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—Original Article—

Production of transgenic cloned pigs expressing the far-red fluorescent protein monomeric Plum

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Abstract. Monomeric Plum (Plum), a far-red fluorescent protein with photostability and photopermeability, is potentially suitable for *in vivo* imaging and detection of fluorescence in body tissues. The aim of this study was to generate transgenic cloned pigs exhibiting systemic expression of Plum using somatic cell nuclear transfer (SCNT) technology. Nuclear donor cells for SCNT were obtained by introducing a Plum-expression vector driven by a combination of the cytomegalovirus early enhancer and chicken beta-actin promoter into porcine fetal fibroblasts (PFFs). The cleavage and blastocyst formation rates of reconstructed SCNT embryos were 81.0% (34/42) and 78.6% (33/42), respectively. At 36–37 days of gestation, three fetuses systemically expressing Plum were obtained from one recipient to which 103 SCNT embryos were transferred (3/103, 2.9%). For generation of offspring expressing Plum, rejuvenated PFFs were established from one cloned fetus and used as nuclear donor cells. Four cloned offspring and one stillborn cloned offspring were produced from one recipient to which 117 SCNT embryos were transferred (5/117, 4.3%). All offspring exhibited high levels of Plum fluorescence in blood cells, such as lymphocytes, monocytes and granulocytes. In addition, the skin, heart, kidney, pancreas, liver and spleen also exhibited Plum expression. These observations demonstrated that transfer of the Plum gene did not interfere with the development of porcine SCNT embryos and resulted in the successful generation of transgenic cloned pigs that systemically expressed Plum. This is the first report of the generation and characterization of transgenic cloned pigs expressing the far-red fluorescent protein Plum.
Key words: Fluorescent protein, Plum, Somatic cell nuclear transfer, Transgenic cloned pig

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Recent studies in various fields of biomedical research, particularly translational research, have begun to use pigs as an animal model because of their physiological and anatomical similarities to humans [1, 2]. Indeed, disease models of cystic fibrosis [3], diabetes mellitus [4, 5], Alzheimer's disease [6] and retinitis pigmentosa [7] have been developed in pigs. In addition, pigs with genetic modifications have also been developed to act as organ donors for xenotransplantation [8, 9], and further studies are underway to develop methods for clinical transplantation from pigs to humans [10].

One of the advantages of using genetically modified animals is that it is possible to visualize (and monitor) the expression of particular genes using fluorescent proteins [11]. Systemic or tissue-specific expression of a gene encoding a fluorescent protein in the animals is achieved using either a ubiquitous or a tissue-specific promoter [12–16]. This approach has been exploited in not only small animals, such as mice [11] and rats [17], but also large animals, such as pigs [18–20] and cattle [21]. To date, transgenic (Tg) animals that express green (GFP), blue (BFP), yellow (YFP), or red fluorescent proteins (RFP) have been generated [22–25]. The availability of such fluorescent proteins has made it feasible to monitor gene expression, intracellular protein dynamics and cell behavior in tissues and organisms. Thus, fluorescent proteins have become essential tools in cell biological and molecular biological research [26]. However, fluorescent proteins do have some technical limitations. For example, proteins that respond to short wavelengths, such as GFP, are more readily influenced by intrinsic (auto-) fluorescence than long-wavelength fluorescent proteins [27, 28]. Another disadvantage of GFP is the absorption of its excitation and emission spectra by hemoglobin. One method for avoiding these technical problems is to use proteins that respond to longer, infrared wavelengths [29, 30].

One such far-red fluorescent protein is monomeric Plum (Plum; excitation 590 nm, emission 649 nm), which was created by directed mutagenesis of monomeric RFP1 (a variant of the red fluorescent protein DsRed) [31]. Plum is photostable and photopermeable [32] and is therefore potentially suitable for *in vivo* imaging and detection

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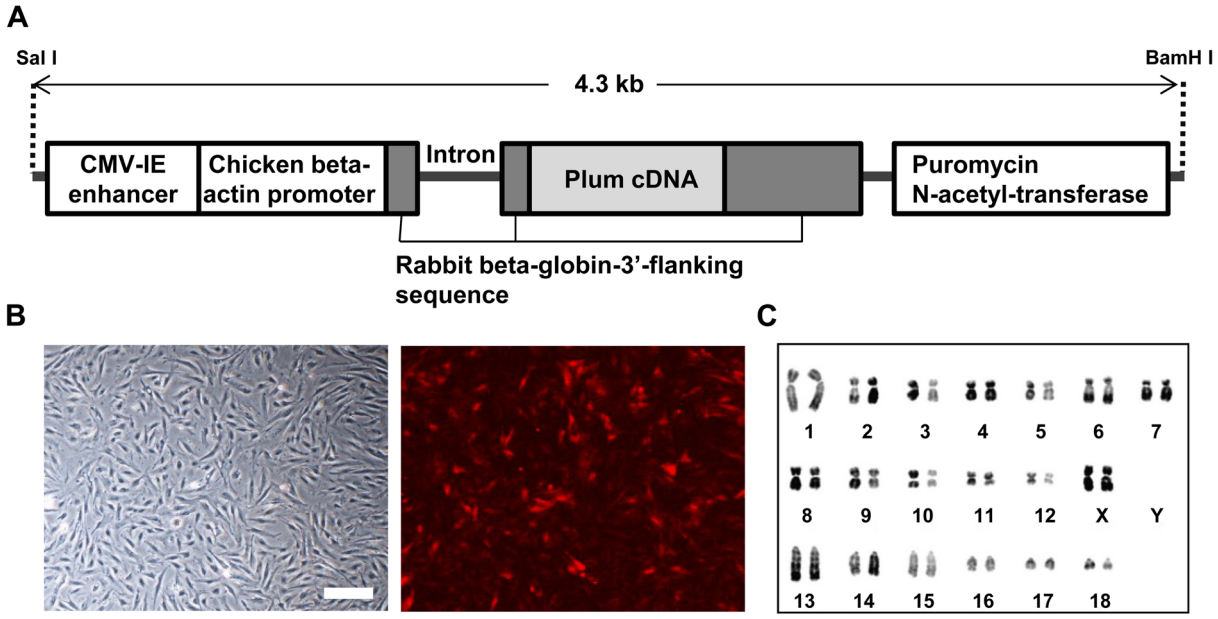

Fig. 1. Construction of the Plum expression vector and preparation of nuclear donor cells for somatic cell nuclear transfer. (A) Schematic representation of pCX-Plum-puroR. The vector consisted of Plum cDNA under the control of a CAG promoter (a linked cytomegalovirus enhancer and chicken beta-actin promoter) and included puromycin N-acetyl-transferase. (B) Preparation of nuclear donor cells. The pCX-Plum-puroR construct was introduced into PFFs to establish nuclear donor cells (Plum-PFFs). Bright-field (left) and fluorescence images (right) are shown. Scale bar = 200 μm. (C) The number of chromosomes in Plum-PFFs. The female cells normally have 38 chromosomes (36+XX).

of fluorescence in body tissues [33-36].

In previous studies, we used somatic cell nuclear transfer (SCNT) technology to generate Tg cloned pigs that express enhanced GFP (EGFP) [37] and humanized Kusabira-Orange (huKO; a red fluorescent protein) [23]. Here, we generated Tg cloned pigs that systemically expressed monomeric Plum using the same technique and analyzed the potential of this fluorescent tag for monitoring gene expression in Tg embryos and adult pigs.

Materials and Methods

Animal care and chemicals

All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IAUCU-12-0008). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Construction of the Plum expression vector

The Plum expression vector used in the present study consisted of a chicken beta-actin promoter with a cytomegalovirus enhancer (CAG promoter), Plum cDNA, rabbit beta-globin 3'-flanking sequence including a polyadenylation signal, and the puromycin N-acetyltransferase gene driven by the phosphoglycerate kinase (PGK) promoter (Fig. 1A). The Plum expression vector was constructed based on the pCX-GFP vector [38]. Briefly, Plum cDNA was amplified by polymerase chain reaction (PCR) from a plasmid containing the Plum coding sequence (pmPlum Vector, Takara Bio, Shiga, Japan). The amplified product was inserted into the *Eco*RI restriction sites of the pCX-GFP vector to replace the GFP fragment with Plum using a cloning kit (In-Fusion HD Cloning Kit, Takara Bio) to produce pCX-Plum. For antibiotic selection, a 1.1-kb fragment including the puromycin N-acetyltransferase gene under the control of the PGK promoter (puroR) was inserted into the *Hind*III site of the constructed pCX-Plum vector. Finally, the constructed Plum expression vector, designated pCX-Plum-puroR (Fig. 1A), was verified by sequencing using a 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The transgene fragment was excised from the plasmid vector by enzymatic digestion using *Sal*I (Takara Bio) and *BamH*I (Takara Bio), separated by gel electrophoresis, and purified using a gel extraction kit (QIAquick Gel Extraction Kit, QIAGEN, Hilden, Germany).

Preparation of nuclear donor cells

A primary culture of porcine female fetal fibroblasts [39] was prepared for nuclear donor cells. The porcine fetal fibroblasts (PFFs) were cultured in minimum essential medium (MEM Alpha, Life Technologies) supplemented with 15% fetal bovine serum (FBS, Nichirei Bioscience, Tokyo, Japan) and antibiotic-antimycotic solution (Life Technologies) with type I collagen-coated dishes (AGC Techno Glass, Shizuoka, Japan) in a humidified atmosphere containing 5% CO_2 at 37 C.

For transfection, PFFs were cultured to 70–90% confluence, washed twice with Dulbecco's phosphate-buffered saline (DPBS) and collected after treatment with 0.05% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Life Technologies). The collected cells (6.0×10^5) were then resuspended in 60 μl of resuspension buffer supplied

as part of a Neon Transfection System kit (Life Technologies), and 1.5 µg of pCX-Plum-puroR was added. The cells were then electroporated under the following conditions: pulse voltage, 1,100 V; pulse width, 30 ms ; and pulse number, 1. Forty-eight hours after electroporation, the cells were transferred to medium containing 2.5 µg/ml puromycin. At 12 days in culture, Plum-positive cells (1.0×10^5 cells) were collected from puromycin-resistant cells using a BD FACSAria III cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) equipped with a 561-nm (Yellow-Green) laser and were seeded onto a collagen-coated dish (AGC Techno Glass). Plum-positive cells (Plum-PFFs) were grown to confluence within 2–3 days and were then cryopreserved for later use as nuclear donor cells to generate Tg fetuses expressing Plum.

For chromosome counting, metaphase chromosome spreads from pig fibroblast cells were prepared according to standard procedures [40]. Cells were treated with 20 ng/ml colcemid (demecolcine) for 14 h and harvested. After treatment with 0.075 M KCl for 20 min at room temperature (RT), cells were fixed by exposure to MeOH:acetic acid (3:1) three times, and fixed cells were spread on slides. Chromosome images were captured using a Leica DC350FX cooled CCD camera (Leica, Wetzlar, Germany) mounted on a Leica DMRA2 microscope and analyzed using the Leica CW4000 FISH software.

SCNT

SCNT was performed as described previously [23] with slight modifications. Briefly, *in vitro* matured oocytes containing the first polar body were enucleated by gentle aspiration of the polar body and the adjacent cytoplasm using a beveled pipette in Tyrode lactose medium containing 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone (HEPES-TL-PVP) in the presence of 0.1 µg/ml demecolcine, 5 µg/ml cytochalasin B (CB) and 10% FBS.

Nuclear donors were used following cell cycle synchronization by serum starvation for 2 days. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. The donor cell-oocyte complexes were placed in a solution of 280 mM mannitol (pH 7.2; Nacalai Tesque, Kyoto, Japan) containing 0.15 mM $MgSO_4$, 0.01% (w/v) polyvinyl alcohol (PVA) and 0.5 mM HEPES and then held between two electrode needles. Membrane fusion was induced with a somatic hybridizer (LF201, Nepa Gene, Chiba, Japan) by applying a single direct-current (DC) pulse (267 V/mm, 20 µsec) and a pre- and post-pulse alternating current (AC) field of 2 V at 1 MHz for 5 sec. The reconstructed embryos were cultured in porcine zygote medium-5 (PZM-5; Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 4 mg/ml bovine serum albumin (BSA) for 1–1.5 h, followed by electrical activation. For induction of electrical activation, the reconstructed embryos were aligned between two wire electrodes (1.0 mm apart) of a fusion chamber slide filled with activation solution consisting of 280 mM mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% (w/v) PVA. A single DC pulse of 150 V/mm was applied for 100 µsec using an electrical pulsing machine (Multiporator, Eppendorf, Hamburg, Germany). After activation, the reconstructed embryos were cultured in PZM-5 for 3 h in the presence of 5 µg/ml CB and 500 nM Scriptaid, followed by culture with 500 nM Scriptaid for another 12–15 h. After these treatments, the cloned embryos were cultured in PZM-5 for 7 days to assess their *in vitro* development.

Embryo culture was performed under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 C. Beyond the morula stage, the embryos were cultured in PZM-5 supplemented with 10% FBS.

Transfer of cloned embryos into recipient pigs

Crossbred prepubertal gilts (Large White/Landrace × Duroc) weighing 100–105 kg were used as recipients of the cloned embryos. The gilts were given a single intramuscular injection of 1,000 IU of equine chorionic gonadotropin (eCG, ASKA Pharmaceutical, Tokyo, Japan) to induce estrus. Ovulation was induced by an intramuscular injection of 1,500 IU of human chorionic gonadotropin (hCG, Kyoritsu Pharmaceutical, Tokyo, Japan) given 66 h after the injection of eCG. The cloned embryos were cultured for 5 or 6 days and were surgically transferred into the uterine horns of the recipients approximately 146 h after hCG injection.

Generation of Plum-expressing offspring and analysis of the embryos, fetuses and offspring

We analyzed the expression of the Plum in Tg embryos at the one-cell (day 1), two-cell (day 2), four-cell (day 2), eight-cell (day 3), morula (day 4) and blastocyst (days 5 and 7) stages using confocal microscopy (FV-1000, Olympus, Tokyo, Japan). Some of the day-7 blastocysts were mounted on glass slides (Matsunami Glass Ind., Osaka, Japan) in HEPES-TL-PVP containing 20% ethylene glycol (Nacalai Tesque) and 5 mg/ml Hoechst 33342; these embryos were examined by fluorescence microscopy (TE-300 microscope, Nikon, Tokyo, Japan) to count cell numbers.

Recipient pigs to which SCNT embryos had been transferred were euthanized at day 36–37 of gestation, and the fetuses were collected. These fetuses were used to confirm the expression of Plum and to determine transgene copy numbers; additionally, the fetuses were used to generate rejuvenated porcine fetal fibroblasts (Neo Plum-PFFs) for the next round of SCNT to produce offspring. We used SCNT to generate Plum-expressing offspring using Neo Plum-PFFs as nuclear donor cells. On day 2 after birth, we also screened for Plum expression in the tissues and organs of piglets. Fluorescence was analyzed using a fluorescence microscope (MVX10, Olympus; excitation, 532.5–587.5 nm; emission, 607.5–682.5 nm).

Flow cytometric analysis of blood cells from Tg pigs expressing Plum was performed using a BD FACSAria III cell sorter. Freshly isolated blood cells were lysed with BD Pharm Lyse (Becton, Dickinson and Company) reagent to remove erythrocytes. Each cell population was selected by gating strategies based on forward and side scatter properties.

Female Tg cloned offspring were euthanized at 10 months of age. The ovaries were removed, and cumulus-oocyte complexes were collected as described previously [23]. The expression of Plum in the cumulus-oocyte complexes was confirmed using a confocal microscope (FV-1000, Olympus).

Estimation of transgene copy number by Southern blot analysis

Genomic DNA was extracted from skin samples of Tg cloned fetuses using a DNA purification kit (DNeasy Blood & Tissue Kit, QIAGEN). The purified genomic DNA (5 µg) was digested with *Pst*I (Takara Bio), separated by gel electrophoresis, and transferred

Table 1. *In vitro* development of SCNT embryos

Donor cells	No. of embryos cultured	Embryonic development		Cell numbers in the blastocysts (means ± SEMs)
		Cleaved (%)	Blastocysts (%)	
Plum-PFFs	42	34/42	33/42	88.3 ± 6.0
		(81.0)	(78.6)	
PFFs	40	37/40	30/40	99.9 ± 8.8
		(92.5)	(75.0)	

onto a nylon membrane (Hybond N^+, GE Healthcare Bio-Sciences, Uppsala, UK). The membranes were blocked for 30 min at room temperature with blocking reagent (Blocking One, Nacalai Tesque). After blocking, the membranes were incubated in hybridization solution (DIG Easy Hyb, Roche Diagnostics, Basel, Switzerland) and hybridized with a digoxigenin (DIG)-labeled Plum probe prepared by PCR using a DNA-labeling reagent (DIG DNA Labeling Mix, Roche Diagnostics). The blot was developed using chemiluminescent reagent (DIG Luminescent Detection Kit, Roche Diagnostics), and the signal was detected and imaged with an ImageQuant LAS-4000 system (GE Healthcare Bio-Sciences). The number of transgene copies integrated into the porcine genome was determined by comparison of the hybridization signal with that of the copy-number control, which was diluted to make a standard series (1–10 copies per diploid genome).

Multicolor fluorescence imaging using Plum

To determine whether Plum-expressing PFFs could be distinguished from PFFs expressing different fluorescent proteins (e.g., EGFP and huKO), we carried out both *in vitro* fluorescence observations and flow cytometry analyses. PFFs that express EGFP [36] and huKO [22] were generated previously; we cocultured these PFFs with fibroblasts derived from Plum-expressing fetuses. The three cell types were inoculated onto a 35-mm dish (7.0×10^4 cells for each cell type) and observed by confocal microscopy (FV-1000, Olympus) at 24 h after the start of culture. To confirm that the three cell types could be fractionated using their different fluorescence colors, a cell mixture (1.0×10^6 cells of each cell type) was sorted using a BD FACSAria III cell sorter. For each cell type, 2.0×10^5 cells were sorted and inoculated onto a 35-mm dish; 24 h later, cells were screened using a confocal microscope (FV-1000, Olympus). The excitation wavelengths and emission wavelengths of the three fluorescent proteins were as follows: EGFP, 488 nm/495–515 nm; huKO, 559 nm/561–610 nm; and Plum, 559 nm/ 650–750 nm.

Statistical analysis

Experimental results were expressed as the mean ± standard error of the mean (SEM). The data were analyzed using the SPSS 16.0 software (SPSS, Chicago, IL, USA). For proportional data, differences between groups were analyzed using the χ^2 test. For blastocyst cell number data, differences between groups were determined by Student's *t*-test. The level of significance was set at $P < 0.05$.

Results

Generation of Plum-expressing SCNT embryos

Plum-PFFs obtained by transferring pCX-Plum-puroR showed no abnormalities in shape or chromosome number (Fig. 1). The cleavage and blastocyst formation rates in SCNT embryos reconstructed using Plum-PFFs (Plum embryos) were 81.0% (34/42) and 78.6% (33/42), respectively. In comparison, the rates for control SCNT embryos reconstructed using PFFs without the *Plum* gene were 92.5% (37/40) and 75.0% (30/40), respectively (Table 1). The average number of cells in Plum blastocysts was 88.3 ± 6.0 compared with 99.9 ± 8.8 in control blastocysts. Our results indicated there was no significant difference in *in vitro* development ability between the two groups of embryos ($P < 0.05$).

Analysis of fluorescence in Plum embryos confirmed weak expression of Plum at the eight-cell stage. Higher expression was observed as development progressed (Fig. 2).

Production and analysis of Plum-expressing cloned fetuses

One hundred three SCNT embryos reconstructed using Plum-PFFs as nuclear donor cells were transferred to a single recipient pig; three fetuses were obtained at 36–37 days of gestation (3/103, 2.9%) (Table 2). Plum fluorescence was observed systemically in all three fetuses and was visible in fibroblasts (Neo Plum-PFFs) derived from the fetuses. In contrast, wild-type fetuses showed only autofluorescence (Fig. 3). Southern blot analysis revealed that Plum fetuses had 1–5 copies of the transgene (Fig. 3E).

Characterization of Plum-expressing cloned offspring

To generate offspring expressing Plum, we used Neo Plum-PFFs from one of the three cloned fetuses (Fig. 3D and D') as nuclear donor cells. One hundred seventeen SCNT embryos were transferred to a single recipient pig; four cloned offspring and one stillborn cloned offspring were produced (5/117, 4.3%) (Table 2). At birth, the piglets weighed 911.0 ± 115.8 g and had a body length of 27.3 ± 1.3 cm. The offspring developed normally to adulthood. Expression of Plum protein was confirmed in lymphocytes, monocytes and granulocytes and in 15 different tissues and organs (Figs. 4 and 5). Plum expression was identified in cumulus-oocyte complexes from the ovaries collected after euthanasia at 10 months of age (Supplementary Fig. 1: online only).

Function of Neo Plum-PFFs in cells expressing other fluorescent proteins

The three types of PFFs expressing EGFP [37], huKO [23] and

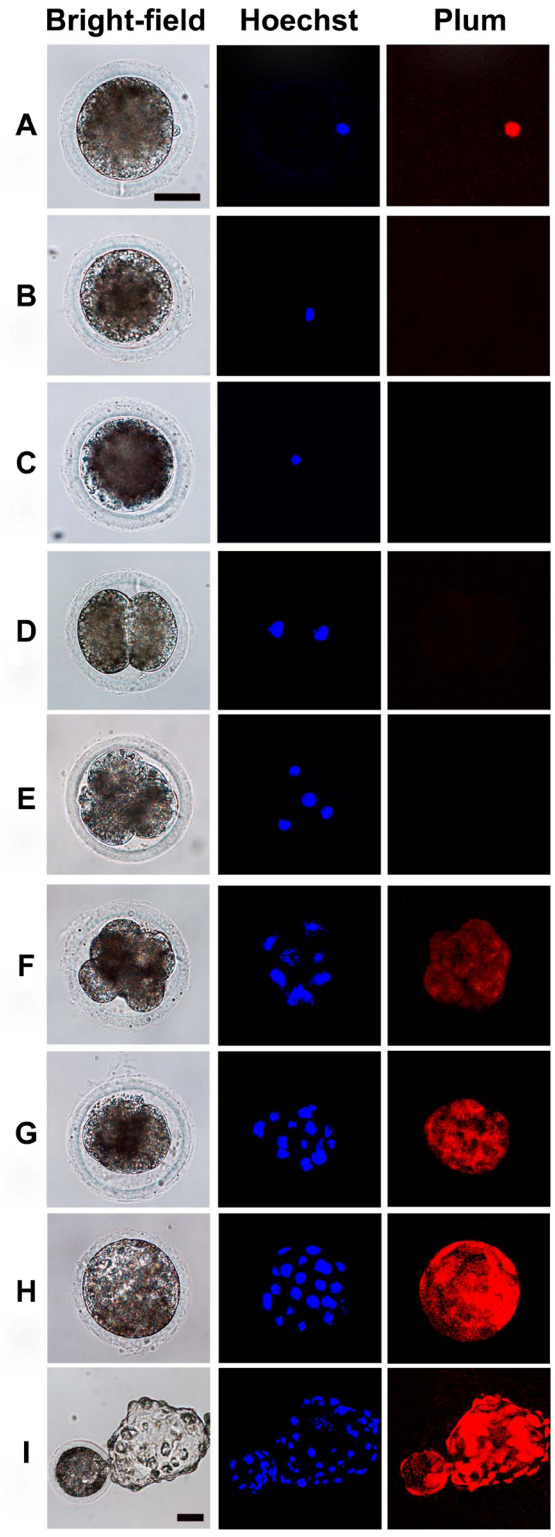

Fig. 2. Plum fluorescence in Plum-expressing SCNT embryos. (A–I) Plum expression in SCNT embryos at various developmental stages. (A) Reconstructed oocytes before fusion with the nuclear donor cell, (B) 2 h after fusion, (C) at the one-cell stage, (D) at the two-cell stage, (E) at the four-cell stage, (F) at the eight-cell stage, (G) at the morula stage and (H, I) at the blastocyst stage. (Left) Bright-field. (Middle and right) Confocal fluorescence microscopic images. Scale bar = 50 µm.

Plum were clearly distinguished in culture by confocal microscopy based on excitation wavelengths and emission wavelengths. In addition, using a flow cytometer, we found that a mixture of the three cell types was distributed at wavelengths for EGFP, huKO and Plum (Fig. 6B). Subsequently, the three cell types were precisely sorted at the appropriate wavelength ranges corresponding to the emission wavelength of the expressed fluorescent protein (Fig. 6C).

Discussion

In this study, we demonstrated that it is feasible to generate Tg cloned pigs that systemically express Plum, a long-wavelength far-red fluorescent protein, using SCNT technology. These results support the use of transgenic cloned pigs as an animal model for various biological fields based on the ability to express fluorescent markers systemically.

Previous studies have shown that animals cloned using somatic cells can be efficiently produced by serial cloning when fetus-derived fibroblasts are used as nuclear donor cells [41, 42]. The collection of PFFs at an early stage of development has two principal advantages. First, selection of nuclear donor cells with genetic modifications involves the use of drug treatment in long-term culture. Such cultures are inevitably associated with a high likelihood of cell exhaustion and aging senescence [43]. Exhausted or aged cells are more likely to carry chromosomal aberrations [44]. For this reason, we chose to produce SCNT embryos using rejuvenated PFFs that possessed a high rate of proliferation and the appropriate competence as nuclear donor cells. Second, it is a relatively simple matter to confirm expression of the target gene in these cells. In this study, transgene-derived Plum expression was confirmed systemically in the three Tg fetuses obtained (day 36–37 of gestation). The cloning efficiency for generating offspring (4.3%) with rejuvenated cells was higher than that for producing fetuses (2.9%) without rejuvenated cells. Thus, serial cloning may contribute to an increase in the cloning efficiency for generating offspring [41].

An increase in the copy number of a transgene generally increases the expression level of a transgene but may also influence the growth of cells and organs [23, 45]. Fluorescent proteins likewise affect cell division and organogenesis [46-48]. In our preliminary study, we used nuclear donor cells that carried 25 transgene copies for SCNT but did not obtain any fetuses (data not shown). This result suggests that a high copy number of the transgene may have a detrimental influence on fetal development. The three Tg cloned fetuses obtained in this study were confirmed by Southern blot analysis to have between 1 and 5 copies of the transgene, i.e., a relatively low number. The use of cells with a low copy number of the Plum-expressing transgene as nuclear donor cells resulted in the generation of Plum embryos with developmental characteristics similar to those of control embryos. We concluded that it is important to first confirm the transgene copy number in the genome for the generation of Tg clones using SCNT in order to determine whether the cells can function optimally as nuclear donor cells.

Embryonic genome activation (EGA) occurs at the two-cell stage in mice [49]. Okabe *et al.* reported that EGFP expression could be detected at the four-cell stage and onwards in mouse embryos that expressed EGFP under a CAG promoter [12]. In contrast, EGA has

Table 2. Production of Plum-expressing cloned fetuses and offspring

	Donor cells	No. of embryos transferred	Recipients	Pregnancy	Fetuses or offspring obtained (stillborn)	Production efficiency of cloned fetuses or offspring (%)*
Fetuses	Plum-PFFs	103	1	+	3	2.9
Offspring	Neo Plum-PFFs	117	1	+	5 (1)	4.3

* The production efficiency was calculated as the number of fetuses or offspring obtained divided by the number of embryos transferred times 100.

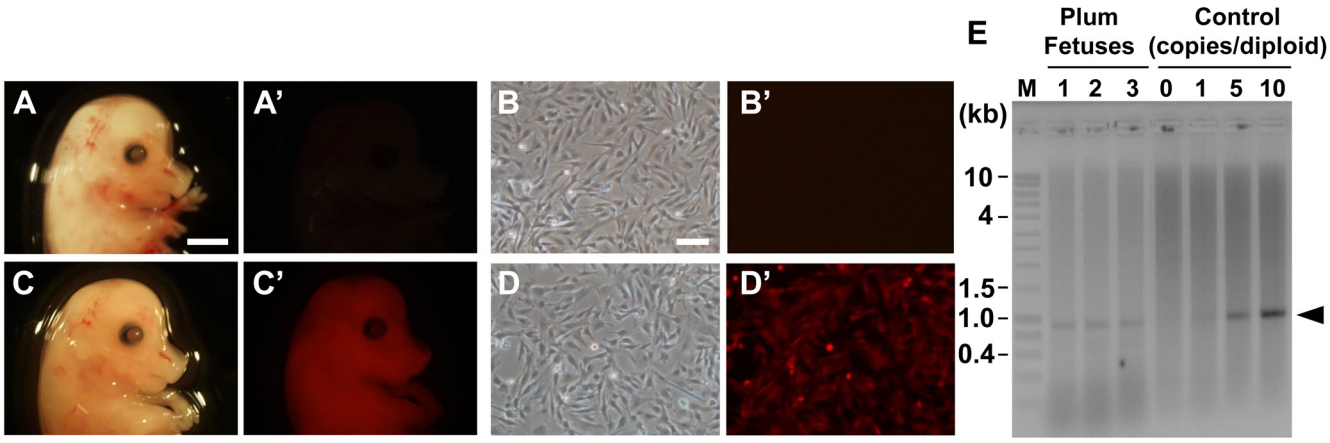

Fig. 3. Characterization of Plum-expressing cloned fetuses generated by SCNT using Plum-PFFs. (A–D) Macroscopic view of the fetus and microscopic images of PFFs. Bright-field (A, C) and fluorescence stereomicroscopic images of a fetus (A', C'). Phase contrast (B, D) and fluorescence images of fibroblasts (B', D'). A wild-type fetus (A, A') and fetal fibroblasts from the wild-type fetus (B, B'). Scale bar in A = 5 mm. Scale bar in B = 100 μm. A Plum-expressing cloned fetus (C, C') and fetal fibroblasts from the Plum-expressing cloned fetus (D, D'). Plum fluorescence was systemic in all fetuses, and expression of Plum was confirmed in all fibroblasts (D'). (E) Southern blot analysis of Plum-expressing cloned fetuses. The copy number of the integrated transgene in each fetus was determined by comparison with the signal intensity of the copy number control. The arrowhead indicates the expected band. M: DNA size marker.

been reported to occur at the four-cell stage in pig embryos [50]. In this study, we did not detect Plum florescence until the eight-cell stage, i.e., slightly later than the expected onset of EGA in pig embryos. This gap might be due to the time necessary to produce the fluorescent protein after EGA and to the need to accumulate sufficient protein for detection.

Cells and tissues that express fluorescent proteins are of value for analyzing cell-to-cell interactions, cell behavior during organogenesis, cell fusion and cell lineages during development. Ohtsuka *et al.* generated aggregation chimeric mice that displayed multicolor fluorescence through use of monomeric cyan fluorescent protein (CFP), tandem dimer tomato (tdTomato) and EGFP; they used these embryos to investigate early embryogenesis and to track the clonality of cells during organogenesis [51]. Similarly, Webster *et al.* generated multi-Tg pigs that expressed BFP, EGFP and DsRed2 and showed that these animals offered a powerful tool for medical, agricultural, and pharmaceutical studies [24]. Here, we demonstrated that three cell types expressing different fluorescent proteins could be clearly distinguished in culture and accurately sorted by flow cytometry using the differences in their fluorescence colors. Thus, the long-wavelength far-red fluorescent protein, Plum, which is distinguishable from other proteins with shorter wavelengths, was used.

In conclusion, this is the first report to describe the generation and characterization of SCNT Tg cloned pigs that expressed a far-red fluorescent protein (Plum). The cells, tissues and organs of animals that express Plum will be of value in future studies in regenerative medicine, transplantation medicine and other medical fields.

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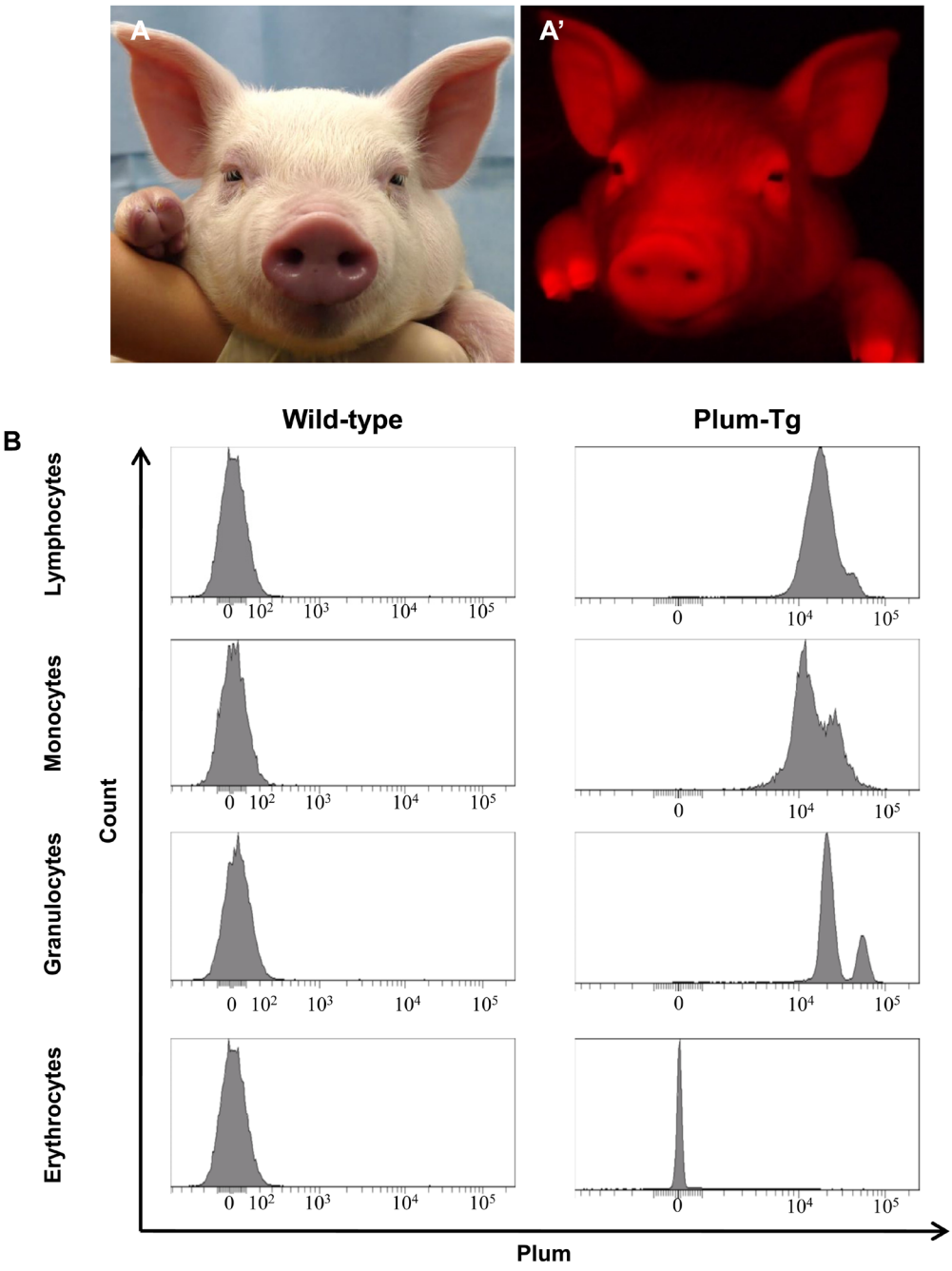

Fig. 4. Expression of Plum in Tg cloned pigs generated by SCNT using rejuvenated PFFs. (A, A') Bright-field and fluorescence images of a 1-month-old Tg pig. (B) Flow cytometric analysis for expression of Plum in blood cells. Lymphocytes (top panel), monocytes (second panel), granulocytes (third panel) and erythrocytes (bottom panel) were analyzed for wild-type (left) and Tg pigs (right). The X- and Y-axes in the histogram represent Plum fluorescence intensity and cell counts, respectively.

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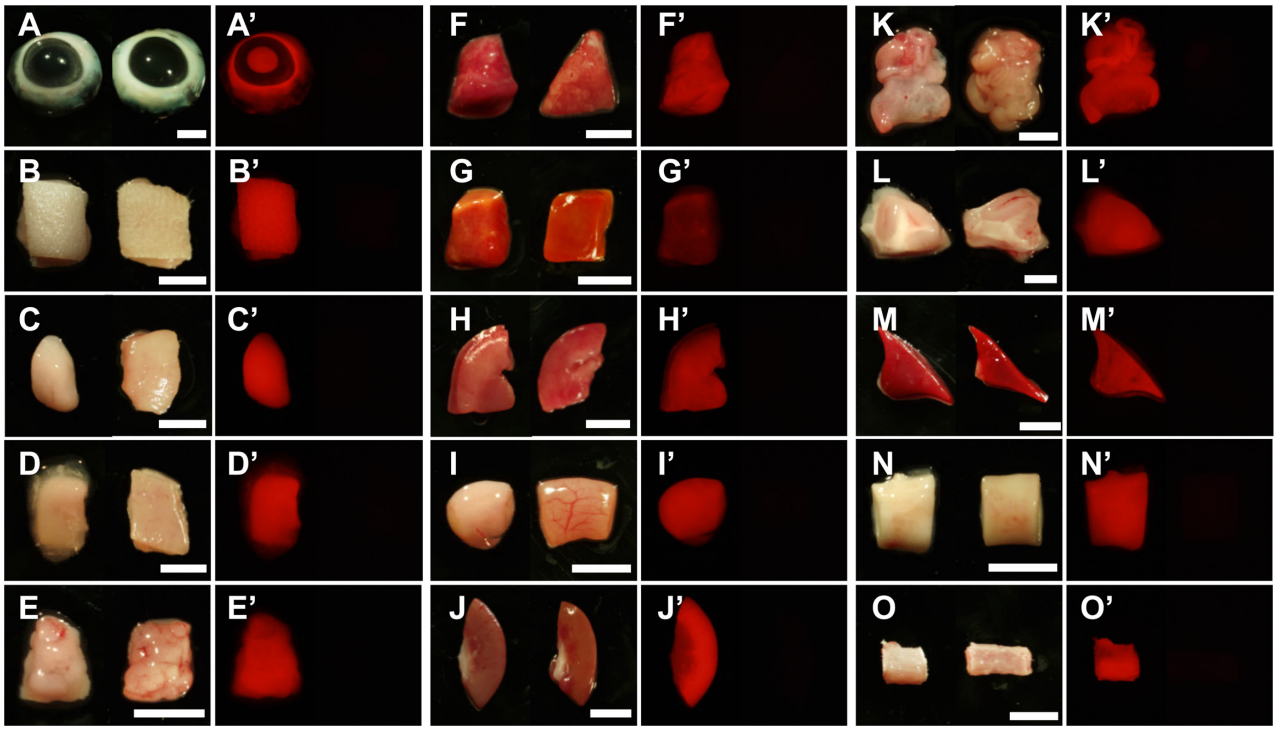

Fig. 5. Systemic expression of Plum in Tg cloned pigs. (A–O) Bright-field stereomicroscopic images of tissues from a Tg cloned pig (left) and wild-type control (right). (A'–O') Fluorescence stereomicroscopic images demonstrated that tissues from the Tg cloned pig (left) exhibited Plum fluorescence, while none of the control tissues (right) showed fluorescence, including background autofluorescence. (A, A') eye, (B, B') skin, (C, C') skeletal muscle, (D, D') stomach, (E, E') pancreas, (F, F') lung, (G, G') liver, (H, H') heart, (I, I') intestine, (J, J') kidney, (K, K') reproductive tract, (L, L') brain, (M, M') spleen, (N, N') blood vessel, (O, O') rib. Scale bar = 5 mm.

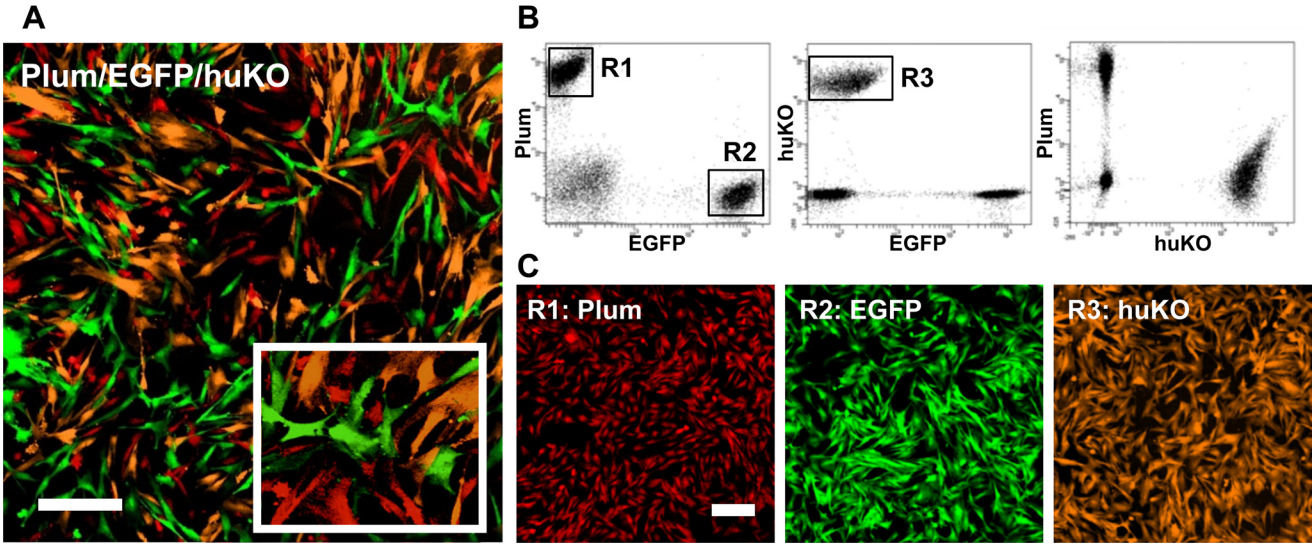

Fig. 6. Multicolor fluorescence imaging of a mixed culture of PFFs expressing EGFP, huKO or Plum. (A, C) Confocal fluorescence microscopic images of the fibroblasts. (A) Fluorescence image of the culture showing all three cell types. Scale bar = 200 µm. (B) Fluorescence-activated cell sorting analysis of the mixed culture. In each distinctive gate (R1–R3), each fibroblast type was separated by the wavelength of emitted fluorescence, and the cells were then sorted by a cell sorter. (C) Confocal fluorescence microscopic images of the sorted fibroblasts. Scale bar = 200 µm.

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