Characterizations of Cell Lineage Markers in the Bone Marrow Cells of Recloned GFP Pigs for Possible Use of Stem Cell Population

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ABSTRACT

Two piglets and one juvenile pig were used to investigate closely what types of cells express green fluorescent protein (GFP) and if any, whether the GFP-tagged cells could be used for stem cell transplantation research as a middle-sized animal model in bone marrow cells of recloned GFP pigs. Bone marrow cells were recovered from the tibia, and further analyzed with various cell lineage markers to determine which cell lineage is concurrently expressing visible GFP in each individual animal. In the three animals, visible GFP were observed only in proportions of the plated cells immediately after collection, showing 41, 2 and 91% of bone marrow cells in clones #1, 2 and 3, respectively. The intensity of the visible GFP expression was variable even in an individual clone depending on cell sizes and types. The overall intensities of GFP expression were also different among the individual clones from very weak, weak to strong. Upon culture for 14 days *in vitro* (14DIV), some cell types showed intensive GFP expression throughout the cells; in particular, in cytoskeletons and the nucleus, on the other hand. Others are shown to be diffused GFP expression patterns only in the cytoplasm. Finally, characterization of stem cell lineage markers was carried out only in the clone #3 who showed intensive GFP expression. SSEA-1, SSEA-3, CD34, nestin and GFAP were expressed in proportions of the GFP expressing cells, but not all of them, suggesting that GFP expression occur in various cell lineages. These results indicate that targeted insertion of GFP gene should be pursued as in mouse approach to be useful for stem cell research. Furthermore, cell- or tissue-specific promoter should also be used if GFP pig is going to be meaningful for a model for stem cell transplantation.

(Key words : Recloned GFP pig, GFP expression, Cell lineage markers, Bone marrow cells)

INTRODUCTION

Generation of reporter gene-inserted animals has provided a wide applications in the fields of basic biology and applications. Thus, the studies on feasibility of foreign gene insertion, consistency of the inserted gene expression in various tissues, cell lineage and tagging of specific cells and tissues and etc. have been reported. In traditional gene injection into fertilized eggs, there may be some mosaic expression in the expression due to the latency of gene incorporation during early cleavage of the embryo. Therefore, cloning through nuclear transfer by using somatic cells transfected with foreign genes, and followed by visual verification of gene expression may avoid such a complication in interpreting expression of transgene in the embryos and their resulting animals. Since the reported animal cloning, in particular, pig have been examined for their gene expression in whole body and various tissue in a macroscopically, convincing ubiquitous gene expression using green fluorescent protein (GFP) gene in recent years (Park *et al.*, 2001a; Cabot *et al.*, 2001; Kang *et al.*, 2007). However, the low resolution for GFP visualization in such studies may mask details of GFP expressions in cells and specific cell lineages of cells. Furthermore, it has not been determined whether primary cells recovered from the cloned GFP pigs are expressing GFP in all cell types or whether there is any differential expressions among the cell type, and lastly, whether these GFP expressions persists *in vitro* conditions where the three dimensional structure of the cells are destroyed during

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in vitro culture. In this study, we examined such possibilities and if there are some consistencies in GFP expression in bone marrow cells of re-cloned GFP pigs because the marrow contains many somatic stem cells including hematopoietic stem cells, mesenchymal stem cells and other mesodermal derivatives. This data may evaluate usefulness of cloned GFP pig in establishing preclinical research model for stem cell transplantation.

MATERIALS AND METHODS

Animals

The use of animals and the procedure of recovering tissue and cells in these experiments were approved by the Committee Review Board of Animal Experimentation, Korea University. Recloned animals or tissue were provided from MGEN Inc. (Seoul). These pigs were produced by using somatic cell nuclear transfer, and ear fibroblasts of GFP pigs (Kang *et al.*, 2007) were used as donor somatic cells. Bone marrow cells were collected from three animals including two neonatal Landrace piglets (7 days old) and one juvenile Landrace pig (80 days old) tissues.

Recovery of Bone Marrow Cells

Bone marrow cells (BMCs) were recovered from tibia using phosphate buffered saline in a sterile manner. Cells were dissociated with a brief exposure to trypsin-EDTA in PBS and collected after centrifugation at 1,000 rpm for 7 min, followed by two more washes with PBS. The individual cell pellets were either used for examination for GFP expression or culture for further determination of expression patters during *in vitro* culture up to 2 weeks as described below.

Cell Culture

Suspended MBCs were cultured in 100 mm plastic dishes containing Dulbecco's modified Eagle's medium (DMEM, 12100/046-GibcoBRL), including 10% fetal bovine serum (FBS, PAA) in an atmosphere of 5% CO_2 in air under saturated humidity at 37℃ for up to 14 days. The medium was changed every 2 days, and the cells were subcultured at every 4 days in. To analyze cell lineages among the GFP-expressing BMCs, aliquots of cells were cultured on gelatin-coated coverslips in 24-well dishes for 2 weeks before the analysis.

Determination of GFP Expression in BMCs

Immediately after recovery from the cell pellets, individual BMCs were examined for visible GFF expression patterns among cell types and intracellular GFP distributions. Randomly selected field of the cells were recorded under a fluorescence microscope (see below). Five recorded image were used for dada analysis for counting cells and presenting typical GFP expression patterns among the primary BMCs from each individual animal. The number of cells expressing GFP was presented as mean ± standard deviation in a graphic form by counting over 600 cells from each experiment.

Immunocytochemistry for Cell Lineages

Cells on coverslips were washed and fixed in 4% para-formaldehyde in PBS for 20 min at room temperature, followed by extraction using 0.05% Triton X-100 in HPEM buffer for 1 h for cytoskeletal visualization. After extensive washing three times for 15 min each in PBS containing 0.02% Tween-20, the cells were incubated with cell lineage marker antibodies, including SSEA-1, SSEA-3, CD34, nestin and GFAP (1:200 dilution; Sigma), and then with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200 dilution; Sigma). Nuclei were then stained with 20 μg 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma)/ml at a final concentration for 20 min at room temperature. The cells were observed after application of an antifading agent, 20 mg/ml *p*-phenylenediamine in carbonate buffer (pH 8.3) containing 10% (v/v) glycerol, for immunofluorescence analysis under a fluorescence microscope (Zeiss, Oberkochen, Germany). Cell images were recorded with a digital camera (CoolPix 990, Nikon Co., Japan) attached to the microscope. The images were used to quantitate cells showing specific staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and PCR of Genomic DNA

Total RNAs were isolated from the livers of cloned and wild type pigs due to the lack of BMC for further analysis to verify GFP insertion and expression. The cells were washed twice with diethyl pyrocarbonate-treated PBS by centrifugation, then lysed in 100 μl TRIzol Reagent (Invitrogen, Groningen, The Netherlands). The mixtures were then processed with chloroform, isopropanol, and 75% ethanol before adjustment of the RNA concentration and storage as aliquots at −70℃ until use (Chomczynski and Sacchi, 1987).

To examine the expression of GFP, total RNA was treated with 1 U RQ1 DNAse (Promega, Madison, WI, USA) for 15 min in DNase buffer (400 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 100 mM NaCl) to eliminate any contaminating genomic DNA. After heat-inactivation, 20 μl of DNase-treated RNA, 4 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTPs, 2 μl of 10×RT buffer, 20 U recombinant RNasin ribonuclease inhibitor (Promega), 5 μg of oligo(dT), and 12 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega) were mixed and reacted at 42℃ for 1 h.

Total RNAs from equal numbers of cells subjected to different treatments were reverse transcribed to pro-

duce complementary DNA (cDNA). RT products were used as the templates for PCR with GFP (forward: 5'-GGCGACGTAAACGGCCACA-3'; reverse: 5'-CGGTCA-CGAACTCCAGCAGG A-3') and GAPDH primer pairs (forward: 5'-CATCATCCCT GCTTCTACCG-3'; reverse: 5'-CCTGCTTCACCACTTTCTTG-3') under standard conditions to amplify the transcripts of selected genes involved in lipid metabolism. Briefly, 1 μl RT product, 2.5 μl 10× buffer, 2 μl of 2.5 mM dNTPs, 1.5 μl of 25 mM $MgCl_2$, 1.0 μl of 20 μM forward and reverse primers (Bio-Rad, Hercules, CA, USA), and 0.1 μl of *Taq* DNA polymerase (Promega) were mixed, and the volume of the reaction mixture was adjusted to 25 μl with distilled water. PCR reactions were performed under similar conditions, except that annealing temperatures differed: denaturation at 94℃ for 5 min, 30 cycles of annealing at 94℃ for 30~65 sec depending on the primer pair, extension at 72℃ for 10 min, followed by an extra final 10 min of extension. The RT-PCR products were separated electrophoretically and the bands on the gels were recorded with a Gel-Doc 2000 (Bio-Rad Laboratories, Milan, Italy).

Gnomic DNA was also isolated from the liver tissue by a standard method and similarly used as in cDNA for amplification using the same GFP primers as in RT-PCR. The PCR products recorded were processed for semi-quantitation by Image J (NIH, http://rsbweb.nih.gov/ij/ download.html).

Western Blotting Analysis for GFP

To determine whether the tissues from the recloned GFP-pigs, some abundant tissues such as skin and liver were selected due to the lack of BMC cells for further analysis to verify GFP expression. One of primary cells (porcine ear skin fibroblast, pEF) was also used for analysis after expansion and storage. Total proteins were prepared from cell and tissue lysates in a lysis buffer containing 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1× protease inhibitor cocktail. Equal amount of protein lysates were used to quantify released total proteins by Bradford dye-binding procedure (Bradford, 1976) using a Bio-Rad kit (Bio-Rad Laboratories, Inc.) and followed by separation on 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis. After transferring to nitrocellulose membranes, anti-β-actin, anti-GFP mouse monoclonal antibody, and GAPDH rabbit polyclonal antibodies were used, followed by a rabbit anti-mouse antibody or goat anti-rabbit antibody conjugated to alkaline phosphatase (Abcam Plc., Cambridge, U.K.). As an internal control, anti β-actin antibody was also similarly used to compare relative GFP expression between clones and wild type pigs. The immunoreactive bands were detected using a color reaction solution containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) by additions of NBT and BCIP within 15 min. The bands were quantified as relative ratios of GFP/β-actin or GAPDH expression using Image J.

RESULTS

Different Extent of GFP Expression among the Recloned GFP Pigs

With the advent of utilizing a simple visible reporter gene, GFP, many applications would be possible. In this study, we focus on possible use of GFP-tagged stem cell population in the bone marrow where many different stem cell populations are known to colonize in recloned GFP pigs.

To determine firstly the extent of GFP expression in the recovered BMCs of individual recloned pig, and secondly, compare the GFP expression among three recloned pigs, the cells were examined and used for quantitation for GFP expression immediately after the recovery. Typical GFP expressions of the BMCs from three clones of pigs are summarized in Fig. 1. The first thing noticed was that the extent of the GFP intensity is varying in the BMCs of an individual clone ranging from strong to no visible GFP expression (Fig. 1A). The GFP expression was also different among the three clones, ranging from scarce to abundant GFP-expressing cells found in different animals. To compare the GFP expression in the BMCs of the clones, visible GFP expressions were scored in recorded images of 5 random fields of the cell preparations in three replicates. The total numbers of cells expressing GFP scored over 600 cells were compared and presented in a graphic form (Fig. 1B). The three different clones showed markedly different proportions of GFP expression, ranging from only 2% to over 90% depending on individual clone.

Cellular Distribution of GFP Expressed in GFP-positive Cells

From above experiments, we found cellular distribution of GFP expressed appears to be variable. Therefore, GFP expression was examined more closely at intracellular level to determine whether there is any predominant GFP expression pattern within cells. The cells were examined in detail for GFP distribution within the positive cells regardless of cell morphology. After examining the images recorded, three typical GFP expression patterns were found: diffused expression in whole cytoplasm with strong expression in the nucleus, restricted expression in Golgi complex and diffused expression throughout the cytoplasm and the nucleus (Fig. 2). These three expression patterns are found in all three clones although the attempt was not made to quantify

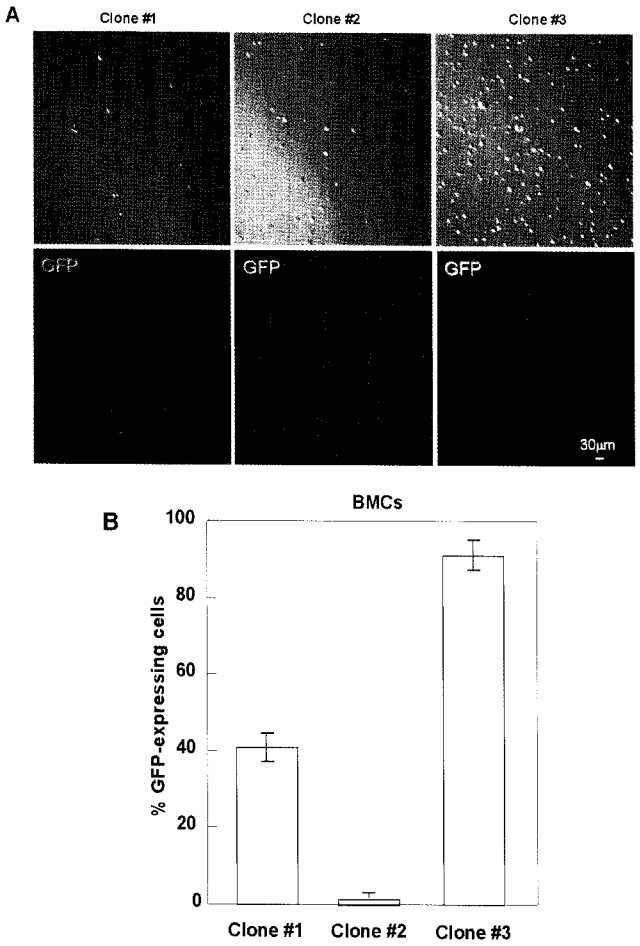

Fig. 1. GFP expression in the bone marrow cells of the recloned pigs immediately after collection. Tibial bone marrow cells were recovered from each animal and washed three times with PBS, followed by washing twice with DMEM containing 10% fetal bovine serum. Representative bright and the corresponding fluorescence fields were recorded and shown here (A). For quantitaion of visible GFP-expressing cells five fields were randomly selected, scored for GFP expression over 600 cells in each count and expressed as a graph (B). The experiments are carried out in three replicates.

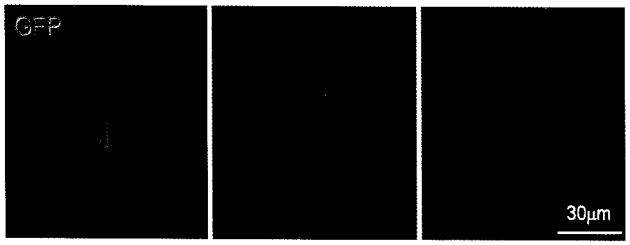

Fig. 2. Typical expression patterns of GFP found in the bone marrow cells of clone pigs used. Three major patterns of the expressed GFP distribution were found in cultured bone marrow cells. Each figure was magnified from Fig. 2, 4 and 5, respectively. Both strong in the nucleus and moderate expression in the cytoplasm (left), marked peripheral expression in the endoplasmic reticulum (middle), and diffused expression throughout the cytoplasm (right) can be drawn from various cell types, respectively.

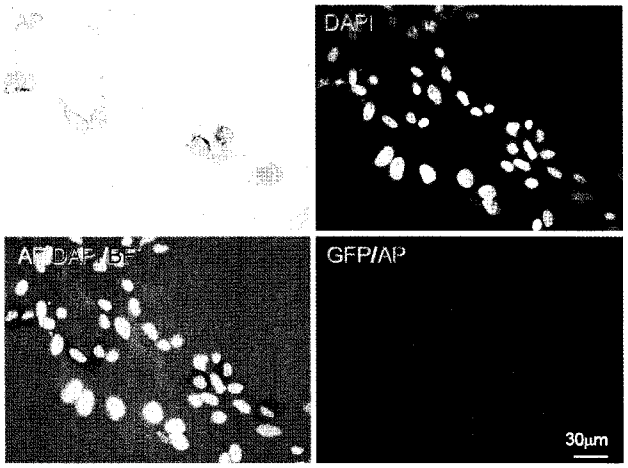

Fig. 3. Concurrent expression of alkaline phosphatase (AP) and weak GFP in bone marrow cells at 14 DIV. Bone marrow cells were recovered from a recloned juvenile pig and cultured for 2 weeks. They were then examined for cell lineage by cytochemical staining of AP, followed by a DAPI counter staining (top and bottom left). Cytoplasmic staining peripherally in fibroblast-like mesenchymal cells expressed GFP and AP concurrent only in the peripheral vesicles within cells with no GFP expression in the nucleus (top and bottom right). Among the weak GFP-expressing cells, only fibroblast-like mesenchymal cells are AP-positive. The brightfield (BF) images were obtained either alone (top left) or with a fluorescent field together under a dim light as in bottom left.

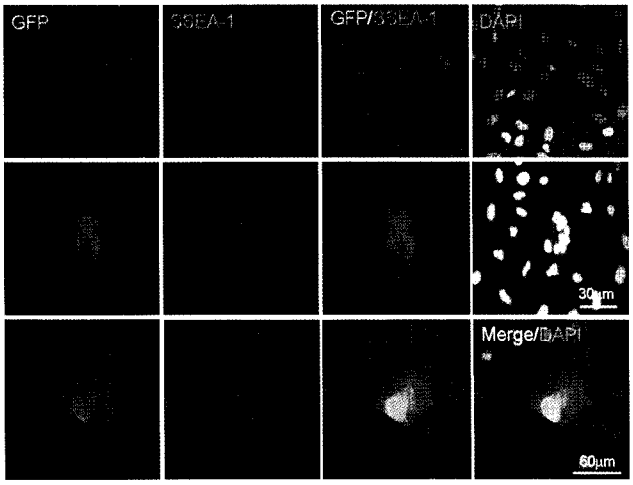

Fig. 4. Concurrent expression of SSEA-1 in GFP-positive bone marrow cells at 14 DIV. Bone marrow cells were recovered from a recloned juvenile pig and cultured for 2 weeks. They were then examined for cell lineage by staining with an SSEA-1 antibody, followed by a secondary antibody conjugated to TRITC (red). Representative cells depicting concurrent expression of GFP and SSEA-1, in which different types of cells are expressing GFP only in the peripheral vesicles within cells without any GFP expression in the nucleus (upper panel). On the contrary, the bone marrow cells are expressing GFP intensively within whole cytoplasm, including fiber-like structures and their nuclei (lower panel).

them. However, the first pattern is rare, and the rest of patterns are frequent in GFP-positive cells of the BMC examined.

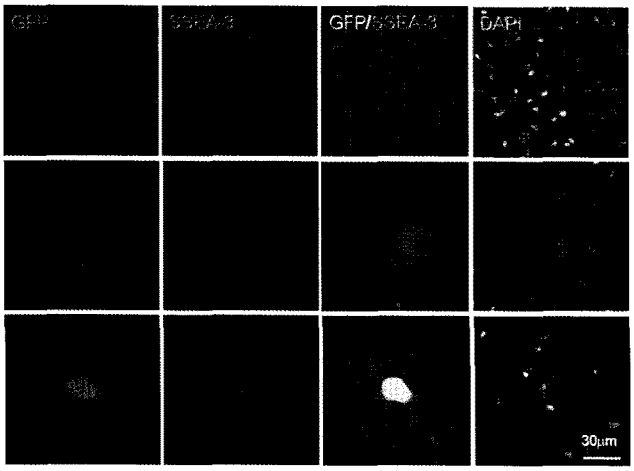

Fig. 5. Concurrent expression of SSEA-3 in GFP-positive bone marrow cells at 14 DIV. Bone marrow cells were similarly treated as in the legend of Fig. 4. They were then examined for cell lineage by staining with an SSEA-3 antibody, followed by a secondary antibody conjugated to TRITC (red). Mild GFP expression was found in a cell population of large cell nucleus with fibrous cytoskeletal structures in the middle panel. Strong expression of GFP was also seen in a different cell population within whole cells including the nucleus and cytoskeletal components in the bottom panel.

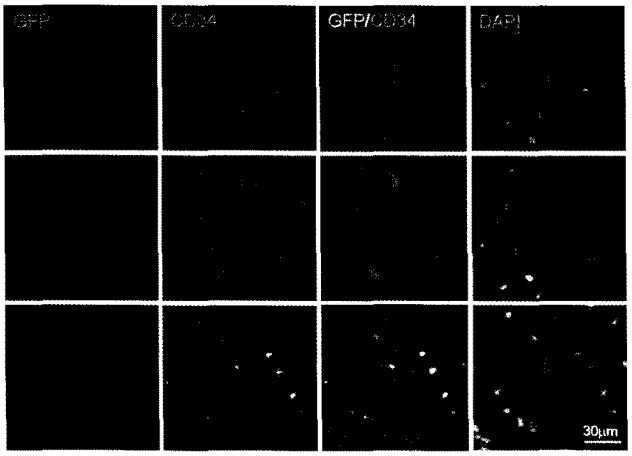

Fig. 6. Different expression patterns of CD34 in GFP-positive bone marrow cells at 14 DIV. Bone marrow cells were similarly treated as in the legend of Fig. 4. They were then examined for hematopoietic stem cell lineage by staining with a CD34 antibody, followed by a secondary antibody conjugated to TRITC (red). Representative cells depicting concurrent expression of moderate GFP and CD34, in which different types of cells are expressing GFP only in the peripheral vesicles within cells without any GFP expression in the nucleus (top panel). On the contrary, weak GFP- expressing cells are also show with concurrent CD34 expression variably depending on the cell types in the bottom panel.

Expressions of Cell Surface Markers, SSEA-1, SSEA-3 and CD34 in GFP-positive Cells

To evaluate possible use of GFP-tagged BMCs for a pre-clinical stem cell transplantation as a animal model, the cells from clone 3 were cultured up to 14 days *in vitro* (DIV) on gelatin-coated coverslips prior to analyzing for alkaline phosphatase expression using cytochemical localization of the enzyme. Alkaline phosphatase was concurrently expressed among GFP-expressing BMCs. The proportions of the coexpressed cells were small, and their GFP expression was weak among other cells.

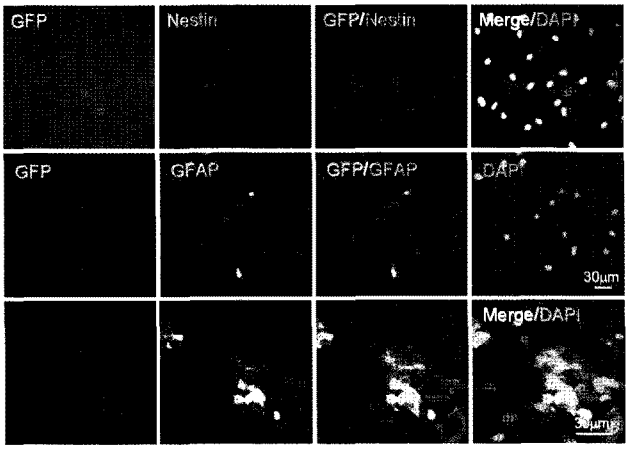

Fig. 7. Patterns of nestin expression in GFP-negative bone marrow cells, and concurrent GFAP expression in GFP-positive cells at 14 DIV. Bone marrow cells were similarly treated as in the legend of Fig. 4. They were then examined for neural cell lineage markers, including nestin and GFAP. Representative cells show no expression of GFP, but with nestin expression (top panel). However, GFAP were concurrently seen in either cells with diffused GFP expression throughout the cytoplasm (middle panel) or those with peripheral GFP expression in the cytoplasm (bottom panel).

To examine stem cell surface antigens prevail in proliferative cells, SSEA-1 and SSEA-3 were chosen, the cells on coverslips were stained with anti-SSEA-1 antibody, followed by labeling with a secondary antibody conjugated to TRITC. GFP-positive cells with different patterns of expression showed basically a similar pattern of SSEA-1 expression in their Golgi complex regardless of GFP expression patterns (Fig. 4). All the cells with three different GFP expression patterns were strong expression of SSEA-1. Similarly, GFP-positive cells with different patterns of expression showed a similar pattern of SSEA-3 expression in their Golgi complex to those of SSEA-1 regardless of GFP expression patterns (Fig. 5).

Another cell lineage surface marker, CD34, was examined similarly in GFP-expressing cells since this marker is known for hematopoietic stem cells. Small proportions of cells gave a similar staining pattern of CD-34 expression to those of SSEA-1 and SSEA-3 expression, showing restricted expression in their Golgi complex (Fig. 6). However, majority of the cells expressing CD34 showed confined membrane distribution of CD34 on their cell surfaces. The CD34-positive cells on the cell surfaces appeared smaller in their size. However, two types of cells were found CD34-positive cells with microglia-like cell and round shapes of cells. Both types of cells shared a small nucleus with strong expression

of CD34 with intermediate intensity of GFP expression.

Expressions of Neural Cell Lineage Markers in GFP-positive Cells

To define whether GFP-positive cells are expressing any neural cell lineage markers, cells were also stained for nestin and GFAP antibodies since they are known to express prevalently in BMCs. Similarly prepared cells were extracted with 0.05% Triton X-100 in HPEM buffer, followed by antibody labeling. No nestin-positive cells expressed GFP; however, GFAP-positive ones were found in two types of GFP-expressing cells (Fig. 7). Similar morphology of cells to those CD34-positive cells was one type with GFP distribution within whole cell, and the other type was the cells with GFP distribution within Golgi complex.

Verification of Insertion and Expression of GFP Gene in the Tissues of Recloned Pigs

Verification of genetic components (GFP) in the cells and tissues of the clones demonstrated that positive PCR products of GFP fragment targeted from genomic DNAs, RT-PCR products from mRNAs, and protein were demonstrated and expressed similarly in the cells and tissues examined (Fig. 8).

DISCUSSION

Since pig cloning have been reported for the first time (Betthauser *et al.*, 2000; Onishi *et al.*, 2000), genetically modified somatic cells were used to evaluate and develop technologies involving somatic cell transfection, selection and cloning of the selected somatic cells. In this study, we evaluated whether the GFP-tagged BMCs of recloned pig would be useful for stem cell population present in the bone marrow as a potent cell source in a preclinical animal model for stem cell therapy (Burt *et al.*, 2003: Moelker *et al.* 2006; Park *et al.*, 2009; Herbst *et al.*, 2009). The usefulness of GFP-tagged stem cells has been addressed in many previous reports in the mouse, allowing easy visualization of robust GFP expression, trace of cell fate for transplanted GFP-tagged cells, and determination of the capacities of the transplanted cells for repair or regeneration of damaged tissues in live state of cell culture and tissues and organs *in vivo* (Göthert *et al.*, 2005; Bratincsák *et al.*, 2007; Klassen *et al.*, 2008; Mchedlishvili *et al.*, 2007).

Over the recent years, many GFP pigs have been produced by either gene injection or viral infection, and cloning of the transfected somatic cells for the expectations of contributing to biomedical researches including feasibility study for the working complex technologies involved in transgenesis, stem cell and xenotransplantation studies (Cabot *et al.*, 2001; Park *et al.*, 2001a; Park *et al.*, 2001b; Park *et al.*, 2001c; Kang *et al.*, 2007; Kurome *et al.*, 2006; Klassen *et al.*, 2006; Vodicka *et al.*, 2008). However, no study has been forwarded to demonstrate distribution of GFP expression at cellular level and whether the ubiquitous promoters used for GFP expression, including cytomegalovirus (CMV) and chicken actin promoters (CAG), are robustly working

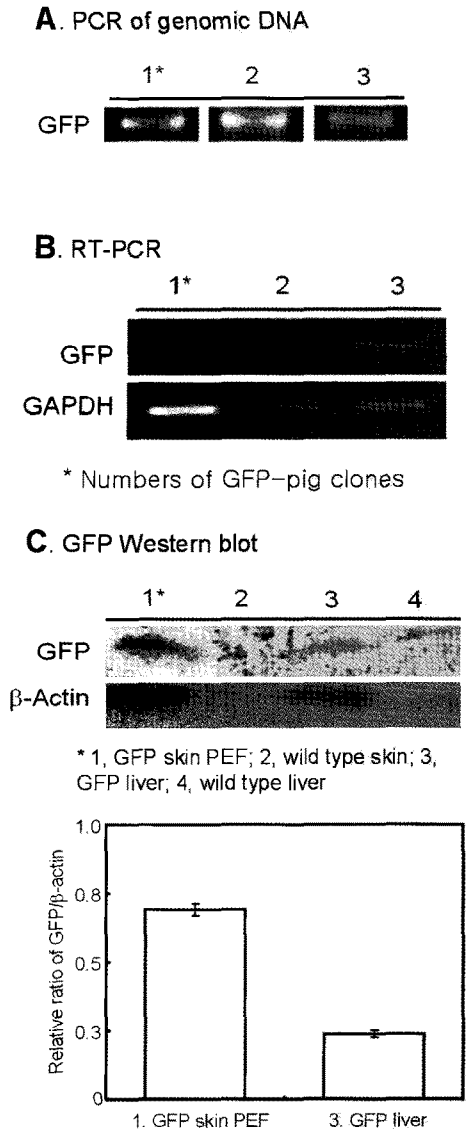

Fig. 8. Verifications of GFP insertion and GFP transcription in genomic DNAs and mRNAs of the recloned pigs, respectively. Genomic DNA was amplified using a pair of specific GFP primers at 30 cycles (A). The identical primers were used for RT-PCR of the animals (B). After SDS-PAGE and subsequent transfer, the nitrocellulose membrane was incubated with an anti-GFP antibody, followed by a secondary antibody conjugated to alkaline phosphatase which was detected by NBT and BCIP for color reaction. The positive bands were quantitated (C).

as traceable in all tissue and cells in GFP pig produced although visualization of GFP expression in whole animals, tissues and organs have been studied at low resolutions (Park *et al.*, 2001a; Kang *et al.*, 2007; Klassen *et al.*, 2008).

Our results reveal for the first time the presence of heterogeneity of the GFP expression among different cell populations in the BMCs, and varying GFP distribution within cells of individual recloned pig in great detail (Figs. 1 and 2). Although the three recloned pigs were derived from single ear skin fibroblasts of different cloned animals, it would be thought that the cells should show similar GFP expressions in one type of cells and in the distribution of GFP expressed within cells. It is expected that all the cells harboring the pCAG-GFP gene be identical in copy number of the transgene gene and methylation status at initial stage of embryonic development. As cell differentiation undergoes during embryonic and fetal development, the methylation status may be regulated in accordance with directions of cell differentiation, thus generating different GFP expression among different cell types of tissues, organs of both cloned and recloned animals. However, it would be difficult to explain why cellular distribution of GFP expression varies. Normally, GFP expressed is delivered to the cytoplasm, but the nucleus unless an NLS sequence is inserted within the vector or there is other binding proteins carrying GFP to the nucleus (Stochaj *et al.*, 2000).

Since porcine bone marrow cells were analyzed for their phenotypes and cell surface proteins (Summerfield and McCullough, 1997), subsequent studies characterized bone marrow-derived cells and their functions and mesenchymal stem cells (Baffour *et al.*, 2004; Zou *et al.*, 2008; Peterbauer-Scherb *et al.*, 2010). From our study for GFP-coexpressing cells, BMCs cultured for 14 days *in vitro* (14DIV), alkaline phosphatase, SSEA-1 and SSEA-3 expressions were demonstrated in varying degree of cell types depending on the cell lineage markers (Figs. 3, 4 and 5). Alkaline phosphatase activity was known to present in a hematopoietic stem cell progenitors, granulocytic precursors in the pig (Westen and Bainton, 1979). In our study, alkaline phosphatase-positive cells in culture are not expressing GFP or very dim fluorescence, if no at all (Fig. 3). Some mesenchymal stem cells derived from BMCs are also known to express alkaline phosphatase strongly in stromal cells (Zou *et al.*, 2008; Böhrnsen *et al.*, 2009; Wilson *et al.*, 2010). The cell surface makers showed either localized expression in the Golgi complex or whole surfaces, suggesting that some of cells with Golgi expression may undergo post-translational process at the time of immunocytochemistry. These patterns were also observed with a hematopoietic stem cell marker, CD34 that is cell surface marker, too (Fig. 6) as reportedly in the pig BMCs (Copland *et al.*, 2008; Herbst *et al.*, 2009).

In the experiments trying to demonstrate neural cell lineage markers, nestin, a neural stem cell marker was not found markedly in GFP-expressing cells although GFAP-positive cells coexpressing GFP were found abundantly, According to previous reports, highly selected bone marrow-derived MSCs were demonstrated to express pluripotency-associated stem cell markers, such as Oct4, nanog, alkaline phosphatase and SSEA-4, constitutively (Battula *et al.*, 2007; Pacini *et al.*, 2010). Since it is well known that adherent non-hematopoietic MSCs to culture dish are multipotent progenitor cells, the BMC cultured at the end of the experiment appears to be mixture of stromal cells and MSCs (Gregory *et al.*, 2005).

Despite the varying GFP expression in the BMCs examined in the experiments, examination of genomic DNA insertion, mRNA expression and the protein expression by Western blotting revealed that all the tissues from the GFP pigs are indeed GFP pig although there may be instability of the inserted gene or different epigenetic status among cells and tissues or translational instability. From these studies, it was proposed that for robust GFP tagging should use targeting strategy as used in mouse in either ubiquitous or cell-specific tagging for stem cell research in this preclinical model.

It was also clear that although cell types and clones showed different extents of GFP expressions, they all contain the fragments of genomic GFP, GFP mRNA, and immunoreactive GFP protein at expected molecular size in Western blot.

Qin *et al.* (2010) compared common ubiquitous promoters including SV40, CMV, UBC, EF1A, PGK and CAG for mammalian systems in different cell types, demonstrating that their GFP or RFP expression degrees are different among the cell types. In addition, it has been suggested that CMV, CAG and other promoters appear to be different in their epigenetic states as evidenced by different methylation patterns of CpG dinucleotides in the promoter depending on the inserted sites and cell types in various recent reports (Bauer *et al.*, 2010).

It was also suggested from our results that disappearance of GFP was notified in *in-vitro* cultured cells or upregulation of GFP expression temporally in certain cell types of BMCs. This may be the destruction of three dimensional niches of the cells *in vivo*, leading to different state of cell differentiation or altering extracellular molecules, causing different gene expression. Even in the case of using a specific promoter (Challen and Goodell, 2008). Thus, the usefulness of GFP-tagged cells for transplantation may be dependent completely on the inserted genomic sites and subjected to distal gene regulation by the long-spanning long genes. Therefore, GFP-tagging techniques should be targeted to certain genomic region where such a regulation is absent.

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