—Original Article—

A transgenic-cloned pig model expressing non-fluorescent modified Plum

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Abstract. Genetically modified pigs that express fluorescent proteins such as green and red fluorescent proteins have become indispensable biomedical research tools in recent years. Cell or tissue transplantation studies using fluorescent markers should be conducted, wherein the xeno-antigenicity of the fluorescent proteins does not affect engraftment or graft survival. Thus, we aimed to create a transgenic (Tg)-cloned pig that was immunologically tolerant to fluorescent protein antigens. In the present study, we generated a Tg-cloned pig harboring a derivative of Plum modified by a single amino acid substitution in the chromophore. The cells and tissues of this Tg-cloned pig expressing the modified Plum (mPlum) did not fluoresce. However, western blot and immunohistochemistry analyses clearly showed that the mPlum had the same antigenicity as Plum. Thus, we have obtained primary proof of principle for creating a cloned pig that is immunologically tolerant to fluorescent protein antigens.
Key words: Modified Plum, Non-fluorescent protein, Somatic cell nuclear transfer, Transgenic-cloned pig

Animals that express fluorescent proteins, such as green or red fluorescent proteins (GFP or RFP, respectively), have become indispensable biomedical research tools in recent years [1]. Experimental and domestic animals have hitherto been developed to express a variety of fluorescent proteins [2–6]. Animals with tissue-specific [7, 8], cell cycle-dependent [9], and other types of fluorescent protein expression have been reported.

Fluorescent proteins are extremely effective for the *in vivo* analysis of cell behavior and function [10, 11]. With the development of regenerative medicine in particular, fluorescent proteins are increasingly being used in studies involving cell and tissue transplantation, where visual tracking of processes such as graft survival, post-graft propagation, and tumorigenesis is important. When using fluorescent proteins as cell markers, the characteristics of the fluorescent protein itself have an undeniable effect on experimental results. The

cytotoxicity of fluorescent proteins compromises the *in vitro* and *in vivo* evaluations of cell behavior and propagation [12]. Furthermore, it is important to sufficiently understand the optical properties of fluorescent proteins used in research. For example, the fluorescence of GFP is often compromised in animal tissues containing large amounts of blood, such as the liver, where fluorescence absorption by hemoglobin hinders observations [13, 14]. In such cases, the long wavelength fluorescence of proteins such as DsRed [15] and Plum [16, 17] has features that are less affected by background, such as autofluorescence in animal tissue.

On the other hand, when using fluorescent proteins in cell or tissue transplantation studies, it is necessary to consider the possibility that the xeno-antigenicity of fluorescent proteins affects engraftment and graft survival. For example, when transgenic (Tg) rat skin [18] or limbus [19] expressing GFP was transplanted into syngenic inbred rats, the antigenicity of the fluorescent proteins was thought to be the cause of the reported tissue rejection. Similar rejection was reported when GFP-expressing lymphocytes [20] and myoblasts [21] were transplanted into syngenic inbred mice. Based on these examples, exogenous fluorescent proteins and their genetic derivatives are thought to have antigenicity in mammals [22–24]. Therefore, information obtained through cell and tissue transplantation studies would improve considerably if experiments could be performed in the absence of fluorescent protein-based immune rejection. Thus,

we designed a Tg-cloned pig that intrinsically gained immunological tolerance to fluorescent protein antigenicity. Our ultimate goal is to produce two types of individuals that share a syngenic genetic background, one that expresses the fluorescent protein and one that expresses the derivative, non-fluorescent protein. This pig model will be ideal for transplant experiments in which immunologic rejection does not occur.

The present study aimed to create a Tg-cloned pig that expressed a gene encoding a modified Plum (mPlum), a non-fluorescent protein with the same antigenicity as the far-red protein, Plum. mPlum was generated through the substitution of a single amino acid in the Plum chromophore. Furthermore, we report here the fluorescent properties of the cells and tissues of mPlum Tg-cloned pigs and the antigenic properties of mPlum protein.

Materials and Methods

Animal care and chemicals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Meiji University (IAUCU-11-0002, -12-0008). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Construction of the mPlum expression vector

The mPlum expression vector was constructed based on the pCX-Plum-puroR vector as previously reported [25]. The expression vector used in the present study consisted of (1) a chicken beta-actin promoter with a cytomegalovirus immediate early (IE) enhancer (CAG promoter), mPlum cDNA, rabbit beta-globin 3′-flanking sequence including a polyadenylation signal (poly A), and (2) the puromycin N-acetyltransferase gene under the control of the phosphoglycerate kinase (PGK) promoter and a herpes simplex virus thymidine kinase poly (A) site (Fig. 1A). We chose to modify the chromophore (Met^{67}-Tyr^{68}-Gly^{69}), which is a key part of the molecule responsible for Plum fluorescence based on the amino acid sequences of other fluorescent proteins. Tyr^{68} is essential for the formation of an *n*-conjugated system structure that confers fluorescence [26, 27]. Therefore, Tyr^{68} was replaced with Gly, which is the smallest aliphatic amino acid, to maintain the conformation of Plum. The Y68G mutation was introduced into the expression vector containing the Plum cDNA by using a standard site-directed mutagenesis technique with the appropriate primers (5′-CCTCAGATCATGGGCGGCTCCAAGGCC-3′ and 5′-GGCCTTGGAGCCGCCCATGATCTGAGG-3′). Finally, the constructed mPlum expression vector, designated pCX-mPlum-puroR (Fig. 1A), was verified by sequencing using a 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The 4.3-kb transgene fragment was excised from the plasmid vector by enzymatic digestion using *Sal*I (Takara Bio, Shiga, Japan) and *Bam*HI (Takara Bio), separated by gel electrophoresis, and purified using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany).

Preparation of nuclear donor cells

A primary culture of porcine female fetal fibroblasts was prepared for nuclear donor cells as previously described [28]. The porcine fetal fibroblasts (PFFs) were cultured in minimum essential medium (MEM Alpha, Life Technologies) supplemented with 15% fetal
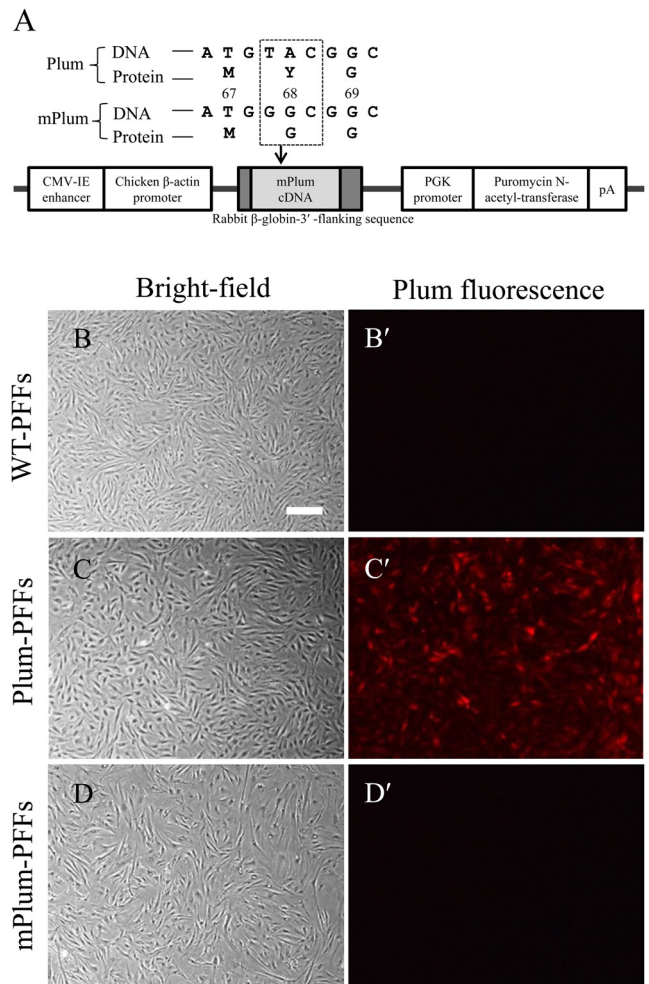

Fig. 1. Modified Plum (mPlum) protein expression vector and porcine fetal fibroblasts (PFF) transformed with the mPlum transgene. (A) Structure of the mPlum expression vector (pCX-mPlum-puroR). In the mPlum cDNA, TAC (Tyrosine), the 68th amino acid of Plum, was replaced with GGC (Glycine) (Y68G). CMV-IE enhancer; cytomegalovirus immediate early (IE) enhancer. pA; herpes simplex virus thymidine kinase poly (A) site. (B–D, B′–D′) Bright-field (B–D) and fluorescent images (B′–D′) of the WT fetal fibroblasts (WT-PFFs; B, B′), PFFs transfected with the pCX-Plum-puroR (Plum-PFFs; C, C′), and PFFs transfected with the pCX-mPlum-puroR (mPlum-PFFs; D, D′). Note that the mPlum-PFFs (D, D′) did not emit fluorescence, while the Plum-PFFs (C, C′) exhibited red fluorescence. Scale bar = 200 μm.

bovine serum (FBS, Bovogen Biologicals Pty, Victoria, Australia) 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 linearized pCX-mPlum-puroR was added. The cells were then electroporated under the following conditions: pulse voltage, 1,100 V; pulse width, 30 msec; and pulse number, 1. Forty-eight hours after the electroporation, the cells were transferred to medium containing 2.5 μg/ml puromycin. At 12 days in culture, puromycin-resistant cells were collected and seeded onto a type I collagen-coated dish (AGC Techno Glass). These cells (mPlum-PFFs) were grown to confluence within 2–3 days and then were cryopreserved for later use as nuclear donor cells to generate Tg fetuses expressing mPlum. Control PFFs expressing the Plum transgene (Plum-PFFs) previously produced [25], were also cultured.

Flow cytometric analysis

Fluorescence of the established transformed fibroblast cells and blood cells from Tg-cloned fetuses were analyzed using a BD FACSAria III cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), equipped with a 561-nm (Yellow-Green) laser. Whole blood cells were treated with BD Pharm Lyse (Becton, Dickinson and Company) reagent to remove erythrocytes. The lymphocyte population was selected by gating strategies based on forward and side scatter properties.

Somatic cell nuclear transfer (SCNT)

SCNT was performed as described previously [5, 25] 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 donor cells 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.

Embryos were cultured in 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

Cross 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, cultured for 5 or 6 days, were surgically transferred into the uterine horns of the recipients approximately 146 h after hCG injection.

Generation of Tg-cloned embryos, fetuses and piglets

Some of the day-7 cloned blastocysts were mounted on glass slides (Matsunami Glass Ind., Osaka, Japan) in HEPES-TL-PVP containing 20% ethylene glycol (Nacalai Tesque) and 5 μg/ml Hoechst 33342; these embryos were examined by fluorescence microscopy (TE-300 microscope, Nikon, Tokyo, Japan) to evaluate their cell numbers.

Recipient gilt to which SCNT embryos had been transferred were euthanized at day 37–38 of gestation to recover the cloned fetuses. These fetuses were used to examine the integration and expression of the mPlum transgene. The fetuses were also used to generate rejuvenated fibroblasts (Neo-mPlum-PFFs). Rejuvenated fibroblasts were established from minced skin tissue of the Tg-fetuses by routine cell culture technology and were maintained in the medium described above. After 3–4 passages, the cells were stored frozen until they were used for nuclear transfer. SCNT was performed using the Neo-mPlum-PFFs as nuclear donors to produce cloned piglets expressing the mPlum. Fluorescence in the tissues/organs of the cloned piglets was examined using a fluorescence microscope (MVX10, Olympus; excitation, 532.5–587.5 nm; emission, 607.5–682.5 nm).

Estimation of transgene copy number by Southern blot analysis

Genomic DNA was extracted from skin samples of Tg-cloned fetuses using a DNeasy Blood & Tissue Kit (QIAGEN). The purified genomic DNA (5 μg) was digested with *Pst*I (Takara Bio), separated by gel electrophoresis, and transferred onto a nylon membrane (Hybond N^+, GE Healthcare Bio-Sciences, Uppsala, UK). The membranes were blocked for 30 min at 25°C 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 mPlum probe prepared by polymerase chain reaction (PCR) using a DNA-labeling reagent (DIG DNA Labeling Mix, Roche Diagnostics). The blot was developed using a 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 comparing the hybridization signal with that of the copy-number control, which was diluted to make a standard series (1–10 copies per diploid genome).

Genotyping

The mPlum transgene was amplified by PCR from blastocysts directly or from extracted genomic DNA, as described above, using MightyAmp DNA polymerase (Takara Bio). The primers sequences are 5′-CTACAAGACCGACATCAAGCTG -3′ and 5′-ACAGCTATGACTGGGAGTAGTCAGG -3′. PCR was performed under the following conditions: 98°C, 30 sec; 30 cycles of 98°C, 10 sec; 60°C, 15 sec; 68°C, 1 min. Nested PCR was then performed using PrimeSTAR HS DNA polymerase (Takara Bio) with the appropriate primers (5′-ACGAGGACTACACCATCGTGG -3′ and 5′-TGTTCATGGCAGCCAGCATATGG -3′) under the following conditions: 95°C, 1 min; 25 cycles of 95°C, 30 sec; 68°C, 20 sec; 72°C, 45 sec. The expected product size of nested PCR was 382 bp.

Western blot analysis

Skin and liver of the cloned fetuses were homogenized in lysis and extraction buffer (RIPA buffer, Thermo Scientific, MA, USA) with a protease inhibitor cocktail (Nacalai Tesque), centrifuged (12,000 × g) at 4°C for 5 min, and the supernatants were collected. The protein concentrations of the samples were quantified using a DC protein assay (Bio-Rad, CA, USA) based on the Lowry method. Approximately 10 μg of protein from the extracts was subjected to 10% SDS-PAGE and transferred by electroblotting to a Hybond-P PVDF membrane (GE Healthcare Bio-Sciences). The membranes were blocked for 30 min at 25°C with Blocking One (Nacalai Tesque). After blocking, the membranes were incubated with an anti-DsRed antibody that recognizes the Plum protein (1:1,000 dilution; Takara bio) for 1 h at 25°C, and were subsequently incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:10,000 dilution; Santa Cruz Biotechnology, CA, USA) for 1 h at 25°C. The blot was developed using ECL Western Blotting Detection Reagents (GE Healthcare Bio-Sciences). The signal was detected and imaged with an ImageQuant LAS-4000 system.

Immunohistochemistry

The kidney and heart tissues dissected from the cloned piglets obtained were fixed in a 4% paraformaldehyde solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin, sectioned, and stained with hematoxylin using standard methods. The fixed sections were also incubated with blocking solution (2% BSA/DPBS) for 1 h and then treated with a rabbit anti-DsRed antibody (1:200 dilution) for 1 h at 25°C. After removal of the excess antibody, the sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:300 dilution, Life Technologies) for 2 h at 25°C. The slides were visualized using a Biorevo BZ9000 microscope (Keyence, Osaka, Japan).

DNA methylation analysis

Genomic DNA extraction and bisulfite conversion were performed as described previously [29]. Briefly, genomic DNA was purified from fibroblasts isolated from fetuses using the DNA purification kit described above, and digested with the restriction enzyme *Hin*dIII (Takara Bio). After purification of digested genomic DNA with a QIAquick gel extraction Kit (QIAGEN), bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA, USA). Bisulfite-treated genomic DNA was amplified with BioTaq HS DNA polymerase (Bioline, London, UK) using specific primers for chicken beta-actin promoter (Forward, 5′-TTTGTGGTTGYGTGAAAGTTTTG-3′; Reverse, 5′-CCACACCCCCTACTCACC-3′). PCR was performed under the following conditions: 95°C, 10 min; 40 cycles of 95°C, 30 sec; 60°C, 30 sec; 72 C, 1 min; final extension 72°C, 2 min. Amplified PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA), and 6–8 clones were sequenced to determine DNA methylation status.

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

Construction of nuclear transplanted embryos

After transfection and subsequent selection with puromycin, mPlum-PFFs and wild-type (WT)-PFFs showed similar morphological shapes and proliferative capacities (Fig. 1D, D′).

A total of 127 nuclear transfer embryos were reconstructed using mPlum-PFFs as the nuclear donors. The rates of normal cleavage and blastocyst formation were 81.9% (104/127) and 75.6% (96/127), respectively. The average number of cells in the blastocysts was 109.8 ± 4.5 (n = 96). Embryonic development and blastocyst cell number were comparable to those of the SCNT embryos produced in our previous study using the same non-transfected and Plum-transfected PFFs [25].

Fluorescence was not detected in any of the cloned blastocysts (Fig. 2C, C′), while they were confirmed to carry the mPlum transgene by PCR analysis (data not shown).

Production of Tg-cloned fetuses harboring the mPlum transgene

A total of 71 nuclear transfer embryos were transplanted into one recipient pig, from which eight (11.3%) fetuses on day 37–38 of gestation were collected by laparotomy (Table 1). These fetuses did not emit Plum fluorescence under excitation (Fig. 3C, C′). Skin from the cloned fetuses was sampled to establish a primary culture of fibroblasts (Neo-mPlum-PFFs) as rejuvenated donor cells (Fig. 3H, H′). Southern blotting analysis indicated that 1-10 copies of the transgene were integrated into the fibroblasts (Fig. 3D). Western blotting analysis showed that the mPlum protein was expressed only in Tg-cloned fetus #2 (Fig. 3E). Analysis using flow cytometry confirmed that the fibroblasts established from fetus #2 did not express Plum fluorescence, similar to the WT fetus (Fig. 3I).

We also performed DNA methylation analyses of the CAG promoter region using the fibroblasts established from the eight fetuses. The results showed that the CAG promoter region was highly methylated in Tg-cloned fetuses #6, #7, and #8 (Fig. 4), suggesting that expression of the mPlum protein was inhibited in these fetuses. However, the cloned fetuses with no detectable mPlum expression, as determined
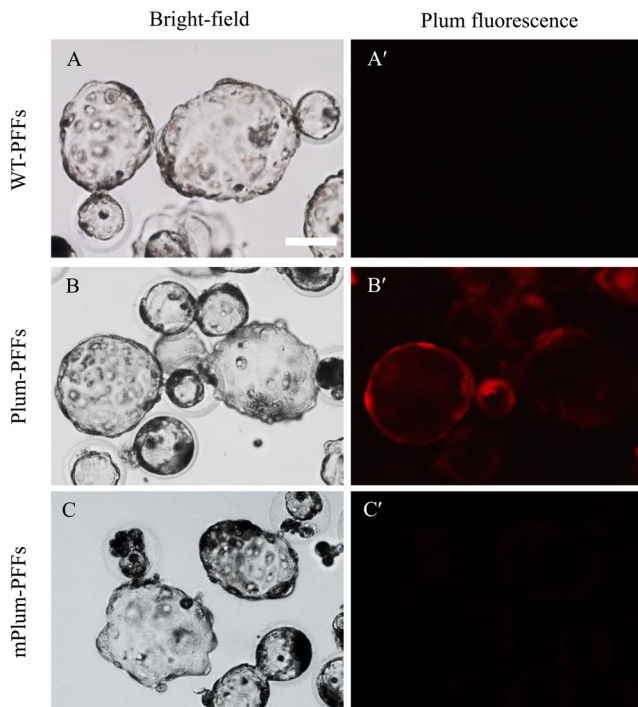
Fig. 2. Somatic cell nuclear transfer embryos using mPlum-PFFs as the nuclear donor. (A–C, A′–C′) Morphology of the blastocysts developed from the nuclear transfer embryos. Bright-field (A–C) and fluorescent images (A′–C′) of the cloned blastocysts. (A, A′) Cloned blastocysts reconstructed with WT-PFFs, not emitting fluorescence under excitation. (B, B′) Cloned blastocysts generated using Plum-PFFs, emitting red fluorescence. Unpublished pictures of the cloned blastocysts produced in our previous study [25]. (C, C′) Cloned blastocysts obtained using mPlum-PFFs, showing no fluorescence under excitation. Scale bar = 100 μm.

by western blotting, included those with low methylation in the CAG promoter (#1, #3, #4, and #5), suggesting that the transgene was mutated or integrated into a heterochromatic region with low transcriptional activity.

Production of Tg-cloned piglets harboring the mPlum transgene

SCNT embryos were generated using the rejuvenated donor cells established from the #2 fetus (Neo-mPlum-PFFs-2). The transgene copy number of Neo-mPlum-PFFs-2 was approximately 3–4 (Fig. 3D). These embryos were transplanted into one recipient pig, resulting

in four offspring; however, all were stillborn (4/89, 4.5%; Table 1). These offspring were confirmed by PCR analysis to harbor the mPlum transgene (Fig. 5A).

In cloned offspring harboring the mPlum transgene, Plum fluorescence was not observed in any of the 11 types of cells, tissues, and organs tested, which included lymphocytes, skin, kidney, pancreas, blood vessels, heart, skeletal muscle, liver, lung, spleen and intestine (Figs. 5C, 6). However, western blotting analysis using anti-DsRed indicated that mPlum was produced in the liver of all four offspring (Fig. 5B). Immunostaining of kidney and heart tissue using anti-DsRed also demonstrated that Plum protein was expressed in these organs (Fig. 5D–5I′).

Discussion

The present study provided primary proof of principle for creating a cloned pig that is immunologically tolerant to fluorescent protein antigenicity and demonstrated that the mPlum systemically expressed in this pig had the same antigenicity as Plum.

Tg animals naturally become immunologically tolerant to transgenic proteins. Therefore, the Tg-cloned pig created in the present study is assumed immunologically tolerant to the protein product of the introduced transgene, mPlum. Nevertheless, the present study demonstrated that mPlum cross-reacts with anti-DsRed antibodies, indicating that Plum and mPlum have the same antigenicity. Therefore, it is likely thought that the Tg-cloned pig created is immunologically tolerant, not only to mPlum, but also to Plum.

Immunorejection does not occur when cell and tissue transplantation occurs between donor and recipient individuals that share a syngenic genetic background, such as inbred mice and rats. This concept is applicable to cell and tissue transplants between cloned pigs created from identical cells [30–32]. Our ultimate goal was to produce two types of animals, individuals that expressed Plum and individuals that expressed mPlum, in a group of cloned pigs sharing a syngenic background. We propose that by using cloned pigs that express Plum and syngenic cloned pigs that express mPlum as donors and recipients, respectively, ideal transplant experiments can be performed in which immunologic rejection does not occur.

To express the mPlum protein that does not emit fluorescence but maintains the antigenicity of the Plum protein, a minimum number of amino acids were replaced in the transgene to disrupt the structure of the chromophore. Of the amino acids that constitute the Plum chromophore (Met[67]-Tyr[68]-Gly[69]), Tyr[68] is essential for the formation of an n-conjugated system structure [26, 27]. We replaced Tyr[68] with Gly, which is the smallest of the aliphatic amino acids, to produce mPlum, which does not emit fluorescence.

Table 1. Production efficiency of cloned fetuses and offspring harboring the mPlum transgene

	Donor cells	No. embryos transferred	Recipients	Pregnancy	Fetuses or offspring obtained (stillborn)	Production efficiency (%) *
Fetus	mPlum-PFFs	71	1	+	8	11.3
Offspring	Neo-mPlum-PFFs-2	89	1	+	4 (4)	4.5

* The production efficiency was calculated as the number of fetuses or offspring obtained divided by the number of embryos transferred multiplied by 100.
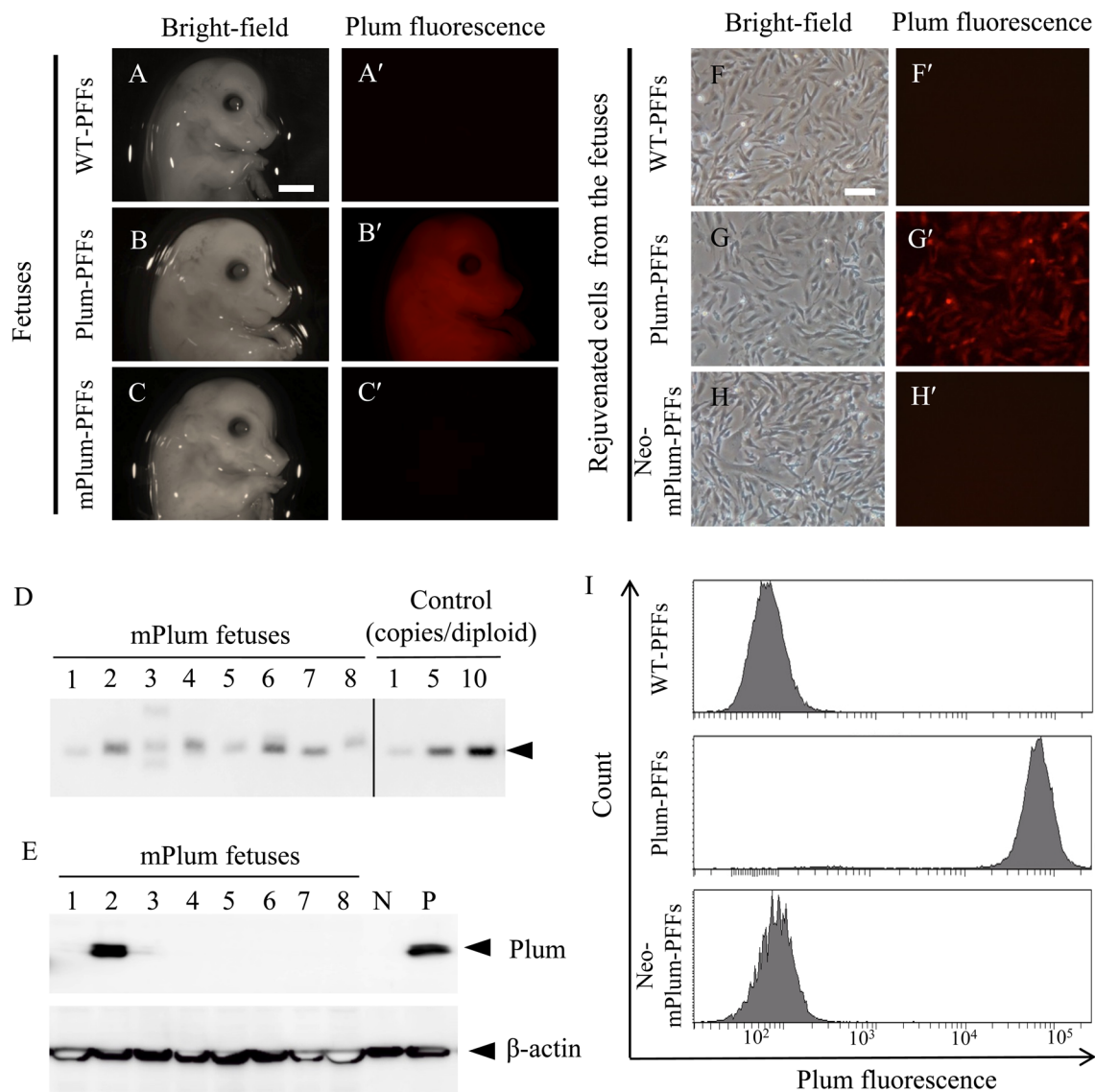
Fig. 3. Analysis of cloned fetuses developed form the nuclear transfer embryos reconstructed with the mPlum-PFFs. (A–C, A′–C′) Bright-field (A–C) and fluorescent images (A′–C′) of the cloned fetuses. (A, A′) Cloned fetus generated from the WT-PFFs. Unpublished picture of an age-matched cloned fetus produced in our separate study (unpublished). (B, B′) Cloned fetus developed from the Plum-PFFs. Unpublished picture of an age-matched cloned fetus produced in our previous study [25]. (C, C′) Cloned fetus produced from the mPlum-PFFs. Scale bar = 5 mm. (D) Southern-blot analysis of the cloned fetus developed from the mPlum-PFFs. The copy number of the transgene integrated into each fetus was defined by comparison with the band intensity of the control lanes. The arrowhead indicates the detected band. (E) Western blot analysis of the fibroblasts established from the cloned fetus generated from the mPlum-PFFs. β-actin was used as the control. N: WT-PFFs were used as the negative control. P: Cells established in our previous study [25] from a cloned fetus harboring the Plum transgene were used as the positive control. The arrowheads indicate the predicted bands. (F–H, F′–H′) Bright-field (F–H) and fluorescent images (F′–H′) of fibroblasts established from the WT fetus (F, F′), Tg fetus harboring the Plum transgene (G, G′), and the mPlum Tg fetus (H, H′) presented in A–C. Scale bar = 100 μm. (I) Flow cytometry analysis of the fibroblasts established from the WT fetus (top), the Plum Tg fetus (middle), and the mPlum Tg fetus (bottom). The X- and Y-axis show the fluorescent intensity and number of the cells, respectively.

In the present study, we performed SCNT using fetal fibroblasts harboring the mPlum transgene as the nuclear donor cells and generated eight fetuses. Variety in the integration and expression pattern of the transgene may be ascribed to the heterogeneity of the nuclear donor cells. Southern blotting analysis of genomic DNA from two of the cloned pigs showed multiple bands and long-chain bands. In

these two fetuses, it is possible that the mutations occurred in the transfected vectors or that transgene was integrated into different sites in the chromosomes.

Furthermore, western blotting analysis indicated that mPlum was produced in only one of eight cloned fetuses. The effect of epigenetic control caused by SCNT may explain inhibited expression of the
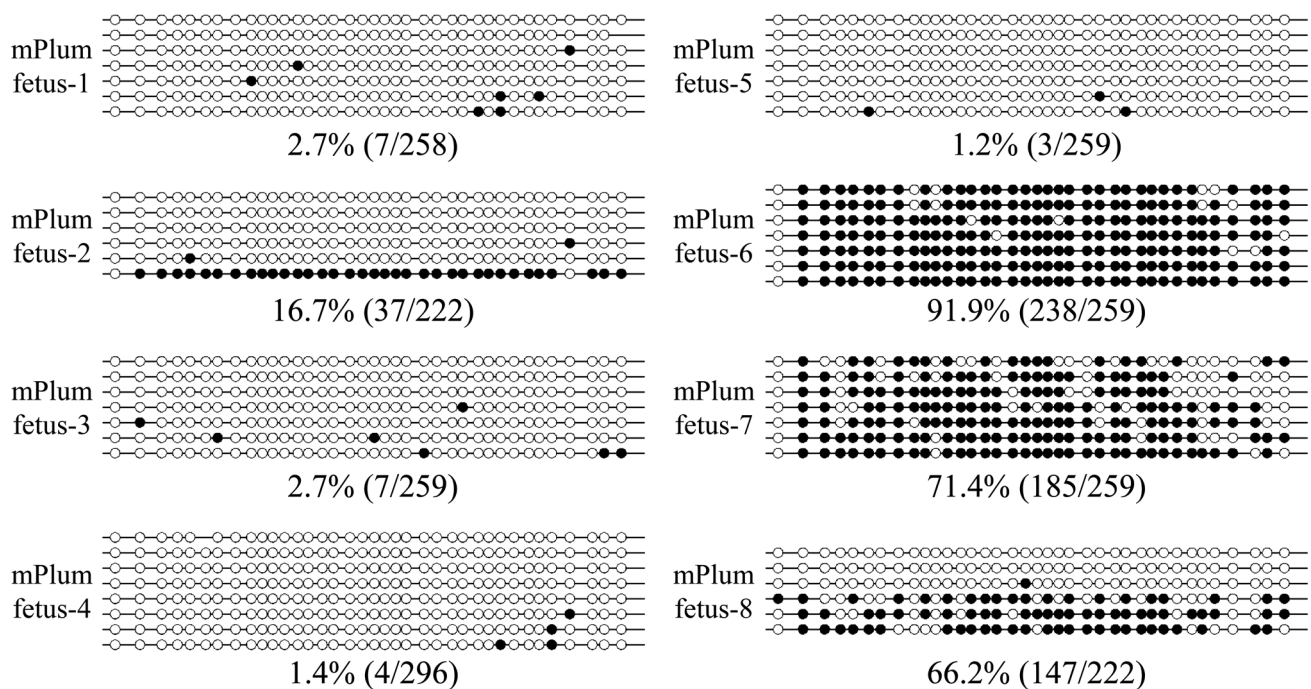
Fig. 4. DNA methylation analysis of the CAG promoter of the fibroblasts established from the mPlum Tg-cloned fetuses generated from the mPlum-PFFs. White and black circles indicate non-methylated and methylated CpGs, respectively.

transgene [33]. In fact, our data showed that the CAG promoter region of the transgene was highly methylated in three of eight cloned fetuses. However, the results of the western blotting analysis of the remaining four fetuses did not explain the inhibited protein production. This will be examined in a future study through acetylation analysis of histones and identification of transgene integration sites by FISH analysis.

In the present study, the production efficiency of the Tg-cloned piglets harboring the mPlum gene was equal to that of Tg-cloned pigs harboring the Plum gene generated in our previous study [25]. However, all of the cloned offspring were stillborn. Because the body weights of the stillborn piglets were similar to the average weight of the previously cloned piglets [25, 34], death may have occurred as a result of an intrapartum accident. Overexpression of mPlum might also have manifested cytotoxicity. It is known that overexpression of xenogeneic transgenes affects the propagation of cells and the growth of the animals due to multiple copy integration [12, 25, 35]. It has also been reported that the excessive expression of fluorescent proteins is detrimental to the growth of cells and individuals [36–39]. The non-fluorescent mPlum used in this study is likely to be as cytotoxic as the original Plum protein. The optimum expression level of mPlum protein has not been determined. Alternatively, we cannot rule out the possibility of insertional mutagenesis that disrupted a necessary gene. Nevertheless, the present study provided the characteristics of pig organs/tissues in which non-fluorescent mPlum proteins were expressed.

In cell/tissue transplantation therapy, including pancreatic islet transplantation, determining the appropriate transplantation site is a vital issue [40]. A transplantation test model comprised of fluorescent donor cells and syngeneic recipient pigs that tolerate the antigenicity of the fluorescent protein will allow for innovative research.

Acknowledgments

References

1. **Day RN, Davidson MW.** The fluorescent protein palette: tools for cellular imaging. *Chem Soc Rev* 2009; **38**: 2887–2921. [Medline] [CrossRef]

2. **Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y.** 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 1997; **407**: 313–319. [Medline] [CrossRef]

3. **Hakamata Y, Tahara K, Uchida H, Sakuma Y, Nakamura M, Kume A, Murakami T, Takahashi M, Takahashi R, Hirabayashi M, Ueda M, Miyoshi I, Kasai N, Kobayashi E.** Green fluorescent protein-transgenic rat: a tool for organ transplantation research. *Biochem Biophys Res Commun* 2001; **286**: 779–785. [Medline] [CrossRef]

4. **Bordignon V, Keyston R, Lazaris A, Bilodeau AS, Pontes JHF, Arnold D, Fecteau G, Keefer C, Smith LC.** Transgene expression of green fluorescent protein and germ line transmission in cloned calves derived from in vitro-transfected somatic cells. *Biol Reprod* 2003; **68**: 2013–2023. [Medline] [CrossRef]

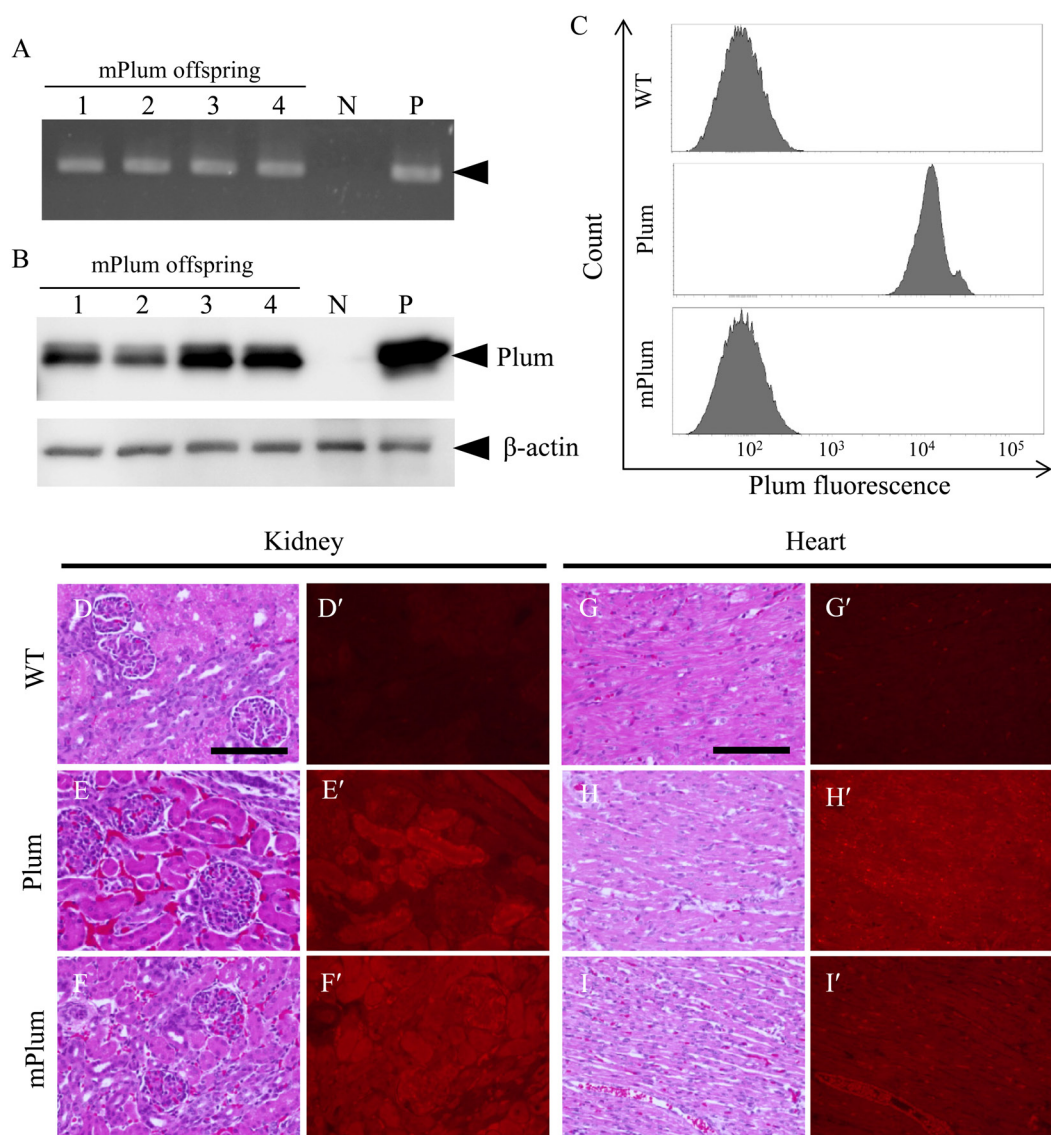5. **Matsunari H, Onodera M, Tada N, Mochizuki H, Karasawa S, Haruyama E,

Fig. 5. Phenotypic analysis of the cloned piglets generated from the Neo-mPlum-PFFs-2 as the nuclear donors. (A) PCR analysis of the Tg-cloned offspring harboring the mPlum transgene. N: Negative control (WT). P: Positive control (mPlum expression vector; pCX-mPlum-puroR). Arrowhead indicates the detected band. (B) Western blot analysis of the Tg-cloned offspring. β-actin was used as the control. N: negative control (WT). P: Positive control (cells of a Tg-cloned pig harboring the Plum transgene [25] was used). The arrowhead indicates the detected band. (C) Flow cytometry analysis of the lymphocytes obtained from the WT (top), the Plum Tg (middle), and the mPlum Tg offspring (bottom). The X- and Y-axis show the fluorescent intensity and number of the cells, respectively. (D–I′) HE and immunohistochemical staining of the kidney (D–F′) and heart (G–I′) tissues of the WT (top), Plum Tg (middle), and mPlum Tg pigs (bottom). Note that the tissues of the mPlum Tg-cloned pig (F′, I′) show positive anti-DsRed antibody staining similar to the tissues expressing the Plum transgene (E′, H′). Scale bar = 100 μm.

Nakayama N, Saito H, Ueno S, Kurome M, Miyawaki A, Nagashima H. Transgenic-cloned pigs systemically expressing red fluorescent protein, Kusabira-Orange. *Cloning Stem Cells* 2008; **10**: 313–323. [Medline] [CrossRef]

6. **Matsunari H, Nagashima H.** Application of genetically modified and cloned pigs in translational research. *J Reprod Dev* 2009; **55**: 225–230. [Medline] [CrossRef]

7. **Holland AM, Micallef SJ, Li X, Elefanty AG, Stanley EG.** A mouse carrying the green fluorescent protein gene targeted to the Pdx1 locus facilitates the study of pancreas development and function. *Genesis* 2006; **44**: 304–307. [Medline] [CrossRef]

8. **Matsunari H, Kobayashi T, Watanabe M, Umeyama K, Nakano K, Kanai T, Matsuda T, Nagaya M, Hara M, Nakauchi H, Nagashima H.** Transgenic pigs with pancreas-specific expression of green fluorescent protein. *J Reprod Dev* 2014; **60**: 230–237. [Medline] [CrossRef]

9. **Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A.** Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 2008; **132**: 487–498. [Medline] [CrossRef]

10. **Klassen H, Warfvinge K, Schwartz PH, Kiilgaard JF, Shamie N, Jiang C, Samuel M, Scherfig E, Prather RS, Young MJ.** Isolation of progenitor cells from GFP-transgenic pigs and transplantation to the retina of allorecipients. *Cloning Stem Cells* 2008; **10**: 391–402. [Medline] [CrossRef]

11. **Shigeta T, Hsu HC, Enosawa S, Matsuno N, Kasahara M, Matsunari H, Umeyama K, Watanabe M, Nagashima H.** Transgenic pig expressing the red fluorescent protein kusabira-orange as a novel tool for preclinical studies on hepatocyte transplantation. *Transplant Proc* 2013; **45**: 1808–1810. [Medline] [CrossRef]
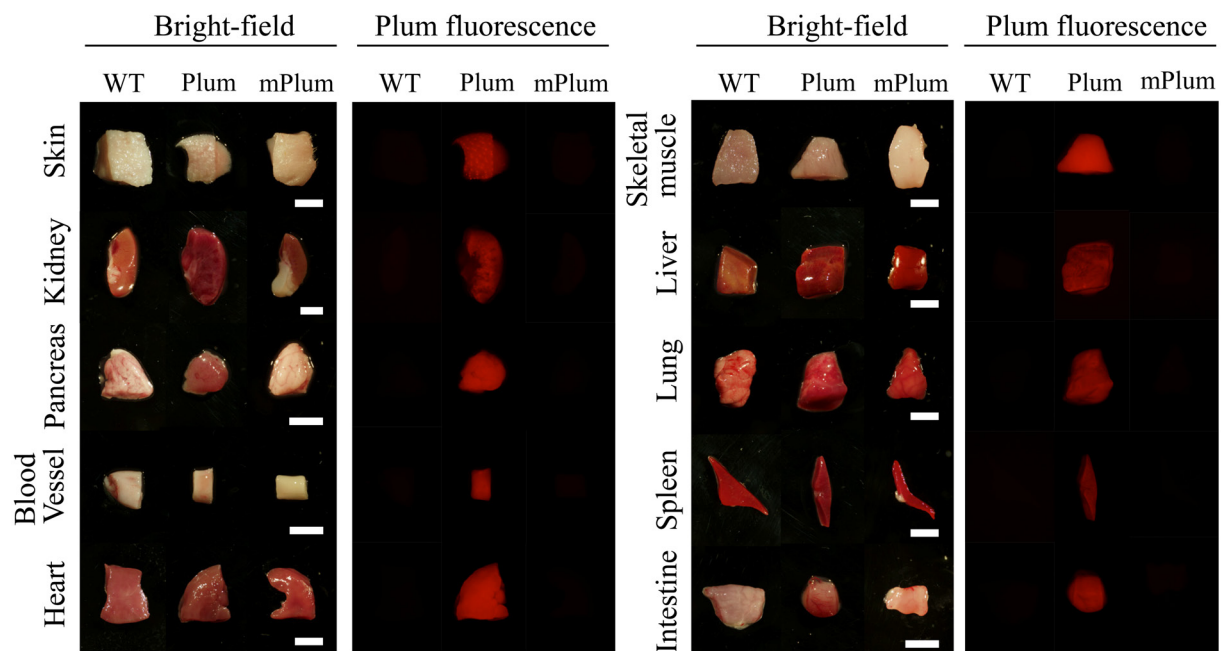
Fig. 6. Analysis of fluorescence expression in various tissues of the Tg-cloned offspring expressing the mPlum transgene. Tissues of WT, the Plum Tg (Plum), and the mPlum Tg (mPlum) pigs were presented in the left, middle, and right lane of each panel. Note that the tissues of the mPlum Tg pig show no fluorescence under excitation. Scale bar = 5 mm.

12. **Strack RL, Strongin DE, Bhattacharyya D, Tao W, Berman A, Broxmeyer HE, Keenan RJ, Glick BS.** A noncytotoxic DsRed variant for whole-cell labeling. *Nat Methods* 2008; **5**: 955–957. [Medline] [CrossRef]

13. **Colin M, Moritz S, Schneider H, Capeau J, Coutelle C, Brahimi-Horn MC.** Haemoglobin interferes with the ex vivo luciferase luminescence assay: consequence for detection of luciferase reporter gene expression in vivo. *Gene Ther* 2000; **7**: 1333–1336. [Medline] [CrossRef]

14. **Lin MZ, McKeown MR, Ng HL, Aguilera TA, Shaner NC, Campbell RE, Adams SR, Gross LA, Ma W, Alber T, Tsien RY.** Autofluorescent proteins with excitation in the optical window for intravital imaging in mammals. *Chem Biol* 2009; **16**: 1169–1179. [Medline] [CrossRef]

15. **Vintersten K, Monetti C, Gertsenstein M, Zhang P, Laszlo L, Biechele S, Nagy A.** Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 2004; **40**: 241–246. [Medline] [CrossRef]

16. **Wang L, Jackson WC, Steinbach PA, Tsien RY.** Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc Natl Acad Sci USA* 2004; **101**: 16745–16749. [Medline] [CrossRef]

17. **Shcherbo D, Merzlyak EM, Chepurnykh TV, Fradkov AF, Ermakova GV, Solovieva EA, Lukyanov KA, Bogdanova EA, Zaraisky AG, Lukyanov S, Chudakov DM.** Bright far-red fluorescent protein for whole-body imaging. *Nat Methods* 2007; **4**: 741–746. [Medline] [CrossRef]

18. **Pan XC, Deng YB, Sugawara Y, Makuuchi M, Okabe M, Ochiya T, Sugiura W, Kitazawa Y, Fuji N, Li XK, Miyamoto M, Kimura H.** Immunological behavior of enhanced green fluorescent protein (EGFP) as a minor histocompatibility antigen with a special reference to skin isograft and specific regulation of local graft-versus-host reaction (GvHR). *Immunol Lett* 2009; **123**: 103–113. [Medline] [CrossRef]

19. **Keijser S, de Keizer RJW, Prins FA, Tanke HJ, van Rooijen N, Vrensen GFJM, Jager MJ.** A new model for limbal transplantation using E-GFP for follow-up of transplant survival. *Exp Eye Res* 2006; **83**: 1188–1195. [Medline] [CrossRef]

20. **Stripecke R, Carmen Villacres M, Skelton D, Satake N, Halene S, Kohn D.** Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther* 1999; **6**: 1305–1312. [Medline] [CrossRef]

21. **Yang Z, Wang Y, Li Y, Liu Q, Zeng Q, Xu X.** Options for tracking GFP-Labeled transplanted myoblasts using in vivo fluorescence imaging: implications for tracking stem cell fate. *BMC Biotechnol* 2014; **14**: 55. [Medline] [CrossRef]

22. **Rosenzweig M, Connole M, Glickman R, Yue SP, Noren B, DeMaria M, Johnson RP.** Induction of cytotoxic T lymphocyte and antibody responses to enhanced green fluorescent protein following transplantation of transduced CD34(+) hematopoietic cells. *Blood* 2001; **97**: 1951–1959. [Medline] [CrossRef]

23. **Beagles KE, Peterson L, Zhang X, Morris J, Kiem HP.** Cyclosporine inhibits the development of green fluorescent protein (GFP)-specific immune responses after transplantation of GFP-expressing hematopoietic repopulating cells in dogs. *Hum Gene Ther* 2005; **16**: 725–733. [Medline] [CrossRef]

24. **Eixarch H, Gómez A, Kádár E, George M, Martínez N, Espejo C, Pétriz J, Gimeno R, Barquinero J.** Transgene expression levels determine the immunogenicity of transduced hematopoietic grafts in partially myeloablated mice. *Mol Ther* 2009; **17**: 1904–1909. [Medline] [CrossRef]

25. **Watanabe M, Kobayashi M, Nagaya M, Matsunari H, Nakano K, Maehara M, Hayashida G, Takayanagi S, Sakai R, Umeyama K, Watanabe N, Onodera M, Nagashima H.** Production of transgenic cloned pigs expressing the far-red fluorescent protein monomeric Plum. *J Reprod Dev* 2015; **61**: 169–177. [Medline] [CrossRef]

26. **Miyawaki A, Nagai T, Mizuno H.** Mechanisms of protein fluorophore formation and engineering. *Curr Opin Chem Biol* 2003; **7**: 557–562. [Medline] [CrossRef]

27. **Karasawa S, Araki T, Nagai T, Mizuno H, Miyawaki A.** Cyan-emitting and orange-emitting fluorescent proteins as a donor/acceptor pair for fluorescence resonance energy transfer. *Biochem J* 2004; **381**: 307–312. [Medline] [CrossRef]

28. **Kurome M, Hisatomi H, Matsumoto S, Tomii R, Ueno S, Hiruma K, Saito H, Nakamura K, Okumura K, Matsumoto M, Kaji Y, Endo F, Nagashima H.** Production efficiency and telomere length of the cloned pigs following serial somatic cell nuclear transfer. *J Reprod Dev* 2008; **54**: 254–258. [Medline] [CrossRef]

29. **Arai Y, Ohgane J, Fujishiro SH, Nakano K, Matsunari H, Watanabe M, Umeyama K, Azuma D, Uchida N, Sakamoto N, Makino T, Yagi S, Shiota K, Hanazono Y, Nagashima H.** DNA methylation profiles provide a viable index for porcine pluripotent stem cells. *Genesis* 2013; **51**: 763–776. [Medline] [CrossRef]

30. **Shimada A, Tomii R, Kano K, Nagashima H.** Transplantation and differentiation of donor cells in the cloned pigs. *Biochem Biophys Res Commun* 2006; **344**: 455–462. [Medline] [CrossRef]

31. **Martin MJ, Yin D, Adams C, Houtz J, Shen J, Chong AS, Sharma A, Byrne GW, Wiseman BS, Logan JS.** Skin graft survival in genetically identical cloned pigs. *Cloning Stem Cells* 2003; **5**: 117–121. [Medline] [CrossRef]

32. **Yokote S, Matsunari H, Iwai S, Yamanaka S, Uchikura A, Fujimoto E, Matsumoto K, Nagashima H, Kobayashi E, Yokoo T.** Urine excretion strategy for stem cell-generated embryonic kidneys. *Proc Natl Acad Sci USA* 2015; **112**: 12980–12985. [Medline] [CrossRef]

33. **Rideout WM 3rd, Eggan K, Jaenisch R.** Nuclear cloning and epigenetic reprogramming of the genome. *Science* 2001; **293**: 1093–1098. [Medline] [CrossRef]

34. **Umeyama K, Watanabe K, Watanabe M, Horiuchi K, Nakano K, Kitashiro M, Matsunari H, Kimura T, Arima Y, Sampetrean O, Nagaya M, Saito M, Saya H, Kosaki K, Nagashima H, Matsumoto M.** Generation of heterozygous fibrillin-1 mutant cloned pigs from genome-edited foetal fibroblasts. *Sci Rep* 2016; **6**: 24413. [Medline] [CrossRef]

35. **Naruse K, Ishikawa H, Kawano HO, Ueda H, Kurome M, Miyazaki K, Endo M, Sawasaki T, Nagashima H, Makuuchi M.** Production of a transgenic pig expressing human albumin and enhanced green fluorescent protein. *J Reprod Dev* 2005; **51**: 539–546. [Medline] [CrossRef]

36. **Tao W, Evans BG, Yao J, Cooper S, Cornetta K, Ballas CB, Hangoc G, Broxmeyer HE.** Enhanced green fluorescent protein is a nearly ideal long-term expression tracer for hematopoietic stem cells, whereas DsRed-express fluorescent protein is not. *Stem Cells* 2007; **25**: 670–678. [Medline] [CrossRef]

37. **Hadjantonakis AK, Macmaster S, Nagy A.** Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. *BMC Biotechnol* 2002; **2**: 11. [Medline] [CrossRef]

38. **Furuta T, Tomioka R, Taki K, Nakamura K, Tamamaki N, Kaneko T.** In vivo transduction of central neurons using recombinant Sindbis virus: Golgi-like labeling of dendrites and axons with membrane-targeted fluorescent proteins. *J Histochem Cytochem* 2001; **49**: 1497–1508. [Medline] [CrossRef]

39. **Long JZ, Lackan CS, Hadjantonakis AK.** Genetic and spectrally distinct in vivo imaging: embryonic stem cells and mice with widespread expression of a monomeric red fluorescent protein. *BMC Biotechnol* 2005; **5**: 20. [Medline] [CrossRef]

40. **Lau J, Mattsson G, Carlsson C, Nyqvist D, Köhler M, Berggren PO, Jansson L, Carlsson PO.** Implantation site-dependent dysfunction of transplanted pancreatic islets. *Diabetes* 2007; **56**: 1544–1550. [Medline] [CrossRef]