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Establishment of protocol for preparation of gene-edited bovine ear-derived fibroblasts for somatic cell nuclear transplantation

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ABSTRACT

Recently, gene-editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technique has attempted to utilize fibroblasts of livestock animals for somatic cell nuclear transfer. In this study, we establish the procedure for preparing skin fibroblast clones whose genes were edited by the CRISPR/Cas9 technique. After isolating fibroblasts from earlobes of Japanese Black cattle, subsequent collagenase-digestion and extensive wash procedures enabled us to avoid contamination of fungi. Electroporation using NEPA21, rather than lipofection using commercially available liposome reagents, allowed us to perform more efficient transfection of plasmid constructs. Although bovine ear-derived fibroblasts were not able to proliferate in single cell cultures in Dulbecco's modified Eagle medium containing 10% fetal calf serum, supplementation with insulin-transferrin-selenium mixture, human recombinant epidermal growth factor, or human recombinant basic fibroblast growth factor promoted proliferation of the cells, even in a single cell culture. Taking advantage of our established protocol, we eventually obtained eight ear-derived fibroblast clones with a recessive mutation in the isoleucyl-tRNA synthetase gene corrected by the CRISPR/Cas9 technique.

Recently, genome editing has been widely performed in biomedicine to target or modify genes of interest in various organisms (3, 13, 20, 36, 45). The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) method is the most popular gene-editing technique because of its efficiency and ease of use (43). This technique employs target-recognizing short guide RNA (sgRNA) and Cas9 nuclease, which was originally isolated from *Streptococcus pyogenes* (28). During the gene-editing process, the addition of donor DNA enables us to reconstitute genes of interest (22). Taking advantage of this technique, a greater number of genome-edited human embryos and livestock animals have been generated to overcome inherited diseases or to confer resistance against pathogens (5, 7, 19, 24).

Japanese Black cattle are one of the popular beef breeds in Japan owing to their flavor richness. Since Japanese Black cattle have been bred within closed populations for more than 60 years (26), several recessive mutations have accumulated in their genomic DNA. Isoleucyl-tRNA synthetase (IARS) syndrome is one of the most common inherited diseases in Japanese Black cattle. The c.235G>C (p. Val79Leu) substitution in the *IARS* gene causes protein synthesis defects, resulting in neonatal weakness or abor-

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Table 1 *Cattle and fibroblasts used in this study*

Cattle ID	Gender	Age	*IARS* Allele	fibroblast ID
13409-8450-0	female	4 years 6 months	wt/wt	B8450
13409-8457-6	female	3 years 8 months	wt/wt	B8457
13596-8493	female	2 years 6 months	wt/mut	B8493
13646-8510-0	female	1 year 5 months	wt/mut	B8510

wt; wildtype allele, mut; mutant allele.

tion (10). To overcome this disease, Ikeda and his colleagues (11) generated gene-edited individuals through combinatory techniques of CRISPR/Cas9-depenent gene-editing and somatic cell nuclear transfer (SCNT). Although this pioneer study suggests the possibility of using gene editing for IARS syndrome, there are many technical issues when applying gene editing to bovine somatic cells. Ikeda *et al.* (11) utilized fibroblasts isolated from fetuses, which were obtained from pregnant cows around 35–40 days after fertilization. Although this approach is acceptable in well-equipped experimental stations, the high costs mean most laboratories struggle to perform this technique. Moreover, qualified adults, rather than fetuses, may be a better source of fibroblasts because the purpose of livestock SCNT is to generate good quality clones (39). In this study, we attempt to establish a procedure for gene editing fibroblasts isolated from bovine ear tissue, which can be obtained by relatively easy and low-invasive treatments. We show a stable method for preparing sterilized bovine ear-derived fibroblasts. Moreover, we also evaluate transfection procedures and cloning of cells. By using these techniques, we eventually obtained fibroblast clones where the mutations in the *IARS* allele were corrected.

MATERIALS AND METHODS

Isolation and maintenance of bovine ear-derived fibroblasts. The ID number, age, and genotype of the four female Japanese Black cattle (Rakuno Gakuen Field Education Center) used in this study are listed in Table 1. The cattle were anesthetized with xylazine (0.1 mg/kg i.m.). After shaving and sterilizing ear lobes, 1 cm cube was cut from the auricle. The protocol for animal treatment was approved by the Animal Ethics Committees of Rakuno Gakuen University (permit number: VH16C16).

Isolated tissue slices were subsequently cut into pieces with scissors in ice-cold phosphate buffered saline (PBS). In some experiments, the tissues were put underneath cover slides in dishes and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 200 U/mL penicillin and 200 μg/mL streptomycin, 500 ng/mL amphotericin B in rat collagen-coated dishes with 37°C, 5% CO_2, and 95% humidity. Some tissues were digested with 40 μg/mL collagenase (Sigma Aldrich, St. Louis, MS, USA) in Tyrode's solution at 37°C for 30 min with strong agitation. After centrifugation at 1,000 × *g* for 10 min, pellets were extensively washed with PBS containing 200 U/mL penicillin and 200 μg/mL streptomycin with 500 ng/mL amphotericin B at least two times. After removing debris with a cell strainer (70 μm; Corning, New York, NY, USA), cells were cultured in DMEM containing 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin, and 250 ng/mL amphotericin B in rat collagen-coated dishes with 37°C, 5% CO_2, and 95% humidity.

Cell lines. Human-derived HeLa cells were obtained from RIKEN Bioresource Center (Tsukuba, Japan) and maintained according to manufacturer's instructions. MDBK and BEK cells were kindly provided from Dr. Shinichi Hatama (National Agriculture and Food Research Organization, Sapporo, Japan) and were grown in DMEM containing 10% FCS.

Plasmids and donor DNAs. pSpCas9(BB)-2A-GFP or pSpCas9(BB)-2A-puro plasmids were purchased from Addgene (Cambridge, MA, USA). sgRNA was designed by referencing the deposited DNA sequence data of the bovine *IARS* gene (Accession no. ENSBTAG00000000974), consisting of a 3-bp proto-spacer adjacent motif (PAM) located near the *IARS* mutation site and 20-bp sequence upstream of the PAM (see Fig. 3A). The double-strand oligo DNA coding for the sgRNA (Hokkaido System Science, Sapporo, Japan) was ligated into *Bbs*I-digested pSpCas9(BB)-2A-puro plasmid with T4 DNA ligase (Takara Bio, Otsu, Japan) at 4°C overnight. After the resultant plasmid (pSpCas9(BB)-bIARSgRNA-1-puro) was cloned using *E. coli* DH5α, the plasmid was isolated using a GenElute HP Endotoxin-Free Plasmid Midiprep Kit (Sigma-Aldrich). DNA sequence of the plasmid was confirmed by Hokkaido

System Science. All donor DNA elements were synthesized by FASMAC (Atsugi, Japan). Seventy-one bp (Donor 1 and Donor 2)- and 121 bp (Donor 3)-long donor DNAs were designed based on sequencing of the bovine *IARS* sequences. Donor 1 (Fig. 3A) was completely matched to 81–151 bp of exon 3 of the bovine *IARS* gene. Donors 2 and 3 (Figs. 4A and C) had one and four synonymous mutations in the region.

Transfection of DNA. Liposomal transfections, or "lipofection", were performed using Lipofectamine 2000 (Thermo Fisher Scientific), Lipofectamine LTX (Thermo Fisher Scientific), Fugene 6 (Promega, Madison, WI, USA), Fugene HD (Promega), ScreenFect (Wako, Osaka, Japan), TransFectin (BioRad, Hercules, CA, USA), and LipoTrust EX (Hokkaido System Science) following manufacturer's instructions. At transfection, the cells were cultured by densities of 2×10^5 cells/mL in 1.5 mL of Opti medium (Thermo Fisher Scientific). At 4 h after transfection, the medium was exchanged with DMEM containing 10% FCS with or without growth factors.

Genes were introduced by electroporation using a NEPA21 instrument (Nepagene, Chiba, Japan). Trypsinized bovine fibroblasts (1×10^6 cells), 10–14 µg of pSpCas9(BB)-2A-GFP plasmid, or 14 µg of pSpCas9(BB)-bIARSgRNA-1-puro plasmid, with 7 µg of donor DNA, was added to 100 µL of Opti medium, then pulsed by NEPA21. The parameters were as follows: voltage, 125 V; pulse length, 5 ms; pulse interval, 50 ms; number of pulses, 1; decay rate, 10%; polarity + as poring pulse and voltage, 20 V; pulse length, 50 ms; pulse interval, 50 ms; number of pulses, 5; decay rate, 40%; and polarity +/− as transfer pulse. Subsequently, the mixtures were rapidly transferred to a dish with the culture medium.

Microscopic observation. GFP expression in transfected cells was observed using a fluorescence inverted microscopy IX71 (Olympus, Tokyo, Japan) with a cooled CCD camera DP73 (Olympus). Transfection efficiencies were calculated by number of GFP^+ cells divided by that of all cells in microscopic fields (100× magnification).

Growth factors. Insulin-transferrin-selenium (ITS) solution, human recombinant epidermal growth factor (EGF), and human recombinant basic fibroblast growth factor (bFGF) were purchased from Wako Pure Chemical (Osaka, Japan), PeproTech (Rocky Hill, NJ, USA), and DS Pharma Biomedical (Osaka, Japan), respectively.

Cell proliferation assay. Proliferation of cultured cells was evaluated by reduction of 3-(4,5-di-methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole (MTT), or 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT). An XTT assay was performed using the Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN, USA). For the MTT assay, cells were incubated with 500 µg/mL MTT (Nacalai) for 4 h, and then produced formazan salt was dissolved with a lysis solution containing 10 mM HCl and 10% SDS. Absorbance at 450 and 540 nm was read using an iMark microplate reader (BioRad) to measure the concentration of XTT- and MTT-reduced formazan, respectively.

Genotyping the IARS gene. Genomic DNA was extracted with a lysate buffer [50 mM Tris-HCL (pH 8.0), 20 mM EDTA, 100 mM NaCl, 1% SDS] and proteinase K (100 µg/mL; New England Biolabs, Ipswich, MA, USA) at 65°C for 2 h. After phenol-chloroform-isoamyl alcohol (25 : 24 : 1, pH 5.2) extraction, DNA was precipitated and washed in 70% ethanol, then dissolved in nuclease-free water (Life Technologies).

For restriction fragment length polymorphism (RFLP) analysis, a partial DNA element of *IARS* gene was amplified by polymerase chain reaction (PCR) using Quick *Taq* HD DyeMix (Toyobo, Osaka Japan) with an *IARS* primer pair: 5'-TTACCTTCT ATGATGGGCCTC-3' and 5'-TTAACATCCCTGCC CTATGAC-3'. A PCR reaction was performed using a Gene-Atlas E (Astec, Fukuoka, Japan) as follows: 94°C for 2 min (pre-PCR), 55 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s. After purification with FastGene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan), the PCR product was digested with *Hinc*II (10 U/mL, Takara Bio) at 37°C overnight. Subsequently, phenol-chloroform extracted DNA was loaded in 2% agarose gel and stained with 0.5 µg/mL ethidium bromide (Nacalai). DNA bands were visualized using IN-6W-CPS (Oriental Instruments, Tokyo, Japan) and captured by Coolpix S3700 (Nikon, Tokyo, Japan).

In some experiments, we also amplified a DNA fragment corresponding to the *IARS* locus having artificial synonymous mutations using primers: 5'-GG TTTCACGTCGACCGTC-3' (DnA Primer) and 5'-T GGGTTTCACGTCGACCGTC-3' (DnB Primer).

Genomic DNA was also evaluated via direct sequencing analysis by Hokkaido System Science. For

direct sequencing, primers with the sequence 5'-TTA CCTTCTATGATGGGCCTC-3' were used.

Statistical analysis. Statistical analyses were performed using Microsoft Excel 2013 (ver. 15.0.4859. 1000, Microsoft, Redmond, WA, USA) with Ekuseru-Toukei 2012 (ver. 1.0, Social Survey Research Information, Tokyo, Japan).

RESULTS

Establishment of the procedure for preparation of bovine ear-derived fibroblasts

The "cell migration method" has been widely used to prepare fibroblasts from cattle dermis (25, 34, 38). Thus, we first tried to isolate fibroblasts from ear tissue of Japanese Black cattle using this method. After cutting and washing ear tissues into small pieces (~1 mm^3), the epidermis and dermis were put under a cover glass and cultured in DMEM containing amphotericin B at high concentrations (500 µg/mL). Although we observed fibroblasts on day 3 of the culture, severe contamination of fungi was observed in all dishes. We next validated the "collagenase-disperse" method referenced in previous reports (14, 32). We treated the pieces of ear tissue with 40 µg/mL collagenase at 37°C for 30 min and extensively washed the dispersed cells with 10–20 mL of amphotericin B (500 ng/mL)-containing PBS at least two times (Fig. 1). Cells obtained by the "collagenase-disperse method" showed growth on day 3 and reached a semi-confluent state on day 11. There was no visible contamination of fungi and bacteria at least by one month. By using the "collagenase-disperse method", we eventually isolated fibroblasts from four cattle. Two cattle had a heterozygous mutation at the *IARS* locus, while the others showed wild-type sequences (Table 1).

Evaluation of transient transfection methods for bovine ear-derived fibroblasts

For the application of gene editing in livestock, utilization of recombinant virus vectors is not desirable. Conversely, our preliminary data suggest that bovine fibroblastic cell lines, such as MDBK and BEK, showed low transfection efficacy (<0.1%) of plasmid vectors using commercially available lipofection reagents; Lipofectamine 2000, Lipofectamine LTX, Fugene 6, Fugene HD, ScreenFect, TransFectin, and LipoTrust. Similarly, transfection efficacy of bovine ear-derived fibroblasts was less than 0.1% using these reagents. Conversely, electroporation using a NEPA21 enabled us to transfect pSpCas9

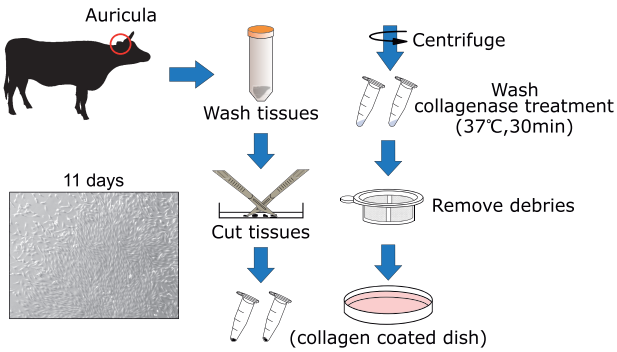

Fig. 1 Isolation of fibroblasts from the ears of adult Japanese Black cattle using the collagenase-dispersal method. Schematic presentation of the collagenase-dispersal method. Representative microscopic images of the bovine ear-derived fibroblasts on day 11 of cell culture are shown. Note: By using the collagenase-dispersal method, the severe contamination of fungi was not observed in the dishes for at least several months.

(BB)-2A-GFP to 0.35 ± 0.1% ($n = 4$) of B8510 cells. Therefore, electroporation is a relatively effective technique to transfect plasmids into bovine ear-derived fibroblasts.

Selection of supplementary substances for cloning of single cattle fibroblasts

For nuclear transfer of gene-edited somatic cells, we have to amplify a single cell fibroblast to a sufficient number of cells. To check whether density influences proliferation of bovine ear-derived fibroblasts, we serially diluted B8450 cells in 96 well plates and monitored cell numbers until 14 days. On day 7, a remarkable increase of cells was detected in wells where more than 100 cells were seeded (Fig. 2A). Conversely, 10 cells thoroughly increased in number by day 14, although cell numbers were not high enough to detect on day 7. In contrast, the single cell culture failed to provide a significant XTT signal on day 14. Insufficient proliferation of single B8450 cells was observed even after more than one month. Considering doubling time (~3 days) of B8450 cells in high density culture conditions, certain supplementary substances seem to be required for proliferation of bovine ear-derived fibroblasts in low density cell cultures.

We next verified effects of growth factors on B8450 cells. As shown in Fig. 2B, supplementation with ITS (insulin 1 g/L, transferrin 550 mg/L, selenium 67 µg/L) significantly promoted proliferation of cells, which were seeded at 100 cells/mL. Similar accelerated proliferation was also observed after treatment with EGF (50 ng/mL) and bFGF (50 ng/

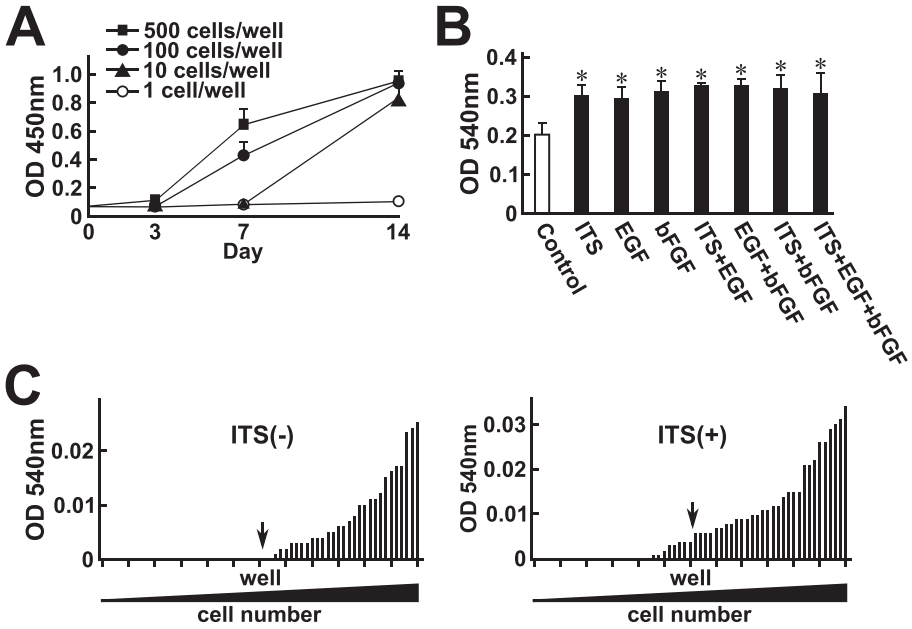

Fig. 2 Screening of supplementary materials to maintain a single fibroblast derived from Japanese Black cattle. Cell proliferation was assessed by the XTT (A) or MTT (B, C) method using B8450 cells. Proliferation activity was determined on (A) day 0, 3, 7, and 14, or (B, C) day 7. (**A**) Growth curves of the bovine ear-derived fibroblasts seeded at different densities. Values are means ± SD of 2–3 wells. (**B**) Effects of insulin-transferrin-selenium (ITS), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) on proliferative activity of the bovine ear-derived fibroblasts. One hundred cells were seeded in the well at the beginning of the culture. Values are means ± SD of 8–10 wells. $^{*}P < 0.01$ vs medium control. (**C**) Single cell culture of the bovine ear-derived fibroblasts maintained in (left) 10% FCS-containing DMEM or (right) 10% FCS- and ITS-containing DMEM. We attempted to seed cells as one per well. Thus, wells where absorbance showed a median value seemed to have one cell at the beginning of the culture. Sixty wells were used in this analysis. Arrows indicate the 30th and 31st wells sorted by ascending order.

mL). Synergism of the supplements on cell proliferation was not evident. Since ITS is beneficial and cost-effective, we employed ITS for further studies.

Fig. 2C shows colorimetric data of an MTT assay of B8450 cells in the presence or absence of ITS. Seeding attempted to place one cell per well in a 96 well plate. Thus, wells showing median absorbance seemed to have one cell in the well at the beginning of the culture. Without supplementation of ITS, the formazan salt-derived color was not detected in wells whose absorbance showed a median of 60 wells. In sharp contrast, ITS stimulated a significant increase in B8450 cells, allowing the median to reach the detectable level. Therefore, ITS is a useful supplement for amplifying bovine ear-derived fibroblasts.

Preparation of bovine ear-derived fibroblasts clones with gene-editing at the IARS locus

Taking advantage of our established protocol, we attempted to obtain gene-edited bovine ear-derived fibroblastic clones. In this study, we edited the mutated site of the *IARS* gene in B8510 cells. As shown in Fig. 3A, B8510 has a heterozygous mutation (c.235G>C), resulting in a conversion of leucine to valine 79 amino acids from the N-terminus. We transfected a single-strand DNA fragment 'Donor 1' and a sgRNA encoding plasmid (pSpCas9(BB)-bIARSgRNA-1-puro) into 1×10^6 B8510 cells using a NEPA21 system (Figs. 3A and B). After two days, we selected transfected cells with 2 μg/mL of puromycin for 3 days, then obtained more than 300 cells. Subsequently, the resultant 300 cells were serially diluted and cultured in ITS-containing medium. After one month of culture, we obtained a total of 71 proliferative clones. Resultant clones did not have altered cell morphologies compared to parental B8510 cells (Fig. 3C). RFLP of the PCR product with *Hinc*II digestion indicates at least 8 clones displayed a low signal intensity ratio of 392 to 24 bp bands, which correspond to mutated and wild-type alleles, respectively (Fig. 3D). In agreement, direct sequencing of the *IARS* locus of one of the gene-edited clones (B8510-a6) exhibited a wild-type pattern (Fig. 3E).

To more clearly ensure the gene editing in our procedure, we transduced a synonymous mutation

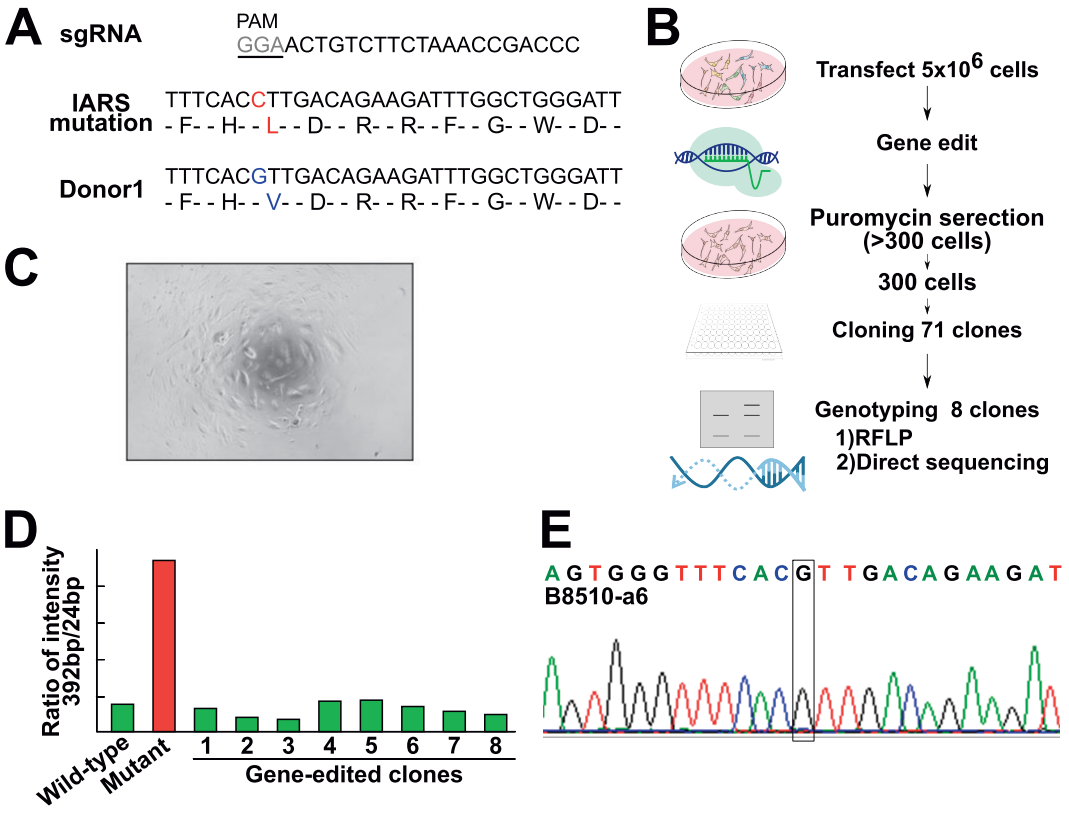

Fig. 3 Gene editing of the bovine ear-derived fibroblasts showing the *IARS* heterozygous mutation. (**A**) Alignment of DNA sequences of donor/wild-type and mutated *IARS* gene. Corresponding amino acid sequences and sequences of guide RNA are also shown. The PAM sequence is underlined. (**B**) Schematic summary of preparation of gene edited bovine ear-derived fibroblasts. (**C**) A microscopic image of an *IARS* gene-edited clone B8510-a6. (**D**) Restriction fragment length polymorphisms (RFLP) analysis of the gene-edited clones. Ratios of electrophoretic band intensities are shown. Note: DNA of cells with gene editing was clearly digested with *Hinc*II, resulting in a low 392 bp/24 bp ratio. (**E**) DNA sequence of a part of the *IARS* gene (104–128 bp of the 3rd exon, Accession no. ENSBTAG00000000974) of B8510-a6 cells. Restored sequence "G" is boxed.

(c. 237C>T) in donor DNA (Donor 2, Fig. 4A). As shown in Fig. 4B, gene editing using Donor 2 introduced a T to C mutation in B8510 cells (clone B8510-b3). Moreover, Donor 3, which includes four synonymous mutations (c. 235C>T, 239C>A, 241T>A, 242C>A), was introduced by gene editing, which can be distinguished by conventional PCR using donor specific primers; primers DnA and DnB. Thus, our procedure successfully obtained gene-edited bovine ear-derived fibroblast clones (Fig. 4C).

Finally, we also checked whether undesired plasmid-derived fragments had also been integrated into the genomic DNA of the gene-edited cells. For this purpose, we checked for the presence of puromycin resistance genes in genomic DNA, because cells having this element in their genomic DNA are likely to be selected by the puromycin-containing medium. Immediately after transfection, a PCR band corresponding to a puromycin resistant gene was detectable in a genomic DNA fraction of B8510 cells, as well as HeLa cells. On the contrary, after extensive passages, cloned cells did not exhibit the amplicon even after 40 × cycle reactions (Fig. 5). Therefore, the gene-edited clones, B8510-a1 and B8510-b3, do not seem to contain the plasmid-derived fragment in their genomic DNA.

DISCUSSION

Recently, *IARS*-restored Japanese Black cattle were generated by the CRISPR/Cas9 method and SCNT using fetal fibroblasts (11). To generate higher quality cattle, skin fibroblasts from qualified adults rather than unqualified embryos are more desirable. So far, several reports have prepared skin fibroblasts using the cell migration method (25, 33, 41). However, we failed to prepare fibroblasts using this method due to contamination of fungi. Since hairs cannot be com-

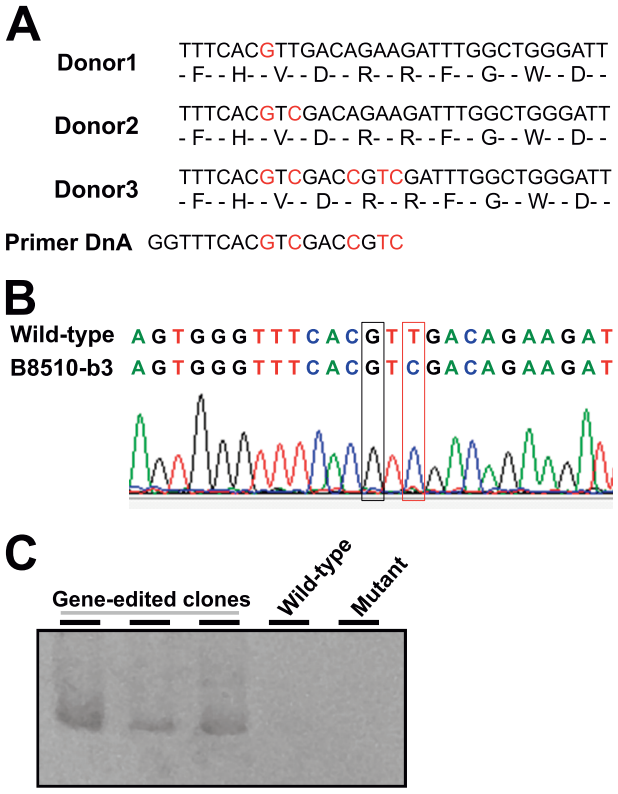

Fig. 4 Incorporation of synonymous mutation into the *IARS* locus. (**A**) Sequence alignments of Donors 1, 2, and 3. Donors 2 and 3 have one and four synonymous mutations (red letters), respectively. Sequence of the forward primer for genotyping (Primer DnA) is also shown. (**B**) Sequence of *IARS* allele of gene-edited cells. B8510-b3 cells are edited with Donor 2. Restored site at 235 bp and synonymous mutated site at 237 bp are boxed by black and red lines, respectively. (**C**) A representative image of genomic PCR to detect synonymous mutations introduced by Donor 3. PCR was performed using DnA and DnB primers (see Results).

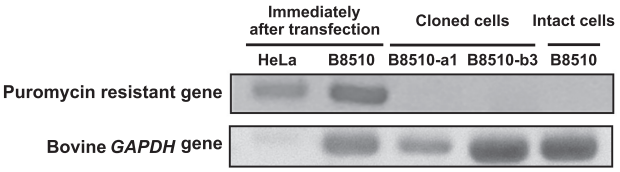

Fig. 5 Validation of exogenous gene fragments in edited fibroblasts. Genomic DNA was prepared from transient-transfected, subsequently cloned (B8510-a1 and B8510-a3), and intact cells. PCR for puromycin-resistant genes, which were included in the plasmid vector, was performed. As a reference, the bovine *GAPDH* gene was also amplified.

pletely removed from tissue pieces, fungi attached to hair rapidly grew in the culture medium. Conversely the "collagenase-disperse" method enabled us to prepare skin fibroblasts under sterilized conditions. Using the collagenase-disperse method, cells were extensively washed with a large amount of PBS, including an anti-fungal agent. Moreover, hairs were completely removed by filtration. Given the clean state of normal livestock farms compared to public experimental stations, the collagenase-disperse method is better for preparing fibroblasts from cattle ear.

In this study, we evaluated transfection efficacies of bovine ear-derived fibroblasts using lipofection and electroporation methods. In contrast to mouse and human fibroblasts (15, 16), bovine ear-derived fibroblasts, as well as bovine fibroblast cell lines, showed extremely low transfection efficacy using seven commercial available reagents. Electroporation using NEPA21 stably introduced plasmids to the cells; however, efficacy was not high. Electroporation is still a better way to introduce plasmids to the bovine ear-derived fibroblasts at this point in time. The mechanisms underlying the differences in transfection between lipofection and electroporation are not obscure. Using the lipofection method, a positively charged liposome and plasmid DNA form a complex, which is accepted by endocytosis or membrane fusion. Membrane charge is one of the factors determining the uptake efficiency of liposomes. Since membrane charge is diverse among cells (40), the charge of bovine cells might not be adequate for the lipofection method using commercially available liposome reagents.

In the absence of growth factors, B8450 did not proliferate from a single cell, although it was cultured for more than one month. Conversely, a small increase in cell numbers per well led to better growth. These results suggest that cell-to-cell contact rather than humoral factors sustain proliferative activity of bovine ear-derived fibroblasts. In accordance with this idea, several molecules confer proliferative activity to culture fibroblasts in a juxtacrine manner. For instance, heparin-binding EGF, a membrane-integrated ligand, contributes to promoting proliferation and activation of fibroblasts (18, 29). Although ITS enables us to obtain proliferative bovine fibroblasts, the promotion of proliferation was limited. Therefore, identification of cell membrane ligands participating in proliferation may lead to a more effective method of obtaining fibroblastic clones in a short time period.

So far, ITS, EGF, and bFGF are capable of stimulating proliferation of fibroblasts isolated from several species (6, 8, 14, 21, 37). The current study demonstrated that all these factors are applicable in maintaining bovine ear-derived fibroblasts. Of interest, these three factors represent comparable prolif-

erative effects on bovine ear-derived fibroblasts. Moreover, there was no obvious synergism in proliferative effects between factors. These results collectively imply that these factors stimulate proliferation of the bovine fibroblasts through the same signaling pathway. Insulin, EGF, and bFGF bind receptors with a tyrosine kinase domain in the intracellular region and activate both the PI3K/Akt/mTOR pathway and Ras/MEK/MAPK pathway, both of which contribute to proliferation of various cell types (1, 9, 35). Further studies are required to elucidate whether these signaling pathways participate in proliferation of bovine ear-derived fibroblasts.

In this study, 71 puromycin-resistant clones were obtained. Of them, 8 clones (11.3%) exhibited gene editing at the expected site. In previous studies, gene editing in HEK293 and mouse embryonic stem cells took place at 21.2% and 16.7%, respectively (42, 44). The precise reasons for the low efficiency of gene editing in this study are currently unclear. In any case, labors lowering the false-positive rate seem to be necessary. Since the design of gRNA and donor DNA is a known determinant of gene-editing rates (23), a larger number of tools should be validated in the future. Alternatively, it is also possible that selection of transfected cells may be insufficient in the current study. Since ear-derived fibroblasts were not resistant to conventional FACS sorting in our preliminary study, we aimed to select transfected cells by antibiotic resistance. In this trial, we selected transfected cells with relatively lower concentrations (2 μg/mL) of puromycin since we considered that cells transfected with a small number of plasmids may not be resistant to higher doses of puromycin. Therefore, application of higher concentrations of puromycin (*e.g.* 5 μg/mL) may decrease the false-positive rates of gene editing.

Aberrant and normal alleles of *IARS* are usually distinguished by RFLP. Although RFLP using *Hinc*II is a powerful approach for genotyping Japanese Black cattle (10), this method does not seem to be the best way to check the genome of gene-edited ear-derived fibroblasts. In practice, preparation of enough DNA for *Hinc*II digestion is time-consuming and labor-intensive as it requires genotyping a large number of cells to successfully obtain edited cells. In this study, we used donor DNA showing four synonymous mutations. Resultant cells edited with this donor had unique genomic DNA that could be distinguished from wild-type and naturally mutated cells only by conventional PCR. Although the introduction of synonymous mutations into genomic DNA is a debatable and ethical argument, a donor having synonymous mutations seems to be beneficial in regards to expense and labor.

This study establishes a protocol to prepare a sufficient number of gene-edited somatic cells, which are isolated from adult cattle. Since somatic cell nuclear transfer is not applicable to human embryos for ethical reasons, our established method is not directly used for gene therapy in the medical field. However, our data provide fundamental information for human biomedical research. Ruminant arteries have been widely used as experimental models for circulation research (30, 31). Since fibroblasts maintain arterial function incorporating endothelial cells and smooth muscle cells of blood vessels (12), methods for preparation and modulation of cattle fibroblasts may be useful in this research area. Conversely, production of tissues and proteins from humanized livestock animals is desirable for obtaining tools for transplantation and medication (17, 27). In this field, cattle fibroblasts having human-type gene(s) are considered useful for nuclear transfer. In the industrial field, bovine skin has been used as a major source of cosmetic and food collagen (4). Immunogenicity and transmission of diseases are issues to address for utilization of collagen (2, 4). To improve the safety of collagen obtained from bovine skin, gene-edited cattle fibroblasts may become valuable tools.

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