Possible application of muscle specific conditional mouse-derived induced pluripotent stem cells for muscle research

Tohru Hosoyama

Department of Regenerative Medicine, National Center for Geriatrics and Gerontology, 7-430 Morioka-cho, Obu, Aichi, 474-8511, Japan

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ABSTRACT

The Cre-driver mouse line, which allows for *in vivo* regulation of target gene(s) in specific cells, is an indispensable tool for recent muscle research. In this study, I aimed to explore new applications of muscle specific Cre-driver mouse line in muscle research. For this purpose, I generated an iPS cells from a myofiber specific conditional mouse with tamoxifen inducible GFP expression, and then I checked whether homologous recombination was induced in the iPS-derived myogenic cells by tamoxifen administration. Fibroblasts were isolated from the tails of $Myf6^{CE/wt}$::CAG-*EGFP* mice, which expressed GFP specifically in Myf6 lineages by tamoxifen injection, and then iPS cells was generated by transfection with a vector based on sendai-virus and containing OSKM genes. Muscle specific conditional mouse-derived iPS cells (mCM-iPSCs) were successfully differentiated to myogenic cells, such as $Pax7^+$ muscle progenitors, $MyoD^+$ myoblasts, and MHC^+ myotubes, under myogenic differentiation conditions. Using this model, I examined whether homologous recombination was induced in mCM-iPSC-derived myotubes by 4-hydroxytamoxifen (4OH-TAM) administration. As a result, multinucleated myotubes showed GFP expression, while no GFP signals were detected in both $Pax7^+$ muscle progenitor and non-myogenic cells. These results indicated that homologous recombination could be induced in mCM-iPSC–derived myotubes by tamoxifen administration, and that this system operated normally even in reprogrammed cells. Also, I evidenced that GFP reporter was expressed in myoblasts in addition to multinucleated myotubes when tamoxifen-pulse was applied at an early phase of myogenesis. Taken together, $Myf6^{CE/wt}$::CAG-*EGFP* mouse-derived iPS cells reproduced at least in part Myf6 expression during mouse myogenesis. This study demonstrated a novel application of muscle specific conditional mouse in addition to *in vivo* application, and mCM-iPSCs could also be used in *in vitro* investigations with muscle specific conditional knock-out mouse.

1. Introduction

Today, a number of different conditional mouse lines, which allows for *in vivo* regulation of expression of target gene(s) in specific cells, have been generated worldwide, and these mouse lines have been indispensable tools for recent research, including muscle-related research [1]. In these mouse lines, the Cre/loxP system was used to control target gene(s) with temporal and tissue/cell specific recombination. For example, in muscle research, paired box protein 7-CreER ($Pax7^{CreER}$) mouse lines, which induce tamoxifen-mediated recombination, enable to trace lineages and/or to knock-out specific genes of muscle stem cells in postnatal muscles [2–4]. Also, muscle fiber specific conditional mouse lines, such as muscle creatine kinase-Cre (MCK^{Cre}), and myosin light chain 1-Cre ($Myl1^{Cre}$) mice have provided important information in development, growth, and atrophy of muscle [5,6]. However, the use

of conditional mice has drawbacks, such as costs, breeding space, and animal welfare, in addition to its applicability in research.

Induced pluripotent stem (iPS) cells, which were established initially in 2006 from mouse, and in 2007 from human, in theory could give rise to any type of cells except for extraembryonic tissues, such as trophoblasts [7–9]. Importantly, iPS cells are generated from somatic cells but not from embryos, indicating that iPS cells have much lower ethic hurdle than embryonic stem (ES) cells. As is the case with other types of cells, myogenic cells can be generated from both mouse and human iPS cells, although differentiation protocols for human iPS cells are more established than those for murine iPS cells [10–12]. Additionally, several types of muscle diseases have been modeled by iPS cell technology [13,14], suggesting that genetic mutations or modifications are transferred from patients with muscle diseases to iPS-derived myogenic cells.
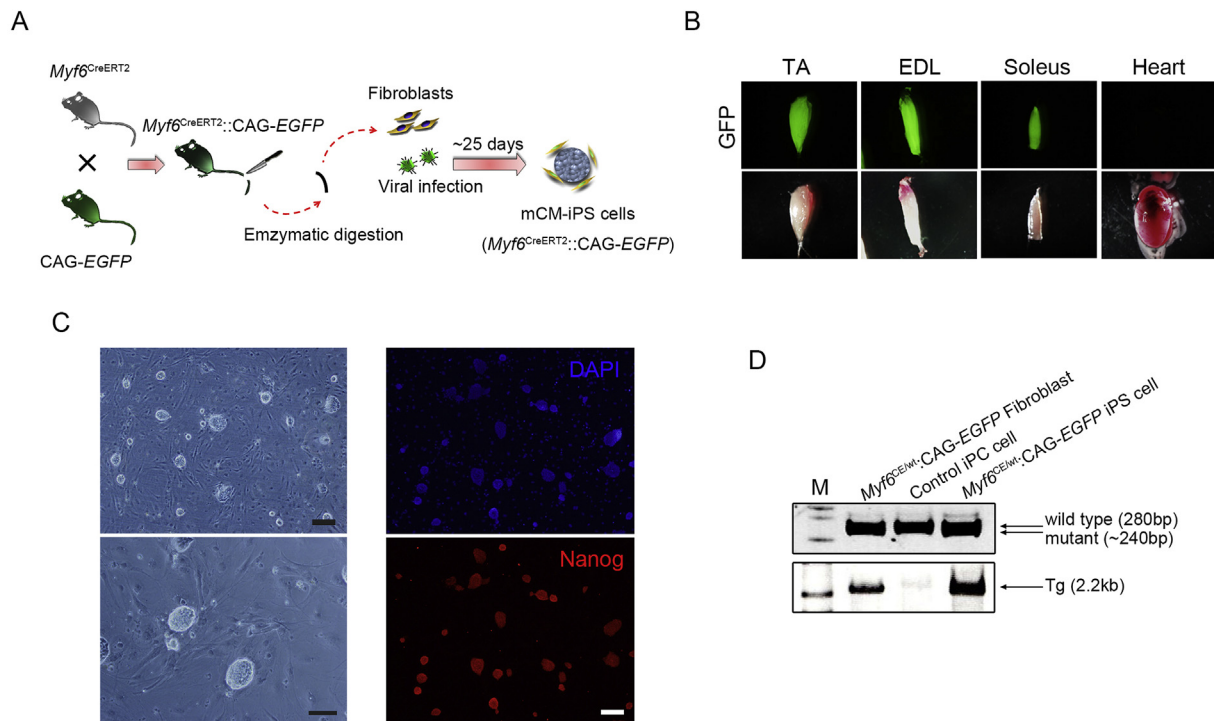
Fig. 1. Generation of muscle specific conditional mouse-derived iPS (mCM-iPSC) cells
(A) Strategy to generate mCM-iPSCs. (B) Confirmation of myofiber specific recombination in *Myf6*[CE/wt]::CAG-*EGFP* mouse. Stereoscopic fluorescence microscope images; upper panels indicate GFP-filtered images (4 weeks after tamoxifen injection). (C) Representative images of mCM-iPSCs from *Myf6*[CE/wt]::CAG-*EGFP* mouse. Left panels: Phase contrast images of mCM-iPSCs (low and high magnifications). Right panels: Immunocytochemistry for pluripotent stem cell marker, Nanog. (D) Genotyping of *Myf6*[CE/wt]::CAG-*EGFP* mouse-derived mCM-iPSCs, indicating that mCM-iPSCs possessed Myf6-CreER and EGFP reporter alleles. iPS-MEF-Ng-492B-4 cell (Control iPS cell) was used as negative control for CreER and reporter alleles. M: DNA ladder marker. Scale bar = 100 μm.

Here, I aimed to investigate whether iPS cells were able to be generated from tamoxifen-inducible Cre-driver mouse and to fine novel *in vitro* application of muscle specific conditional mouse. For this purpose, I generated iPS cells from myofiber specific conditional mouse, myogenic factor 6-CreER (*Myf6*[CreER]), with tamoxifen-inducible GFP expression [15]. Myf6, also known as MRF4, is a member of muscle regulatory factors (MRF) as well as MyoD and Myogenin, and its expression is known to be restricted in myofibers in postnatal muscle [16]. On the other hand, it has been reported that Myf6 was also expressed in muscle progenitors during embryogenesis, suggesting bimodality of Myf6 expression/function in myogenesis [17–19]. Here, I investigated using a bimodality of Myf6 expression whether muscle specific conditional mouse-derived iPS cells (mCM-iPSCs) can be available for muscle researches.

2. Materials and methods

2.1. Animals

Myf6[CreERT2] (*Myf6*[CE]) and B6.Cg-Tg (CAG-cat,-EGFP) 39Miya (CAG-*EGFP*) mouse lines were provided by the Jackson Laboratory (Bar Harbor, ME, USA) and RIKEN BRC (Tsukuba, Ibaragi, Japan), respectively [15,20]. These mice were crossed to generate *Myf6*[CE/wt]::CAG-*EGFP* mouse. Tamoxifen (1 mg/10 g body weight) was intraperitoneally injected for 5 days to induce Cre-mediated recombination. To check the presence of the recombination in skeletal muscle fibers, GFP signal was observed in dissected muscles, including *tibialis anterior* (TA), *extensor digitorum longus* (EDL), *soleus*, and heart of tamoxifen-injected mice using stereoscopic fluorescence microscopy. All mice were maintained, crossed, genotyped, and sacrificed in accordance with the Institutional Animal Care and Use Committee (IACUC) in the National Center for Geriatrics and Gerontology.

2.2. Isolation of tail fibroblasts

Mouse fibroblasts were isolated from tails of *Myf6*[CE/wt]::CAG-*EGFP* mouse (4-month old, male) according to a previous study [21]. Briefly, the mouse tail (about 3-cm) was dissected and cut into small pieces, following incubation in 70% ethanol for 5 min. Tail was digested in Collagenase D/Pronase (Roche, Basel, Switzerland) solution at 37 °C for 90 min. Digested tail was washed with 10% fetal bovine serum (FBS)/ Roswell park memorial institute 1640 media (RPMI 1640) and then vigorously homogenized by pipetting. After removing digested tail tissue using a cell strainer, the homogenate was centrifuged and then the collected fibroblasts were plated onto collagen-coated cell culture dish. Fibroblasts were cultured until sub-confluent density.

2.3. Generation of iPS cell

To generate the iPS cell, tail fibroblasts from *Myf6*[CE/wt]::CAG-*EGFP* mouse were infected with Sendai-virus, carrying the human Oct4/ Sox2/Klf4/c-Myc (OSKM) genes, for 24 h (Cytotune 2.0; ID Pharma, Tokyo, Japan) [22]. Then, infected cells were seeded onto mouse embryonic fibroblasts (MEF) in GS2-M media, including LIF and 2i (Takara, Shiga, Japan). Presence of mCM-iPSC colonies were confirmed by Day 25 (P0 generation), and P5-7 generations of mCM-iPSCs were used for experiments in this study. The murine iPS cell, iPS-MEF-Ng-492B-4, was used as a control (RIKEN BRC).

2.4. Myogenic differentiation

To induce myogenic differentiation of mCM-iPSCs, the protocol using CHIR was applied (modified from Ref. [23]) (*see* Fig. 2A). Briefly, mCM-iPSCs were cultured in serum free differentiation media (SFD: 75% Iscove's modified Dulbecco's Media (IMDM)/25% F12/1% B27 (w/
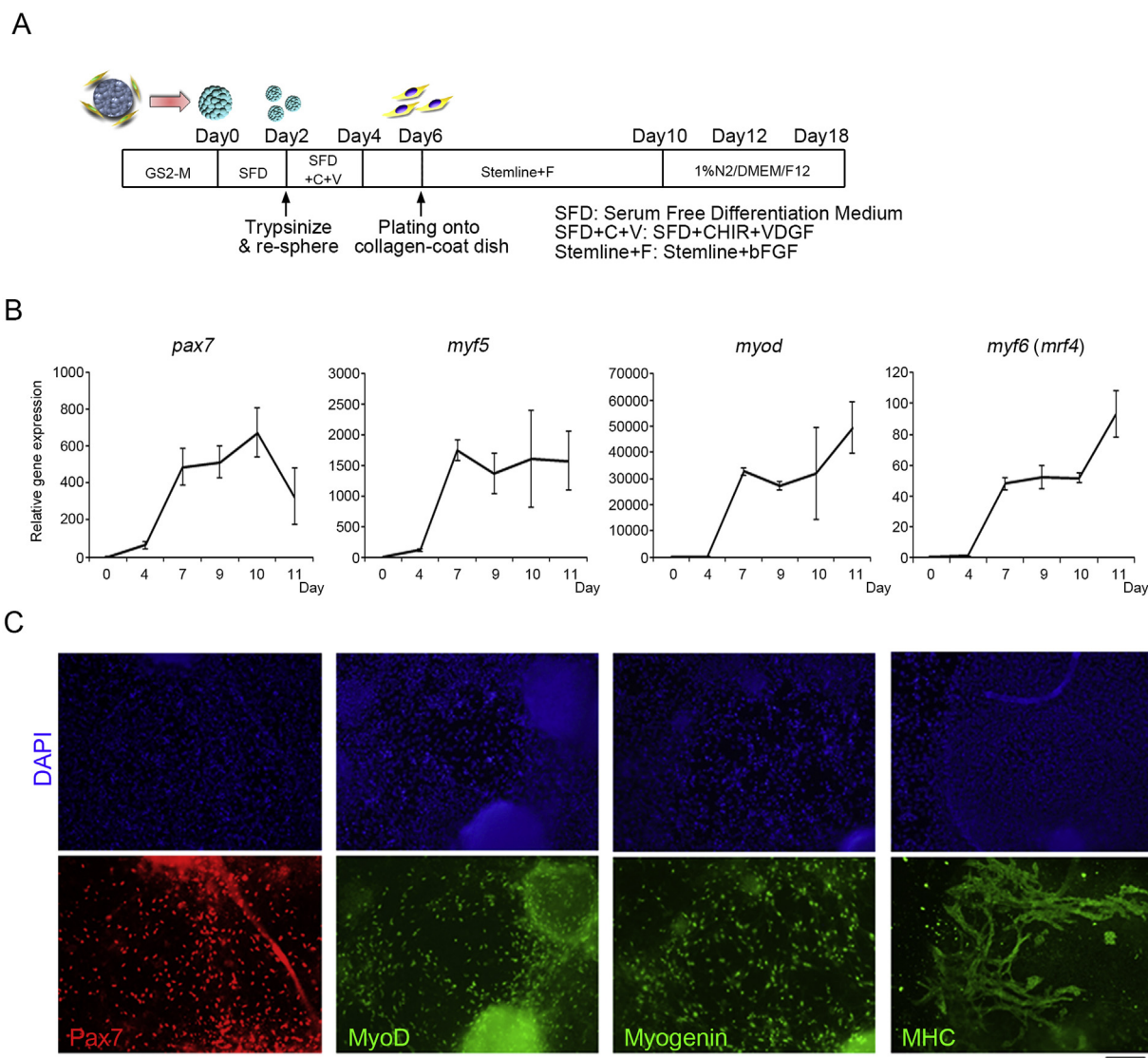
A

Fig. 2. Myogenic differentiation of mCM-iPSC cells
(A) Strategy for myogenic differentiation of mCM-iPSCs. (B) Gene expression profile of myogenic markers during myogenesis of mCM-iPSCs. (C) Immunocytochemistry for myogenic markers at Day 18 of myogenic differentiation. Scale bar = 100 μm.

o RA)/1% antibiotics/0.5% BSA/0.5% N2 supplement/0.45 mM Monothioglycerol/50 mg/ml Ascorbic acid) for 2 days in free floating condition. After 2 days, developed embryoid bodies were once trypsinized and then re-cultured in SFD media including 10 μM CHIR and 5 ng/ml VEGF (SFD + C + V). Embryoid bodies were further cultured for 2 days in Stemline (Sigma-Aldrich, St. Louis, MO, USA) media with 10 ng/ml bFGF (Stemline + F), and then plated onto collagen-coated cell culture dish. After additional 4 days in cultured with Stemline + F media, the media was replaced to 1% N2/F12/DMEM and cells were cultured for another 15–18 days. At day 7 or Day 14, tamoxifen (100 nM 4OH-TAM) was added into the media for 1 day to induce Cre-mediated GFP expression in Myf6$^+$ cells.

2.5. RT-PCR

Total RNA was extracted from cells at Day 0, 4, 7, 9, 10, 11 of myogenic differentiation (n = 3 for each time point) and cDNA was synthesized using the SuperPrep II Cell Lysis Kit (TOYOBO, Osaka, Japan). Polymerase chain reaction was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the following primer sets: *pax7*; 5′-CTCAGTGAGTTCGATTAGCCG -3′ and

5′- AGACGGTTCCCTTTGTCGC -3′, *myf5*; 5′- TGAGGGAACAGGTGGAG AAC -3′ and 5′- AGCTGGACACGGAGCTTTTA -3′, *myod*; 5′- AGCACTA CAGTGGCGACTCA -3′ and 5′-GCTCCACTATGCTGGACAGG -3′, *myf6*; 5′- GGGCCTGGTGATAACTGCT -3′ and 5′- AGAAAGGCGCTGAAGACTG -3′, *gapdh*; 5′-GTGAAGGTCGGTGTGAACG -3′ and 5′- ATTTGATGTTA GTGGGGTCTCG -3′.

2.6. Immunocytochemistry

For immunocytochemistry, cells were cross-linked with 4% paraformaldehyde (PFA) for 15 min at room temperature. After washing with phosphate-buffered saline (PBS), cells were blocked with 4% normal goat serum/0.1% Tween 20/PBS for 1 h at room temperature and then incubated with primary antibodies. Primary antibodies used in this study were: anti-Pax7 (DSHB, Iowa City, IA, USA, 1:100), anti-MyoD (5.5A; Santa Cruz, Carlsbad, CA, USA, 1:100), anti-Myogenin (F5D; Santa Cruz, 1:200), anti-Myosin Heavy Chain (MF20; eBioscience, San Diego, CA, USA, 1:100), anti-GFP (MBL, Nagoya, Aichi, Japan, 1:200), CD144 (VE-cadherin; 1:100, eBioscience), and Nanog (Reprocell, Yokohama, Kanagawa, Japan, 1:100). The secondary antibodies used in this study were: Alexa Fluor 488 or Alexa Fluor 594
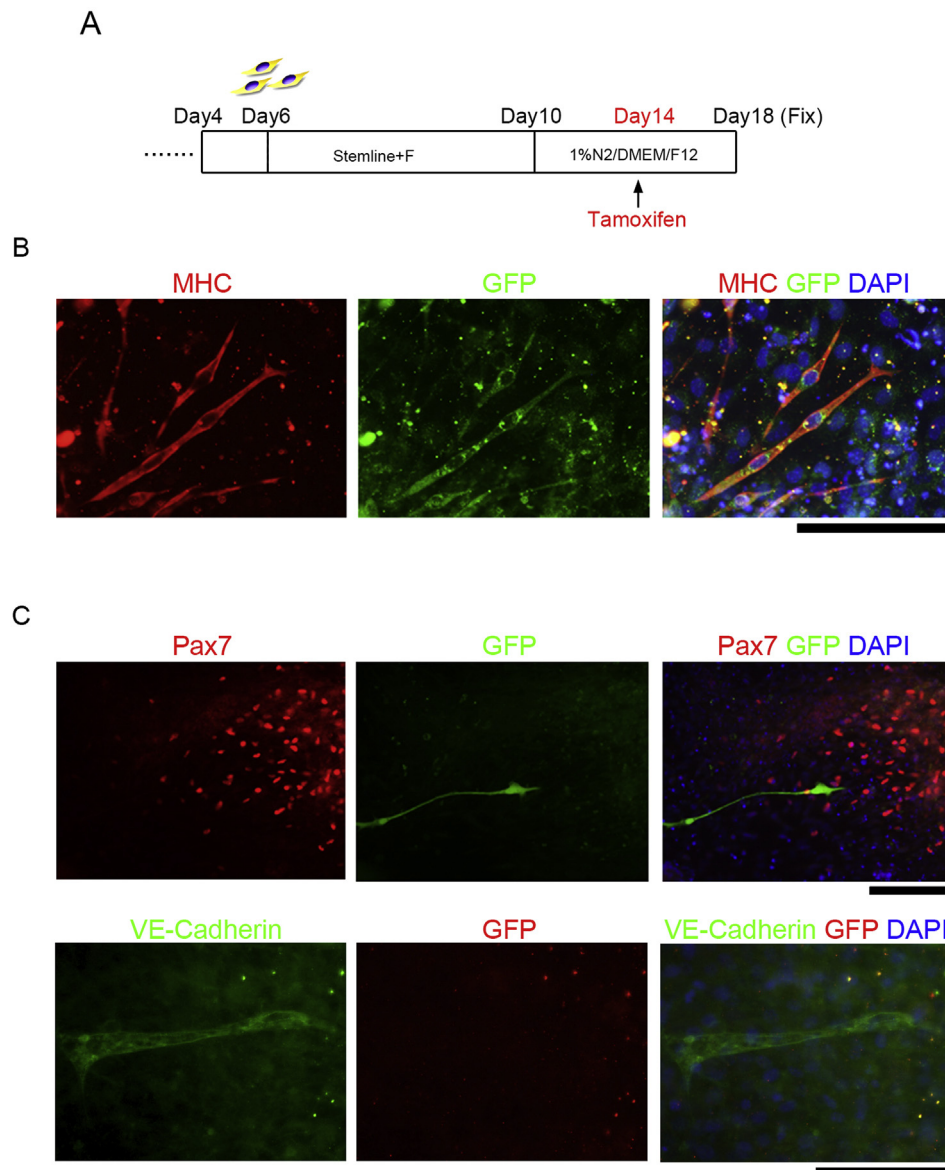
Fig. 3. Tamoxifen induced Cre-mediated recombination in mCM-iPSC-derived myogenic cells.
(A) Strategy for Cre-mediated recombination in mCM-iPSC-derived Myf6 lineages. Tamoxifen was added into media at Day 14 (late TAM). (B) Immunocytochemistry for MHC and GFP at Day18. (C) GFP expression was not observed in Pax7$^+$ muscle progenitors and VE-cadherin$^+$ endothelial cells. Scale bar = 100 μm.

goat anti-mouse IgG, and Alexa Fluor 488 goat anti-rabbit IgG (Abcam, Cambridge, UK), all used at a 1:400 dilution. DAPI in anti-fading reagent was used for nuclear staining.

3. Results

3.1. Generation of muscle specific conditional mouse-derived iPS cells

Myf6^{CreERT2} mouse and CAG-*EGFP* mouse were crossed to generate *Myf6*$^{CE/wt}$::*CAG-EGFP* mouse (Fig. 1A). To confirm skeletal muscle specific recombination after tamoxifen injection, three different areas of skeletal muscles (TA, EDL, and Soleus) and heart were dissected and GFP expression was observed *in vivo*. Fluorescence signals were detected in both fast (TA and EDL) and slow (Soleus) muscles, while heart did not show any GFP signal (Fig. 1B). Because Myf6 is generally expressed in both types of fibers in the postnatal muscle but not in heart [15], these observations confirmed a limited Cre-mediated recombination in skeletal muscle. Next, I generated the iPS cell line from fibroblasts harvested from *Myf6*$^{CE/wt}$::CAG-*EGFP* mouse tails. After

transfection of conditional mouse-derived fibroblasts, cells were cultured in specific media including 2i, and iPS-like colonies were observed within 25 days (Fig. 1C). Because these colonies expressed pluripotent stem cell markers, such as Nanog, I confirmed that these colonies were iPS cells and named the muscle specific conditional mouse-derived iPS cells as mCM-iPSCs. I also detected the two alleles, Myf6-CreER and GFP-floxed, in the genome of this mCM-iPSC (Fig. 1D).

3.2. Myogenic differentiation capacity of mCM-iPSCs

Next, I investigated whether generated mCM-iPSCs possessed myogenic differentiation capacity. *Myf6*$^{CE/wt}$::CAG-*EGFP* mouse-derived mCM-iPSCs were subjected to myogenic differentiation conditions, as described in a previous study [23] (Fig. 2A). Under this culture condition, mCM-iPSCs gradually expressed muscle-related genes, such as *myod,* along with the time (Fig. 2B), suggesting that mCM-iPSCs differentiated into myogenic cell lineages. Importantly, differentiated mCM-iPSCs expressed major markers for myogenic lineages even at the protein level. Particularly, the presence of multinucleated myotubes
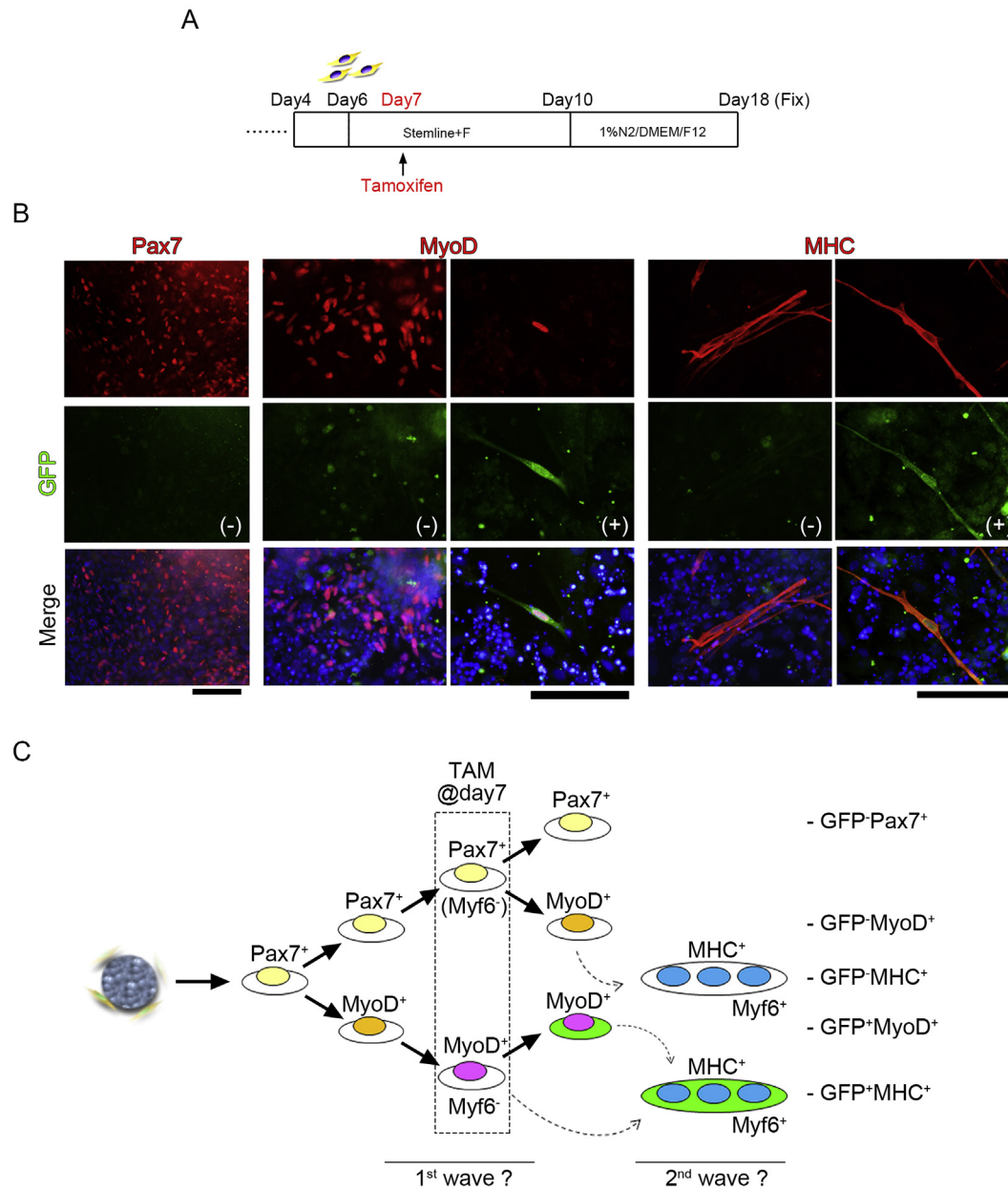
Fig. 4. Lineage tracing of Myf6 expression during the early stage of myogenesis in iPS cell line.
(A) Strategy for lineage tracing of Myf6 progeny during myogenesis using mCM-iPSCs. Tamoxifen was added at Day 7 (early TAM), and then cells were fixed at Day 18. (B) Pax7$^+$ cells did not colocalize with GFP$^+$ cells. In contrast, MyoD$^+$ and MHC$^+$ cells showed heterogeneity for GFP expression. Scale bar = 100 μm. (C) An *in vitro* model for bimodal expression of Myf6 during myogenesis.

expressing myosin heavy chain indicated the myogenic differentiation capacity of mCM-iPSCs (Fig. 2C).

3.3. Homologous recombination in mCM-iPSCs

As mentioned above, after *Myf6*$^{CE/wt}$::*CAG-EGFP* mouse showed muscle specific reporter expression due to tamoxifen injection, I next investigated whether muscle specific Cre-recombination was induced in mCM-iPSCs from this mouse line. Tamoxifen was added into the culture at day 14 of myogenic differentiation, and then GFP expression in multinucleated myotubes was investigated at day 18 of myogenic differentiation (Fig. 3A). As a result, almost all myotubes showed GFP reporter expression, indicating that tamoxifen-mediated Cre-recombination successfully induced recombination in mCM-iPSCs (Fig. 3B). I also confirmed no GFP expression in Pax7$^+$ muscle progenitor cells and in VE-cadherin$^+$ vascular endothelial-like cells

(Fig. 3C). Taken together, these results clearly demonstrated the specificity of Cre-recombination in mCM-iPSCs.

3.4. Application of mCM-iPSCs in tracing myogenesis

In this study, I investigated whether mCM-iPSCs were capable of inducing *in vitro* myogenesis using the lineage tracing technique. Briefly, iPS cells from *Myf6*$^{CE/wt}$::*CAG-EGFP* mouse were subjected to myogenic differentiation, and then tamoxifen was added into the media at Day 7 of *in vitro* myogenesis, which is an earlier phase than Day 14 (Fig. 4A). As a result, I detected GFP expression in MyoD$^+$ myoblasts and MHC$^+$ myotubes at Day 18, in addition to a GFP negative population in Pax7$^+$ muscle progenitor cells, MyoD$^+$ myoblasts, and MHC$^+$ myotubes (Fig. 4B). These results indicated that Myf6 was already expressed in an early phase of myogenesis, suggesting the 1st wave of Myf6 expression (Fig. 4C).

4. Discussion

Established *Myf6*^{CE/wt}::*CAG-EGFP* mouse–derived iPS-like cells revealed morphology similar to the typical murine iPS cells, and expression of pluripotent marker Nanog, indicating successful generation of iPS cells from conditional mouse using a regular protocol. However, the current protocol to generate iPS cell lines using tail fibroblasts is time consuming (~25 days to find iPS cell-like colonies); therefore, the development of a protocol that allows for faster results is required. To this end, we may shorten the time if peripheral blood mononuclear cells from Cre-driver mouse are used as source of iPS cells, because of the previously established protocol in human cells [24].

Generated mCM-iPSCs from *Myf6*^{CE/wt}::*CAG-EGFP* mouse can give rise to myogenic lineages, including embryonic muscle progenitors, myoblasts, and multinucleated myotubes. This indicates that conditional mouse derived-iPS cells are available for *in vitro* muscle research in addition for conventional *in vivo* studies. Although other type of cells, such as vascular endothelial-like cells, were simultaneously generated with this modified protocol, it proved that Cre-recombination occurred specifically in Myf6$^+$ myogenic lineages but not in other cell types. The protocol for myogenic lineages has not been fully established in murine iPS cells, in contrast to human cells [10,11,23,25], causing lower myogenic differentiation of mCM-iPSCs. To maximize mCM-iPSCs for muscle research, hereafter, we need to establish proper protocols to efficiently induce myogenic cells from murine iPS cells.

Myf6 (MRF4) is highly expressed in postnatal skeletal muscle and also in embryonic muscle progenitors. During embryogenesis, Myf6 acts as one of determination factors for muscle identity and priming factor for adult muscle stem cells [17,18]. In addition, Myf6 also negatively regulates postnatal muscle growth through repression of MEF2 transcriptional factor [26]. Thus, Myf6 functions in multi-aspects of the muscle development and growth. In this study, I focused on a bimodal expression pattern of Myf6 during embryogenesis as an indicator for mCM-iPSC's application. Judging from the gene expression profile, myogenic differentiation started from nearby Day 4–7 in this differentiation condition, and *myf6* was highly expressed in a later stage of differentiation (Day 10~). Actually, tamoxifen pulse in the stage of differentiation resulted in GFP labeling in multinucleated myotubes. Because Pax7$^+$ cells and other types of mononuclear cells did not show reporter expression, Cre-recombination was driven specifically in *myf6*-expressing myotubes. On the other hand, tamoxifen pulse in the early phase of myogenic differentiation resulted in GFP expression in MyoD$^+$ myoblasts but not in all cells, and this mosaic GFP expression pattern was also observed in MHC$^+$ myotubes. As mentioned above, Myf6 expression is bimodal during embryogenesis, which is called as 1st wave and 2nd wave [17]. Therefore, results in this study indicated the possibility that mCM-iPSCs precisely reproduced both waves *in vitro* during early and late myogenesis (Fig. 4C). If this is correct, a lineage tracing embryonic muscle progenitors using mCM-iPSCs would provide novel information during mouse myogenesis, because this phenomenon has been observed only at an individual level. While, I also detected GFP$^-$/Pax7$^+$ cells after tamoxifen administration at an early stage of myogenesis. When it is considered with exception for recombination efficiency after tamoxifen administration, results in this study suggested that Pax7$^+$ muscle progenitors emerged from the Myf6 (MRF4)$^-$/Pax7$^+$ pool, which have been argued in previous study [17]. Because an induction protocol for satellite cells from iPS cells has not been established neither in mouse nor in human, I could not track embryonic muscle progenitors to adult stem cells in this study. Although further investigations are needed in this regard, I believe that muscle development from embryo to adult including satellite cell formation would be fully reproducible by mCM-iPSC technology.

As far as I know, this is the first study that generated an iPS cell line from tamoxifen-inducible muscle-specific conditional mouse, and I demonstrated its possible applicability for muscle research. Although only lineage tracing of Myf6$^+$ cells during myogenesis was performed in this study, mCM-iPSC technology can be used to understand the molecular mechanisms of muscle development and diseases by using conditional knock-out mice.

CRediT authorship contribution statement

Tohru Hosoyama: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The author has no conflicting interests to declared.

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