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Research Article

# Electroporation-Based Non-Viral Gene Delivery to Adipose Tissue in Mice

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**Abstract**

Adipose tissue is distributed throughout the body as fat depots. The amount of adipose tissue increases with age. In mice, epididymal fat depots in males and gonadal fat depots in females are associated with the reproductive system. Regarding fat depots in females, the adipose tissue under the skin can be easily exposed via surgery when the ovary, oviduct, and uterus are pulled out and exposed. As handling adipose tissue is relatively easy, adipocytes might be

good targets for genetic manipulation (including gene delivery to the adipose cells). To examine this possibility, we injected 1 µL of dye (e.g., trypan blue or India ink) into the gonadal fat depots of female mice using a breath-controlled micropipette under a dissecting microscope. The injected dye remained at the injection site for at least one day. The injection of *piggy*Bac (PB) transposons containing an enhanced green fluorescent protein (EGFP)-expressing unit and subsequent *in vivo* electroporation (EP) at the injection site resulted in the successful transfection of adipocytes. The introduction of the PB transposons caused chromosomal integration of the gene of interest. The introduction of a vector containing an octamer-binding transcription factor-3/4 promoter-directed *EGFP* cDNA expression unit helped to identify stem-like cells. These results supported the feasibility of our EP-based non-viral gene delivery system to transfect murine adipocytes *in vivo*. Using this approach, several applications such as the local production of therapeutically useful proteins, plasmid-based vaccinations, and the acquisition of immortalized adipose-derived stem cells might be possible.

**Keywords**
*In vivo* gene delivery; adipose tissue; electroporation; plasmid; enhanced green fluorescent protein; tdTomato; Oct-3/4; adipose-derived stem cells; *piggy*Bac transposon; chromosomal integration; gene of interest

## 1. Introduction

Adipose tissue, also known as fat tissue, is a connective tissue consisting of lipid-rich cells (adipocytes) [1]. Adipose tissue was traditionally considered to be an energy storage organ, but its role as an endocrine organ to mediate physiological processes related to health and disease has been recently reported [2]. Adipose-derived stem cells (ASCs) are a part of the adipose tissue and were first identified as mesenchymal stem cells (MSCs) in the adipose tissue in 2001 [3]. Zuk et al. [3] minced adipose tissue (abundant in subcutaneous tissues and usually discarded after surgery) and performed enzymatic digestion. After centrifugation, the resulting pellet containing ASCs and other types of cells was grown *in vitro* after the plastic-adherent population was cultured overnight. Because ASCs can differentiate into multiple cell lineages, they are valuable and might be useful in regenerative medicine [4]. ASCs release various growth factors, and when ASCs were transplanted into mice with cell carriers that adsorb these growth factors, an angiogenic effect was observed at the transplantation site in mice [5].

Gene delivery is a useful tool to elucidate the role of a gene of interest (GOI) and produce genetically modified cells that are used for therapeutic purposes. The delivery of genes in cultured ASCs *in vitro* includes transfection using chemical reagents [6], electroporation (EP) (or nucleofection) [7-9], and viral infection [8, 10-13]. Furthermore, *in vivo* adipocyte transfection has been attempted multiple times [12-25]. For example, Smith et al. [14] injected an amphotropic murine packaged retroviral vector carrying the *lacZ* gene (encoding β-galactosidase) into the epithelium-divested mammary fat pads of syngeneic mice. After 12 months, they observed stable recombinant gene expression *in vivo* in the mammary gland. Granneman et al. [17] injected a solution containing an enhanced green fluorescent protein (EGFP)-expressing plasmid into the

subcutaneous adipose pad of adult C57BL/6 mice using a needle and a Hamilton microliter syringe. Immediately after injection, the injection site was flanked with Gerald bipolar forceps and electroporated. Gene expression occurred at the injected sites 1-3 days after gene delivery. These results suggested that adipose tissue facilitates the transient expression of the GOI.

We developed novel techniques, such as the *g*enome-editing via *o*viductal *n*ucleic *a*cids *d*elivery (GONAD) technique and the improved GONAD (*i*-GONAD) technique [26, 27]. These are simple and convenient methods for obtaining genome-edited preimplantation murine embryos *in situ* through exposure to the adipose tissue/ovary/oviduct/uterus complex outside the body. These methods enable the injection of a nucleic acid (NA)-containing solution using a breath-controlled micropipette under a dissecting microscope into the lumen of an oviduct of a pregnant female and the subsequent *in vivo* EP in the oviduct [26-29]. We suggested that the adipose tissue (gonadal fat depots) associated with the reproductive organs (i.e., ovary, oviduct, and uterus) might be a promising target for gene delivery if an approach similar to the GONAD/*i*-GONAD method is applied to the tissue.

In this study, we described a technique that enabled chromosomal integration of a GOI in adipocytes near the ovary through the direct injection of *piggy*Bac (PB)-based plasmid DNA (facilitates chromosomal integration), with subsequent *in vivo* EP at the injection site. We also marked the ASCs present in adipose tissues by injecting a plasmid containing an octamer-binding transcription factor-3/4 (*Oct-3/4*) promoter-directed *EGFP* cDNA expression unit into the adipose tissue before *in vivo* EP. We called this novel technique "EP-based non-viral gene delivery to adipose (EPGDA)."

## 2. Materials and Methods

### *2.1 Animals and Cells*

Adult (four-to-six-month-old) and young (four-week-old) female B6C3F1 mice (a hybrid between C57BL/6 and C3H/He; CLEA Japan, Ltd., Tokyo, Japan) were used for EPGDA. P19 cells [30] are embryonal carcinoma cells (ECCs) derived from C3H/He mouse post-implanted embryos. The NIH3T3 cells were a gift from Dr. Tadashi Furusawa (National Institute of Agrobiological Sciences, Japan). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Co., Ltd, St. Louis, MO, USA) (supplemented with 10% heat-inactivated fetal bovine serum, 50 units of penicillin, and 50 µg/mL of streptomycin) at 37 °C and 5% $CO_2$. Animal experiments were performed based on the guidelines of the Kagoshima University Committee on Recombinant DNA Security and approved by the Animal Care and Experimentation Committee of Kagoshima University (Permit no. 25035 and 25036; August 8, 2013). Surgeries were performed after anesthetization using three agents (medetomidine, midazolam, and butorphanol), and efforts were made to minimize animal pain and suffering.

### *2.2 Plasmid DNA*

The four types of plasmid DNA (pTrans [31], pT-EGFP [31], ptdTomato [31], and pdOEN-2) used in this study are shown in Figure 1A and Figure 2A. Briefly, pTrans is a vector that enables the systemic expression of PB transposase under the action of the chicken β-actin gene-based promoter *CAG* [32]. pT-EGFP is a PB-based vector containing an *EGFP* cDNA expression unit [*CAG* promoter +

*EGFP* cDNA + poly(A) sites] flanked by the two inverted repeats (ITR; also called PB acceptors). ptdTomato is a PB-based vector carrying a tandem dimer Tomato (*tdTomato*) cDNA (provided by Dr. Roger Tsien) under the *CAG*. The pdOEN-2 was constructed by inserting ~3 kb mouse *Oct-3/4* promoter (corresponding to the proximal portion of the *Oct-3/4* promoter in pO4P-19 [33]) into the 5' end of the *EGFP* cDNA in pEGFP-N1 (Invitrogen Co., Carlsbad, CA, USA).

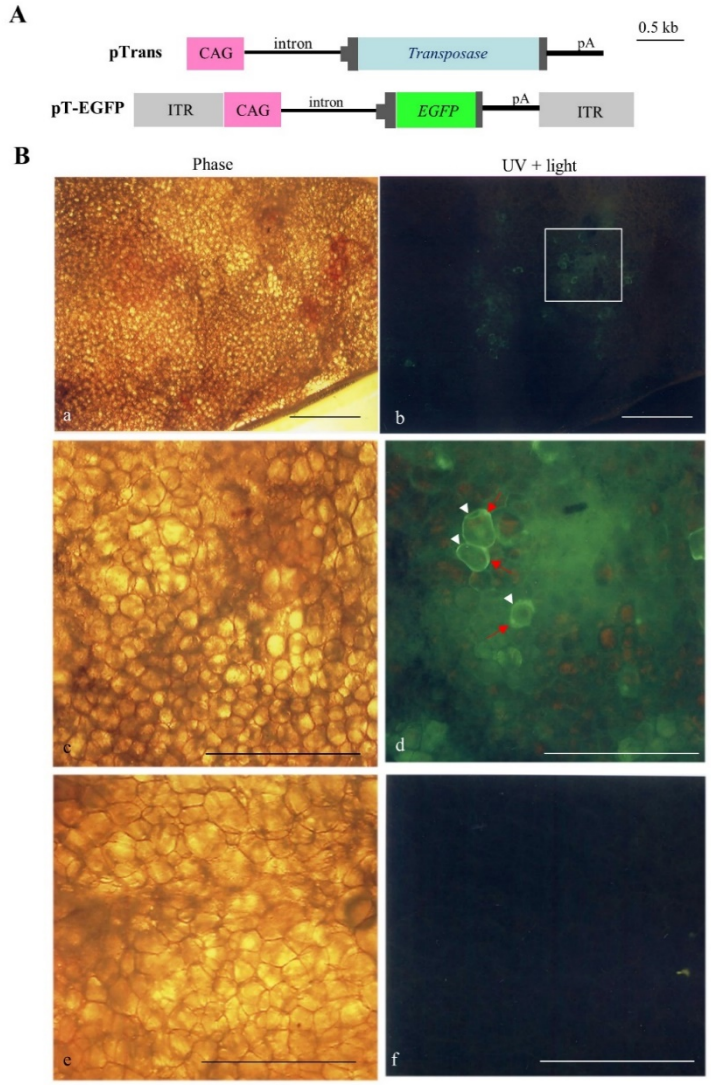


**Figure 1** EPGDA using the *piggy*Bac (PB)-based transposon system. **A.** Schematic representation of the PB-based transposon vectors (pTrans and pT-EGFP) used in this study. The plasmid backbone is not shown. CAG: cytomegalovirus enhancer + chicken β-actin promoter; pA: poly(A) sites; *EGFP*: enhanced green fluorescent protein cDNA; ITR: inverted terminal repeat as an acceptor site in the PB system; transposase: PB transposase gene. **B.** Detection of EGFP-derived green fluorescence in adipocytes after EPGDA. One day after gene delivery, the tissue at the injection site (identified based on the presence of TB) was dissected to evaluate EGFP-derived fluorescence using a fluorescence microscope under light (a and c) or ultraviolet (UV) + light (b and d). The inset shown in b is enlarged in d. The arrowheads indicate the cell membrane of fluorescent adipocytes, and the red arrows indicate the nuclei of fluorescent adipocytes. There was no fluorescence in the control adipose tissue (e and f). Bar: 100 μm.

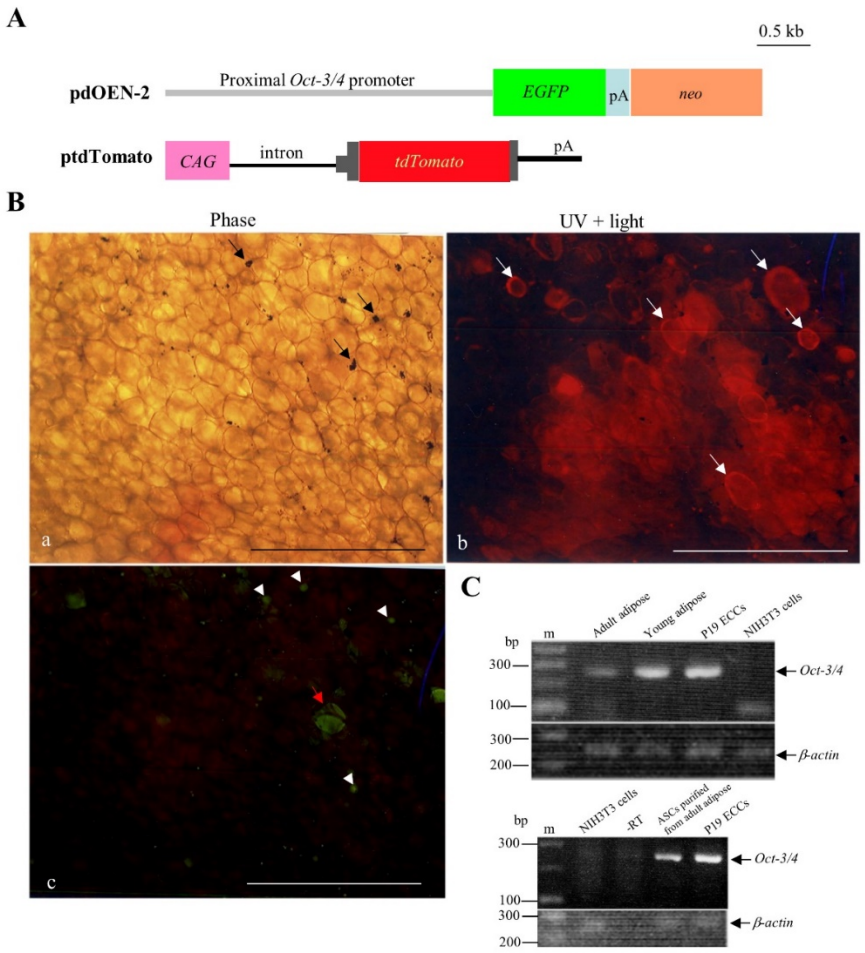


**Figure 2** EPGDA for marking adipose-derived stem cells (ASCs). **A.** Schematic representation of the plasmid vectors (pdOEN-2 and ptdTomato) used in this study. The plasmid backbone is not shown. CAG: cytomegalovirus enhancer + chicken β-actin promoter; pA: poly(A) sites; *EGFP*: enhanced green fluorescent protein cDNA; *tdTomato*: tandem dimer Tomato cDNA; *neo*: neomycin resistance gene expression unit (comprising a mouse phosphoglycerate kinase promoter, a neomycin resistance gene, and poly(A) sites). **B.** Detection of fluorescence in adipocytes after EPGDA. One day after gene delivery, the tissue at the injection site (identified based on the presence of India ink) was dissected to evaluate tdTomato-derived or EGFP-derived fluorescence using a fluorescence microscope under light (a) or ultraviolet (UV) + light (b and c). When the injected site was assessed for tdTomato expression, many adipocytes were found to be fluorescent (indicated by arrows in b). However, when the site was assessed for EGFP expression, some adipocytes were found to exhibit spot-like fluorescence (indicated by arrowheads), although only a few cells exhibited membranous fluorescence (indicated by red arrows). Bar: 100 μm. **C.** RT-PCR analysis of murine adipose tissue to detect *Oct-3/4* and *β-actin* mRNA. The upper panel shows the adipose tissue isolated from young (one-month-old) and adult (five-month-old) females; P19 embryonal carcinoma cells (ECCs) were used as the positive control, and NIH3T3 cells were used as the negative control. The lower panel shows the adipose stem cells (ASCs) purified from adult adipose tissue with P19 ECCs and NIH3T3 cells. "-RT" indicates RT-PCR reactions without template cDNA, and "m" denotes ladder markers of 100 bp.

The plasmids were amplified in *Escherichia coli* DH5α and purified using the MACHEREY-NAGEL plasmid purification kit (#740609.10; Takara Bio Inc., Shiga, Japan), following the method described by Sato et al. [34].

### *2.3 EPGDA*

The schematic representation of the EPGDA procedure is shown in Figure 3A. Before conducting the procedure, a micropipette with a pointed end was made by pulling a glass capillary tube (#GDC-1; Narishige Scientific Instrument Lab., Tokyo, Japan) using a micropipette puller (P-97/IVF; Sutter Instrument Company, CA, USA). Then, the tip of the micropipette was cut using microscissors (#MB-53; NAPOX, Natsume Co., Ltd., Tokyo, Japan) under a dissecting microscope (SZX10, Olympus, Tokyo, Japan) to obtain an inner diameter of 10-30 μm. Then, a solution containing the plasmid DNA (circular; final concentration of 0.5 μg/μL for each plasmid) and 0.1% (v/v) trypan blue (TB; Trypan Blue Stain 0.4%; Invitrogen Co.) or India ink (#Kaimei bokuju B01020; ODA, Nagoya, Japan) (for monitoring the injected solution) in $Ca^{2+}$, $Mg^{2+}$-free Dulbecco's modified phosphate-buffered saline (hereinafter D-PBS(−)) was placed in a 1.5 mL-tube using a 200 μL tip. Then, approximately 1 μL of the solution was drawn in using an injection micropipette connected to a mouthpiece (Figure 3A-a). The procedure was conducted under a dissecting microscope (Figure 3A-b).

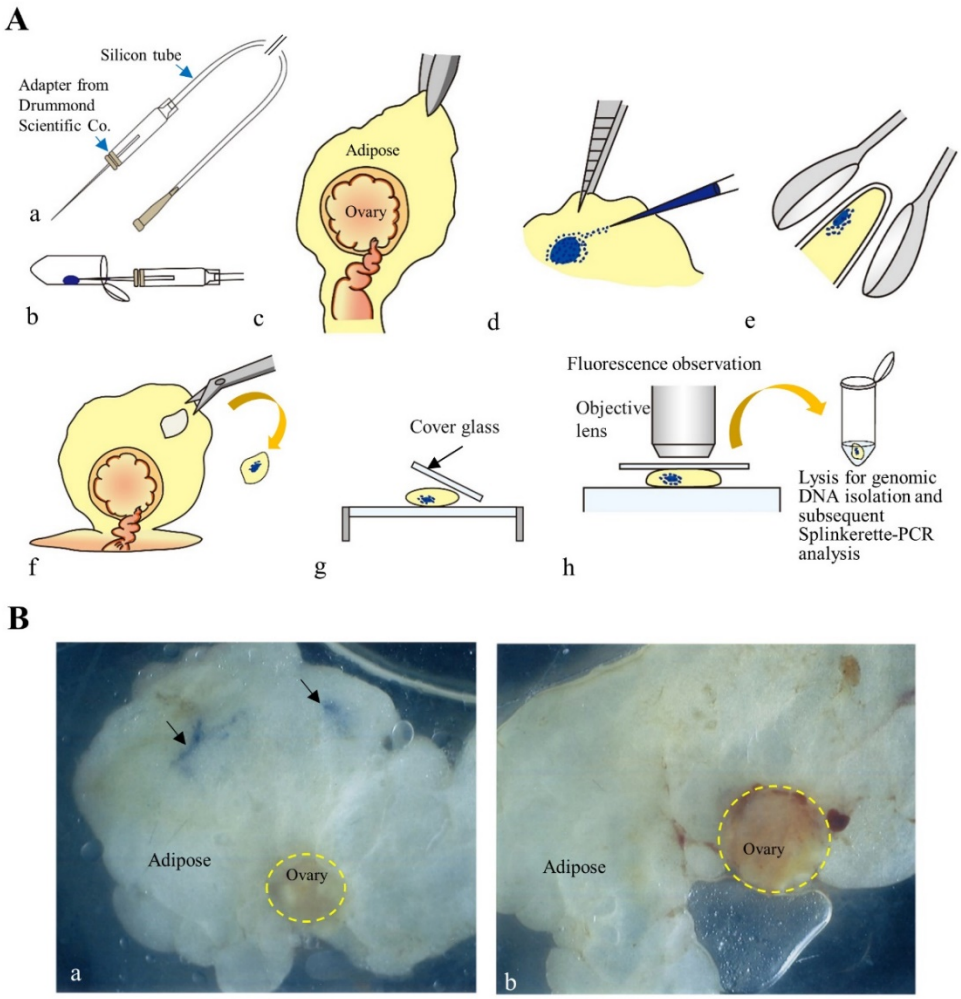


**Figure 3** The outline of electroporation-based non-viral gene delivery to adipose (EPGDA), a technique enabling transfection of adipocytes *in vivo*. **A.** Schematic illustration of EPGDA. Nucleic acids (NAs) suspended in a solution containing trypan blue (TB) were drawn in using a glass micropipette connected to a pipette holder (shown in a) while observing under a dissecting microscope (shown in b). In female mice, the ovary, oviduct, and uterus were exposed under anesthesia, and the exposed fat depots were held in position using an Aorta-Klemme (shown in c). The injection was performed by inserting a micropipette into the fat depots that were held in position using forceps (shown in d). In general, two injections were performed. Then, the injection site was covered with wet paper (e). Immediately after covering, the injection site was compressed between the two tweezer-type electrodes and electroporated (e). One day after EPGDA, the fat depots containing the injection site, which was easily discernible by the presence of residual TB, were dissected using microscissors (f). Next, the sample was squashed gently with a coverslip (g). After detecting fluorescence in the sample, it was lysed for DNA analysis (h). **B.** The fate of TB after EPGDA. When TB was injected into the fat depots (under a dissecting microscope), the injected dye remained at the injection sites (arrows in a), whereas the control adipose tissue remained intact (b).

Next, adult female B6C3F1 mice were anesthetized by intraperitoneal injection of three combined anesthetics (medetomidine, midazolam, and butorphanol), as described by Sato et al. [28]. A small incision was made on the left dorsal skin, and fat depots associated with the ovary, oviduct, and uterus were exposed (Figure 3A-c). The exposed fat depots were held in position using an Aorta-Klemme (#C-17-40-2; Natsume Co., Ltd.) to prevent retraction (Figure 3A-c). The injection micropipette was inserted into the internal part of the fat depots while observing under a dissecting microscope. Subsequently, 1 µL of D-PBS(−) containing the plasmid DNA and dye was injected (Figure 3A-d). As a negative control, 1 µL of D-PBS(−) containing only the dye was similarly injected. The injection process could be easily visualized because a rapid color change (from white to blue or black) occurred at the injection site. After the injection, the injected sites were covered with a small piece of D-PBS(−)-dipped wet paper (KimWipe; Jujo-Kimberly Co. Ltd., Tokyo, Japan) (Figure 3A-e), compressed between a pair of disc-shaped tweezer-type electrodes (5 mm inner diameter; #LF650P5; BEX Co., Ltd., Tokyo, Japan) (Figure 3A-e), and electroporated using the square-pulse generator NEPA21 (Nepa Gene Co., Ltd., Chiba, Japan) that generates two types of pulses, which include the poring pulse (Pp) and the transfer pulse (Tp). In this study, *in vivo* EP was performed using conditions that included 4 Pp (2.5 ms wavelength/50 ms duration/50 V) and 8 Tp (50 ms wavelength/50 ms duration/20 V). The procedure was repeated once near the site of the first injection. Thus, the adipose tissue in each mouse received two injections. After the *in vivo* EP, the electroporated fat depots were returned to their original position, and the wound was closed. The anesthetized mice recovered after receiving an injection of atipamezole (a medetomidine antagonist), following the method described by Sato et al. [28].

One day after gene delivery, the injected site (approximately 3 $mm^3$; easily discernible because of the presence of residual co-injected dye) was dissected using microscissors (Figure 3A-f). The dissected sample was squashed by placing a coverglass onto a plastic surface (Figure 3A-g). It was then checked for fluorescence under an Olympus BX60 fluorescence microscope, as described below and in Figure 3A-h, and photographed. After observation, genomic DNA was isolated from the sample, and Splinkerette-PCR analysis was performed, as described below and in Figure 3A-h.

### *2.4 Analyzing the Genomic Integration Site Using Splinkerette-PCR*

Genomic DNA was extracted by adding 300 µL of lysis buffer [31] to a section of adipose tissue (~3 $mm^3$) in a 1.5 mL tube and then incubating at 37 °C for several days, followed by phenol/chloroform extraction and isopropanol-precipitation. The purified genomic DNA was finally dissolved in 6 µL of sterile water.

To assess the potential integration sites within the DNA-introduced adipose tissue, Splinkerette-PCR was conducted using the "Splinkerette Protocol" (http://www.cmhd.ca/protocols/genetrap_pdf/Splinkerette%20Protocol%20Single%20Clone.pdf# search =' splinkerette') (based on Potter and Luo [35]). Briefly, genomic DNA (~3 µg) digested with *Sau* 3AI was ligated using a splinkerette adapter produced by annealing two oligonucleotides, HMSpAa and HMSpBb. Junction fragments were amplified using the primer set (HMSp1 and PB-R-Sp1) in a PC708 thermal cycler (Astec, Fukuoka, Japan) using the following conditions: initial denaturation (10 min at 92 °C), followed by 40 cycles of denaturation (10 s at 96 °C), annealing (1 min at 56 °C), and polymerization (2 min at 72 °C), with a final extension (5 min at 72 °C) using *Taq*

polymerase (TaKaRa Taq; #R001A; Takara Bio Inc.). Nested PCR was performed using the primer set (HMSp2 and PB-L-Sp2) under the conditions used for the first PCR.

The PCR products were visualized by performing 2% agarose gel electrophoresis and staining with ethidium bromide to check the size of each PCR product. The desired PCR products containing over 300 bp were excised from the gel and cloned into the TA cloning vector pCR2.1 (#451641; Thermo Fisher Scientific Inc., Waltham, MA, USA). The recombinant colonies underwent PCR using the universal primers, M2 and RV, as described by Inada et al. [31]. The PB integration sites were considered where the genomic sequence started immediately after the terminal TTAA sequence at the end of the 3'-ITR sequence and showed a sequence identity of 93% at the genomic locus.

### *2.5 RT-PCR*

The progenitor cells (ASCs) were collected from the adipose tissue (~1 g) of adult B6C3F1 females using the Adipocyte Progenitor Isolation Kit (#130-106-639; Miltenyi Biotec Inc., Auburn, CA, USA) in combination with the Adipose Tissue Dissociation Kit for mouse and rat (# 130-105-808; Miltenyi Biotec Inc.).

Total RNA was isolated from ASCs, adipose tissue of young and adult B6C3F1 females, P19 ECCs, and NIH3T3 cells using an ISOGEN kit (#317-02503; Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Then, the total RNA (4 μg) was reverse-transcribed into cDNA using SuperScript® II Reverse Transcriptase (#18064022; Thermo Fisher Scientific Inc.) and Oligo$(dT)_{20}$ primer (#FSK-201; Toyobo Co., Ltd., Osaka, Japan) by performing a cDNA synthesis reaction (20 μL). The cDNA template (1 μL) underwent a 20 μL PCR following 40 cycles of denaturation (45 s at 94 °C), annealing (45 s at 58 °C), and extension (60 s at 72 °C) in a thermal cycler. The primers used for the first RT-PCR (mO4-S, mO4-RV, βA-S, and βA-RV) are listed in Table S1. In some cases, the nested RT-PCR was performed using 1 μL of the first RT-PCR products in 20 μL of the solution under the same PCR condition as that used for the first RT-PCR. The primers (mO4-2S and mO4-2RV) used for the nested RT-PCR are shown in Table S1. The RT-PCR products were visible after performing 2% agarose gel electrophoresis and staining with ethidium bromide.

## 3. Results

### *3.1 Dye Injected into Adipose Tissues was Successfully Entrapped*

The schematic representation of the EPGDA procedure is shown in Figure 3A and explained in detail in Section 2.3. Interestingly, when EPGDA was performed for transfecting the adipocytes, the solution injected into the fat depots did not easily spread beyond the injection site. If the DNA-containing solution gets entrapped at the injection sites, gene delivery efficiency to adipocytes may increase at injection sites. To test this hypothesis, we first injected a solution (1 μL) containing 0.1% (v/v) TB into adult female adipose tissue located near the ovary twice, and one day after the injection, the adipose tissue and the ovary were excised to check TB distribution under a light microscope. The injected dye was visible at the injection site (arrows in Figure 3B-a), suggesting that the dye did not spread beyond the injection site in fat depots. In the intact control adipose tissue, no blue deposit was discernible (Figure 3B-b).

### *3.2 Gene Delivery of PB Vectors into Adipose Tissues*

To determine PB-mediated chromosomal integration of the GOI (*EGFP* cDNA expression unit) and cellular distribution of the GOI in adipocytes, a 1 µL-solution containing 0.5 µg/µL of each plasmid DNA (pTrans and pT-EGFP; shown in Figure 1A) and 0.1% TB was injected into the fat depots of adult B6C3F1 mice, and *in vivo* EP was immediately applied to the injection site. One day after EPGDA, the injected site in the adipose tissue (which was easily discernible because of the presence of TB) was excised using microscissors and inspected for EGFP-derived fluorescence (as shown in Figure 3A). Intense fluorescence was observed at the injection site, but little or no fluorescence was noted at the periphery of the injection site when the specimens were examined under a microscope at low magnification (Figure 1B-a, b). When the fluorescent area (enclosed in the box in Figure 1B-b) was enlarged, distinct fluorescence was discernible in cell membranes/cytoplasm and probably also in the nuclei of some adipocytes with a characteristic globular shape (arrows and arrowheads, respectively, Figure 1B-c, d). However, no fluorescence was found when a plasmid-free solution was injected into the adipose tissue (Figure 1B-e, f). When green fluorescent cells were calculated, 54% of the cells (73/135) in an area of $4.0 \times 10^{-8}$ $m^2$ were positive for fluorescence.

### *3.3 Determining Chromosomal Integration of GOI after PB-Based Gene Delivery*

The PB-based integration events are preceded by the formation of a junction between the ITR and the TTAA sequence on the chromosomes [36, 37]. Thus, we examined whether the GOI introduced into adipose tissues through the EPGDA technique was integrated into the host chromosomes by performing Splinkerette-PCR [35], which is useful for assessing junctional sequences between host genomes and the transgene. The fluorescent green sample shown in Section 3.2 was dissected under a fluorescence dissecting microscope using microscissors (as shown in Figure 3f) and lysed before genomic DNA isolation. The isolated genomic DNA was used for Splinkerette-PCR and was subcloned into a TA cloning vector; 13 clones were isolated. PCR was performed with all the clones using universal primers (M2 and RV) to amplify the insert. In Figure S1, the data obtained after conducting 2% agarose gel electrophoresis are shown. Several clones with inserts of different sizes (ranging from ~250 to ~350 bp) were identified. These PCR products were sequenced, and we found that 57% of the clones were the same as those (that were identified as clone Spl-1) encoding *Mus musculus* mRNA for the pancreatitis-associated protein (*PAP*) homologous protein (located on chromosome 6) and 29% were the same as those (that were identified as clone Spl-3) encoding *Mus musculus* GATA-binding factor 6 (*GATA6*) gene, exon 1 (located on chromosome 18) (Table 1). The remaining 14% of the clones had irrelevant sequences, probably of unknown contaminants generated during the cloning process. Notably, two clones were identified that did not possess the desired consensus sequence (TTAA) and the subsequent host (murine) chromosomal sequence (Table 1).

**Table 1** The Splinkerette-PCR analysis.

| Clone | Sequence (5'-3')[1] | Reference[2] |
|---|---|---|
| Spl-1 | **GGGCCC**AATTCGCCCTATAG TGAGTCGTATTACAATTCACT GGCCGTCGTTTTACAACGTC GTGACTGGGA | D13509.1 *Mus musculus* mRNA for pancreatitis-associated protein (PAP) homologous protein, complete cds; located on chromosome 6; identities = 65/65 (100%) |
| Spl-3 | **TACCAA**GCTTGGCGTAATCA TGGTCATAGCTGTTTCCTG | AB119275.1 *Mus musculus* GATA-binding factor 6 (GATA6) gene, exon 1; located on chromosome 18; identities = 33/33 (100%) |

[1] Sequences that are similar to TTAA are shadowed. Sequences corresponding to the end of ITR are shown in bold.

[2] Sequence homology analysis was performed using the BLASTN program (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

### *3.4 ASCs in Adipose Tissue May be Transfected with An Octamer-Binding Transcription Factor-3/4* (Oct-3/4) *Promoter-Directed Plasmid pdOEN-2*

According to Tai et al. [38], somatic stem cells (i.e., stem cells from the epithelium, bone marrow, and liver) express Oct-3/4. To mark the ASCs in female fat depots, we injected a solution containing pdOEN-2 (carrying an *Oct-3/4* promoter-directed *EGFP* cDNA expression unit; Figure 2A) and ptdTomato (carrying a *CAG* promoter-directed *tdTomato* cDNA expression unit; Figure 2A) into adult B6C3F1 fat depots and immediately conducted *in vivo* EP at the injection site. India ink was co-introduced instead of TB. If EGFP expression was observed at the injection site, the fluorescent cells could be considered to be ASCs. When a small portion (~3 $mm^3$) of the India ink-containing adipose tissue, which was easily discernible under a light microscope (arrows in Figure 2B-a), was dissected and inspected for tdTomato-derived red fluorescence, many adipocytes exhibited fluorescence in their cell membrane and cytoplasm (arrows in Figure 2B-b). Green fluorescence was confined to the corner (probably corresponding to a nucleus) of the cells (indicated by arrowheads in Figure 2B-c), although a few cells exhibited membranous fluorescence (indicated by red arrows in Figure 2B-c). When green fluorescent cells were calculated, 24% of the red fluorescent cells (20/83) in an area of $3.91 \times 10^{-8}$ $m^2$ were estimated to be Oct-3/4-positive cells.

To confirm that adipose tissue could express *Oct-3/4* mRNA, RT-PCR was performed using young (one-month-old) and adult (five-month-old) fat depot samples. We used P19 ECCs and NIH3T3 cells as positive and negative controls, respectively. Both types of fat depot samples exhibited a 232 bp-band for mouse *Oct-3/4* mRNA (arrow in the upper panel of Figure 2C), although the young sample expressed *Oct-3/4* mRNA more strongly than the adult sample. This was also confirmed using ASCs collected from the adipose tissue of adult female mice (lower panel of Figure 2C).

## 4. Discussion

For *in vivo* gene delivery to adipocytes, a non-viral method using plasmid DNA [6-9] and multiple viral methods involving adenovirus [10], adeno-associated virus [11-13], and retroviral vectors [14-16] are used. For safety and to prevent the occurrence of cellular toxicity due to immunological reactions, non-viral gene delivery is preferred [25]. Among the non-viral methods, EP is the most feasible and convenient one and has been used by several researchers to deliver genes *in vivo* to the liver, pancreas, testis, ovary, kidney, skin, bladder, oviduct, and skeletal muscle [39].

Transfection of plasmid DNA into the subcutaneous adipose pad [17, 24] and adipose tissue by EP-based gene delivery in the male reproductive system [19] has been reported. Granneman et al. [17] injected 7 µL of a solution containing 1 µg/µL of plasmid (to express EGFP-perilipin fusion proteins targeted to distinct subcellular domains) into the subcutaneous adipose pad of adult C57BL/6 mice, using a 26-gauge needle and a Hamilton microliter syringe. Immediately after injection, the injection site was flanked (1-2 mm on either side) with Gerald bipolar forceps (1.5 × 8 mm) that were used to hold the tissue and maintain a gap of 1 mm. *In vivo* EP was performed with seven square-wave pulses (20 ms wavelength/20 ms duration/50 V) over three seconds with the internal resistance of the stimulator set at 250 Ω. Gene expression was observed at the injection sites 1-3 days after gene delivery, and the technique was called "adiporation." This facilitated the examination of subcellular targeting and translocation *in vivo*. Endo and Kobayashi [19] injected a 10 µL-solution containing a plasmid (1 µg/µL) carrying a functional mouse thyroid-stimulating hormone (*TSH*) receptor gene using a 27-gauge needle into the epididymal white adipose tissue of mutant *hyt/hyt* mice (C.RF-Tshr$^{hyt/hyt}$ mice). These mice had hypothyroidism and severely hypoplastic thyroid glands. The experiment was conducted to assess the roles of TSH on lipid metabolism. Electric pulses (two 50 ms pulses of 70 V, followed by two pulses of the opposite polarity at a rate of 1 pulse/s) were delivered using an electric pulse generator (Square Electroporator CUY21; Nepa Gene Co., Ltd.) with a pair of stainless electrode needles. Endo and Kobayashi detected gene expression at the injection sites three weeks after gene delivery and called this technique "plasmid injection combined with electroporation (PICE)." Furthermore, Fisher et al. [24] injected 50 µL of a solution containing 50 µg of plasmid encoding a green fluorescent protein (GFP) into the interscapular subcutaneous fat pads of guinea pigs using a 22-gauge needle. The area was then clamped between electrode plates (the distance between the electrodes was 12 mm, and the electrode plate faces were 20 × 20 mm), and a voltage of 50-200 V was maintained. Fisher et al. [24] observed widespread gene expression that persisted for at least 60 days *in vivo*. However, these experiments focused on transient expression (but not chromosomal integration) of plasmids carrying the GOI.

In this study, we focused on *in vivo* EP-based gene delivery to murine adipose tissue (fat depots) associated with the female reproductive system. This approach is very convenient, as genes can be efficiently delivered to the adipose tissue after it is exposed through an incision in the skin and the dorsal muscular layer, under anesthesia (Figure 3A). In this study, we used a breath-controlled glass micropipette (Figure 3A-a), instead of a needle, for injecting into the adipose tissue because the solution being injected could be better controlled by the micropipette while observing under a dissecting microscope. We injected 1 µL of NA-containing solution and subsequently performed *in vivo* EP (with Pp: 50 V and Tp: 20 V) after flanking the injection site with tweezer-type electrodes (Figure 3A-e). High voltage (Tp: >30 V) may cause irreversible damage to the adipose tissue. When

the injection site was inspected 24 h after gene delivery, the expression of the GOI was detected in the cell membrane, nucleus, and cytoplasm of adipocytes, with an increase in the fluorescence in the cell membrane and the nucleus (arrows and arrowheads in Figure 1B-d).

The expression of a GOI in a plasmid that is introduced *in vivo* is generally transient. In most cases, the expression ceases within one week after EPGDA. To enable the continuous expression of the GOI *in vivo*, chromosomal integration of the GOI is a prerequisite. To determine whether the transfected cell continues to express the GOI, the expression of the gene should be checked after one week. Transposons are ideal for producing stable transfectants, and several laboratories have used Tol2, PB, and Sleeping Beauty for transfection and gene integration [39]. For example, we detected GOI integration into chromosomes 1, 2, 5, 7, 8, 9, 10, 14, 16, and Y when a solution containing PB-based plasmids (pTrans + pT-EGFP) was introduced into murine pancreatic cells using the same technique described in this study; the injection sites were assessed by Splinkerette-PCR analysis [40]. In our previous experiments, the PB-based chromosomal integration occurred within 24 h after transfection. We found at least two integration sites on chromosomes 6 and 18 when adipose samples were examined 24 h after EPGDA (Table 1). The number of integration sites was low, compared to the sites of transfection in murine pancreatic cells, probably due to fewer sampled injection sites (~3 $mm^3$).

Generally, PB-based chromosomal integration of the GOI is preceded by a TTAA consensus sequence on the chromosomal DNA [41, 42]. Thus, the TTAA sequence is usually found at either end of the GOI integrated into a chromosome [41, 42]. However, in our previous experiment (where we delivered genes to the pancreas using the PB system *in vivo*), seven Splinkerette-PCR-derived clones were tested, and four (57%) failed to exhibit a TTAA sequence at the end of the GOI, exhibiting chromosomal integration [40]. Similarly, the resulting Splinkerette-PCR-derived clones obtained through EPGDA were those that did not have the TTAA sequence (Table 1). When we used a similar strategy to examine the GOI integration sites in cultured porcine transfected clones, we found that they all had the TTAA sequence at the end of the chromosomally integrated GOIs [43]. The mode of PB-based chromosomal integration of the GOI may differ between *in vivo* and *in vitro* procedures. The PB-based gene delivery system relies on random integration events. Therefore, occasional abnormal growth of adipose tissue near injection sites might occur. To avoid this problem, long-term monitoring after EPGDA may be required.

Adipose tissues contain various types of cells, including adipocytes, ASCs, endothelial cells, endothelial progenitor cells, pericytes, smooth muscle cells, leukocytes, and erythrocytes [5]. Since CAG promoter-based plasmids are used for EPGDA, transfection into and the expression of the GOI in the abovementioned cells is highly possible. While observing cell fluorescence using unfixed adipose samples, we only detected fluorescence in adipocytes due to their globular morphology (see Figure 1B-d). Thus, histological analysis of the injected sites is required for the precise localization of the cells mentioned.

One of the goals of this experiment was to enrich ASCs isolated from the fat depots of females, as ASCs can differentiate into various cell types (including osteoblasts, chondrocytes, myocytes, neurocytes, and other cell types) and are a promising resource for the regenerative medical field [5, 44]. According to Tai et al. [38], many somatic stem cells express Oct-3/4. To specifically mark the putative ASCs possibly present in the adipose tissue, we introduced pdOEN-2, a plasmid conferring EGFP expression under the control of the mouse *Oct-3/4* promoter, along with ptdTomato, a plasmid conferring tdTomato expression under the control of the ubiquitous *CAG* promoter, into

the adipose tissue using EPGDA. When the injected portions of the fat depots were dissected 24 h after gene delivery and inspected for fluorescence, massive expression of tdTomato-derived red fluorescence was observed (arrows in Figure 2B-b). In the red fluorescent area, spot-like green fluorescence was observed (arrowheads in Figure 2B-c), although a few cells showed membranous fluorescence (red arrow in Figure 2B-c). The spot-like green fluorescence indicated the nucleus, and the cells that showed green fluorescence were ASCs or ASC-like stem cells. The expression of *Oct-3/4* mRNA in ASC-containing adipose tissue was confirmed by performing RT-PCR using adipose mRNA isolated from young (one-month-old) and adult (five-month-old) female mice (see Figure 2C). The expression of *Oct-3/4* mRNA or protein in the adipose tissue was also reported in ASCs [45] of other species, including horses [46] and rats [47]. To further confirm that these *Oct-3/4*-positive cells marked by transfection with pdOEN-2 are ASCs or ASC-like stem cells, primary cultivation of these cells is required to show that they still have the multi-differentiation ability *in vitro* or immunocytochemical staining of these cells with ASC-specific marker antibodies should be performed to check for certain markers in the cells. Similar to our findings in another system (the *in vivo* PB-based gene delivery system targeting hepatocytes) [48], the findings of this study suggested the possibility of the *in vivo* acquisition of gene-engineered ASCs through the delivery of a PB transposon carrying the GOI (linked to the mouse *Oct-3/4* promoter) and pTrans by the EPGDA technique.

In this study, we efficiently transfected a GOI into adipocytes (associated with the female reproductive system) *in vivo* and performed chromosomal integration of the GOI using a PB-based gene delivery system. For this, we used 1 µL of NA-containing solution for EPGDA. However, under this condition, only a few adipocytes could be transfected. To overcome this issue and increase the efficiency of the method, the NA-containing solution may be injected into multiple sites in a small area (as suggested by Granneman et al. [17]), or a large volume of NA-containing solution (e.g., >10 µL) can be injected using a needle (as suggested by Endo and Kobayashi [19]) before performing EP *in vivo*.

The EPGDA technique has numerous applications, including the *in situ* visualization of the three-dimensional cellular morphology, lineage tracing (i.e., marking of specific cell types in adipose tissue [17]), the acquisition of immortalized ASCs through *in vivo* transfection with immortalization genes [48], functional analysis (i.e., production of therapeutically valuable substances [19], and plasmid or mRNA-based vaccination [24, 49]).

## 5. Conclusion

We injected a solution containing PB-based plasmids into the gonadal fat depots of female mice using a breath-controlled micropipette under a dissecting microscope. EP was immediately performed at the injection site *in vivo*. Consequently, successful transfection of adipocytes was observed at the injection area. Furthermore, the GOI was integrated into the chromosome, and the putative ASCs were marked after the transfection of a plasmid with an EGFP-expressing gene under the transcriptional control of the mouse *Oct-3/4* promoter. The advantages of the *in vivo* EP-based method over other virus-based methods include the ability to transfect adipocytes (including ASCs) at specific sites by observing under a dissecting microscope and using a breath-controlled pipette.

This approach is safer and has a lower possibility to induce immunogenicity compared to virus-based methods. The disadvantages are that the area of adipose tissue that can be transfected is

very small, and only a small volume of solution can be injected into the target site. Using this technique, it might be possible to manipulate the size of the adipocytes or induce overexpression of exogenous genes such as adipokines. Furthermore, besides female gonadal fat depots, there are several target sites for EPGDA, including epididymal fat depots in male mice and the interscapular brown adipose tissue.

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**Author Contributions**

Conceptualization, M.S.; methodology, Y.K., E.A., I.S.; investigation, S.N., S.W.; writing—original draft preparation, M.S.; writing—review and editing, E.I. All authors have read and agreed to the published version of the manuscript.

**Competing Interests**

The authors have declared that no competing interests exist.

**Additional Materials**

The following additional materials are uploaded at the page of this paper.

1. Figure S1: Two percent gel electrophoresis of Splinkerette-PCR products.
2. Table S1: Primer sets used for RT-PCR analysis.

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