells (mGSCs) such as gonocytes and SSCs carry tremendous research value because of their potential to efficiently produce transgenic offspring possessing the donor haplotype when genetically modified and transplanted into a recipient testis by the spermatogonial transplantation technique [8, 9]. In the case of livestock species such as pigs, mGSCs are of great commercial value because of their potential to generate transgenic animals with improved productivity traits or for biomedical purposes, as embryonic stem cell lines with germline characteristics have not been found to date.

Despite the clear importance of mGSCs, information regarding their biochemical and molecular characteristics is limited, partly due to insufficiency of mGSCs. While SSCs comprise 0.03% and 0.2% of adult mouse and rat testicular cells, respectively, gonocytes comprise 1%–2% of neonatal testicular cells [10–13]. Therefore, further biological and molecular analyses are needed among highly pure and enriched populations of mGSCs to delineate their full range of phenotypic and genotypic properties.

Identification of surface markers enables highly enriched gonocyte and SSC populations to be easily produced without significant cell loss using fluorescent-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) [14–18]. For transplantation of mGSCs into large animal models, it is essential to sufficiently prepare a large quantity of cells with minimum damage using an appropriate enrichment technique [19, 20]. However, FACS and MACS are limited in delivering desired numbers of cells for transplantation into a large animal recipient testis. Therefore, they are unsuitable for enrichment of

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large numbers of mGSCs from testes of large animals, including porcine.

Previous studies [14, 21, 22] have applied the associability of mGSCs with the basement membrane to isolate rodent or pig SSCs. The basement membrane consists of thin layers of extracellular matrix (ECM) components such as laminin, collagen type IV, and fibronectin, among others. The SSCs were observed to show highest affinity to laminin [14, 21]. Some differentiated cells, including SSCs, have β_1- and α_6-integrins that take part in binding to laminin [23–25]. Alternatively, in rodents differential adhesion to ECM components such as collagen and laminin was used to enrich spermatogonia [26]. Therefore, differential plating may serve as a method to enrich mGSCs. Gelatin, a denatured derivative of collagen, is also a cell adhesion protein associated with seminal plasma proteins in bovine, goat, and boar that interacts with choline phospholipids on the sperm surface [27]. During cell culture, gelatin optimizes cell surface interaction when plated on a glass or plastic surface, which collectively raises its potential as a possible selection tool for various cell types.

To apply differential plating, it is important to select an appropriate donor testis from which a maximum number of mGSCs can be isolated. A relatively large quantity of mGSCs can be found in neonatal and prepubertal testes before the onset of spermatogenesis. Therefore, immature testes serve as superior donors to isolate mGSCs. Furthermore, the distinct morphology of gonocytes compared with SSCs facilitates their isolation and thus gives a selection advantage over SSCs. The functionality of selected gonocytes can be demonstrated by establishing donor-derived expansion of germ cell colonies in a recipient testis through the spermatogonial transplantation technique [13]. In rodents, transplantation of gonocytes and SSCs into seminiferous tubules of recipient nude mouse testes has generated germ cell colonies through proliferation of these donor cells [13, 28]. The transplantation of SSCs is also known to restore the fertility of an infertile recipient animal, which passes the donor haplotype to the offspring [8]. Similar transplantation techniques have been applied in evolutionarily more distant animals such as rabbits, dogs, boars, and bulls, in which xenogeneic transplantation resulted in colonization of SSCs in the testes of recipient mice [29, 30].

Spermatogonial transplantation is also an essential technique for performing SSC-mediated transgenesis [26, 31, 32]. Genetic modification was achieved using various methods, including many viral transduction attempts. Moloney murine leukemia virus (MMLV), which belongs to the family of oncoretrovirus, was used as a major vector to transport foreign genes into the target cells by incorporating recombinant proviral DNA into the host genome [33]. However, because proviral DNA of MMLV cannot translocate past the nuclear membrane, it requires active cell division by the host where the nuclear membrane dissociates [33]. This is a significant limitation because germline stem cells divide comparably slower than other cells [34]. Therefore lentivirus, a subclass of retrovirus, has been introduced to efficiently transport foreign genes into nondividing cells with a nuclear localization sequence and a matrix protein to allow active transduction [35]. Lentiviral transduction can be used in various cell types because it is equipped with the glycoprotein of vesicular stomatitis virus in place of the envelope protein that recognizes abundant membrane phospholipid as a receptor [36, 37].

In this study, we hypothesized that gonocytes would remain in the center of the seminiferous cords away from interaction with the basement membrane. Therefore, we developed an efficient negative selection method to choose and enrich gonocytes, which are the only germ cells in neonatal pig testis. This method utilized ECM molecules and gelatin based on our hypothesis and the morphological uniqueness of gonocytes. After enrichment, gonocytes were genetically modified using lentiviral transduction. Then, xenogeneic transplantation into the seminiferous tubules of mouse recipients was performed to demonstrate the functional viability of gonocytes.

MATERIALS AND METHODS

Acquisition of Testes and Preparation of Cell Suspensions

Testes of crossbred piglets (Landrace × Large White Yorkshire × Duroc) aged 10–14 days were obtained from a local farm. For gonocyte isolation, the testes were washed with Dulbecco PBS (DPBS; Gibco, catalog no. 14200-075) and transported on ice in DPBS within 1 h to the laboratory. All animal procedures were approved by the Animal Care and Use Committee of Chung-Ang University.

To harvest donor testis cells, the testis cell tissues were initially dissociated using a sequential enzymatic digestion procedure as previously described [19, 38] with minor modifications. Briefly, all visible connective tissue and the tunica albuginea were removed, followed by digestion of exposed seminiferous cords with collagenase (1 mg/ml, type IV; Sigma) and hyaluronidase (1 mg/ml; Sigma) in DPBS at 37°C for 15 min with constant agitation. Testis tissue was washed with DPBS and further digested with a 4:1 mixture of 0.25% trypsin-EDTA (Gibco BRL), and DNase I (7 mg/ml in DPBS; Sigma). Ten percent fetal bovine serum (FBS; HyClone, catalog no. SH30070.03) was added to stop further enzymatic digestion. The resultant cell suspension was filtered through a strainer (pore size, 40 μm; BD Biosciences) and centrifuged at 600 × *g* for 7 min at 4°C. The pellet was resuspended in basic medium (Dulbecco modified Eagle medium; Gibco-BRL) containing 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and gonocytes were identified based on their morphology (large size, prominent nuclei, and low intracellular complexity) and counted under an inverted microscope (TE2000; Nikon).

Gonocytes were initially separated from cellular debris and red blood cells (RBCs) present in collected testis tissues using discontinuous Percoll density gradient as described previously [39] with minor modifications. Two Percoll densities, 20% and 40% (Sigma), were created in a 15-ml conical tube (Falcon). An iso-osmotic Percoll suspension was prepared by the addition of 40% Percoll solution, 10% 10× DPBS, 1% FBS, 0.5% antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin), and 48.5% ultrapure water. The 20% Percoll was prepared by diluting the iso-osmotic 40% Percoll suspension with DPBS supplemented with 1% FBS and antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin). The cell suspension (2 ml of 5×10^6 cells/ml) was loaded on the top of the gradient and centrifuged at 600 × *g* for 10 min at 4°C. Top, bottom, and interface layers were all collected and analyzed for the presence of gonocytes. Gonocytes were located at the interface between 20% and 40% Percoll layers. The interface was diluted with basic medium to be used for further selection, and the number of gonocytes was counted.

Enrichment of Gonocytes Using ECM Molecules and Gelatin

Gonocytes were separated from testis cell populations using fibronectin, collagen type IV, laminin, and gelatin. Briefly, for ECM molecules 60-mm Petri dishes (Falcon) were incubated overnight at 37°C with fibronectin, collagen type IV, and laminin (all from Sigma) at a concentration of 20 μg/ml in DPBS. The incubated dishes were rinsed with DPBS and were preincubated with 0.5% bovine serum albumin (BSA) solution for 1 h at 37°C to prevent nonspecific binding. For gelatin, Petri dishes were incubated for 1 h at room temperature with 0.2% gelatin (Sigma) and were washed in DPBS. For each selection, 1.0×10^7 cells were suspended in 3 ml of basic medium, loaded onto the dishes, and incubated at 37°C for 20 min for ECM molecules and 12 h for gelatin.

For sequential selection with laminin and gelatin, the cells were initially incubated with laminin, and the unbound cell population was transferred onto a gelatin dish for further selection. The dishes were washed three times with DPBS to recover unbound cells, and the bound cells were collected by trypsin (0.25%)-EDTA (1 mM) digestion for 5 min, followed by vigorous pipetting.

The number of gonocytes was counted in both unbound and bound populations of cells to calculate the purity of gonocytes (number of gonocytes in either unbound or bound population divided by total number of cells × 100%). The recovery rate of gonocytes was also calculated as follows: number of recovered gonocytes divided by (total number of loaded cells × gonocyte purity of loaded cells) × 100%. In terms of purity, data were compared using an

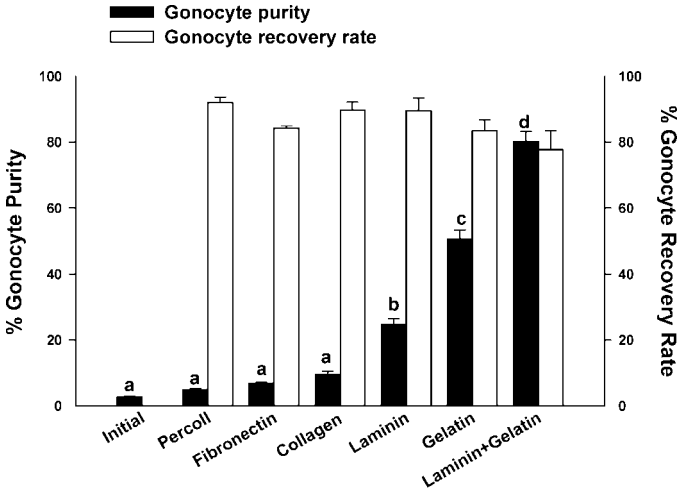

FIG. 1. Selection and enrichment of gonocytes from porcine testes using ECM molecules and gelatin demonstrate a measure of porcine gonocyte purity (solid bars) and its recovery rate (open bars). To harvest donor testis cells, for each experiment the testes tissues obtained from two to four testes (1.5 ± 0.2 g, $\bar{x}$ ± SEM, n = 16 testes) were separated using a sequential enzymatic digestion procedure. Pig gonocytes were initially selected using a Percoll gradient before further selection. Values are $\bar{x}$ ± SEM (n = 5). The difference in purity between negative selection and control values was significant ($P < 0.05$) for and among laminin, gelatin, and laminin plus gelatin (sequential negative selection with laminin and gelatin). No significant difference was observed for recovery rate between selections. The initial gonocyte purity of the unselected cell population served as the control; thus, the recovery rate was not recorded. Gonocyte purity values for the ECM molecules and gelatin selections with different lowercase letters were significantly different by Tukey HSD ($P < 0.05$).

unselected population of cells as the control. For comparison of recovery rates, the Percoll-selected population of cells, which was the first method of selection, served as the control.

Immunofluorescence Procedures

Cells were stained with protein gene product 9.5 (PGP 9.5) and *Dolichos biflorus* agglutinin (DBA) antibodies to analyze the expression of PGP 9.5 and DBA in Percoll-selected (recovered cells from the midlayer of the Percoll gradient), unbound (unbound cell population on the gelatin plate after negative selection with laminin), and bound (bound cell population on the gelatin plate after negative selection with laminin) cell populations. For immunocytochemical detection of GATA4, bound and unbound cell populations were stained with GATA4 antibody.

Standard immunofluorescence was performed for each marker. Dilutions of primary and secondary antibodies were performed in PBS containing 5% (w/v) BSA (Sigma). Briefly, after checking viability by trypan blue exclusion, 100 μl of the cell suspension (5×10^5 cells/ml) was plated on poly-L-lysine slides (Polyscience, Inc.) and incubated for 30 min at 37°C. Bound cells on the slides were fixed in 4% paraformaldehyde for 15 min at room temperature and blocked using 20% (v/v) donkey serum and 5% (w/v) BSA or 5% (w/v) BSA in PBS for 30 min. The cells were incubated with rabbit anti-human PGP 9.5 (1:200; AbD Serotec) and DBA-rhodamine (1:100; Vector Laboratories) at 4°C overnight, washed several times with PBS, and incubated with donkey anti-rabbit Alexa Fluor 488 (1:200; Molecular Probes) for PGP 9.5-stained slides. For immunocytochemical detection of GATA4, the cells were incubated with goat anti-GATA4 (1:200; Santa Cruz Biotechnology) overnight at 4°C, washed several times with PBS, and incubated with donkey anti-goat Alexa Fluor 488 (1:200; Molecular Probes) for 1 h. Following the staining procedure, samples were mounted with VectaShield mounting media containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and analyzed using a Nikon TE2000 microscope with NIS Elements imaging software (Nikon). The numbers of PGP 9.5-, DBA-, and GATA4-positive cells were counted in five random microscopic fields for each cell population, and the percentages expressing PGP 9.5, DBA, and GATA4 were calculated by dividing the number of labeled cells by the total number of DAPI-positive cells in the same fields.

Recipient Preparation and Transplantation Procedure

Recipients were prepared by injecting BALB/c nude mice aged 4–6 wk with the chemotoxic drug busulfan (44 mg/kg). Endogenous spermatogenesis was abolished with busulfan, and the recipients were treated with busulfan at least 5 wk before transplantation. The gonocyte suspension was prepared by diluting to 1×10^7 cells/ml and measured for cell survival using trypan blue exclusion. Gonocytes were labeled with 32×10^{-6} M fluorescent dye PKH26 (PKH26 Red Fluorescent Membrane Linker Dye; Sigma), which inserts long aliphatic tails into the lipid region of the cell membrane, and the residual dye was washed with basic medium [19]. PKH26-labeled gonocytes were placed on ice until transplantation within 2 h. Recipient mice were anesthetized with ketamine (75 mg/kg, i.p.) and medetomidine (0.5 mg/kg, i.p.) for transplantation. At transplantation, 10 μl ($0.5–1.0 \times 10^7$ cells/ml) of labeled gonocytes per testis was microinjected into the seminiferous tubules via the efferent duct of the recipient mice. After transplantation, the surface tubules of the testes were filled about 80%, and trypan blue was used to confirm the success of the injection.

The spermatogonial transplantation system, in which transplanted donor gonocytes are defined by their ability to produce germ cell colonies in recipient testes, is commonly used to evaluate germline stem cell activity and biological characteristics. Each colony is thought to be clonally derived from a single stem cell [40, 41]. Recipient testes were examined for 4 mo following transplantation with a fluorescence microscope by removing tunica albuginea and gently dispersing the tubules to determine the colony number and colony length of the PKH26-labeled germ cell colonies derived from the transplanted gonocytes. Colonies were identified based on length and shape. Therefore, colonies with measured length of 1 mm or longer and simultaneously those that coated the seminiferous tubule along the inner surface were counted [40].

Lentiviral Transduction of Gonocytes and Transplantation

The lentiviral vector (pLV-TH), which contained the enhanced green fluorescent protein (eGFP) gene driven by the elongation factor 1 (EF1) promoter, was used for transduction [42]. The lentiviral vector was produced according to the method described by Watson and Wolfe [43] with minor modifications using in vitro culture and infection procedures as described previously [32, 37]. To summarize, 1×10^6 of the purified cells were placed in a 4-cm^2 tissue culture well in a feeder-free environment. Donor testis cells were then treated with the pLV-TH virus supernatant in the presence of 4 μg/ml of Polybrene (Sigma) for 7 h at a multiplicity of infection (MOI) of 5, 10, or 20 at 37°C in an incubator with 5% CO_2 in air. After incubation, cells were recovered by vigorous pipetting, washed three times in basic medium, and resuspended in basic medium for transplantation. Transduced gonocytes were transplanted as already described. Recipient testes were examined for 6 mo following the transplantation to examine eGFP expression of the donor gonocyte-derived germ cell colonies. The colonies were analyzed and counted as already described.

Statistical Analysis

All statistical analyses were conducted using SPSS version 15 software (SPSS, Inc.). ANOVA was used to test for differences among groups in gonocyte purity, gonocyte recovery, survival rates, and colony number per 10^5 viable transplanted cells for three different MOIs. Significant differences between means were determined using Tukey honestly significant difference (HSD).

RESULTS

Derivation of Selection and Enrichment Technique Using ECM Molecules and Gelatin

For each negative selection procedure, the purity of gonocytes and their recovery rate were measured (Fig. 1). We first counted the number of gonocytes based on their unique morphology in an unselected testis cell sample that comprised only 2.0% ± 0.2% ($\bar{x}$ ± SEM, n = 5) of the testis cell population (Figs. 1 and 2A). The unselected testicular cell population was then initially selected using discontinuous Percoll density centrifugation with two density layers (20% and 40%). Initially, a broader range of Percoll gradient separation was conducted using 20%, 30%, 40%, 50%, and 60% Percoll, but the majority of gonocytes were collected between the 30%–

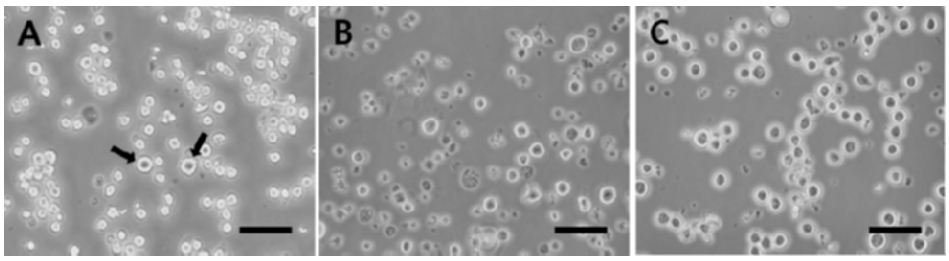

FIG. 2. Phase-contrast micrograph of freshly selected cells from each enrichment procedure. **A**) Unselected testis cells (gonocytes are indicated by arrows). **B**) Cells from negative selection with laminin. **C**) Sequential negative selection with laminin and gelatin. Bars = 50 μm.

40% layer with a purity of approximately 5%. Therefore, the overall Percoll gradient was simplified into a two-layered gradient of 20% and 40%. The gonocyte population was localized to the midlayer, while RBCs and other cell debris were located at the bottom and top, respectively. Gonocyte purity and recovery rates after Percoll selection were 4.9% ± 0.3% and 92.0% ± 1.6%, respectively, and a significant increase in purity was not observed.

In the seminiferous cords of pigs, gonocytes reside in the center until differentiation and show little or low affinity for the basement membrane, while other testis cells display higher affinity for the basement membrane. Therefore, ECM molecules and gelatin, a derivative of collagen, were used for negative selection and enrichment of gonocytes. When the bound population of cells was examined for each selection, only 0%–2% of gonocytes bound to ECM- and gelatin-coated plates, and as hypothesized, most gonocytes remained unbound to ECM- and gelatin-coated plates.

Among the ECM molecules used for negative selection, laminin performed the best and increased gonocyte purity to 24.6% ± 1.9% ($P < 0.05$), with a high recovery rate of 89.6% ± 2.8% (Figs. 1 and 2B). Similar measurements with fibronectin and collagen type IV showed only a marginal increase in purity compared with the unselected cells but maintained a high recovery rate. Negative selection with fibronectin yielded a gonocyte purity of 6.8% ± 0.4%, with a recovery rate of 84.2% ± 0.8%, and the purity and recovery rate for collagen type IV were 9.4% ± 1.0% and 89.8% ± 2.4%, respectively (Fig. 1). Negative selection with gelatin yielded significantly higher gonocyte purity (50.6% ± 2.7%) ($P < 0.05$) compared with laminin, with a high recovery rate of 83.4% ± 3.3% (Fig. 1). Also, survival rates of negative selected cell populations with ECM molecules or gelatin (94.6% ± 2.2% to 95.2% ± 0.8%, n = 5) were not significantly different from the initial cell population (97.8% ± 0.4%, n = 5).

From this result, we hypothesized that we could obtain higher purity by sequential negative selection of gonocytes with laminin and gelatin. As a result, the final gonocyte purity was significantly increased to 80.2% ± 3.0% ($P < 0.05$), with a recovery rate of 77.7% ± 5.8% (Figs. 1 and 2C).

Immunofluorescence of Pig Gonocytes

The effectiveness of the negative selection technique was verified using PGP 9.5 and DBA markers to detect and confirm the isolation of porcine gonocytes. In pigs, PGP 9.5 and DBA are used as germ cell markers to distinguish gonocytes within a testis cell population. PGP 9.5, also known as UCHL 1, is a ubiquitin C-terminal hydrolase and a marker for undifferentiated germ cells in pigs regardless of age [22]. DBA is a lectin expressed only by primitive germ cells in neonatal pig testes and a marker for porcine gonocytes that are progenitor-type stem cells [3]. Immunofluorescence with pig-specific antibodies against PGP 9.5 (green) and DBA (red) showed staining in the cell cytoplasm (Fig. 3, A–D). The stained cells had a large nucleus, and the intensity of stain varied between gonocytes as described by Goel et al. [3]. After Percoll selection, the PGP 9.5- and DBA-positive cell fractions represented 4.6% ± 0.2% and 4.8% ± 1.2% ($\bar{x}$ ± SEM, n = 3), respectively. In contrast, the PGP 9.5-positive fractions represented 74.2% ± 2.7% in unbound and 1.4% ± 1.1% (n = 3) in bound populations (Fig. 3, A and B) on the gelatin plates after negative selection with laminin and were similar in DBA-positive fractions (79.7% ± 5.0% in unbound and 1.0% ± 0.4% in bound [n = 3]) (Fig. 3, C and D). There was no significant difference between morphological (Figs. 1 and 2C) and immunocytochemical (Fig. 3, A–D) evaluation results of gonocyte purities. Also, GATA4 expression was examined in unbound and bound populations on the gelatin plates after negative selection with

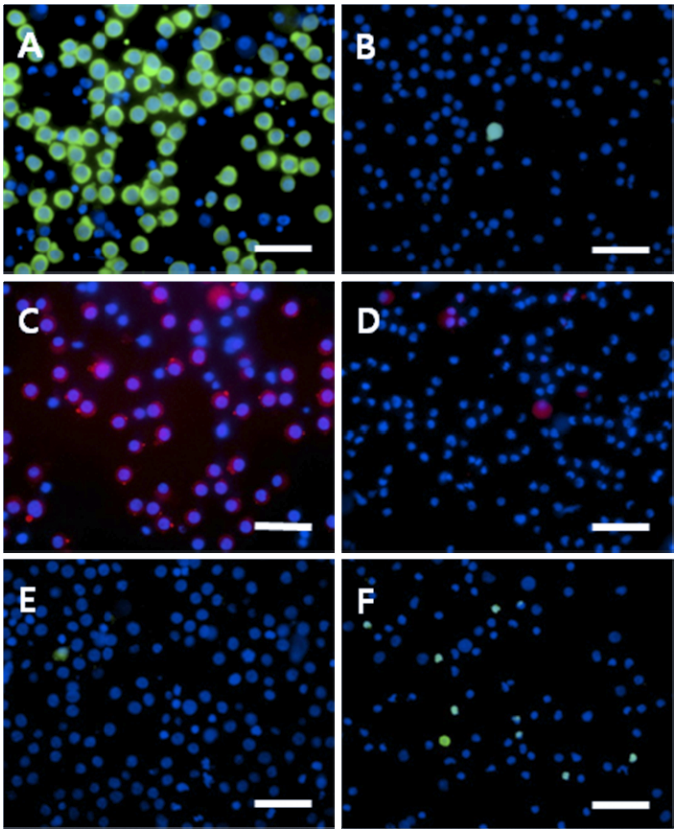

FIG. 3. Immunocytochemical characterization of pig gonocytes. After sequential selection with laminin and gelatin, unbound (unbound cell population on the gelatin plate after negative selection with laminin) and bound (bound cell population on the gelatin plate after negative selection with laminin) cell populations were stained with pig-specific antibody PGP 9.5 (green), DBA (red), or GATA4 (green) and counterstained with DAPI (blue). Images of PGP 9.5-stained cells (cytoplasm) were obtained in unbound (**A**) and bound (**B**) populations. Similarly, DBA-stained cells (cytoplasm) were visualized in unbound (**C**) and bound (**D**) populations. GATA4-stained cells (nuclear) were visualized in unbound (**E**) and bound (**F**) populations. Bars = 50 μm.

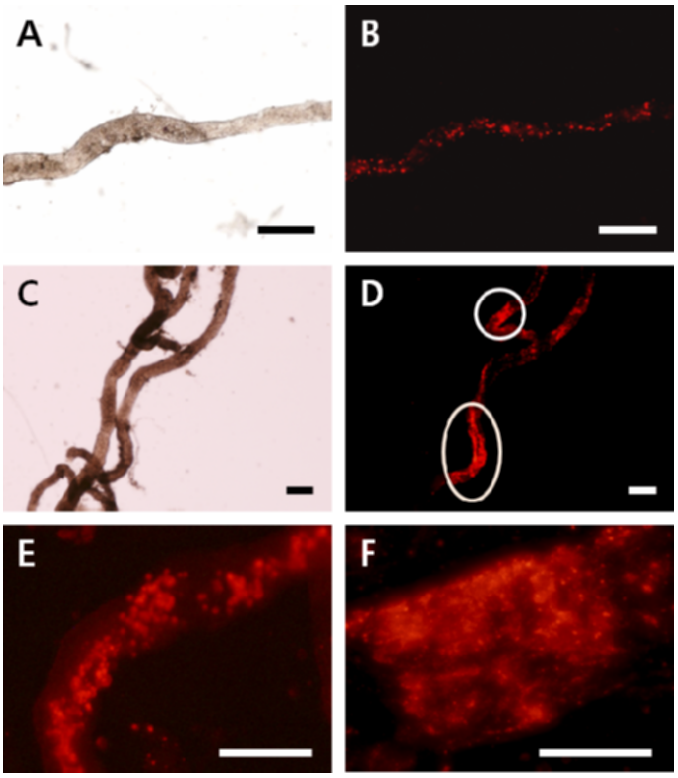

FIG. 4. Detection of donor gonocyte-derived germ cell colonies using PKH26 marker. Microscopic images of PKH26-labeled gonocytes (red) in the seminiferous tubules of nude mouse recipients were obtained at 1 day after transplantation under light (**A**) and fluorescent (**B**) microscopes. Similarly, recipients were analyzed at 2.5 mo after transplantation under light (**C**) and fluorescent (**D**) microscopes (PKH26-labeled germ cell colonies derived from donor gonocytes are circled). The presence of colonies could be easily distinguished at 2.5 mo (**E**), whereas at 4 mo (**F**) after transplantation, the germ cell colonies could not be counted. Bars = 300 μm (**A**–**D**), 200 μm (**E**), and 100 μm (**F**).

laminin. GATA4 is a putative Sertoli cell marker expressed in the nuclei of Sertoli cells but not germ cells [44]. The GATA4-positive (green) fractions represented 1.2% ± 1.0% in unbound and 6.6% ± 1.0% in bound populations (n = 3) (Fig. 3, E and F). As a result, almost all Sertoli cells were eliminated by sequential negative selection with laminin and gelatin, thus confirming the efficiency of the gonocyte purification method.

Transplantation of Purified Pig Gonocytes into Recipient Mice

To ascertain the activity of purified porcine gonocytes, enriched populations of gonocytes from the sequential negative selection with laminin and gelatin were transplanted into the seminiferous tubules of recipient nude mice, and their ability to colonize the recipient testes was examined. PKH26-labeled gonocytes were observed over a 4-mo period after transplantation (Fig. 4).

After PHK26 staining, the survival rate of gonocytes was 80.1% ± 1.0% (32×10^{-6} M PKH26, n = 3), and strong red fluorescence was found in most gonocytes. In a proof-of-principal experiment, after staining with different concentrations of PKH26 dye, the survival rates of gonocytes were found to be 87.4% ± 0.8%, 88.1% ± 0.4%, and 85.2% ± 0.6% (n = 3) at 4, 8, and 16×10^{-6} M, respectively. Although the survival rate increased slightly as the fluorescence dye (PKH26) concentration decreased, a lower concentration of dye did not provide suitable fluorescence intensity for labeling.

TABLE 1. Colonization of recipient mouse testes by transplanted porcine gonocytes.

Treatment[a]	Time of analysis after transplantation (mo)	No. of colonized testes per total transplanted testes	Colonies per 10^5 cells tranplanted[b]
PKH26	2–3	14/14	143.7 ± 9.9
eGFP			
MOI 5	3–6	5/6	20.2 ± 2.6
MOI 10	3–6	6/8	16.5 ± 5.7
MOI 20	3–6	7/8	12.6 ± 3.7

[a] PKH26: n = 7 mice and 14 testes; eGFP: n = 11 mice and 22 testes.
[b] Results (mean ± SEM) from three separate experiments normalized to 10^5 viable transplanted cells/testis.

Initially at the time of transplantation, single cells of gonocytes were dispersed and scattered along the seminiferous tubules (Fig. 4B). Gonocytes then proliferated, and by 2 mo after transplantation, they had formed chains in a z-mesh structure along the seminiferous tubule, which was similar to the colonization pattern described for SSCs by Nagano et al. [40] and Dobrinski et al. [30] for mouse, boar, and bull germ cells (Fig. 4, D and E). Gonocytes colonized seminiferous tubules of all recipient mouse testes between 2 and 3 mo, and 143.7 ± 9.9 colonies ($\bar{x}$ ± SEM, n = 14 testes and three experiments) were formed per 1×10^5 viable transplanted cells (Table 1). The transplanted gonocytes were functionally active and formed colonies that could be detected over the course of a 4-mo period following transplantation (Fig. 4, D–F). During the 4-mo period, many of the PKH26-labeled gonocyte-derived germ cell colonies became dissipated or diluted, and the colonies were detectable only in limited sections of the seminiferous tubule. Furthermore, the remaining PKH26-labeled colonies could not be clearly distinguished and appeared as a blurry aggregate of cells (Fig. 4F). The presence of colonies could not be confirmed later than 4 mo after transplantation.

Lentiviral Vector Transduction of Gonocytes and Transplantation

To evaluate the transduction efficiency of porcine gonocytes, a lentivirus-mediated gene transfer technique was used to introduce eGFP as a donor cell marker [32, 37]. The mouse recipients in which the transduced gonocytes were administered were analyzed over the course of 3–6 mo following transplantation (Fig. 5 and Table 1). The survival rates of gonocytes transduced with the EF1-eGFP lentiviral vector were 94.0% ± 1.5%, 91.0% ± 1.5%, and 92.7% ± 1.8% ($\bar{x}$ ± SEM, n = 3) at MOIs of 5, 10, and 20, respectively. No significant differences were found among the three MOIs (P = 0.47). Also, the numbers of eGFP germ cell colonies generated after transplanting 10^5 viable transplanted cells were 20.2 ± 2.6 (n = 6 testes), 16.5 ± 5.7 (n = 8 testes), and 12.6 ± 3.7 (n = 8 testes) at MOIs of 5, 10, and 20, respectively (Table 1). Consequently, the average number of eGFP colonies from the three MOIs was 16.0 ± 2.4 colonies. Although increasing the MOI slightly decreased the number of eGFP colonies, the decrease was not significant (P = 0.48). The number of transduced colonies was significantly lower compared with the number of PKH26-labeled colonies (143.7 ± 9.9) (Table 1). The transduction efficiency was estimated by comparison with the result obtained for PKH26-labeled colonies and was found to be approximately 11.1% (16.0 of 143.7) at MOIs of 5–20.

The pattern of colonization was as described for PKH26-stained germ cell colonies, but the eGFP colonies along the seminiferous tubules were visible beyond 4 mo and continued to elongate (Fig. 5, B, D, and F).

DISCUSSION

In this study, we integrated the functional biology of ECM molecules and porcine gonocytes to develop a selection and enrichment method to yield both high purity and a high recovery rate of gonocytes. Previously, neonatal porcine gonocytes were successfully isolated using a discontinuous Percoll gradient in which the result produced approximately 70% purity [3], but the recovery rate of gonocytes was not reported. When we tried to select gonocytes using a Percoll gradient as described by Goel et al. [3], we found it difficult to enrich gonocytes, possibly because of technical and handling differences. In our study, we simplified the Percoll procedure using two layers (20% and 40%). This served as an effective method in initially separating gonocytes from RBCs and other cellular debris. To specifically select gonocytes, a negative selection method was employed using ECM molecules. This was based on evidence that gonocytes localize at the center of seminiferous cords in neonatal testes, while Sertoli cells and other spermatogenic cells reside in the basement membrane of prepubertal and adult testes and thus display affinity for ECM molecules [25]. Laminin is known to associate with postmigratory PGCs, collagen type IV binds keratinocytes, and fibronectin binds bone marrow stem cells [45–47]. A similar negative relationship was hypothesized between gonocytes and gelatin because gelatin is a derivative of matrix constituents, assists in attachment of cells to a surface, and allows them to proliferate [48, 49].

In our study, the selection with laminin resulted in the highest purity among the ECM molecules tested. A previous study [14] in mouse identified α_6- and β_1-integrins as surface markers on SSCs. These integrins act as a laminin receptor [50] and mediate cell attachment to laminin. In particular, laminin-binding β_1-integrin shows affinity to Sertoli cells and several stages of germ cells, including SSCs. Because gonocytes remain in the center of seminiferous cords and do not show affinity to the basement membrane, while other cells in the monolayer of seminiferous cords in neonatal pigs do show affinity to it, laminin proved to be an effective negative selection tool.

The serum content of the medium may facilitate the attachment of gonocytes [3] and thus negate the selection method with gelatin. Therefore, gonocytes were initially incubated on gelatin for various periods to determine the period that would yield the highest purity and viability (data not shown). We found that these requirements were fulfilled when gonocytes were incubated for 12 h. Using gelatin, a significantly higher purity was achieved compared with using ECM molecules alone. Gelatin has often been used to coat glass or plastic surfaces and enhance attachment of various cell types for experiments such as in vitro culture of muscle cells, endothelial cells, and some mouse teratomas [48, 49], and gelatin serves a similar role as denatured collagen in conjunction with fibronectin or laminin. Therefore, gelatin may have the capacity to attach more numerous cell types than ECM molecules. However, further studies on properties of gelatin are needed to fully explain the obtained high purity. The possibility of a positive relationship between gonocyte purity and incubation time was ruled out because such a relationship was not observed when tested with ECM molecules.

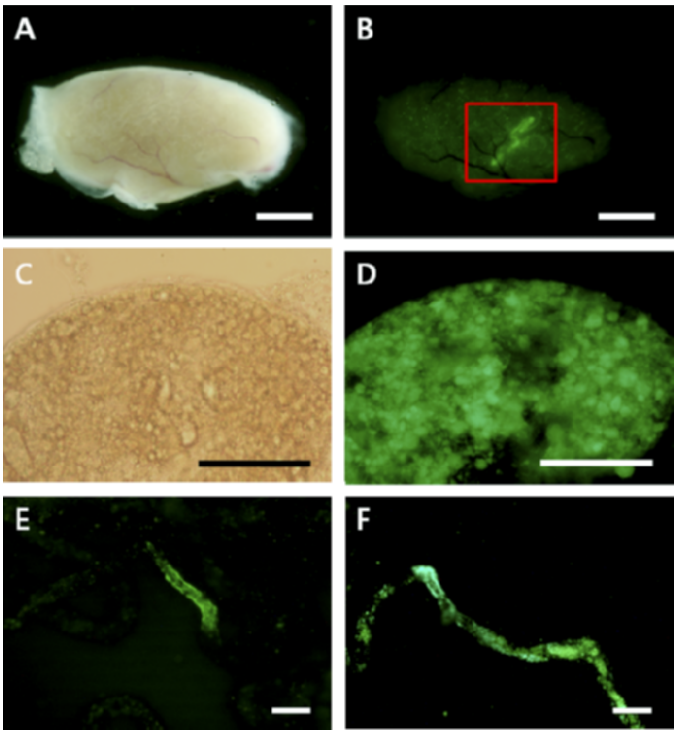

FIG. 5. The eGFP-expressing colonies (green) as a result of donor-derived colonization by lentiviral transduction of pig gonocytes, followed by their transplantation into recipient nude mouse testis. Gonocyte-enriched donor populations were exposed to lentiviral vector EF1-eGFP at 7 h at MOIs of 20 (**A**–**E**) and 5 (**F**), followed by transplantation into recipient nude mouse testes. Whole-mount images of a testis at 6 mo after transplantation were obtained under light (**A**) and fluorescent (**B**) microscopes (the box indicates eGFP colonies). Microscopic images of seminiferous tubules were obtained under light (**C**) and fluorescent (**D**) microscopes at 6 mo after transplantation. The elongation of donor gonocyte-derived germ cell colonies was examined under a fluorescent microscope at 3 mo (**E**) and 6 mo (**F**) after transplantation. Bars = 2 mm (**A** and **B**), 100 μm (**C** and **D**), and 200 μm (**E** and **F**).

Based on these results, an optimal selection and enrichment system was established with combined use of laminin and gelatin in a negative selection method. This two-step selection method yielded a gonocyte purity of 80.2% ± 1.4%, which was similar to the purity of 78% that Marret and Durand [39] obtained after selection with two-layer Percoll (20% and 40%) and negative selection by plating on a culture dish for 12 h but was higher than the purity of 70% described by Goel et al. [3] obtained after selection with a discontinuous Percoll gradient. In terms of the recovery rate of gonocytes, a significant difference was not observed, and a highly pure population of gonocytes could be isolated without significant cell loss.

To further demonstrate the effectiveness of the selection technique for porcine gonocytes, PGP 9.5 and DBA markers were used to identify purified gonocytes. PGP 9.5 is specifically found in reproductive gonads and neuronal cells [51], and its expression pattern of PGP 9.5 differs among rodents, nonrodents, and other species. In rodents such as mice, spermatogonia and Sertoli cells both express PGP 9.5, while in nonrodents such as bovine, PGP 9.5 is expressed in gonocytes and spermatogonia [52–54]. In pigs, PGP 9.5 expression was documented in gonocytes and spermatogonia but not in Sertoli cells [22]. As a result, gonocytes with PGP 9.5 marker were more prevalent, and the effectiveness of the enrichment and negative selection procedure with laminin and gelatin was confirmed. In previous studies [3, 55], DBA was found to be

specific to the surface of porcine PGCs, and this was maintained in gonocytes and primitive spermatogonia. Our results show that DBA is specific to porcine gonocytes, and the proportion of DBA-expressing cells rose in parallel with our morphological observation data after the enrichment. Also, the expression of GATA4, a specific marker for Sertoli cells, was observed in a few cells of the enriched gonocyte population obtained after negative selection with laminin and gelatin.

Spermatogonial transplantation of transgene-expressed donor cells and quantification of colonies in recipient seminiferous tubule [56] represent a common method used to assess the efficiency of enrichment and mGSC functionality in rodents. However, the efficacy of spermatogonial transplantation has been somewhat precluded when applied to pigs because of a lack of suitable transgene-marked donor cells and because large recipient testis hinders quantitative analysis of colonization. Thus, we refined the pig-to-nude mouse xenotransplantation assay based on previously reported transplantation of testis cells from rats [13, 28], rabbits [29], dogs [29], pigs [30], and bulls [30] into recipient mouse testes.

In this study, the activity of purified donor gonocytes was retrospectively characterized through transplantation of fluorescent-labeled gonocytes with PKH26 into the testes of recipient nude mice and analysis of their ability to colonize within the seminiferous tubules. Gonocytes and primitive SSCs are, by functional definition, the only cell type in the testis that may proliferate and expand within the testis of a recipient to form germ cell colonies. Donor pig gonocytes appeared to undergo the first phase of spermatogonial expansion and formed detectable colonies that were visible up to 3 mo after the xenogeneic transplantation, as was the case for other similar studies [29, 30]. Consequently, conservation may exist for molecules required for the interaction between pig gonocytes and mouse Sertoli cells. The number of PKH26-positive cells decreased over time, and after 4 mo the PKH26-labeled cells were no longer visible. It is possible that germ cells derived from the donor gonocytes continued to proliferate beyond 4 mo but could not be visualized because of the limitations of PKH26, which is a fluorescent marker with a transient nature and becomes diluted as the cell divides [57]. Therefore, a longer-lasting or permanent labeling system must be established to be used in donor cells.

To further analyze the ability of gonocytes to be genetically modified, an eGFP transgene was inserted into the genome of gonocytes using lentiviral vectors. The eGFP transgene does not have the limitations described for PKH26 and can be used to detect germ cell colonies derived from donor gonocytes beyond 6 mo. Once gonocytes were transduced and transplanted, they successfully colonized recipient testes, and eGFP colonies proliferated along the length of the seminiferous tubule up to 6 mo after transplantation (Fig. 5). Viral transduction, including lentivirus, was previously successful in modifying the SSC genome in mouse and rat, in which the transduction efficiency was recorded to be between 0.5% and 20% [31, 37, 58, 59]. In our study, the estimated transduction efficiency was calculated by dividing the number of eGFP colonies that were transduced using lentivirus by the total number of PHK26-labeled colonies (Table 1). Although our estimated transduction efficiency of 11.1% falls within the documented range without any notable toxic effects, it is low compared with a previous study [32] among rats, in which the transduction efficiency was approximately 45%. It is unclear whether transducibility of gonocytes differs from that of SSCs because their developmental timing differs. It is possible that the developmental stage of gonocytes may be the limiting factor in terms of transducibility, but further research is needed.

Although the pig-to-nude mouse xenotransplantation in this study appears to be an assay of pig gonocytes as mGSCs, complete spermatogenesis from the transplanted cells was not observed, and the efficiency of the assay could not be measured. Therefore, as a functional end point, complete spermatogenesis of donor pig mGSCs can be demonstrated in a pig-to-pig transplantation paradigm.

In conclusion, the present investigation shows the derivation of an efficient selection and enrichment technique with laminin and gelatin, which yields highly purified active and viable pig gonocytes that can produce germ cell colonies in the seminiferous tubules of immunodeficient nude mice upon xenotransplantation. The purification technique developed in this study has its foundation in the biology of interaction between germ cells and the basement membrane. The selection and enrichment technique of gonocytes, its analysis by transplantation, and genetic modification using lentivirus described in this study represent an efficient and unique method in pigs. These results could be extended to purify and enrich gonocytes of other species for in vitro and in vivo studies and to aid in genetic modification of mGSCs of livestock species.

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