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Transdifferentiation of Muscle Satellite Cells to Adipose Cells Using CRISPR/Cas9-Mediated Targeting of MyoD

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Abstract

Brown adipocytes dissipate energy through non-shivering thermogenesis mediated by UCP1 protein, hence representing a powerful target to overcome obesity due to energy surplus. However, brown adipocytes are scarce in adult humans, especially in obese subjects, urging the development of novel strategies to boost the number of these thermogenic adipocytes from a therapeutical perspective. In this regard, transdifferentiation of myoblasts into brown adipocytes represents a promising approach. Here, we describe a method that we have recently developed to transdifferentiate myoblasts into brown adipocytes through CRISPR/Cas9-mediated targeting of MyoD, the master myogenic regulatory factor.

Keywords

Satellite cell; Myoblast; Adipocyte; Transdifferentiation; Adipogenesis; CRISPR/Cas9

1 Introduction

Brown adipocytes, previously thought to be absent in adult humans, have recently been detected in the neck, supraclavicular, paravertebral, mediastinal, para-aortic, infradiaphragmatic, and suprarenal regions by PET scans and found to be active [1–6]. More recent studies suggest that human brown adipocytes are similar to the inducible thermogenic adipocytes (namely beige or brite adipocytes) found in subcutaneous white adipose tissues (WAT) of rodents [7]. Innervated by the sympathetic nervous system and in response to cold stimuli, brown adipocytes undergo thermogenesis through uncoupled respiration mediated by uncoupling protein-1 (UCP1), a protein that is uniquely found in brown/beige adipocytes and localized to the inner membrane of mitochondria. UCP1 catalyzes the proton leak across the mitochondria membrane thus uncouples the proton from ATPase, rendering energy dissipation as heat instead of generating ATP. Brown adipose tissue (BAT) has received much attention primarily because it is positively correlated with weight loss but negatively correlated to metabolic disorders including insulin resistance and type 2 diabetes, as well as atherosclerosis [7]. However, the abundance of brown adipocytes is low in adult humans, especially in obese subjects, urging the need to increase the number of brown adipocytes in face of obesity prevention and treatment.

Lineage tracing studies in mice indicate that brown adipocytes are derived from a progenitor cell population that expresses Pax7 and Myf5, which are established markers of skeletal muscle progenitors, suggesting a common developmental origin of brown adipocytes and skeletal muscle cells [8, 9]. During development, while the progenitor cells adopting the skeletal muscle cell fate continue to express Pax7, the progenitor cells destined to the brown adipocyte fate ceased to do so [9] and switch on the expression of PR-domain containing protein 16 (PRDM16), a key regulator of brown adipocyte cell fate [8]. The developmental linkage between brown adipocytes and skeletal muscles opens an opportunity to reprogram (transdifferentiate) muscle progenitors into brown adipocytes, especially considering that the skeletal muscle accounts for ~40% of body mass in the adult humans [10]. Efficient transdifferentiation of skeletal muscle progenitors into brown adipocytes would greatly enhance the application of brown adipocytes in the treatment of metabolic syndromes.

Several mechanisms are involved in the cell fate choice between skeletal muscle and brown fat [11], and corresponding strategies to induce the transdifferentiation from skeletal muscle cells into (brown) adipocytes have been reported (Fig. 1). The first strategy is to induce adipogenesis of muscle progenitors through enforcing or activation of canonical adipogenic pathways. These could be achieved through administration of chemical inducers or manipulating expression of adipogenic genes through genetic or epigenetic approaches (Fig. 1A). Forced expression of PRDM16, or its upstream regulator early B cell factor-2 (EBF2) is sufficient to stimulate brown adipogenesis in C2C12 myoblasts [8, 12]. Consistently, it was reported that a retinoid X receptor (RXR) agonist, bexatotene, can potently reprogram myoblasts into brown adipocyte-like cells by activating RXR α and RXR γ , which subsequently upregulate PRDM16 [13]. Peroxisome proliferator-activated receptor gamma (PPAR γ) is a key transcriptional regulator of adipogenesis and its agonists (thiazolidinediones) have been reported to convert myoblasts into adipocyte-like cells [14]. In addition, bone morphogenic protein-2 (BMP2) cooperates with CCAAT-enhancer-binding protein alpha (C/EBP α) to induce adipogenic transdifferentiation of C2C12 myoblasts [15]. Several studies reported that either PPAR γ or C/EBP- α is sufficient to induce adipogenesis in myoblast [15–17]. However, Hu et al. reported that ectopic expression of PPAR γ or C/EBP- α alone does not significantly change the myogenic potentials in C2C12 cells, but co-expression of PPAR γ and C/EBP α induces adipogenic transdifferentiation of myoblasts [18]. Epigenetic regulators such as chromatin modifiers and small noncoding RNA have also been reported to switch the myogenic program of myoblasts to the adipogenic program (Fig. 1). Euchromatic histone-lysine *N*-methyltransferase 1 (EHMT1) directly interacts with PRDM16 to stabilize the PRDM16 transcriptional complex, thus leading to the adipogenic transdifferentiation [19]. Among miRNAs, miR-193b was found to block the myogenic program in C2C12 myoblasts and induce the adipogenic program under adipogenic conditions [20]. MiR-133, a muscle enriched microRNA, was shown to target Prdm16, therefore suppressing the brown adipogenesis in muscle progenitors. Reduction of miR-133 conversely induces the development of brown and beige adipocytes from muscle satellite cells and white adipocyte progenitors [21–23].

The second strategy to induce myoblast to brown adipocyte transdifferentiation is to suppress myogenic program in myoblasts (Fig. 1B). We have recently reported that loss of MyoD converts myoblasts to brown adipocytes through upregulation of Prdm16 [23].

Consistent to our report, An et al. showed that inducible deletion of Pax7, a homeodomain and paired domain containing transcription factor [24–26], leads to a cell fate switch of myoblasts to brown adipocytes [27]. Knockdown of Pax7 downstream genes *MyoD* or *Myf5* phenocopy *Pax7* KO [23, 27]. Mechanistically, MyoD inhibition suppressed the E2F4/p107/p130 axis and upregulated PRDM16 to promote adipogenesis [27]. However, the transdifferentiation of myoblasts to brown adipocytes in vivo was not very efficient [27, 28], possibly due to the lack of key adipogenic cues in the skeletal muscles.

Other signaling pathways have also been targeted to induce fate conversion of myoblasts to brown adipocytes (Fig. 1C). Homeobox containing transcriptional repressor Msx1 induces dedifferentiation of C2C12 myotubes and promotes their adipogenic differentiation [29]. Forced expression of dominant negative mitogen-activated protein kinase kinase 3 (DN MKK3) also transdifferentiates C2C12 cells into adipocytes in the presence of PPAR γ -activator rosiglitazone [30]. Interestingly, the three major subtypes of mitogen differentiation protein kinases (MAPK) namely ERK (extracellular signal regulated kinases), JNK (c-Jun amino terminal kinases), and p38 have distinct effects on the adipogenic transdifferentiation on C2C12 myoblasts. Qi et al. reported that p38 and JNK were negative regulators while ERK1/2 was a positive regulator of adipogenesis in C2C12 [31]. The negative effect of p38 MAP kinase was substantiated by the observation that Shp2 (nonreceptor tyrosine phosphatase Shp2) promotes adipogenesis through inhibition of p38 [32]. Other signaling pathways including IGF-1 signaling were also reported to induce adipogenesis in C2C12 in the presence of Rho kinase inhibitor [33]. Interrupting Wnt signaling by dominant negative TCF4 also promotes the expression of C/EBP- α and PPAR γ , thus switching the myogenic program into adipogenic program [34].

In this article, we describe a protocol to induce the adipogenic transdifferentiation harnessing CRISPR-Cas9 technology to delete MyoD in C2C12 myoblasts. Besides, the adipogenic potential of the primary myoblasts isolated from the MyoD null mice is verified by induced adipogenic differentiation. Both methods manifest a robust switch to the adipogenic program in the myoblasts.

2 Materials

2.1 C2C12 Cell Culture

1. C2C12 cells: C2C12 (ATCC® CRL1772™).
2. Dulbecco's Modified Eagle's Medium (DMEM).
3. Fetal Bovine Serum.
4. Penicillin-streptomycin.
5. Trypsin.
6. 100 mm petri-dish.
7. 24-well culture plate.
8. 48-well culture plate.

9. 50 ml Falcon tube.
10. 15 ml Falcon tube.
11. PBS: 8 g of NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄. Adjust the pH to 7.4 with HCl and bring the solution to a total volume of 1 L.
12. C2C12 culture medium: DMEM, 10% FBS, 1% Penicillin/Streptomycin.

2.2 Generation of Cas9-Expressing C2C12 Myoblasts [35]

1. Plasmid: pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988).
2. Puromycin.
3. Neon transfection system (Invitrogen).

2.3 Immunofluorescence Staining of C2C12 Cells

1. Fixative (4% paraformaldehyde): ddH₂O, HCl, NaOH (1 N), paraformaldehyde powder, 1 × PBS; add 800 ml of 1 × PBS to a beaker in a ventilating hood, heat the PBS to approximately 60 °C. Be careful not to boil the PBS. Add 40 g of paraformaldehyde powder to the PBS solution. Slowly add 1 N NaOH until the solution becomes clear. After cooling and filtering the solution, adjust the volume to 1 L and the pH to around 6.9 with HCl.
2. Blocking Buffer: 5% goat serum, 2% BSA, 0.2% Triton X-100, and 0.1% sodium azide in PBS.
3. FLAG antibody (Catalog No. F1804, Sigma).
4. DAPI.
5. Secondary antibody: Goat anti-mouse IgG1, Alexa Fluor 568 (catalog no. A-21124, Thermo Fisher Scientific).

2.4 Production of gRNAs by In Vitro Transcription

1. Q5 hot start polymerase (Catalog No. M0493S, New England BioLabs).
2. MEGAshortscript T7 transcription Kit (Catalog No. AM1354, Thermo Fisher Scientific).
3. MEGAclear Transcription Clean-Up Kit (Catalog No. AM1908, Thermo Fisher Scientific).
4. Cycle Pure Kit (Catalog No. D6492-01, Omega Bio-Tek).

2.5 Primary Myoblasts Isolation from MyoD^{-/-} Mice

1. MyoD^{-/-} mice [36] were generously provided by Dr. Michael Rudnicki (Ottawa Health Research Institute, Ottawa, Ontario, Canada).
2. Type I collagenase and Dispase B (Roche Applied Science).

3. F-10 Ham's medium.
4. 4 ng/ml basic fibroblast growth factor.
5. Primary myoblasts culture medium: F-10 Ham's medium, 20% FBS, 4 ng/ml basic fibroblast growth factor, 1% penicillin-streptomycin.

2.6 Adipogenic Induction of Myoblasts

1. Adipogenic induction medium: DMEM, 10% FBS, 2.85 μ M insulin, 0.3 μ M dexamethasone, 0.63 mM 3-isobutyl-1-methylxanthine, 1 μ M Rosiglitazone.
2. Adipogenic differentiation medium: DMEM, 10% PBS, 200 nM insulin, 10 nM T3, 1 μ M Rosiglitazone.
3. Oil red O staining: working solution contains 6 ml Oil Red O (ORO) stock solution (5 mg/ml in isopropyl alcohol, Sigma) and 4 ml ddH₂O.

3 Methods

All the experimental procedures are carried out under room temperature unless otherwise stated. Cells are cultured at 37 °C with 5% CO₂.

3.1 Generation of Cas9-Expressing C2C12 Cells

1. Culture C2C12 myoblasts in 100 mm petri-dish containing 5 ml C2C12 culture medium. When the C2C12 cells reach 70% confluency, aspirate the culture medium and wash cells with 5 ml PBS. Then aspirate the PBS and treat the cells with 1 ml of 0.05% trypsin at 37 °C for 3 min (*see Note 1*). Cell detachment can be observed under the microscope. Add 3 ml of culture medium to the culture plate and transfer the cell suspension to a 15 ml Falcon tube. Centrifuge the cell suspension at 300 $\times g$ for 5 min. Aspirate the supernatant and suspend the cell pellet with 1 ml culture medium (*see Note 2*). Cells are counted with hemocytometer. Approximately 10⁶ cells are mixed with 1 μ g of PX459 plasmid. Transfect the plasmids into C2C12 cells using the Neon Transfection system (Invitrogen) at 1500 V, 10 ms, for 3 times in a 100 μ l electroporation tip.
2. After electroporation, seed the C2C12 cells into a 100 mm petri-dish for 2 days in C2C12 culture medium. Then add puromycin (1 μ g/ml) into the culture medium for selection of cells transfected with PX459. During the selection, cells are consecutively treated with puromycin. Change the culture medium every 2 days. Non-transfected cells are usually floating in the culture medium after 2 days of puromycin treatment. Transfected cells will form cell clones.
3. Cell clones should appear at 10 days after puromycin selection. To collect cell clones, replace the culture medium with 1 ml of 0.05% trypsin and incubate for 30s (*see Note 3*). Immediately aspirate the Trypsin. Suck up the cells in one

¹ Monitor the cell morphology under a light microscope when treating C2C12 with Trypsin as exposing C2C12 myoblasts with Trypsin for too long would induce damages to the cell membranes.

² Triturate the pellets thoroughly to avoid any cell cluster in this step.

single cell clone by using a pipette with 10 μ l culture medium. Transfer the cells to 12-well plates. The culture medium is still supplemented with puromycin.

4. Passage the cells in each well into a 100 mm petri-dish when they reach 70% confluency in 12-well plates. In this step, seed a small portion of the cells into 24-well plates for immunostaining for the detection of FLAG tag encoded by PX459 plasmid.
5. For immunostaining of FLAG tag, fix the cells grown in 24-well plates with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 5 min in room temperature. Remove the PFA and wash the cells three times with PBS. Treat the cells with 100 mM glycine for 10 min. Afterward, remove the glycine and wash the cells with PBS once. Incubate the cells in blocking buffer for at least 30 min in room temperature. Remove the blocking buffer and incubate the cells with FLAG antibody (F1804, Sigma) diluted in blocking buffer at 1: 200 overnight at 4 °C. Remove the FLAG antibody and wash the cells with PBS five times for 1 min each. Then incubate the cells with DAPI and secondary antibody diluted in PBS at 1:1000 for 1 h at room temperature. Finally, the cells are washed five times with PBS for 1 min each before checking fluorescence signal under Leica DM 6000B. Only select cell clones expressing FLAG signals.
6. After the positive C2C12 (C2C12^{Cas9}) in the 100 mm petri-dish reached 70% confluency, collect the cells and freeze for later use.

3.2 gRNA Selection (See Note 4)

1. The gRNA targets of MyoD are designed using an online tool (CRISPR Design, <http://crispr.mit.edu/>) (Table 1).
2. The gRNAs are produced by following an online protocol (<http://www.crisprflydesign.org/grnatranscription/>). Briefly, specific primers introducing a T7 promoter and gRNA core sequence are designed and used to amplify PX459. High fidelity polymerase like Q5 hot start polymerase is used in our lab. After PCR amplification, separate the products on a 1.5% agarose gel and purify with Cycle Pure Kit (Omega Bio-Tek). Transcribe the templates in vitro using Ambion T7 shortscript kit according to the manufacturer's instructions. At last, use the Ambion MEGAclear kit to purify the RNA.
3. Then, mix two gRNAs (50 ng each) with 10⁵ C2C12^{Cas9} cells for electroporation (see Note 5). The condition for electroporation is the same as the condition described in Subheading 3.1, **step 1**. For the control group, C2C12^{Cas9} cells are only given the same electric shocks. After electroporation, seed the cells into 6-well plates.

³The timing of Trypsin treatment is crucial as we want to disassociate the cells from the plate so that the cells could be aspirated yet the clone would remain intact.

⁴A schematic overview of validation of sgRNA efficacy is shown in Fig. 2.

⁵Two sgRNAs are designed which targeted at exon 1 of MyoD. The sequences of the two sgRNAs are listed in Table 1.

4. One day after electroporation, collect the cells for DNA extraction using GeneArt Genomic Cleavage Detection Kit according to the manufacturer's instructions. Amplify the region around the target sites by PCR. Clone the PCR products (*see* Note 6) and send for sequencing (*see* Note 7). By analyzing the sequence, we determine which gRNAs worked with Cas9 to edit MyoD.

3.3 MyoD KO Cell Line Selection (See Note 8)

1. Choose the sgRNA that targets close to the 5 prime of MyoD gene (*see* Note 9) and clone into PX459 plasmid. The protocol of cloning of gRNA into PX459 can be found online at Addgene (<http://www.addgene.org/62988/>). Briefly, primers composing sticky ends for restriction enzyme BbsI for the gRNA are synthesized. Linearize the plasmid with BbsI and purify with Cycle Pure Kit. After annealing the primers, ligate the linearized plasmid and primers using T4 ligase. The plasmid is termed PX459^{MyoD}.
2. Electroporate the wild-type C2C12 cells with either PX459^{MyoD} or PX459 (1 µg/10⁶ cells).
3. Two days after electroporation, add 1 µg/ml of puromycin to the culture medium for at least 5 consecutive days. Change the culture medium every 2 days.
4. Collect the remaining cells electroporated with PX459 and survive the selection of puromycin. Freeze the cells as controls for later use.
5. The remaining cells electroporated with PX459^{MyoD} should form cell clones. Collect the cells in cell clones according to the descriptions in Subheading 3.1, **step 3**. Transfer the cells to 12-well plates.
6. When the cells reach 70% confluency, passage the cells into 6-well plates. Use a small portion of the cells for DNA extraction, PCR amplification and sequencing to confirm correct genomic alteration (*see* Note 10). Cells containing predicted genomic alteration are cloned a second time to achieve homogeneity serial dilution.

3.4 Off-Target Detection

1. The gRNA off targets sites are searched using CRISPR Design (<http://crispr.mit.edu/>).

⁶. After transfection of C2C12^{Cas9} cells with sgRNAs and selection with puromycin, PCR is carried out using the genomic DNA to amplify the region composing the target sites of sgRNAs. Since 2 sgRNAs are transfected, we expect to see several hundred base pairs difference in the PCR product. Indeed, by electrophoresis, two cleaved bands are observed and the ~300 bp band was purified and cloned for sequencing [23].

⁷. Sequencing results from C2C12^{Cas9} transfected with sgRNA show a 336 bp deletion in MyoD, indicating the efficacy of sgRNA in editing MyoD [23].

⁸. A schematic overview of the adipogenic transdifferentiation of MyoD^{KO} C2C12 myoblasts and primary myoblasts is derived from MyoD^{-/-} mice (*see* Fig. 3).

⁹. In order to minimize the off target effects, only the sgRNA that targets the proximity of the 5 prime of MyoD exon 1 is cloned to PX459.

¹⁰. Of the 21 clones established from MyoD^{KO} C2C12 cell line, two clones are found out through sequencing that they either have 1 bp or 11 bp frame shifting deletion in both alleles of MyoD (Fig. 4A). MyoD^{KO} 1 has an 11 bp deletion resulting in the termination of translation at 72nd amino acids and a 1 bp deletion which results in the termination of the translation at 55th amino acids. MyoD^{KO} 2 has an 11 bp deletion on both alleles resulting in the termination at 72nd amino acids.

2. Amplify the top five potential off target sites by PCR using genomic DNA as templates (Table 2).
3. The PCR products are subjected to T7EN1 cleavage assay according to the manufacturer's protocol. The potential off target sites yielding typical cleavage bands are considered candidates for off target (*see* Note 11).

3.5 Adipogenic Induction of MyoD KO Cell Line

1. To examine the adipogenic differentiation potential of MyoD^{KO} C2C12 myoblasts, treat the cells with induction medium for 5 days and differentiation medium for 5 days (*see* Subheading 1).
2. After adipogenic induction, use Oil Red O staining to detect lipid droplets. Rinse the cells with PBS and fix with 4% paraformaldehyde for 15 min at room temperature. Then stain the cells with working solution of Oil Red O (ORO) for 1 h. Wash the culture plate with distilled water for 10 min and mount with glycerol mounting medium (*see* Note 12).
3. Meanwhile, the expression profiles of adipogenic and myogenic specific marker genes are examined using qPCR. The deletion of MyoD downregulated the expression of myogenic genes (such as Myog) and upregulated the expression of adipogenic genes (*Fabp4*, *Cebpa*, and *FASN*) (Table 4, also *see* Note 13).

3.6 Adipogenic Induction of Primary Myoblasts from MyoD^{-/-} Mice

1. Dissect the Tibialis Anterior (TA) muscle from MyoD^{-/-} mice of the age of 4–6 weeks old. The protocol for isolating myoblasts has been thoroughly characterized by Motohashi et al. [37]. In brief, thoroughly mince the TA muscles and then digest with type I collagenase and Dispase B mixture at 37 °C for 12 min. Triturate the muscle and digest for another 12 min. Add primary myoblast culture medium to stop the digestion. Filter the digestion resultants with a 0.45 µm filter (*see* Note 14) and then centrifuge at 300 × *g* for 5 min. Culture the cells in primary myoblast culture medium on collagen-coated cell culture plate (*see* Note 15).

¹¹The potential off targets sites are amplified and examined by electrophoresis. T7EN1 cleavage assay shows there aren't genomic modifications to the potential off target sites (Fig. 4B). The off target sites are listed in Table 2 and the primers for detecting off targeting of sgRNA are listed in Table 3.

¹²In control cells, there are only few neutral triglycerides positively stained by Oil Red O whereas in MyoD KO C2C12 cells, there is a substantial region positively stained (Fig. 5B). The deletion of MyoD in C2C12 is confirmed by immunostaining using a well-established MyoD antibody (Fig. 5A).

¹³The deletion of MyoD leads to the significant downregulation of Myog [23], a myogenic marker for muscle differentiation [38], however, adipogenic-specific genes show significant upregulation indicating a switch from myogenic program to adipogenic program. qPCR primers are listed in Table 4.

¹⁴Passing the mechanically minced cells through a strainer helps to eliminate extra cell debris.

¹⁵During this process, if fibroblasts outgrow myoblasts, an extra step of pre-plating could be carried out to get rid of excessive fibroblasts. The cell culture is carefully disassociated with 1 ml of 0.05% Trypsin for about 3 min. 1 ml of culture medium is added into the cell culture plate to stop trypsinization. The cells are centrifuged at 300 × *g* for 5 min. The supernatant is discarded and the cells are suspended with 5 ml primary myoblast culture medium. The suspended cells are seeded in a regular 100 mm culture plate without collagen coating. The cells are incubated in the incubator for 45 min until there is clear morphology of fibroblasts attaching to the culture plate. The culture medium is collected along with the floating cells and transferred to a collagen coated plate. Fibroblasts have better adhesion capacity than do myoblasts, therefore they could easily attach to the culture plate even without collagen coating.

2. Adipogenic induction of the primary myoblasts is characterized in Subheading 3.5, **step 1**.
3. The expression levels of adipogenic markers and myogenic markers are examined as described in Subheading 3.5, **step 3** (*see Note 16*).

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¹⁶The expression profiles of adipogenic and myogenic marker genes are characterized by Wang et al. [23].

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Fig. 1.

Overview of the three adipogenic transdifferentiation pathways. The first strategy is to enforce or activate the canonical adipogenic pathway. The ultimate goal is to increase the expression level of adipogenic master genes *Prdm16*, *Pparg* as well as *C/EBP*. It could be directly manipulating these three genes or through indirect activation by chemical compounds (Thiazolidinediones or Bexarotene), molecules (EBF2, BMP2, or EHMT1), or microRNAs (miR-193b or miR-133). The second strategy is to suppress myogenic program in myoblasts. This strategy mostly focuses on the Pax7-MyoD/Myf5 cascade. It has been reported that MyoD acts through E2F4/p107/p130 regulating axis which in turn upregulates PRDM16. The third strategy involves other signaling pathways including DN MKK3, Msx1, Shp2, MAPK, IGF-1, as well as Wnt signaling pathways

Validation of truncation of MyoD by sgRNA

Fig. 2.

Schematic view of the experiment validating sgRNA efficacy. In brief, a modified C2C12 cell line is established which expresses Cas9 for later validation of sgRNA. Then sgRNAs are electroporated into the modified C2C12. After sequencing of the target MyoD locus, it is revealed that there is a 336 base pairs deletion, indicating the efficacy of sgRNA in editing MyoD

Adipogenic transdifferentiation of C2C12 myoblasts

Fig. 3.

Schematic overview of adipogenic transdifferentiation of C2C12 myoblasts and primary myoblasts derived from *MyoD*^{-/-} mice. The sgRNA that targets at the proximity of 5 prime of *MyoD* is chosen for inducing genomic deletion of *MyoD* in C2C12 cells. sgRNA-1 is cloned into PX459 as described. Then the plasmid is electroporated into C2C12 myoblasts. The C2C12 cells are then subject to puromycin selection. Both the *MyoD*^{KO} C2C12 cells and *MyoD*^{-/-} myoblasts are induced for adipogenesis. Both myoblasts exhibited typical adipogenic features. qPCR of the myogenic and adipogenic marker genes confirmed the shifting of the myogenic program into the adipogenic program, where *Myog* is down regulated yet *Fabp*, *FASN*, and *Cebpa* are upregulated

Fig. 4.

Correct targeting of the two sgRNAs. **(a)** Two sgRNA are designed targeting the exon 1 of MyoD. The DNA sequence maps show the target locus of the control C2C12 myoblasts and the 2 clones transfected with 2 sgRNAs. MyoD^{KO1} has an 11 bp deletion resulting in the termination of translation at 72nd amino acids and a 1 bp deletion which results in the termination of the translation at 55th amino acids. MyoD^{KO2} has an 11 bp deletion on both alleles resulting in the termination at 72nd amino acids. **(b)** Off target detection of the two sgRNAs. The top five potential off target sites subject to T7EN1 assay and amplified by PCR. The electrophoresis result shows that there are not obvious alterations to the off target sites. This figure is adapted from Wang et al., (Ebiomedicine 16:212–223. doi: <https://doi.org/10.1016/j.ebiom.2017.01.015>) licensed under CC BY-NC-ND (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

Fig. 5.

Transdifferentiation of MyoD^{KO} C2C12 myoblasts into adipocytes. **(a)** Immunostaining of the cultured MyoD^{KO} C2C12 cells using a well-established MyoD antibody. Compared with the control group, the positive staining of MyoD is almost obsolete in MyoD^{KO} cells. **(b)** MyoD^{KO} C2C12 is stained by Oil Red O staining for detection of neutral triglycerides. The MyoD^{KO} C2C12 cells show substantial staining of lipids droplets while the control group shows relatively few. This figure is adapted from Wang et al., (Ebiomedicine 16:212-223. doi: <https://doi.org/10.1016/j.ebiom.2017.01.015>) licensed under CC BY-NC-ND (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

Table 1

Sequence of the two sgRNAs designed

	Sequence (5'–3')	Target region on MyoD Exon1 (5'–3')
sgRNA-1	GCGCTTTTTTGAGGACCTGG	92–139
sgRNA-2	GCGCAACGCCATCCGCTACAT	447–508

Table 2

The top five potential off targets of sgRNAs

Position	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	N	G	G	Locus
MyoD	C	C	A	G	G	T	C	C	T	C	A	A	A	A	A	A	G	C	G	C	A	G	G	Chro:start position
OT1	C	C	T	A	G	T	C	C	T	C	A	A	A	A	A	A	G	C	T	C	A	G	G	Chr1:-106859905
OT2	C	T	A	A	G	T	C	A	T	C	A	A	A	A	A	A	G	C	A	C	T	G	G	Chr13:+78086880
OT3	C	C	A	T	C	T	C	A	T	C	A	A	A	A	A	A	G	C	T	C	A	G	G	Chr19:+50585297
OT4	A	C	A	C	G	T	G	C	T	C	A	A	A	A	A	A	G	C	A	C	G	A	G	Chr1:-39340833
OT5	C	C	A	G	G	T	G	C	T	C	C	A	A	A	A	A	G	C	A	C	A	G	G	Chr12:-113574844

PAM is highlighted in grey.

The different bases between the off target sites and MyoD locus were highlighted in red.

Table 3

Primers for amplifying the off target sites (5'–3')

Gene	Sequence
OT1	F: GGCTGAAAGGCTTGACTCG R: GGAGCATTTTCAGGCATCAAT
OT2	F: GGTGGAAAGGCTGTTGATGT R: GCCAGAGTGATCGGAATGAT
OT3	F: TCCCTGGCTATGCAAAATTC R: CATTCATGGAGCCTGTGTGT
OT4	F: ACATGTATGCCCAGATGCAA R: GTGCTCCAACGAGTGAGACA
OT5	F: CCCAAGTTCCATGTCCCTAA R: TGAGCAAGGTTTGCAGTTTG
MyoD	F: AAGCGGCCGCATGGAGCTTCTATCGCCGC R: TTTCTAGATCAAAGCACCTGATAAATC

Table 4

qPCR primers for myogenic and adipogenic marker genes (5'–3')

Gene	Sequence
Gadph	F: TCGGTGTGAACGGATTTG R: GGTCTCGCTCCTGGAAGA
Myog	F: GCAATGCACTGGAGTTCG R: ACGATGGACGTAAGGGAGTG
Fabp4	F: ACAAGCTGGTGGTGAATGTG R: CCTTGGCTCATGCCCTTT
Fasn	F: GGAGGTGGTGATAGCCGGTAT R: TGGGTAATCCATAGAGCCCAG
Cebpa	F: TGC GCAAGAGCCGAGATAAA R: CCTTCTGTTGCGTCTCCACG