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Disruption of exogenous eGFP gene using RNA-guided endonuclease in bovine transgenic somatic cells

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Summary

Genome-editing technologies are considered to be an important tool for generating gene knockout cattle models. Here, we report highly efficient disruption of a chromosomally integrated eGFP gene in bovine somatic cells using RNA-guided endonucleases, a new class of programmable nucleases developed from a bacterial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system. In the present study, we obtained homogenously eGFP-expressing primary fibroblasts from cloned bovine transgenic embryonic tissues and employed them for further analysis. CRISPR/Cas9 plasmids specifically targeting the eGFP gene were transfected into the eGFP fibroblasts by electroporation. After 10 days of culture, more than 40% of the cells had lost eGFP expression in fluorescence activated cell sorting (FACS) analysis. Targeted sequences of the transfected cells were analyzed, and various small indel mutations (6–203 bp deletions) in the target sequence were found. The fibroblasts mutated with the CRISPR/Cas9 system were applied for somatic cell nuclear transfer, and the reconstructed embryos were successfully developed into the blastocyst stage. In conclusion, the CRISPR/Cas9 system was successfully utilized in bovine cells and cloned embryos. This will be a useful technique to develop livestock transgenesis for agricultural science.

Keywords: CRISPR-Cas9, eGFP disruption, PiggyBac, Transgenic cattle

Introduction

In vitro production of cattle embryos is not only important for genetic improvement but also for understanding diseases and basic embryology (Galli *et al.*, 2003; Hasler, 2003; Mapletoft & Hasler, 2005). The technologies of *in vitro* fertilized embryos, stimulation of multiple ovulations, and embryo transfer have been widely used for practical applications (Hasler, 2003). Furthermore, following the birth of cloned or transgenic cattle, several genetically engineered bovine models for bioreactors and disease studies have been produced by gene transfection or knockout technology (Niemann and Lucas-Hahn, 2012).

Knockout of a gene is an effective approach to understanding its function in cell lines or animal models. For this purpose, numerous knockout mice have been produced using embryonic stem cells

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with targeted mutation derived from homologous recombination (HR) (Guan *et al.*, 2010). Unfortunately, this technique cannot be applied in livestock due to the lack of an embryonic stem cell line. Rather than using embryonic stem cells, the somatic cell nuclear transfer (SCNT) technique, which produces cloned animals, can be used for generating knockout livestock. For the SCNT technique, only primary culture cells can be used. However, gene targeting by HR in primary cultured cells is very laborious, time-consuming and inefficient due to the low transfection rate, slow population doubling time, and short life-span (Clark *et al.*, 2000). Therefore, only a few knockout SCNT livestock animals have been reported to date (Lai *et al.*, 2002; Rogers *et al.*, 2008). Subsequently, genome-editing technologies, such as zinc-finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN), have been applied to generate knockout models in mice (Sung *et al.*, 2013), rats (Jacob *et al.*, 2010), pigs (Yang *et al.*, 2011), and cattle (Carlson *et al.*, 2012; Tan *et al.*, 2013). Recently, the CRISPR/Cas9 system has emerged as an improved platform for programmable nucleases in the gene editing field (Cho *et al.*, 2013; Sung *et al.*, 2014). This technology has proven to be an efficient tool to disrupt targeted gene function and to generate knockout animals. However, the CRISPR/Cas9 system has, to date, not been tested with bovine cells. Here, we report the successful generation of gene knockout cloned embryos by SCNT of bovine cells treated with CRISPR/Cas9.

Materials and methods

Isolation and *in vitro* culture of bovine fibroblasts expressing eGFP

The bovine eGFP fibroblasts used in this study were isolated from transgenic embryonic tissues via the *piggyBac* gene delivery system. To obtain the transgenic embryonic tissues, cloned bovine embryos reconstructed with an eGFP transgenic fibroblast were produced and *in vitro* cultured to blastocyst stage, as described in our previous report (Kim *et al.*, 2011). Among the cloned embryos, a single eGFP-expressing blastocyst with good morphology (Fig. 1*A*) was transferred into each recipient. Pregnancy was confirmed using ultrasonography and the conceptus was collected surgically at day 40 of gestation. After enzyme digestion of the skin region of the conceptus with collagenase I, primary fibroblasts were attached to culture dishes and expanded in culture medium, DMEM (Gibco, Carlsbad, CA, USA) supplemented 15% FBS (Gibco), 100 mM beta-mercaptoethanol (Sigma), 1% NEAA (Sigma), and 1% penicillin/streptomycin (Gibco). Expressions of eGFP in the cultured fibroblasts were confirmed by the use of a fluorescence microscope (Nikon, Tokyo, Japan).

Transfection of CRISPR/Cas9 plasmids specifically targeting eGFP gene

For inducing targeted mutation at the eGFP gene in the transgenic fibroblasts, we employed a modified CRISPR/Cas9 system using single guide RNA (sgRNA). The design of the sgRNA specifically targeted the eGFP gene (Fig. 2*B*), and expression vectors of sgRNA with U6 promoter, and Cas9 protein with a cytomegalovirus (CMV) promoter (Fig. 3), were prepared as described previously (Cho *et al.*, 2014). The work flow for using sgRNA is presented in Fig. 2*A*. The plasmids for Cas9 and eGFP-sgRNA (total 10 μg, 1:3 ratio, respectively) were transfected into the eGFP fibroblasts using Nucleofector technology (Neon®, Invitrogen; program #16). After transfection, the fibroblasts were cultured for an additional 10 days in incubators at 38°C, 5% CO_2 in humidified air. Elimination of eGFP expression in the transfected fibroblasts was monitored using fluorescence microscopy. The experiments were replicated three times.

Flow cytometry analysis of eGFP expression after Cas9

The fibroblasts transfected with eGFP-CRISPR/Cas9 DNAs were trypsinized and harvested. After washing with cold phosphate-buffered saline (PBS), the cells were resuspended at a concentration of 5×10^5 cells/ml. The proportion of eGFP-expressed cells in the whole suspension was measured using flow cytometry (FACSCalibur, BD Biosciences, Seoul, BD-Korea) with Cell-Quest software (BD Biosciences). Based on side scatter (SSC) and forward scatter (FSC), only the area with a dense population was gated for analysis. Green fluorescent protein (GFP) expression was displayed as a single parameter histogram, and the number of cells was represented. All flow cytometric data were analyzed with FlowJo software (Tree Star, San Carlos, CA, USA).

Sequence analysis of the targeted region

Following 10 days of transfection, whole genomic DNA were isolated from the cultured fibroblasts containing GFP-negative colony using the G-spin™ Total DNA Extraction Mini Kit (iNtRON, Seoul, Republic of Korea). A 575-bp fragment containing the targeting site was amplified with the following primers: Forward primer 5′-GGACTTCCTTTGTCCCAAATCT-3′ and Reverse primer 5′-TAGCGGCTGAAGCACTGC-3′ using Maxime PCR PreMix (iNtRON). The PCR

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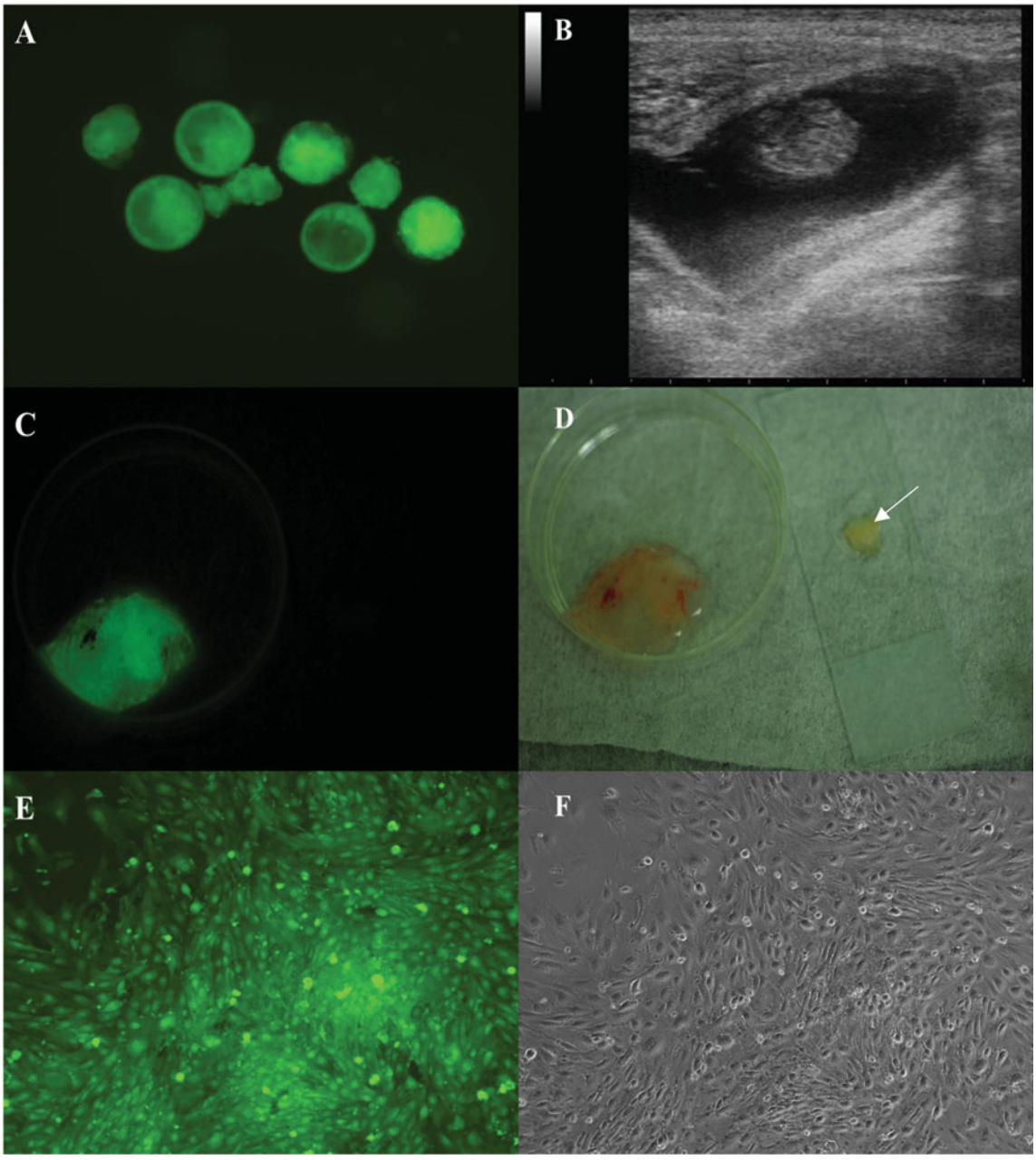

Figure 1 Isolation and culture of a bovine cell line expressing eGFP. (*A*) Cloned blastocyst expressing eGFP. (*B*) Pregnancy image using ultrasonography after embryo transfer. (*C*, *D*) Collected embryonic sac under fluorescence (*C*) and bright light (*D*). (*E*, *F*) Isolated primary cells from embryonic sac under fluorescence (*E*) and bright light (*F*). Arrow indicates a small piece of ovary (control) from a local slaughterhouse.

(Eppendorf, Mastercycler, Hamburg, Germany) conditions were 94°C for 2 min, then 40 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 30 s, with a final extension step at 72°C for 10 min. The PCR product was purified by electrophoresis in 1% agarose gel. Gel-purified PCR products were cloned using the pTOP Blunt kit (Enzynomics, Seoul, Republic of Korea) and sequenced using an M13 primer.

SCNT using CRISPR/Cas9 treated cells

Somatic cell nuclear transfer (SCNT) of CRISPR/Cas9 treated cells was conducted as previously described (Kim *et al.*, 2011), with slight modification. Briefly, after transfection of sgRNA and Cas9, GFP-negative cells were collected and then fused with *in vitro* matured bovine oocytes using electric pulses. The reconstructed

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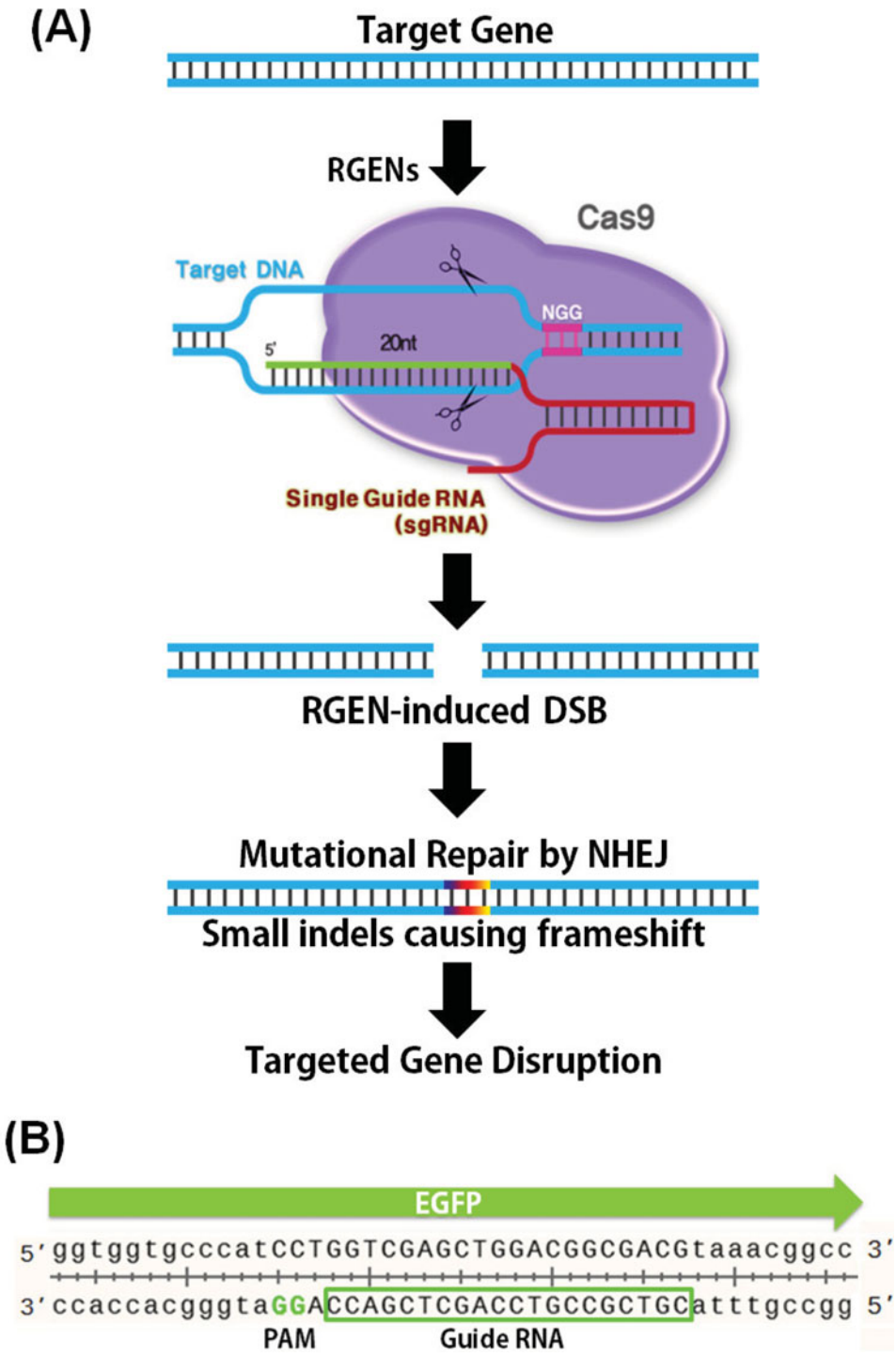

Figure 2 Representative figures for genome editing using CRISPIR/Cas9 DNAs. PAM: protospacer adjacent motif; NHEJ: non-homologous end joining; RGEN: RNA-guided endonucleases.

embryos were activated with ionomycin (Sigma) for 4 min followed by culture for 4 h in 6-DMAP (Sigma). The reconstructed embryos were cultured in 25 μl micro-drops of chemically defined medium overlaid with mineral oil for 7 to 8 days at 39°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 (Jang *et al.*, 2011). Cleaved and blastocyst stage embryos were observed at 24 h and 7 days of culture, respectively. Expressions of eGFP in the reconstructed embryos were recorded at the blastocyst stage.

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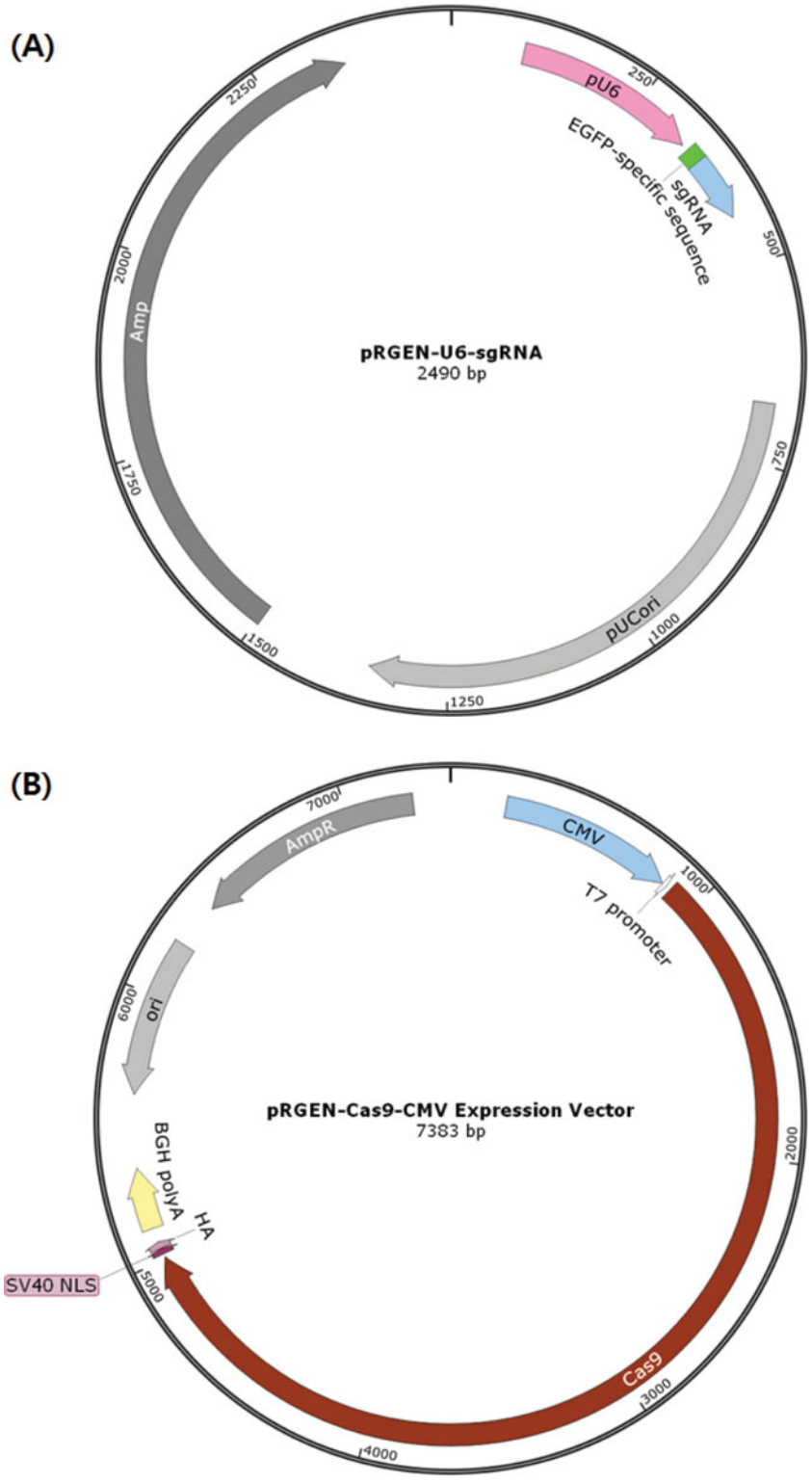

Figure 3 Schematic maps of expression vectors for eGFP-sgRNA (*A*) and Cas9 protein (*B*). (*A*) sgRNA with target sequence from the eGFP gene are expressed by U6 promoter. (*B*) A human codon-optimized Cas9 gene is expressed by CMV promoter. Note that Cas9 gene is translationally fused with a SV40 nuclear localization signal and a haemagglutinin (HA) epitope.

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Results and Discussion

Transgenic bovine embryo research is important for basic agricultural science. There have been several successful efforts to produce transgenic embryos and cattle (Wheeler, 2003). Recently, a DNA endonuclease-based approach has been widely studied in mice, humans and even livestock (Carlson *et al.*, 2012; Cho *et al.*, 2013; Sung *et al.*, 2013; Tan *et al.*, 2013). A few research studies have used ZFN/TALEN to generate knockout cattle (Tan *et al.*, 2013; Yu *et al.*, 2011). However, to date, the CRISPR/Cas9 system has remained to be tested with bovine cells. Here, we successfully generated bovine knockout cells and embryos via the CRISPR/Cas9 system.

In the present study, we produced cloned bovine embryos with stably eGFP-expressing fibroblasts established in our previous study (Kim *et al.*, 2011). In the previous report, we used a *piggyBac* vector to integrate the eGFP gene into the bovine genome and confirmed blastocyst formation of cloned embryos reconstructed with the *piggyBac*-mediated eGFP fibroblasts. In the present study, we observed the cloned eGFP embryos derived from the *piggyBac* system successfully implanted into recipients (Fig. 1*B*) and the resulting conceptus expressing eGFP in all tissues (Fig. 1*C*). Primary cells from the conceptus expressed eGFP homogeneously in *in vitro* culture, as well (Fig. 1*E*). Although pregnancy was not maintained to term, we confirmed that *piggyBac*-derived gene modification can be applied in transgenic studies in cattle up to early pregnancy stages (at least 40 days of pregnancy). However, in the present study, we failed to observe further growth of the cloned embryos. Currently, we are not able to explain why the transferred embryos did not develop further. There are three potential explanations for this phenomenon. Firstly, it was previously reported that *piggyBac* can insert several gene copies into the genome (Ding *et al.*, 2005); this may affect embryonic development. Secondly, because *piggyBac* preferentially integrates near promoter regions, CpG islands, and DNase I hypersensitive sites, it may have an increased potential for activating proto-oncogenes or disrupting tumour-suppressor genes (Huang *et al.*, 2010). Finally, abnormal reprogramming of the somatic cell nucleus in enucleated oocytes may have occurred in the same line having a high incidence of abnormalities in cloned animals (Dean *et al.*, 2001).

We transfected sgRNA targeting eGFP with Cas9 into the cultured transgenic fibroblasts derived from eGFP embryonic tissues. After 10 days of culture, an eGFP-negative population was observed under fluorescence microscope (Fig. 4*a*, *a*′), revealing the disruption of the targeted eGFP sequence. We did not analyze the time lapse of the eGFP signal or changes in the target sequence; however, the CRISPR/Cas9 system works on the genome level and does not directly reduce eGFP at the protein level. Thus, genome editing may be occurring a few days prior to eGFP signal reduction. In our flow cytometry analysis, a loss of eGFP expression in the transfected cells was greater than 40% (Fig. 4*B*). Sequencing of the PCR-amplified target sequence of the eGFP gene showed various sizes of deletions in the target sequence (6–203 bps; Fig. 4*C*). In the previous studies performed with different species, various types of mutations, such as insertion or substitution along with deletion, were observed in the target sequence (Kim *et al.*, 2013). However, in the present study, we only observed deletion type mutations in bovine fibroblasts. Further study is required to confirm that other types of mutations can occur in bovine cells after treatment with the CRISPR/Cas9 system.

To confirm that CRISPR/Cas9 mediated genome editing can be applied for transgenic cattle production, we generated cloned embryos reconstructed with the eGFP-negative fibroblasts. Reconstructed embryos ($n = 146$) successfully cleaved ($n = 118$; 80.82%) and developed into the blastocyst ($n = 35$; 23.97%) stage. While the blastocysts from cloned embryos fused with eGFP-expressing cells expressed the eGFP signal, those embryos from eGFP-negative cells did not express the signal (Fig. 4*A*, *b*, *b*′).

The CRISPR/Cas9 system is a preferred method for generating knockout animals because it has certain advantages, including time- and cost-effective and efficient genome editing (Sung *et al.*, 2014). In the present study, we also observed that 40% efficiency of mutations in bovine cells can be obtained in less than 10 days. Although we only tested the CRISPR/Cas9 system in exogenous integrated genes, the results are very promising for further generation of various genetically engineered cattle.

In conclusion, the data reported here demonstrate that the CRISPR/Cas9 system has been introduced into transgenic bovine cells from a *piggyBac*-derived embryonic tissue to knock out an exogenous gene, eGFP. Genome-editing technologies such as the CRISPR/Cas9 system will be useful techniques to develop livestock transgenesis for applications in agricultural science.

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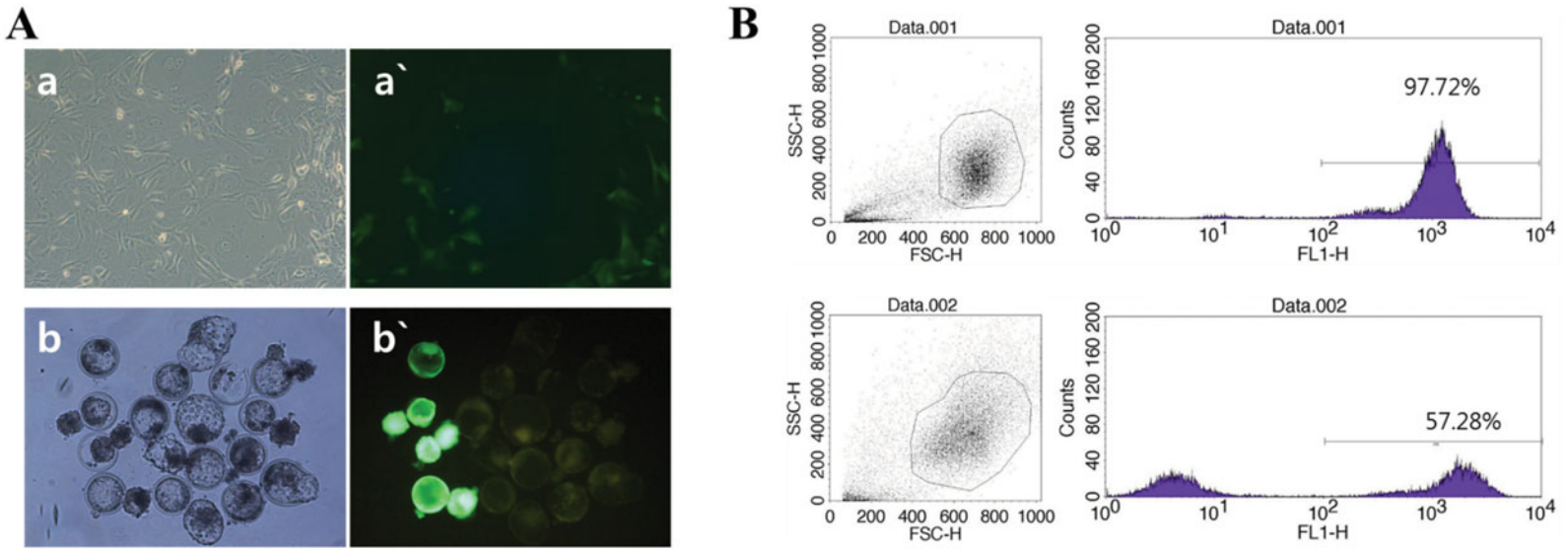

Figure 4 Knockout and sequencing of eGFP-expressing cells. (*A*) After 10 days transfection, non-eGFP-expressing cell populations were observed (*a*: bright light; *a*′: fluorescence) and expressing and non-expressing cells were reprogrammed in an enucleated oocyte and developed into blastocysts. Both blastocysts derived from eGFP-expressing or non-expressing cells were put together and token a capture for eGFP expression comparison (*b*: bright light; *b*′: fluorescence; left blastocysts from eGFP-expressing cells; right blastocysts: non-expressing cells). FACS result shows the ratio of the knockout cell population (*B*). To find deletion sequences, PCR amplicons of the target region were sequenced and some deleted sequences were found (*C*).

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