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Lab Resource: Genetically-Modified Multiple Cell Lines

# Generation of human GAPDH knock-in reporter iPSC lines for stable expression of tdTomato in pluripotent and differentiated culture conditions

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A B S T R A C T

Human induced pluripotent stem cells (iPSCs) can differentiate into multiple cell types and are utilized for research on human development and regenerative medicine. Here, we report the establishment of human GAPDH knock-in reporter iPSC lines (GAPDH-tdT1 and 2), via CRISPR/Cas9-mediated homologous recombination, that stably express tdTomato as a constitutive cell label in both iPSCs and their differentiated derivatives. These cell lines will provide useful tools to trace cell locations and fates in 2D cultures and 3D organoids and will facilitate in vivo experiments.

Resource Table

| | |
|---|---|
| Unique stem cell lines identifier | KUIMSi012-A-1 (https://hpscreg.eu/cell-line/KUIMSi012-A-1)KUIMSi012-A-2 (https://hpscreg.eu/cell-line/KUIMSi012-A-2) |
| Alternative name(s) of stem cell lines | GAPDH-tdT1 (KUIMSi012-A-1)GAPDH-tdT2 (KUIMSi012-A-2) |
| Institution | Takeda-CiRA Joint program (T-CiRA), Fujisawa, Kanagawa, Japan |
| Contact information of the reported cell line distributor | Daisuke Kamiya, kamiya@cira.kyoto-u.ac.jp |
| Type of cell lines | iPSC |
| Origin | Human, 1231A3 iPSC (ref. Nakagawa et al., 2014) |
| Additional origin info *(applicable for human ESC or iPSC)* | Age: 29<br>Sex: Female<br>Ethnicity: African/American |
| Cell Source | Peripheral blood |
| Method of reprogramming | Episomally |
| Clonality | Clonal |
| Evidence of the reprogramming transgene loss (including genomic copy if applicable) | N/A |
| Cell culture system used | Feeder-free culture |
| Type of genetic modification | Heterozygous or homozygous insertion of (a) fluorescent reporter(s) |
| Associated disease | N/A |
| Gene/locus | GAPDH, 12p13.31 |
| Method of modification/site-specific nuclease used | CRISPR/Cas9 ribonucleoprotein (RNP) complex |
| Site-specific nuclease (SSN) delivery method | Plasmid transfection/electroporation |
| All genetic material introduced into the cells | Synthetic tracrRNA and crRNA, pEBMulti-Puro vector, and HDR donor vector |
| Analysis of the nuclease-targeted allele status | Sequencing of the targeted allele |
| Method of the off-target nuclease activity surveillance | N/A |
| Name of transgene | tdTomato (ref. Matz et al., 1999, Lukyanov et al., 2000) |
| Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific) | N/A |
| Inducible/constitutive system details | N/A |
| Date archived/stock date | September 2021 |
| Cell line repository/bank | N/A |
| Ethical/GMO work approvals | This study was approved by the Shonan Health Innovation Park (iPark, No., CS-00001157) |

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| | |
|---|---|
| Addgene/public access repository recombinant DNA sources' disclaimers (if applicable) | N/A |

## 1. Manuscript section expected contents clarification

### 1.1. Resource utility

The reporter iPSC lines GAPDH-tdT1 (KUIMSi012-A-1) and 2 (KUIMSi012-A-2) constitutively exhibit tdTomato fluorescence in both pluripotent and differentiated culture conditions, enabling the monitoring of locations, numbers, shapes, and fates of living iPSCs and their differentiated derivatives in vivo (Table 1).

### 1.2. Resource details

Human *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) is a housekeeping gene that is expressed in all human tissues and cultured cells. It encodes an enzyme essential for glycolysis, which is not only important in metabolic pathways but also plays a role in several non-metabolic pathways such as transcriptional activation and apoptosis. To establish an iPSC line constitutively expressing tdTomato as a fluorescent label, donor DNA designed as an F2A (2A self-cleaving peptides)-tdTomato cassette was inserted at the site of the stop codon in exon 9 of GAPDH (Fig. 1A). This enabled tdTomato to be transcribed and translated with GAPDH as a fusion protein under the control of the intrinsic GAPDH promoter, and then cleaved into independent proteins at the F2A site. Clones showing strong fluorescence were selected, and tdTomato insertion was confirmed with genomic PCR (Fig. 1B). A clone with a tdTomato cDNA heterozygotically inserted into a GAPDH allele was named GAPDH-tdT1 (KUIMSi012-A-1), while a clone inserted with tdTomato homozygotically inserted into a GAPDH allele was named GAPDH-tdT2 (KUIMSi012-A-2). Desirable insertion of tdTomato in GAPDH alleles was confirmed by sequencing the region surrounding the insertion site (Fig. 1A). Both the GAPDH-tdTomato iPSC lines showed normal morphology and strong tdTomato reporter expression (Fig. 1C); additionally, karyotype analysis revealed that the GAPDH-tdTomato lines possessed the same normal karyotypes as the parent 1231A3 iPSC line (Fig. 1D). STR analysis showed that the GAPDH-tdTomato lines had the same origin as the parent line 1231A3 (Supplementary file). The GAPDH-tdTomato lines were confirmed to express pluripotent markers OCT4, SOX2, NANOG, and SSEA4 (Fig. 1E) and were able to differentiate into cells expressing the endoderm marker SOX17, mesoderm marker NCAM, and ectoderm marker PAX6 (Fig. 1F), which indicated that the iPSC lines maintained pluripotency after genome editing. Notably, cells stained with differentiation markers showed tdTomato fluorescence, which indicated that tdTomato was continuously expressed in the differentiated cell types. Additionally, we were able to induce differentiation of the iPSCs to mesenchymal stem/stromal cells (MSCs), a cell type that is expected to be a good source for regenerative medicine. MSCs could be induced from the GAPDH-tdT1 line via neural crest cells, and the expression of cell surface markers of human MSCs was confirmed via FACS after four passages of MSC induction (Fig. 1G). tdTomato expression was maintained in MSCs. These results indicate that these iPSC lines can facilitate monitoring of the locations and fates of living iPSCs and their differentiated derivatives in vitro and in vivo (data not shown) using the fluorescence of tdTomato. GAPDH-tdT2 showed stronger tdTomato fluorescence than GAPDH-tdT1 (Fig. 1C). However, since strong expression of fluorescent proteins sometimes causes cytotoxicity in some cell types, it is desirable to test both of these two cell lines depending on the purpose or cell types to be studied.

## 2. Materials and methods

### 2.1. Cell culture

1231A3 human iPSCs were cultured on cell culture dishes coated with iMatrix-511 (Nippi) in StemFit AK03N (Ajinomoto), as described previously (Nakagawa et al., 2014).

**Table 1**
Characterization and validation.

| Classification (optional *italicized*) | Test | Result | Data |
|---|---|---|---|
| Morphology | Photography | Normal | Fig. 1 panel C |
| Pluripotency status evidence for the described cell line | Immunocytochemistry | Expression of pluripotency markers: OCT4, NANOG, SOX2 | Fig. 1 panel E |
| | Quantitative analysis | SSEA4<br>GAPDH-tdT1: 99.8%<br>GAPDH-tdT2: 99.6% | Fig. 1 panel E |
| Karyotype | Karyotype (G-banding) | 46, XX, inv(9)(p12q13) | Fig. 1 panel D |
| Genotyping for the desired genomic alteration/allelic status of the gene of interest | PCR across the edited site or targeted allele-specific PCR | 5′ (1.1 kb) and 3′ (1.4 kb)<br>Out-out (WT: 1.2 kb, Knock-in: 2.7 kb) | Fig. 1 panel B |
| | Transgene-specific PCR | N/A | |
| Verification of the absence of random plasmid integration events | PCR | PCR detection specific for plasmid backbones | Supplementary file |
| Parental and modified cell line genetic identity evidence | STR analysis | 16 sites tested; all matched | Supplementary file |
| Mutagenesis / genetic modification outcome analysis | Sequencing (genomic DNA PCR or RT-PCR product) | Sanger sequencing | Fig. 1 panel A |
| | PCR-based analyses | N/A | |
| | Southern blotting or WGS; western blotting (for knock-outs, KOs) | N/A | |
| *Off-target nuclease analysis* | PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing | N/A | |
| Specific pathogen-free status | Mycoplasma | Negative (MycoAlert™ Mycoplasma Detection Kit, LONZA, #LT07-21) | Not shown, but available from author |
| Multilineage differentiation potential | Directed differentiation | Differentiation into derivatives of three germ layers: endoderm (SOX17 + cells), mesoderm (NCAM + cells), ectoderm (PAX6 + cells) | Fig. 1 panel F |
| *Donor screening (OPTIONAL)* | HIV 1 + 2, Hepatitis B, and Hepatitis C | N/A | |
| *Genotype - additional histocompatibility info (OPTIONAL)* | Blood group genotyping | N/A | |
| | HLA tissue typing | N/A | |

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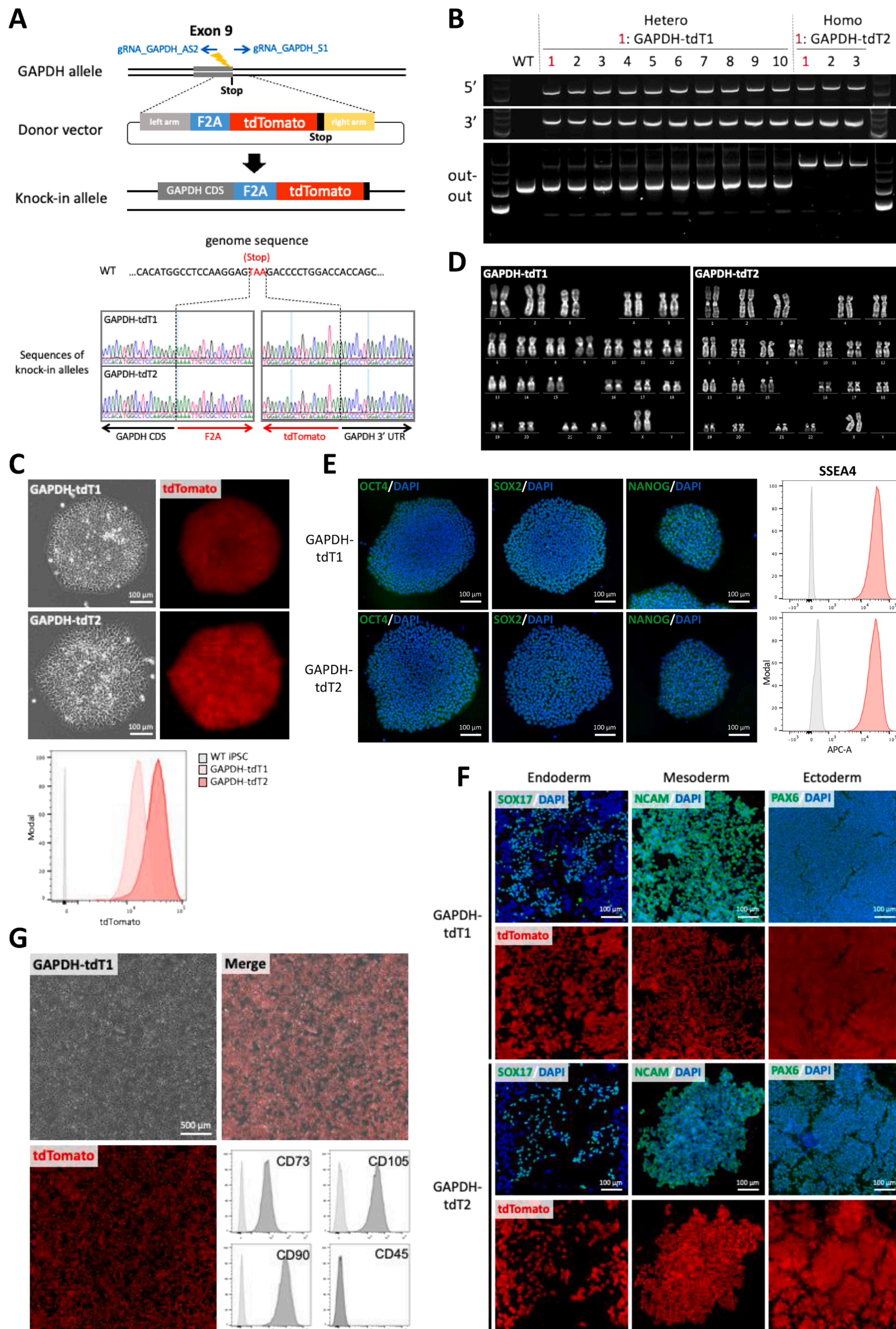

**Fig. 1.** Generation and characterization of GAPDH-tdT iPSC lines.

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### 2.2. Targeting design

The homology-directed repair (HDR) donor vector was designed to insert the F2A-tdTomato cassette flanked by the left and right homology arms of GAPDH (Fig. 1A, synthesized at Eurofins Genomics Inc.) so that F2A-tdTomato cDNA was inserted in-frame at the stop codon of GAPDH in exon 9 (Fig. 1A).

### 2.3. CRISPR/Cas9-mediated knock-in

The SpCas9 protein (Alt-R® S.p. Cas9 D10A Nickase, Cat.# 1081062, IDT), two GAPDH gRNAs (comprising crRNA (IDT) and tracrRNA (Alt-R® CRISPR-Cas9 tracrRNA, Cat.# 1072534, IDT)), HDR donor vector (synthesized at Eurofins Genomics Inc.), and a puromycin-resistance gene expression vector (pEBMulti-Puro, FUJIFILM Wako Pure Chemical) were electroporated into $5 \times 10^5$ 1231A3 iPSCs using the Neon Transfection kit (Thermo Fisher Scientific). The transfected cells were selected by puromycin (0.2 μg/ml) for 2 days and screened visually by the intensity of tdTomato fluorescence as an indicator of successful recombination. Desirable tdTomato insertion in these colonies was confirmed via PCR and sequencing (Fig. 1B), and a clone containing heterozygously inserted tdTomato in a GAPDH locus (GAPDH-tdT1) and a homozygously inserted clone (GAPDH-tdT2) were used for subsequent analyses. The primers used to confirm desirable insertion are listed in Table 2.

### 2.4. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde/PBS at 4 °C for 15 min, washed twice with PBS, incubated with 0.3% Triton-X100 at 4 °C for 30 min, and blocked with 5% BSA/PBS at 4 °C for 1 h. Nuclei were counterstained with DAPI. Images were acquired with a BZ-X700 camera (Keyence). The antibodies used are listed in Table 2.

### 2.5. Flow cytometry

Fluorescence-activated cell sorting (FACS) and flow cytometry were performed using an Aria II instrument (BD Biosciences) and the antibodies listed in Table 2.

### 2.6. Three germ layer differentiation

Three germ layer differentiation was performed using the Stem MACS™ Trilineage Differentiation Kit (Miltenyi Biotec, 130–115-660) according to the manufacturer's instructions. Cells were stained with endoderm, mesoderm, and ectoderm markers. The antibodies used are shown in Table 2.

### 2.7. Mesenchymal stem/stromal cell differentiation

Mesenchymal stem/stromal cell (MSC) differentiation was performed as previously described (Kamiya et al, 2020). Briefly, GAPDH-tdT1 iPSCs were seeded onto iMatrix-511-coated dishes in StemFit AK03N at a density of $3.6 \times 10^3$ cells/cm$^2$ and cultured for 4 days. Cells were cultured in induction medium, StemFit AK03N without bFGF with 10 μM SB431542 and 1 μM CHIR99021, for 10 days to induce neural crest formation. NCCs were stained with the CD271 antibody (BD Biosciences), and CD271+ NCCs were sorted. Sorted cells were cultured onto fibronectin-coated plates at a density of $1 \times 10^4$ cells/cm$^2$ in Basic03 (Ajinomoto) supplemented with 10 μM SB431542, 20 ng/mL EGF, and 20 ng/mL bFGF for 2 weeks. After 2 weeks of culture, the medium was replaced with MSC Expansion medium (PRIME-XV MSC Expansion XSFM, FUJIFILM Irvine Scientific, Inc.) and cultured for further 2 weeks.

**Table 2**
Reagents details RRID. Requirement for antibodies: use http://antibodyregistry.org/ to retrieve RRID for antibodies and include ID in the table, as shown in examples.

| Antibodies and stains used for immunocytochemistry/flow-cytometry | | | |
|---|---|---|---|
| | Antibody | Dilution | Company Cat # and RRID |
| Pluripotency markers | Rabbit anti-OCT4A<br>Rabbit anti-NANOG<br>Rabbit anti-SOX2<br>Alexa Fluor 647 Mouse anti-human EESA-4 | 1/400<br>1/200<br>1/200<br>1/100 | Cell Signaling Technology Cat# 2840, RRID: AB_2167691<br>Thermo Fisher Scientific Cat# PA1-097X, RRID: AB_2539868<br>Abcam Cat# ab92494, RRID: AB_10585428<br>BioLegend Cat# 330408, RRID: AB_1089200 |
| Differentiation markers | Goat anti-SOX17<br>Goat anti-NCAM<br>Rabbit anti-PAX6 | 1/100<br>1/100<br>1/300 | R and D Systems Cat# AF1924, RRID: AB_355060<br>R and D Systems Cat# AF2408, RRID: AB_442152<br>Abcam Cat # ab195045, RRID: AB_2750924 |
| Secondary antibodies | Alexa Fluor 488 Donkey anti-Rabbit IgG (H + L)<br>Alexa Fluor 488 Donkey anti-goat IgG (H + L) | 1/1000<br>1/1000 | Thermo Fisher Scientific Cat# A-21206, RRID: AB_2535792<br>Thermo Fisher Scientific Cat# A-11055, RRID: AB_2534102 |
| Nuclear stain | DAPI | 1:1000 (1 μg/mL) | Thermo Fisher Scientific, Cat# D1306, RRID: AB_2629482 |
| MSC markers (all antibodies are mouse monoclonal antibodies raised against human antigens) | APC Mouse anti-CD45<br>APC Mouse anti-CD73<br>APC Mouse anti-CD90<br>APC Mouse anti-CD105<br>APC Mouse anti-mouse_IgG1_k | 1/100<br>1/100<br>1/100<br>1/100<br>1/100 | BD Pharmingen, Cat# 560973, RRID: AB_10565969<br>BD Pharmingen, Cat# 560847, RRID: AB_10612019<br>BD Pharmingen, Cat# 559869, RRID: AB_398677<br>eBioscience, Cat# 17–1057-42, RRID: AB_1582211<br>BD Pharmingen, Cat# 555751, RRID: AB_398613 |
| **Site-specific nuclease** | | | |
| *Nuclease information* | Nuclease type/ nomenclature | Alt-R® S.p. Cas9 D10A Nickase V3 (INTEGRATED DNA TECHNOLOGIES (IDT), Cat# 1081062) | |
| *Delivery method* | Electroporation | | |
| *Selection/ enrichment strategy* | PCR, transient co-expression of puromycin resistance gene | | |
| **Primers and oligonucleotides used in this study** | | | |
| | **Target** | **Forward/Reverse primer (5′-3′)** | |
| Genotyping | GAPDH_out_F1<br>tdT_in_R1<br>GAPDH_out_R1<br>- tdT_in_F1 | GGGAGGTAGAGGGGTGATGT<br>GCCGCGCATCTTCACCTTGTAGATC<br>GACTTCCTCCACCTGTCAGC<br>TGCAACTGCCCGGCTACTA | |
| Targeted mutation analysis/ sequencing | Sequencing data of the edited allele | | |
| Potential random integration-detecting PCRs | Ampicillin resistance gene (plasmid backbones for targeting events) | TGCAACTTTATCCGCCTCCAT/<br>GGATGGCATGACAGTAAGAGAAT | |
| crRNA sequences | for gRNA_GAPDH_S1<br>for gRNA_GAPDH_AS2 | CCUCCAAGGAGUAAGACCCC<br>AGAAGAUGAAAAGAGUUGUC (Alt-R® CRISPR-Cas9 crRNA, IDT) | |
| Genomic target sequences | | | |

(continued on next page)

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**Table 2** (*continued*)

| Antibodies and stains used for immunocytochemistry/flow-cytometry | | | |
|---|---|---|---|
| | Antibody | Dilution | Company Cat # and RRID |
| | for gRNA_GAPDH_S1 for gRNA_GAPDH_AS2 | CCTCCAAGGAGTAAGACCCCTGG CCTGACAACTCTTTTCATCTTCT (PAM) | |
| *e.g. Top off-target mutagenesis predicted site sequencing (for CRISPR/Cas9 and TALENs) primers* | | | N/A |
| *ODNs/plasmids/ RNA templates used as templates for HDR-mediated site-directed mutagenesis. Backbone modifications in utilized ODNS have to be noted using standard nomenclature.* | | N/A | |

### 2.8. STR analysis

STR analysis was performed by checking 16 sites at BEX (Japan).

### 2.9. Karyotype analysis

Karyotype analysis was performed via quinacrine-Hoechst staining at Chromocenter (Japan).

## 3. Data availability

Data will be made available on request.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgement

We thank Dr. Hirokazu Matsumoto and Ikeya-PJ members for their support throughout this study. We would like to acknowledge Takara Bio USA Inc. for providing tdTomato vector.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102704.

## References

Kamiya, D., Takenaka-Ninagawa, N., Motoike, S., Kajiya, M., Akaboshi, T., Zhao, C., Shibata, M., Senda, S., Toyooka, Y., Sakurai, H., Kurihara, H., Ikeya, M., 2020. Induction of functional mesenchymal stem/stromal cells from human iPCs via a neural crest cell lineage under xeno-free conditions. SSRN. https://doi.org/10.2139/ssrn.3741231.

Lukyanov, K.A., Fradkov, A.F., Gurskaya, N.G., Matz, M.V., Labas, Y.A., Savitsky, A.P., Markelov, M.L., Zaraisky, A.G., Zhao, X., Fang, Y.u., Tan, W., Lukyanov, S.A., 2000. Natural animal coloration can Be determined by a nonfluorescent green fluorescent protein homolog. J. Biol. Chem. 275 (34), 25879–25882.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., Lukyanov, S.A., 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat. Biotechnol. 17 (10), 969–973.

Nakagawa, M., Taniguchi, Y., Senda, S., Takizawa, N., Ichisaka, T., Asano, K., Morizane, A., Doi, D., Takahashi, J., Nishizawa, M., Yoshida, Y., Toyoda, T., Osafune, K., Sekiguchi, K., Yamanaka, S., 2014. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci. Rep. 4, 3594.