Profile of new green fluorescent protein transgenic Jinhua pigs as an imaging source

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Abstract. Animal imaging sources have become an indispensable material for biological sciences. Specifically, gene-encoded biological probes serve as stable and high-performance tools to visualize cellular fate in living animals. We use a somatic cell cloning technique to create new green fluorescent protein (GFP)-expressing Jinhua pigs with a miniature body size, and characterized the expression profile in various tissues/organs and *ex vivo* culture conditions. The born GFP-transgenic pig demonstrate an organ/tissue-dependent expression pattern. Strong GFP expression is observed in the skeletal muscle, pancreas, heart, and kidney. Regarding cellular levels, bone-marrow-derived mesenchymal stromal cells, hepatocytes, and islet cells of the pancreas also show sufficient expression with the unique pattern. Moreover, the cloned pigs demonstrate normal growth and fertility, and the introduced GFP gene is stably transmitted to pigs in subsequent generations. The new GFP-expressing Jinhua pigs may be used as new cellular/tissue light resources for biological imaging in preclinical research fields such as tissue engineering, experimental regenerative medicine, and transplantation. [DOI: 10.1117/1.3241985]

Keywords: Jinhua pigs; green fluorescent protein; biological probes; somatic cell cloning.

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1 Introduction

Imaging has become an indispensable tool in both the biological sciences and medicine. In the past two decades, there has been a huge increase in the number of imaging technologies and their applications. In particular, fluorescent imaging has been most rapidly adapted for *in vitro* and *in vivo* analysis of biological processes.[1] Visualization of processes occurring in

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the complex environment of the cell and/or tissue needs an appropriate cellular marking procedure, and fluorescent dye has often been used as a straightforward technique. However, since fluorescent intensity may decrease during *in vivo* cellular proliferation, the use of fluorescent dye is not always suitable for *in vivo* imaging.[2] Therefore, genetically encoded biological probes serve as stable and high-performance tools to visualize cellular fate in living animals.

The development of genetic molecular tags such as green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*) has mostly accelerated the revolution occurring in this field over the past decade. In fact, GFP as the most popular biological light source has offered important opportunities for the investigation of a wide variety of biological processes in living cells and animals.[3,4] To obtain a stable optic cellular source, we developed GFP-transgenic (Tg) animals, including rats and rabbits.[2,5] These GFP-Tg animals have been employed as valuable cellular and organ sources for cell therapy and transplantation studies.[6–9] For instance, the cultured stem/progenitor cells of GFP-Tg rats transplanted into the spinal cord survived for a long time after transplantation (around 50 days), demonstrating a stable *in vivo* GFP expression.[6] Moreover, application to oligodendrocyte replacement in models of white matter insult and disease also demonstrated the engraftment and survival of GFP-positive oligodendrocytes in the host white matter and cerebral cortex.[9] Thus, the evidence suggests that GFP-Tg animals provide stable cell sources even after cell proliferation and differentiation.

Recent advances in gene manipulation allowed the development of a variety of transgenic animals,[10–12] and the procedure used for the microinjection of animal zygotes has continuously improved.[13,14] However, since expression of an injected expression vector depends on the integration site of the genome and the copy number, it is not always easy to obtain transgenics that ubiquitously express a particular cDNA, even under the general promoter.[15] In fact, our previous results demonstrated that the tissue/organ expression profile depended on the line of established Tg animals.[2] Nonetheless, the characteristics of established Tg animals led to high-demand animal resources for new biomedical translational research fields such as tissue engineering and regeneration medicine. Since the use of authentic transgenic technology through microinjection into zygotes is not always suitable with large transgenic animals, we aimed to generate cloned pigs on the basis of the somatic cell nuclear transfer method[16,17] and the supportive method for reconstructed embryos.[18] In this study we generate new GFP-Tg Jinhua pigs, determine the expression profile of GFP emission light in the Tg pig, and demonstrate a stable reproductive performance. The GFP Tg pig may represent a valuable large-animal resource.

2 Experimental Materials and Methods

2.1 *Plasmid Construction and Polymerase Chain Reaction*

The pEGFPneo expression vector [Fig. 1(a)] was generated by insertion of the PGKneo polyA cassette from the pGKNeoPolyA/pUC19 plasmid into the pCX-GFP vector. The expression plasmids, pCX-GFP and pGKNeo poly-A, were kindly provided by Kashiwazaki (University of Tsukuba, Tsukuba, Japan). GFP cDNA was driven under the chicken β-actin promoter and cytomegalovirus immediate-early 1 gene enhancer.[19] The liner HindIII fragment of the pEGFPneo expression plasmid was transferred into primary fibroblasts of Jinhua pigs (see next).

For confirmation of GFP transduction into the cloned pig, polymerase chain reaction (PCR) was performed using AmpliTaq Gold polymerase (Applied Biosystems Incorporated, Foster City, California). The EGFP sequence was amplified using the following primers: forward, 5′-TGA ACC GCA TCG AGC TGA AGG G-3′; reverse, 5′-TCC AGC AGG ACC ATG TGA TCG C-3′. PCR conditions for each set of primers included initial treatment at 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, followed by extension at 72°C for 2 min. PCR products (307 bp) were analyzed on a 1.5% agarose gel.

2.2 *Animals and Preparation of Transgenic Donor Cells*

Chinese Jinhua pigs were maintained under an experimental protocol approved by the Judging Committee of Transgenic Experiments of Shizuoka Prefectural Swine and Poultry Research Center and Experimental Animal Ethics of Jichi Medical University. Primary fibroblasts from the skin of a 4-day-old female Jinhua pig were grown to confluency in a 100-mm tissue culture dish. Cells (10^6 to 10^7) were trypsinized and transduced with the liner pEGFPneo (10 μg) using an electroporation system [Gene Pulser II; Bio-Rad Company, Limited, Hercules, California; at 0.240 kV, 500 μF in 900 μl of phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}]. Electroporated cells were then cultured in a 100-mm-diam culture dish and maintained with Dulbecco modified Eagle medium (DMEM) (11965-092; Gibco, Carlsbad, California) containing 10% fetal bovine serum (FBS) and 150 μg/ml G418 geneticin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air for 2 weeks. The cells were trypsinized and moved onto 48-well culture plates with one cell per well. GFP-expressing cells were cultured until confluency in 35-mm-diam culture dishes, and thereafter in 60-mm dishes over the course of 6 months. The cells were frozen before nuclear transfer. Donor cells for nuclear transfer were cultured until confluency in a 35-mm dish and produced a synchronized cell cycle by serum starvation (0.5% FBS-DMEM) for 6 days.

2.3 *Somatic Cell Nuclear Transfer*

Mature oocytes and parthenogenotes were produced by methods previously described.[18] Immature oocytes of ovaries collected from a local abattoir were cultured for 40 h, and the maturity of the oocytes was assessed under a stereoscopic microscope. Only oocytes that possessed a distinct first polar body were classified as reaching metaphase 2 and used for nuclear transfer. Nuclear transfer was performed using the microinjection method.[16,17] The nuclei were each introduced into a single enucleated oocyte by piezo-actuated microinjection. Electrostimulation was performed 48 h after the start of maturation (2 to 4 h after nucleus microinjection) in an activation medium containing 280-mM D-mannitol, 0.05-mM $CaCl_2$, 0.1-mM $MgSO_4$, and 0.01% (w/v) polyvinyl alcohol. Pulses were delivered to cells placed between two wire elec-

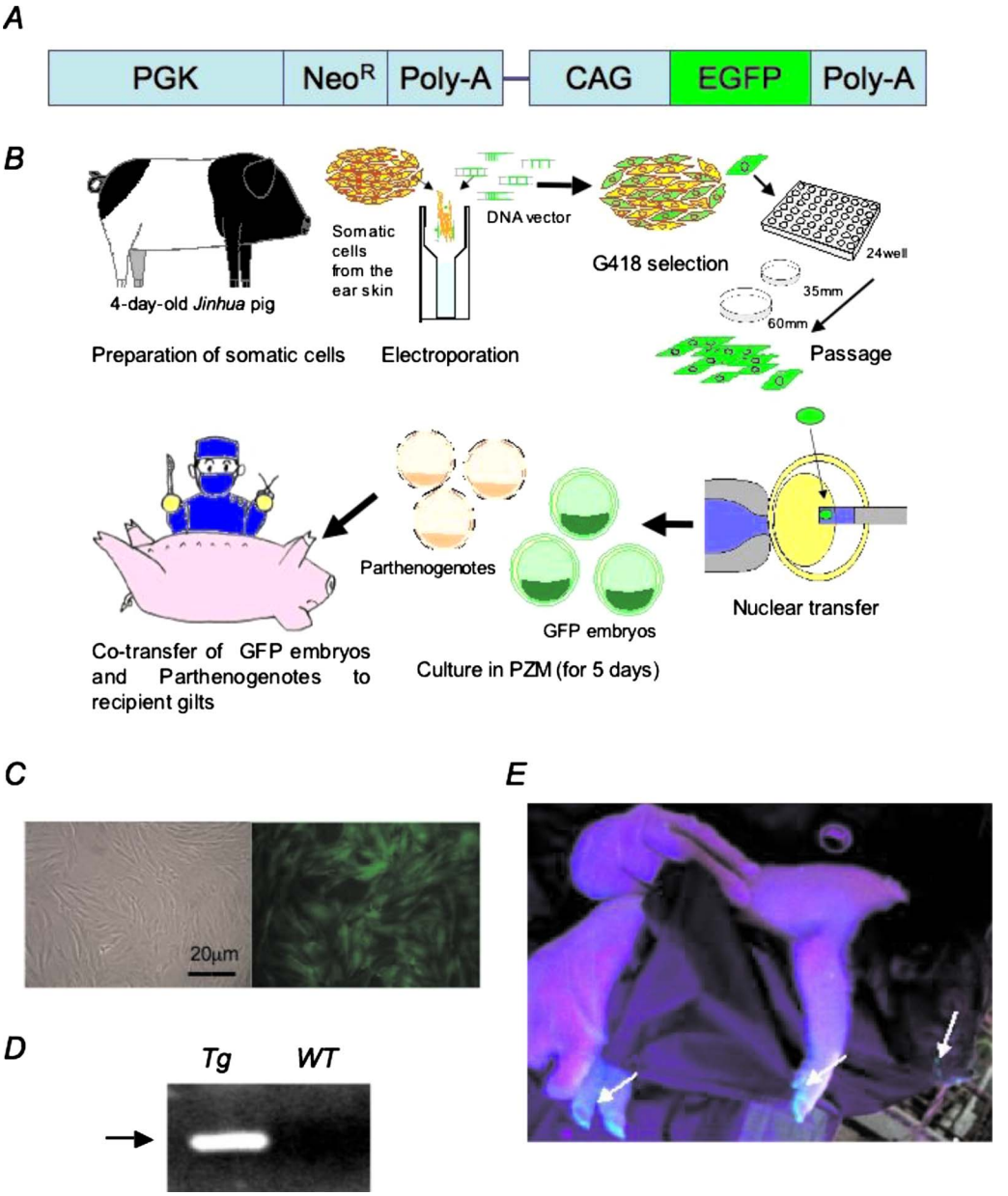

Fig. 1 Establishment of GFP-expressing Jinhua pigs. Representative scheme of the transgene composition. (a) The neomycin-resistant gene (Neo^R) is driven under a mouse phosphoglycerate kinase 1 promoter (PGK), and the enhanced GFP cDNA is driven under the chicken β-actin promoter and cytomegalovirus immediate-early 1 gene enhancer (CAG).[19] (b) Representative scheme of the creation of a cloned pig. (c) GFP expression in cells for nuclear transfer. A G418-resistant single fibroblast was grown in culture. Left panel, visible light; right panel, excitation light. Original magnification 100×. (d) Genotype inspection of a cloned pig. GFP-specific sequences were detected by PCR analysis. Tg, transgenic pig; WT, wild-type pig. (e) Representative image of the generated cloned pig under an excitation light. Arrows indicate parts with strong GFP expression (skin, oral and nasal mucosa, hoof wall).

trodes (1 mm apart) in a fusion dish (CUY5000P1, Nepa Gene Company, Limited, Ichikawa, Japan) by applying a single direct-current pulse of 150 kV/cm for a duration of 99 μsec. The stimulated oocytes were transferred to porcine zygote medium (PZM)[20] supplemented with cytochalasin B (4 μg/ml) for 2 h to prevent cytokinesis, after which the culture was continued in PZM containing 0.3% bovine serum albumin at 38.5°C under 5% O_2 and 5% CO_2 for 110 h. After this period, reconstructed embryos that developed into morula-blastocysts were transferred to the uteri of surrogate sows with parthenogenetic embryos developed at the same stage.[18]

2.4 *Isolation and Culture of Mesenchymal Stromal Cells In Vitro Differentiation Assay, and Hepatocyte Isolation*

Bone marrow cells (BMCs) from cloned pigs were harvested by flushing femurs with ice-cold PBS. Cells were filtered through a 70-μm nylon mesh and plated in T75-cm^2 or T225-cm^2 flasks with DMEM/F-12 (Gibco, Grand Island,

New York) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cultures were kept in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Nonadherent cells were removed after 24 h. Adherent cells were trypsinized with 0.25% trypsin-EDTA (Gibco), harvested and then plated into new flasks at every 90% confluency. Adherent mesenchymal stromal cells (MSCs) from passage 2 were frozen in liquid nitrogen for future use. Early passage cells were examined for their capacity to differentiate in culture.

For the *in vitro* differentiation assay, passage 4 MSCs were tested for their ability to differentiate into osteocytes and adipocytes.[21] For adipocyte differentiation, cells (2×10^5) were cultured with Differentiation Media Bullet Kit-Adipogenic (Lonza, Basel, Switzerland) according to the manufacturer's instructions. MSCs were cultured in 6-well plates with MSC culture medium until they reached confluency. Cells were then exposed to three cycles of adipogenic induction medium alternating with adipogenic maintenance medium. Following three complete cycles of induction/maintenance, the MSCs were cultured for 7 more days in supplemented adipogenic maintenance medium. Cell differentiation to adipocytes was confirmed by Oil Red O (Muto Chemicals, Tokyo, Japan) staining. For osteogenic differentiation, cells (2×10^4) were plated and the culture medium was replaced with Differentiation Media Bullet Kit-Osteogenic (Lonza) until confluence. Cells were stained with alizarin red S (Wako Pure Chemicals).

Primary hepatocytes from swine liver were prepared by the conventional perfusion method with collagenase digestion. Hepatocytes were seeded at 3×10^6 cells per dish in 10-cm-diam plastic dishes and cultured in William's E complete medium (Gibco, Grand Island, New York) supplemented with 10% FBS, 1-nM insulin, and 1-nM dexamethasone in a humidified atmosphere of 5% CO_2/95% air at 37°C.

2.5 *Flow Cytometry*

Peripheral blood cells were isolated from 10 ml of freshly drawn heparinized blood of pigs, and red cells were lysed in a buffer containing 155-mM NH_4Cl, 10-mM $KHCO_3$ and 0.1-mM EDTA. Precipitated white cells were resuspended in 0.1% FBS-PBS, and cells were analyzed using FACSCalibur (Beckton Dickinson, Mountain View, California) and FlowJo analysis software (Tree Star, San Carlos, California).

2.6 *Histological Analysis, Nissl Staining, and Hematoxylin and Eosin staining*

Three male GFP-Tg Jinhua cloned pigs were used for histological observations. Animals (8 to 36 months, weighing approximately 70 to 120 kg) were deeply anesthetized by inhalation of isoflurane (4% 1 L/min, Dinippon Pharmaceutical Company, Osaka, Japan) and intramuscular injection of domitor (0.6 mL, Meiji seika, Tokyo, Japan) and dormicum (0.6 mL, Astellas Pharma, Tokyo, Japan). Tissues (brain, pancreas, skeletal muscle, cardiac muscle, small intestine, stomach, liver, colon, and testis) were isolated from anesthetized Tg animals and fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) with 0.1-M phosphate buffer (pH 7.4). Tissue samples were soaked in 20% sucrose in PBS at 4°C for 2 to 4 days, frozen in OCT compound (Tissue-Tek, Sakura Finetechnical Company, Tokyo, Japan), and then sectioned at a thickness of 10 to 40 μm using a cryostat (Leica CM 1850, Leica Incorporated, Nussloch, Germany). Sections were then mounted onto silane-coated slides.

To identify the cell architecture of the brain, delipidated brain sections were immersed in 0.1% solution of Cresylecht violet (Chroma-Gesellschaft, Munster, Germany) overnight at 37°C (Nissl staining). Slides were dehydrated through graded ethanol baths, delipidated in xylene, and then mounted with Mount-Quick (Daidosangyo Company, Tokyo, Japan).

For hematoxylin and eosin (HE) staining, all samples except for brain were embedded in paraffin (Wako Pure Chemicals) and then sectioned at a thickness of 4 μm using a sliding microtome (REM-700, Yamato Kohki Industrial Company, Saitama, Japan). Sections were deparaffinized, rehydrated to water, and then stained with hematoxylin (Wako Pure Chemicals) and eosin (Wako Pure Chemicals).

All frozen sections except for brain were counterstained with DAPI (Sigma Chemical, Saint Louis, Missouri). GFP fluorescence was examined using a fluorescent microscope Keyence BZ-9000 (Keyence, Tokyo, Japan). Micrographs were taken using a digital camera attached to the same microscope. Digital images were processed with Adobe Photoshop CS2 to adjust the final plates.

2.7 *Survey of Reproductive and Growth Performance in Green Fluorescent Protein Pigs*

A primary GFP Jinhua pig mated with wild-type Jinhua boars and farrowed the second generation of GFP-Tg piglets (three litters). Boars of the second generation mated with five wild-type Jinhua sows and the sows farrowed the third generation.

For surveillance of reproductive and growth performance in GFP-Tg Jinhua pigs, pregnant pigs that conceived GFP pigs entered the farrowing unit 1 week before the expected date of parturition and were housed in an individual section (1.2 × 2.5 m) of a slated barn until weaning. Piglets were weaned at 30 days of age. After weaning, piglets were reared in pens (2.4 × 2.5 m) for each litter. Each pen had a feeder and water cup that allowed free access to feed (18% crude protein, 3080 kcal/kg digestible energy) and water throughout the experiment. The body weight of piglets was recorded every week until they reached two months of age.

2.8 *Statistical Analyses*

All statistical analyses were performed with StatView software (Windows©version 5; SAS Institute Incorporated, Cary, North Carolina). Data concerning born-alive rate (number born alive/number of total born) and weaning rate (number of weaning piglets/number born alive) between GFP-positive and GFP-negative piglets were analyzed by a Fisher's exact test. Data regarding body weight of piglets were analyzed using two-way factorial ANOVA and the Tukey-Kramer multiple range test.

3 Results

3.1 *Generation of Green Fluorescent Protein Expressing Cloned Pigs*

Primary fibroblasts from the skin (4-day-old female Jinhua pig) were isolated and grown for transgenic donor cells. The liner pEGFPneo plasmid DNA (10 μg) was transduced with

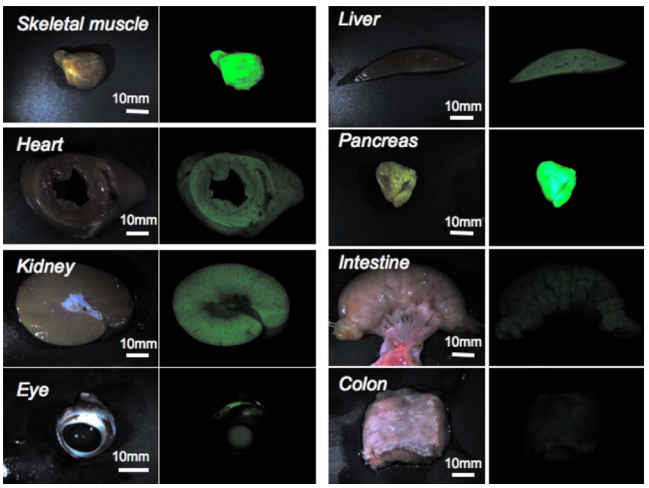

Fig. 2 Macroscopic GFP expression profile in various organs from cloned pigs. Representative organs (skeletal muscle, heart, kidney, eye, liver, pancreas, small intestine, and colon) were removed from GFP-expressing pigs and macroscopically examined under a visible (left) or 489-nm excitation (right) light. Results derived from one of two independent experiments showing similar results.

primary fibroblasts using an electroporation system. After G418 selection for 2 weeks, 212 colonies (18.0%) of 1177-sorted cells were grown to confluence in 35-mm culture dishes. One of the cell lines that expressed a strong GFP signal was used for nuclear transfer [Fig. 1(c)]. 23 (9.2%) of 249 somatic cell nuclear transferred oocytes developed to the morula-blastocyst stage 110 h after nuclear transfer. These morula-blastocysts (average 7.7/recipient) were cotransferred with parthenogenetic embryos (average 17.3/recipient) into three recipient gilts, two of which became pregnant and each farrowed a total of two piglets (8.7%: 2/23) at day 113 after nuclear transfer. Both of the two cloned Jinhua pigs possessed GFP-specific sequences according to PCR analysis [Fig. 1(d)]. Visual inspection under an excitation light demonstrated that GFP-derived fluorescence was clearly expressed in the skin, oral and nasal mucosa, and hoof wall of the transgenic pigs [Fig. 1(e)].

3.2 *Green Fluorescent Protein Expression Pattern in the Newly Created Cloned Pig*

In an effort to examine the expression pattern of newly generated cloned pigs, various organs were removed from the transgenic pigs and their macroscopic expression pattern was determined (Fig. 2). While skeletal muscle and pancreas showed strong GFP expression in the cloned pigs, expression in gastrointestinal tracts and eyes was weak in comparison. GFP signals in the kidney and liver of cloned pigs appeared moderate and heterogeneous.

We further analyzed microscopic GFP expression patterns in various organs (Table 1 and Figs. 3–5). GFP expression sites in the central nervous system (CNS) are summarized in Fig. 3. Nisslstaining was also performed to identify the cell architecture of the brain [Figs. 4(a)–4(k)]. Small-sized and round-shaped GFP-positive cell bodies were found in the olfactory bulb [Figs. 3(a) and 4(d)], lateral ventricle [Figs. 3(b), 3(c), 4(g), and 4(h)], and hippocampus [Figs. 3(c) and 4(f)].

Table 1 Expression profile of GFP-expressing Jinhua pigs. GFP expression was determined microscopically under a 489-nm excitation light.

GFP expression	Tissues/cells
Very strong (+++)	Skeletal muscle, heart, pancreas, thyroid gland
Strong (++)	Epidermis (skin), tongue, oral mucosa, liver, kidney, brain, lung
Moderate (+)	Spleen, stomach, small intestine, colon, thymus, testis, ovary, eye, bone, cartilage, vessels
Negative (−)	Connective tissue (dermis), erythrocyte, sperm

In contrast, large-sized cell bodies were observed in the cerebellum [Fig. 4(j)]. Both large- and small-sized cell bodies were found in the medula oblongata [Fig. 4(l)]. GFP-positive fibers were found in the olfactory bulb [Figs. 4(b) and 4(d)], cerebellum [Fig. 4(j)], and medula oblongata [Fig. 4(l)].

GFP expression of the skin was predominant in the epidermis (the granular layer and stratum spinosum) and the hair follicle, but less so in the dermis [Figs. 5(a) and 5(b)]. Expression in skeletal [Figs. 5(c) and 5(d)] and cardiac muscle [Figs. 5(e) and 5(f)] appeared modestly heterogeneous, but most of the muscle fibers were GFP-positive. Regarding expression in the liver, parenchymal cells appeared GFP-positive and interstitial cells were GFP-negative [Figs. 5(g) and 5(h)]. Acinus cells in the pancreas were strongly GFP-positive [Figs. 5(i) and 5(j)]. In the gastrointestinal tract, GFP was heterogeneously expressed in the epithelium of the stomach, intestine, and colon [Figs. 5(k)–5(p)]. Primary and secondary spermatocytes in the testis weakly expressed GFP, al-

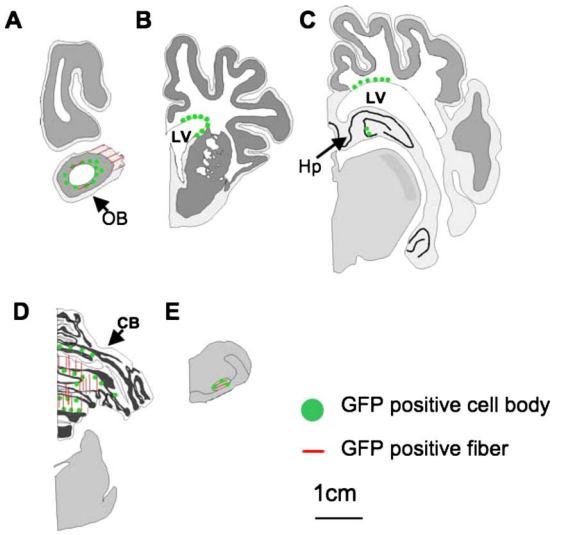

Fig. 3 Schematic illustration of GFP expression sites in the brain of cloned pigs. Coronal sections are illustrated from (a) rostral to (e) caudal. The green circle represents GFP-positive cell bodies and the red line indicates GFP-positive fibers. (Color online only.)

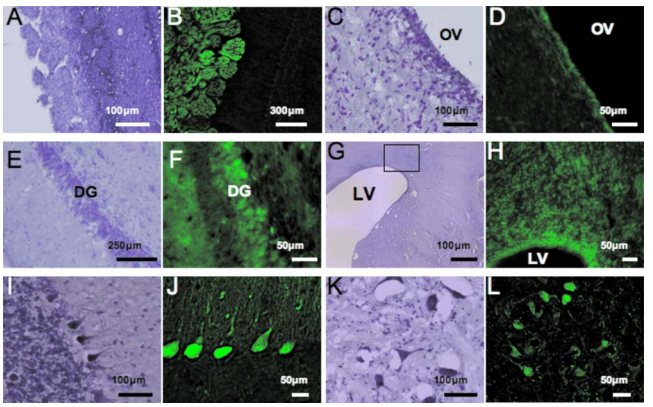

Fig. 4 Photomicrographs of GFP fluorescence and Nissl-staining in the brain of cloned pigs. (a) to (d) olfactory bulb; (e) and (f) hippocampus; (g) and (h) lateral ventricle; (i) and (j) cerebellum; (k) and (l) medula oblongata. (a), (c), (e), (g), (i), and (k): Nissl staining. (b), (d), (f), (h), (j), and (k): 489-nm excitation light.

though sperms were almost totally GFP-negative [Figs. 5(q) and 5(r)]. Since it is important to know the fate of leukocytes in many biomedical studies, we examined GFP expression of peripheral blood cells from cloned pigs [Figs. 5(s) and 5(t)]. Leukocytes were GFP-positive, granulocytes exhibited a particularly strong expression of GFP, and mononuclear cells were also moderately GFP-positive. Notably, GFP expression of erythrocytes was definitely negative. FACS analysis also revealed strong GFP expression in peripheral leukocytes [Fig. 5(u)].

3.3 *Green Fluorescent Protein Expression in Potential Cell Sources*

To restore form and function to damaged tissues, a cell transplantation strategy has emerged as a potential therapeutic approach involving the use of autologous cells. Therefore, we examined GFP expression in the processed cells for transplantation (Fig. 6). Since mesenchymal stem cells (MSCs) possess a high expansion potential and genetic stability[22] and can be easily isolated and transferred from the laboratory to the bedside, we first examined GFP expression in bone-marrow-derived MSCs. As shown in Fig. 6(a), sufficient levels of GFP expression were observed in isolated MSCs. The cells rapidly proliferated and formed colonies, and GFP expression levels were not altered, even within several cell passages (data not shown). These cells were capable of differentiating successfully into osteocytes [Fig. 6(c)], but poorly into adipocytes [Fig. 6(b)]. These results demonstrate that GFP expression was stable in MSCs from cloned pigs and preferentially differentiated into osteocytes. We next addressed GFP expression in cultured parenchymal hepatocytes from the cloned pigs [Figs. 6(d)–6(f)]. Sufficient levels of GFP expression were observed in proliferating hepatocytes, but not in adherent hepatocytes with the contact inhibition. A similar phenomenon was observed in Langerhans islets isolated from cloned pig pancreas [Figs. 6(g)–6(i)].

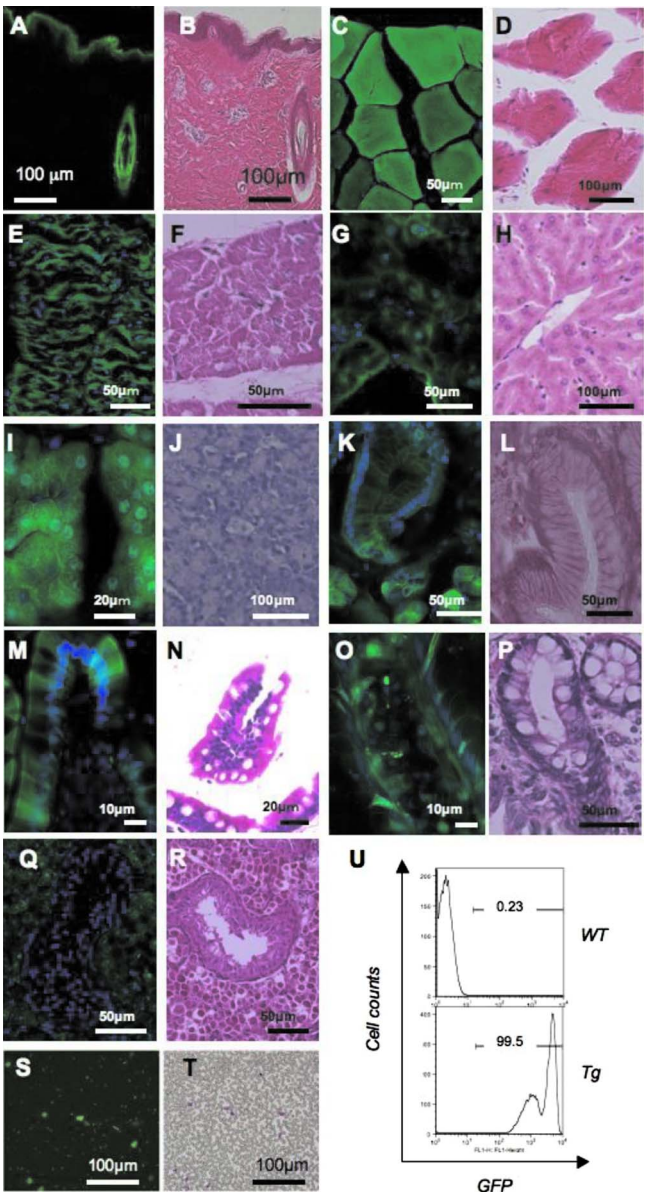

Fig. 5 Microscopic GFP expression in representative tissue sections from cloned pigs other than brain. (a), (c), (e), (g), (i), (k), (m), (o), and (q) Representative tissue sections (skin, skeletal muscle, cardiac muscle, liver, pancreas, stomach, small intestine, colon, and testis) from GFP-expressing pigs were inspected under 489-nm excitation light HE staining: (b), (d), (f), (h), (j), (l), (n), (p), and (r). (s) and (t) Hemogram of peripheral blood from a GFP-Tg pig. Leukocytes (granulocytes and mononuclear cells) expressed GFP, although erythrocytes showed no expression. (s) 489-nm excitation light; (t) visible light. (u) Representative flow-cytometrical GFP-expression pattern in peripheral blood leukocytes. More than 90% of leukocytes were GFP-positive. WT, leukocytes from a wild-type pig; Tg, leukocytes from a GFP-transgenic pig. Results derived from one of two independent experiments showing similar results.

3.4 *Reproductive Performance of Green Fluorescent Protein Jinhua Pigs*

A piglet of GFP-cloned piglets died 2 days after birth, but the remainder grew up normally and expressed estrus. The primary GFP-cloned female mated with a wild-type boar by artificial insemination and farrowed the second generation of

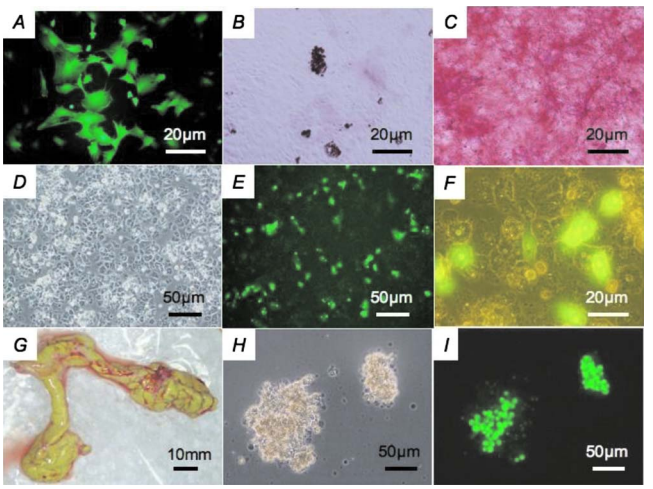

Fig. 6 GFP expression in potential cellular sources. (a) Strong GFP expression in bone-marrow-derived MSCs from the GFP-transgenic pig. Under appropriate differentiation conditions, MSCs were capable of differentiating into (c) adipocytes (stained with Oil red O for lipid droplets) and (d) osteocytes (stained with alizarin red for mineral deposition). Original magnification 20×. (d), (e), and (f) GFP expression in cultured parenchymal hepatocytes from cloned pigs. (d) Visible light, (e) excitation light. (f) Representative merged image of GFP-expressing cultured hepatocytes. (g) Visual inspection of the pancreas of cloned pigs (visible light). Langerhans islets isolated from the cloned pig pancreas were cultured and GFP expression was examined under (h) a visible and (i) 489-nm excitation light (original magnification 200×). (Color online only.)

A

Reproductive performance of GFP cloned pig			
Litter number	Litter size	Number of GFP positive (%)	Number of weaned piglets
1	8	3 (37.5)	8
2	14	8 (57.1)	12
3	11	6 (54.5)	9
Average	11	5.7 (51.5)	9.7

B

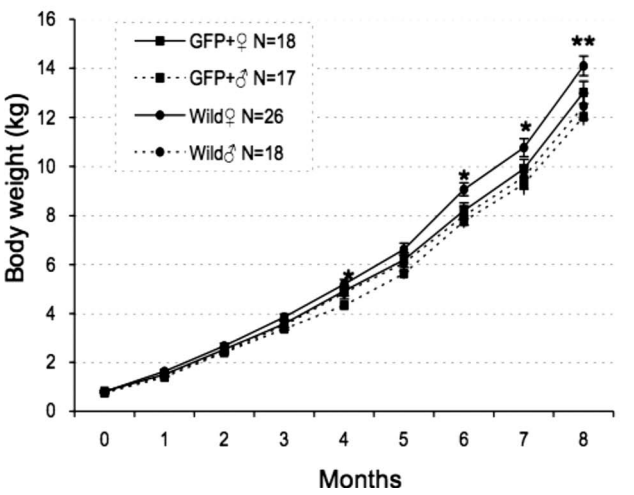

Fig. 7 Reproductive and growth performance of GFP-expressing pigs. (a) Summary of average litter size and average number of weaned piglets in three parities. (b) Growth performance of GFP cloned pigs. GFP, GFP cloned pigs. Wild, wild-type pigs; ♀, female; ♂, male. The female is heavier than the male after reaching 4 months of age (4, 6, and 7 weeks: *, $p < 0.05$; 8 weeks: **, $p < 0.01$).

GFP pigs. The average litter size and average number of weaned piglets in three parities were 11 and 9.7, respectively [Fig. 7(a)]. To examine the GFP expression rate in the second generation, PCR analysis and inspection by excitation light were also performed. The results showed that the GFP expression rate was 51.5% (17/33). Two boars of second-generation GFP Jinhua pigs mated with five females of wild-type pigs. The third generations (total 52 piglets) were born and 22 (42.3%) of these individuals were GFP-positive in genotype and phenotype. These results indicate that reproductive activity is successfully maintained in the established GFP-cloned Jinhua pigs, and that the introduced GFP transgene can be stably transmitted to pigs in subsequent generations.

3.5 *Growth Performance of Green Fluorescent Protein Transgenic Pigs*

We further evaluated the influence of the GFP transgene on growth performance of GFP-Tg pigs. A total of 85 piglets (33 of second-generation and 52 of third-generation piglets) were examined using body weight. The born-alive rate in GFP-positive and -negative pigs was 89.7% (35/39) and 95.7% (44/46), respectively. The weaning rate in GFP positive and negative piglets was 100% (35/35) and 97.7% (43/44), respectively. There were no differences between GFP-positive and -negative piglets for born-alive and weaning rates. The body weights of 79 born-alive piglets did not differ between GFP-positive and -negative piglets, while the female was heavier than the male after 4 months of age (4, 6, and 7 weeks: $P < 0.05$; 8 weeks: $P < 0.01$) [Fig. 7(b)]. As with wild-type Jinhua pigs, the body size of GFP-positive piglets reached plateau levels around 24 months (male: 96.0 ± 4.7 kg [n = 8]; female: 107.6 ± 13.3 kg [n = 12]), and the miniature size was maintained. These results demonstrate that the GFP transgene has less effect on growth performance of Tg pigs.

4 Discussions

We created new GFP-expressing pigs using a somatic cell cloning technique. The remarkable features presented as an imaging source include: 1. the born GFP-Tg pig demonstrated an organ/tissue-dependent expression pattern; 2. it displayed normal growth and fertility; and 3. the introduced GFP gene was transmitted to pigs in subsequent generations. The new GFP-expressing Jinhua pigs could provide new cellular/tissue light sources for biological imaging.

The Jinhua pig is a kind of indigenous Chinese pig.[23] The growth and reproductive traits of this pig were evaluated using microsatellite markers.[24,25] It has been reported that the mean litter size is 11 piglets, and that the pig shows premature growth and high multiplication.[26,27] The adult body weight of Jinhua pigs ranges from 90 to 110 kg (data not shown). Although Jinhua pigs have sufficient reproductive ability, the pigs have not been used as a commercial base resource [due to the low carcass lean content (29 to 30%) compared with large white pigs (53 to 54%).[27] However, since Jinhua pigs

exhibit a middle body size similar to the human body size, organs from this breed may represent an appropriate organ resource of xeno-transplantation for humans.

GFP expression was observed in various organs in this study, although expression levels differed between tissues/organs. It is very important for researchers to know the expression profile for various tissues/organs, because it is impossible to regulate the integration site and the copy number of transgenes into the genome in transgenic animals.[2,22] Since our previous results demonstrated that naked GFP-expression plasmid DNA was successfully expressed in the pig liver,[28] this general promoter and enhancer could be driven in various tissues/organs of cloned pigs. In particular, GFP expression was strong in the skeletal muscle, pancreas, heart, and kidney. These organs are potentially available for organ transplantation experiments as in the case of rats.[2] In terms of experimental cell therapy, it may also be possible to use neural progenitor cells. However, the culture system for pig neural progenitor cells may be required for future *in vivo* animal experiments, whereas the system for cell culture and large-animal experiments remains to be established. At the very least, MSCs, hepatocytes, and islet cells of the pancreas seem to be available for cell transplantation studies.

The present study demonstrated that an introduced GFP gene was very stable in Jinhua pigs, and that it was transmitted to the second and third generations. The first sow transmitted the gene to the second generation (both male and female pigs), and the second-generation male pig transmitted it to the third generation (both male and female pigs), suggesting a stable genotype and phenotype transmission. This also allows us to preserve this animal source as a fertilized egg and/or sperm.

The cells used for the nuclear transfer in this study were grown in culture from one cell of fibroblasts, and they had eventually divided around 20 times. Nevertheless, the evidence that normal individual pigs were successfully generated suggests that somatic cells of the pig may be resistant to gene alterations, including epigenetic mutations, in comparison with other animals such as cows.[29–32] This may represent a species-specific characteristic.

As indicated in Fig. 7(a), the GFP-transmission rate was around 50% from the sow to the second generation. This suggests the transduced gene was integrated into one portion of a chromosome. It was thought that gene transmission by this somatic cell cloning technique was almost totally equivalent to the case of intracytoplasmic sperm injection-mediated DNA transfer.[33] This study also demonstrated that the GFP gene did not influence the growth or reproduction of the cloned pig greatly. Since the GFP-Tg pigs were maintained in hemizygous conditions in the present study, any influence under homozygous conditions remains to be elucidated.

In conclusion, new GFP-expressing pigs showed normal growth and stable reproductive activity. Taking advantage of stable light sources, the GFP expression profile in these pigs may provide useful imaging information in research fields such as tissue engineering, experimental regenerative medicine, and transplantation.

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