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Generation of the Compression-induced Dedifferentiated Adipocytes (CiDAs) Using Hypertonic Medium

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[Abstract] Current methods to obtain mesenchymal stem cells (MSCs) involve sampling, culturing, and expanding of primary MSCs from adipose, bone marrow, and umbilical cord tissues. However, the drawbacks are the limited numbers of total cells in MSC pools, and their decaying stemness during *in vitro* expansion. As an alternative resource, recent ceiling culture methods allow the generation of dedifferentiated fat cells (DFATs) from mature adipocytes. Nevertheless, this process of spontaneous dedifferentiation of mature adipocytes is laborious and time-consuming. This paper describes a modified protocol for *in vitro* dedifferentiation of adipocytes by employing an additional physical stimulation, which takes advantage of augmenting the stemness-related Wnt/β-catenin signaling. Specifically, this protocol utilizes a polyethylene glycol (PEG)-containing hypertonic medium to introduce extracellular physical stimulation to obtain higher efficiency and introduce a simpler procedure for adipocyte dedifferentiation.

Keywords: Mesenchymal stem cells, Dedifferentiation, Adipocytes, Compression, Wnt/β-catenin signaling

[Background] Adipose tissue currently is one of the most appealing sources of mesenchymal stem cells (MSCs), due to its large abundance and relatively less-invasive harvest methods (Shen *et al.*, 2011; González-Cruz *et al.*, 2012; Konno *et al.*, 2013). Adipose-derived MSCs, that isolated from the stromal-vascular fraction of subcutaneous adipose tissue, have been demonstrated to display multilineage potentials both *in vitro* and *in vivo* (Anghileri *et al.*, 2008; González *et al.*, 2009; Gonzalez-Rey *et al.*, 2010; Jumabay *et al.*, 2010; Mao *et al.*, 2017 and 2019; Darnell *et al.*, 2018). To isolate adipose-derived MSCs, the widely-used method is to dissect the stromal-vascular fraction from the adipose tissue, and then sort the MSCs by either fluorescence-activated cell sorting (FACS) or culture selection (Aronowitz *et al.*, 2015; Raposio *et al.*, 2017; Gentile *et al.*, 2019). However, heterogeneous groups of cells are contained in a stromal-vascular fraction of adipose tissue, and limited cell markers are available for MSCs selection; these make it difficult to purify adipose-derived MSCs (Gimble *et al.*, 2011; González-Cruz *et al.*, 2012; Konno *et al.*, 2013).

Alternatively, the adipocytes, rather than the other types of cells in adipose tissue, can spontaneously

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dedifferentiate into multipotent mesenchymal cells named the dedifferentiated fat (DFAT) cells during *in vitro* culturing (Sugihara *et al.*, 1986; Shen *et al.*, 2011; Taniguchi *et al.*, 2016). Because of the multipotency of the DFAT cells and the large abundance of the mature adipocytes, the DFAT cells have been regarded as an ideal source for human postnatal mesenchymal multipotent stem cells (Matsumoto *et al.*, 2008; Shen *et al.*, 2011; Côté *et al.*, 2019). However, the current ceiling culturing for adipocyte dedifferentiation requires a long duration (typically 4 weeks) to enable the adipocytes to spontaneously lose all obvious lipid droplets (Lessard *et al.*, 2015; Taniguchi *et al.*, 2016). Thus, further increasing the efficiency of adipocyte dedifferentiation and shortening its processing time is attractive for its wider applications.

Adipocytes and adipose progenitor cells are also important components in tumor microenvironments (Chandler *et al.*, 2012; Seo *et al.*, 2015; Ling *et al.*, 2020). Recent studies revealed that the dedifferentiation of adipocytes occurred during tumor development, which might be attributed to the activated Wnt signaling (Gustafson and Smith, 2010; Bochet *et al.*, 2013) and Notch signaling (Bi *et al.*, 2016). Recent studies also revealed that the dedifferentiation of adipocytes could occur *in vivo* in mice models (Bochet *et al.*, 2013; Liao *et al.*, 2015; Wang *et al.*, 2018). Tumor progression also largely alters the local physical microenvironments, including elevated osmotic pressure, increased compressive force, and matrix stiffening (Nia *et al.*, 2020). These physical cues largely influence the cell fates of both adipose stromal cells and cancer cells (Guo *et al.*, 2017; Li *et al.*, 2019 and 2020a; Han *et al.*, 2020). Indeed, our recent study reported that the generation of osmotic stress *in vitro* to mimic the elevated osmolarity in *in vivo* tumors could also induce the dedifferentiation of adipocytes (Li *et al.*, 2020b). Consistently, another study also reported that a tough implant *in vivo* drove the dedifferentiation of the local surrounding adipocytes (Ma *et al.*, 2019). Thus, these studies inspired us to develop an alternative protocol to generate multipotent mesenchymal cells by mechanically dedifferentiating adipocytes.

The protocol described here includes the experimental set-ups to induce and verify the reprogramming of adipocytes into multipotent mesenchymal cells using our hypertonic dedifferentiation medium. We also include the procedures to generate adipocytes from preadipocytes or mesenchymal stem cells, and the differentiation assays to confirm the multilineage potentials of the CiDAs.

Materials and Reagents

A. Reagents

1. Minimum Essential Medium Eagle Alpha Modification media (Sigma-Aldrich, catalog number: M8042)
2. Fetal bovine serum (Gibco, catalog number: 10-082-147)
3. Penicillin/streptomycin (Gibco, catalog number: 15140148)
4. Polyethylene glycol 300 (Sigma-Aldrich, catalog number: 8.07484)
5. KnockOut Serum Replacement (Gibco, catalog number: 10828-028)
6. Preadipocyte Growth Medium-2 (Lonza, catalog number: PT-8202)
7. SingleQuots (Lonza, catalog number: PT-9502)

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8. Paraformaldehyde (VWR, catalog number: IC0219998380)
9. PBS (Sigma-Aldrich, catalog number: P5119)
10. Triton-X-100 (Sigma-Aldric, catalog number: X100)
11. Oil Red O (Sigma-Aldrich, catalog number: O0625)
12. DMEM (Sigma-Aldrich, Brand, catalog number: D5546)
13. Horse serum (Gibco, catalog number: 26050070)
14. Dexamethasone (Sigma-Aldrich, catalog number: D4902)
15. Hydrocortisone (Sigma-Aldrich, catalog number: H0888)
16. Hydrogen peroxide (Sigma-Aldrich, catalog number: H1009)
17. Anti-MyoD1 (Abcam, catalog number: ab16148)
18. Donkey anti-Rabbit Alexa 488 (Invitrogen, catalog number: R37118)
19. β-glycerophosphate (Sigma-Aldrich, catalog number: G9422)
20. L-ascorbic acid (Sigma-Aldrich, Brand, catalog number: A4403)
21. ELF-97 (Invitrogen, catalog number: E6588)
22. TGF-β (R&D Systems, catalog number: 240-B)
23. Anti-aSMA (Abcam, catalog number: ab5694)
24. DAPI (Thermo Scientific, Brand, catalog number: 62248)
25. Trypsin (2.5%) (Thermo Fisher Scientific, Gibco™, catalog number: 15090046)

B. Cell culture plasticware
1. T75 and/or T25 flasks (Corning, catalog numbers: 430641U for T75 and 3056 for T25)
2. Centrifuge tubes (15 ml; 50 ml, Corning, catalog numbers: 430790; 430828)
3. Cryovials (STARLAB, catalog number: E3110-6122)
4. Pipette tips (TipOne, STARLAB, catalog numbers: S1111-3700; S1111-1706; S1111-6701)
5. 35-mm cell culture dish (Thermo Fisher Scientific, catalog number: 153066)
6. 6-well plates (Corning, Falcon®, catalog number: 353934)
7. 100 mm cell culture dish (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 150464)

Equipment

1. Centrifuge (Eppendorf, model: 5810)
2. Bright-Line™ Hemacytometer (Sigma-Aldric, catalog number: Z359629)
3. Water bath (Thermo Scientific, catalog number: TSCIR19)
4. Humidified incubator at 37 °C, 5% CO_2 (Thermo Fisher Scientific, Heraeus, model: Heracell™ 150)
5. Leica TCS SP8 Confocal Microscope (Leica)
6. ZEISS Axio Zoom V16 microscope (ZEISS)
7. Xenon Arc Lamp (ZEISS)

8. Hamamatsu Orca Flash 4.0 V3 (Scientifica)
9. Aspirator (Dry vacuum pump/compressor, Welch Vacuum - Gardner Denver, model: 2511)

Software

1. ImageJ (https://imagej.nih.gov/ij/)
2. LAS X (Leica Microsystems, Mannheim, Germany)
3. HCImage (http://www.hamamatsu.com/)

Procedure

A. Cell culture (Figure 1, step 1)
   1. Purchase clonally derived mouse MSCs (OP9) from the American Type Cell Culture (ATCC).
   2. Expand MSCs (OP9) subconfluently in Minimum Essential Medium Eagle Alpha Modification media supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin (complete αMEM) in the condition of 5% CO_2, 37 °C, and 95% humidity.
   3. Assess cell viability using calcein acetoxymethyl and ethidium homodimer-1 (Invitrogen, Eugene, OR) or trypan blue exclusion (Beckman Coulter).
   4. Purchase subcutaneous primary human preadipocytes from Lonza.
   5. Culture primary human preadipocytes at subconfluence in Preadipocyte Growth Medium-2 (Lonza) in the condition of 5% CO_2, 37 °C, and 95% humidity, following the manufacturer's instructions.

B. Generation of adipocytes from preadipocytes or mesenchymal stem cells (Figure 1, step 2)
   1. Induce adipogenesis of OP9 mMSCs by supplementing cells with MEM (Gibco) containing 15% KnockOut Serum Replacement (Gibco).
   2. Induce adipogenesis of human preadipocytes by culturing cells in Preadipocyte Growth Medium-2 (Lonza) supplemented with SingleQuots (Lonza) consisting of insulin, dexamethasone, indomethacin, and isobutyl-methylxanthine, as per the manufacturer's instructions.

C. Sort adipocytes from the mixed cell population
   1. Trypsinize the mixed cell population after adipogenesis induction.
   2. Transfer the cell suspension into a 15 ml centrifuge tube, and centrifuge at a low speed (150 × *g*, 5 min). The differentiated adipocytes are then floating on the top layer of the medium in the centrifuge tube due to their lower density as compared to the culture medium (Figure 1, step 3).
   3. Take up only the differentiated adipocytes from the top layer in the centrifuge tube, and seed 10^5 cells per culture flask (Falcon 3012; 25 cm^2). Incubate cells at 37 °C in a medium consisting of Minimum Essential Medium Eagle Alpha Modification media supplemented with 20% fetal

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bovine serum.

4. Completely fulfill the flask with medium to provide the mixed cells with an air-free environment (Figure 1, step 4).
5. Turn the flask upside down on the first day during culturing, allowing the adipocytes to float up in the medium and adhere to the top inner surface (ceiling surface) of the flasks (Figure 1, step 5).
6. Turn the flask back after cells are fully attached (in most cases 1 day is enough, not exceeding 2 days) so that cells are back on the bottom of the flask again (Figure 1, step 6).
7. To obtain purified and monodispersed adipocyte population, sufficiently digest and pipet the cells.
8. Gently wash away the medium and the residual unattached cells.
9. Culture adipocytes with 5 ml medium contained in one Flask in the condition of 5% CO_2, 37 °C, and 95% humidity. There are also some numbers of undifferentiated mesenchymal stem cells or preadipocytes attached to the ceiling surface of the flask. Without the supplement of a medium, these cells are then exposed directly to the air and die shortly (Figure 1, step 7).

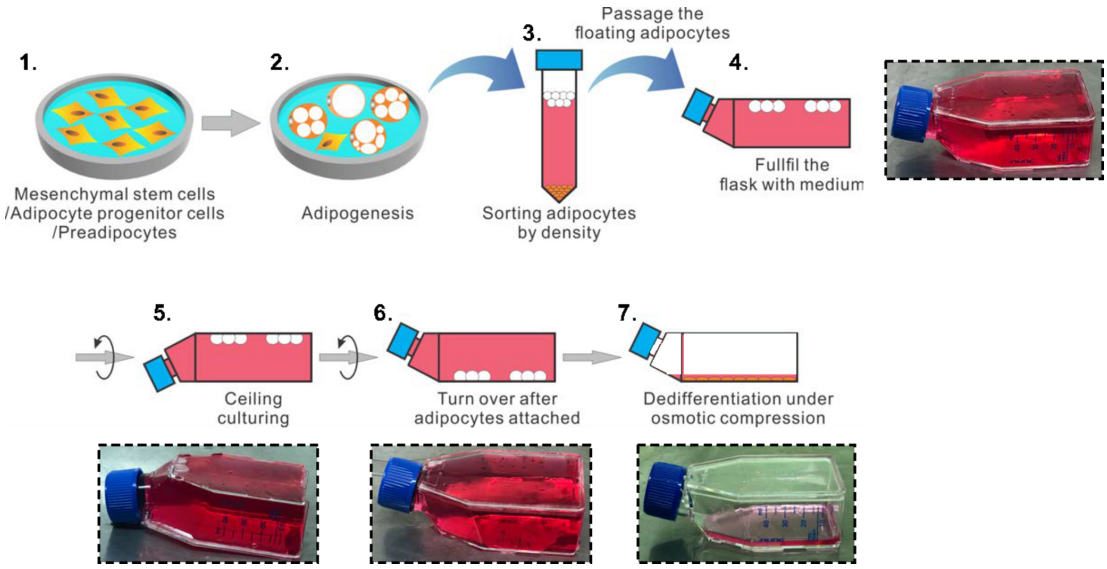

Figure 1. Schematic illustration of the procedure to generate compression-induced dedifferentiation of adipocytes. 1. Mesenchymal stem cells or adipocyte progenitors are homogeneously seeded in tissue culture plate (Step A). 2. Induction of adipogenesis of MSCs or adipocyte progenitors using adipogenesis medium (Step B). 3. Trypsinize the cells and sort generated adipocytes by density (Steps C1-C2). 4. Fulfill the cell culture flask using cell medium and the floating adipocytes (Steps C3-C4). 5. Turn the cell culture flask upside to allow adipocytes attaching to the bottom of the flask by ceiling culturing (Step C5). 6. Turn over the cell culture flask after the adipocytes fully attached to the bottom of flask (Step C6). 7. Induction of dedifferentiation of adipocyte using osmotic compression (Step D).

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D. Induction of compression-induced dedifferentiation of adipocytes

1. Aspire and remove half of the culture medium (2.5 ml) from the flask.
2. Add 2.5 ml hypertonic dedifferentiation medium into the flask [Minimum Essential Medium Eagle Alpha Modification media supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, and 4% PEG-300 (MW: 300, v/v ratio)]. Avoid shaking the flask, and allow the hypertonic medium to slowly diffuse and mix with a residual culturing medium in the flask. Culture the cells in the condition of 5% CO_2, 37 °C, and 95% humidity.
3. Exchange the hypertonic dedifferentiation medium every 3 days. Aspire and remove 4 ml of the medium from the flask, and refill with 4 ml hypertonic dedifferentiation medium [Minimum Essential Medium Eagle Alpha Modification media supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, and 2% PEG-300 (MW: 300, v/v ratio)].
4. Avoid complete removal of the medium and avoid any shear that could be applied to adipocytes. Because of the fragile property of the adipocytes and their contained lipid droplets, any shear or rapid osmotic stress changes may damage the cells.
5. Image the cultured adipocytes during culturing. In 10 days, we likely observe half of the population of the adipocytes transiting and dedifferentiating to mesenchymal stromal cell-like cells (Figure 2). Other adipocytes remain large lipid droplets, many of which would not undergo dedifferentiation.

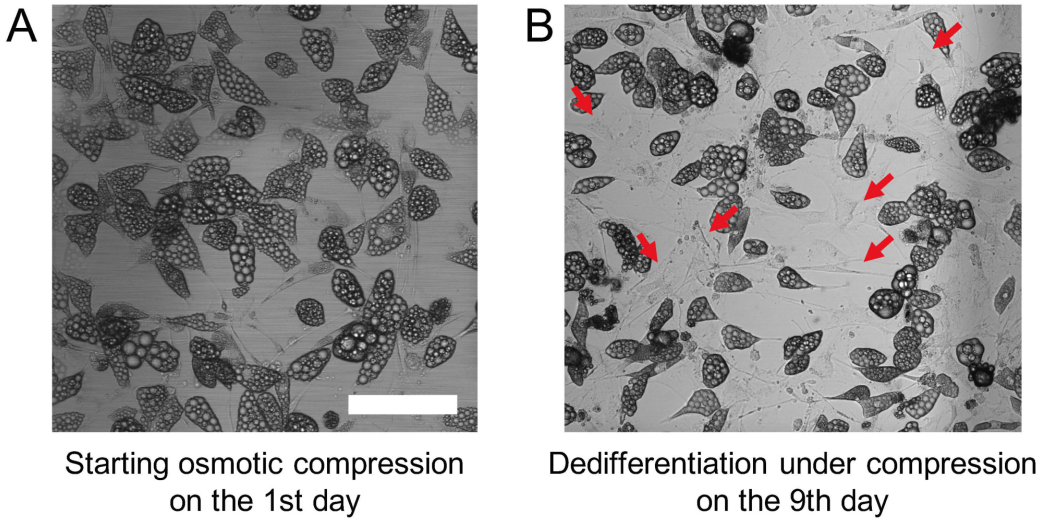

Figure 2. Dedifferentiation of adipocytes before and after 9 days of compression treatment. A. Adipocytes with cellular lipid droplets before osmotic compression-induced dedifferentiation. B. Anticipated results of CiDAs. The red arrows indicate the regions CiDAs are located, while some other adipocytes are remaining their lipid droplets. Scale bar, 100 μm. (Step D)

6. Trypsinize all the cells and transfer them to a 15 ml centrifuge tube. Centrifuge the cells at a speed of 150 × *g* for 5 min.

7. Remove the top layer residual adipocytes and the supernatant. Resuspend the CiDAs in MSCs expansion medium (Minimum Essential Medium Eagle Alpha Modification media supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin) and seed the cells back to a flask with a density of 10^5 cells per culture flask. Culture the cells in the condition of 5% CO_2, 37 °C, and 95% humidity.
8. Exchange the expansion medium every other day, and keep doing this up to 2 weeks until the CiDAs reaching 80% confluence of the flask surface.

E. Inducing osteogenesis of CiDAs to test the multilineage potential of CiDAs
1. Seed the harvested CiDAs in the wells of 6-well plates with a density of 3×10^5 cells per well, and culture them with MSCs expansion medium (5% CO_2, 37 °C, and 95% humidity) until CiDAs reaching more than 90% confluence in the well.
2. Exchange the expansion medium with osteogenic medium (complete DMEM supplemented with 10 mM β-glycerophosphate and 250 μM L-ascorbic acid). Cycle the osteogenic medium every 2 days.
3. To test ALP activity, fix CiDAs after 6 days of culturing in an osteogenic medium. Permeabilize CiDAs with Triton X-100. Stain the fixed CiDAs with ELF-97 (Thermo Fisher Scientific), following the manufacturer's instructions.
4. Image the stained CiDAs using epifluorescence microscopy, consisting of a Xenon lamp, an Axio Zoom V16 microscope, and Hamamatsu Flash 4.0 v3. Anticipate observing over 50% of the cells are positive with the blue color of ALP staining (Figure 3A). The positive ratio of calcium deposition can be accessed using ImageJ, which is defined by the number of pixels positive with red color divided by the total numbers of pixels of the image.
5. To test mineral deposition of osteogenesis of CiDAs, firstly aspirate the medium from the well. Then, fix the cells in ice-cold 70% ethanol for 5 min at room temperature. Aspirate alcohol and rinse cells twice with DI water (5 min each time). Aspirate the water and add 1 ml 2% Alizarin Red S solution, which is adjusted to a pH value of 4.1-4.3 with ammonium hydroxide. Incubate the well plate at room temperature for 3 min. Aspirate Alizarin Red S solution and wash the wells five times with 2 ml water.
6. Image the stained CiDAs using epifluorescence microscopy, consisting of a Xenon lamp, an Axio Zoom V16 microscope, and Hamamatsu Flash 4.0 v3. Anticipate to observe over 50% of the surface is positive with the red color of calcium deposition staining (Figure 3B).

F. Inducing adipogenesis of CiDAs to test the multilineage potential of CiDAs
1. Seed the harvested CiDAs in the wells of 6-well plates with a density of 3×10^5 cells per well, and culture them with MSCs expansion medium (5% CO_2, 37 °C, and 95% humidity) until CiDAs reaching more than 90% confluence in the well.
2. If the CiDAs is originating from mouse MSCs (OP9), exchange the expansion medium with an adipogenic medium (MEM (Gibco) containing 15% KnockOut Serum Replacement (Gibco)).

Cycle the osteogenic medium every 2 days.

3. If the CiDAs is originating from human primary preadipocytes, exchange the expansion medium with an adipogenic medium (Preadipocyte Growth Medium-2 (Lonza) supplemented with SingleQuots (Lonza) consisting of insulin, dexamethasone, indomethacin, and isobutyl-methylxanthine). Cycle the osteogenic medium every 2 days.
4. To test lipid droplet accumulation after 10 days of incubation, fix CiDAs with 4% paraformaldehyde (Thermo Fisher Scientific) in phosphate-buffered saline (PBS) (Gibco) with 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at 25 °C. Wash CiDAs with PBS 3 times. Neutral lipid accumulation was visualized by Oil Red O (Abcam) staining as a functional marker for adipogenesis. Rinse cells with PBS 3 times for 10 min each.
5. Image the stained CiDAs using epifluorescence microscopy, consisting of a Xenon lamp, an Axio Zoom V16 microscope, and Hamamatsu Flash 4.0 v3. Anticipate to observe over 50% of the cells is positive with the red color of Oil Red O staining (Figure 3C). The positive ratio of Oil Red O staining can be accessed using Image J, which is defined by the number of cells positive with red color divided by the total number of the cells.

G. Inducing myogenic of CiDAs to test the multilineage potential of CiDAs
   1. Seed the harvested CiDAs in the wells of 6-well plates with a density of 3×10^5 cells per well, and culture them with MSCs expansion medium (CO_2, 37 °C, and 95% humidity) until CiDAs reaching more than 90% confluence in the well.
   2. Exchange the expansion medium with myogenic medium (complete DMEM supplemented with 5% horse serum (HS) (Gibco), 0.1 μM dexamethasone (Sigma-Aldrich), and 50 μM hydrocortisone (Sigma-Aldrich)) for 10 days. Cycle the myogenic medium every 2 days.
   3. To test myogenesis efficiency after 10 days of incubation, fix CiDAs with 4% paraformaldehyde (Thermo Fisher Scientific) in PBS (Gibco). Wash the fixed sample three times with PBS. Incubate the fixed sample with PBS (Gibco) with 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at 25 °C. Block nonspecific sites in the fixed sample using blocking buffer (PBS, 10% HS, and 0.1% Triton X-100) for an additional 60 min. Wash three times for 5 min each. Incubate the fixed cells with primary antibody anti-MyoD1 (Abcam) in blocking buffer. Rinse cells extensively in blocking buffer and incubate secondary antibody donkey anti-rabbit Alexa 488 (Thermo Fisher Scientific) for 1 h. Rinse cells by PBS 3 times for 10 min each. Incubate cells with DRAQ5 solution for 10 min before imaging.
   4. Image the stained CiDAs using confocal microscopy, with LAS X. Anticipate to observe over 50% of the cells are positive with the anti-MyoD1 staining (Figure 3D). The positive ratio of MyoD1 staining can be accessed using ImageJ, which is defined by the number of cells positive with anti-MyoD1 divided by the total number of the cells.

H. Inducing myofibrogenesis of CiDAs to test the multilineage potential of CiDAs
   1. Seed the harvested CiDAs in the wells of 6-well plates with a density of 3×10^5 cells per well,

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and culture them with MSCs expansion medium (5% CO_2, 37 °C, and 95% humidity) until CiDAs reach more than 70% confluence in the well.

2. Exchange the expansion medium with myofibrogenic medium (complete DMEM supplemented with 2 ng/ml TGF-β (Abcam)) for 7 days. Cycle the myogenic medium every 2 days.
3. To test myofibrogenesis efficiency after incubation, fix CiDAs with 4% paraformaldehyde (Thermo Fisher Scientific) in PBS (Gibco). Wash the fixed sample three times with PBS. Incubate the fixed sample with PBS (Gibco) with 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at 25 °C. Block nonspecific sites in the fixed sample using blocking buffer (PBS, 10% HS, and 0.1% Triton X-100) for an additional 60 min. Wash three times for 5 min each. Incubate the fixed cells with primary antibody anti-α-SMA1 (Abcam) in blocking buffer. Rinse cells extensively in blocking buffer and incubate secondary antibody donkey anti-rabbit Alexa 488 (Thermo Fisher Scientific) for 1 h. Rinse cells by PBS 3 times for 10 min each. Incubate cells with DRAQ5 solution for 10 min before imaging.
4. Image the stained CiDAs using confocal microscopy, with LAS X. Anticipate to observe over 50% of the cells are positive with the anti-α-SMA1 staining (Figure 3E). The positive ratio of α-SMA1 staining can be accessed using ImageJ, which is defined by the number of cells positive with anti-α-SMA1 divided by the total number of the cells.

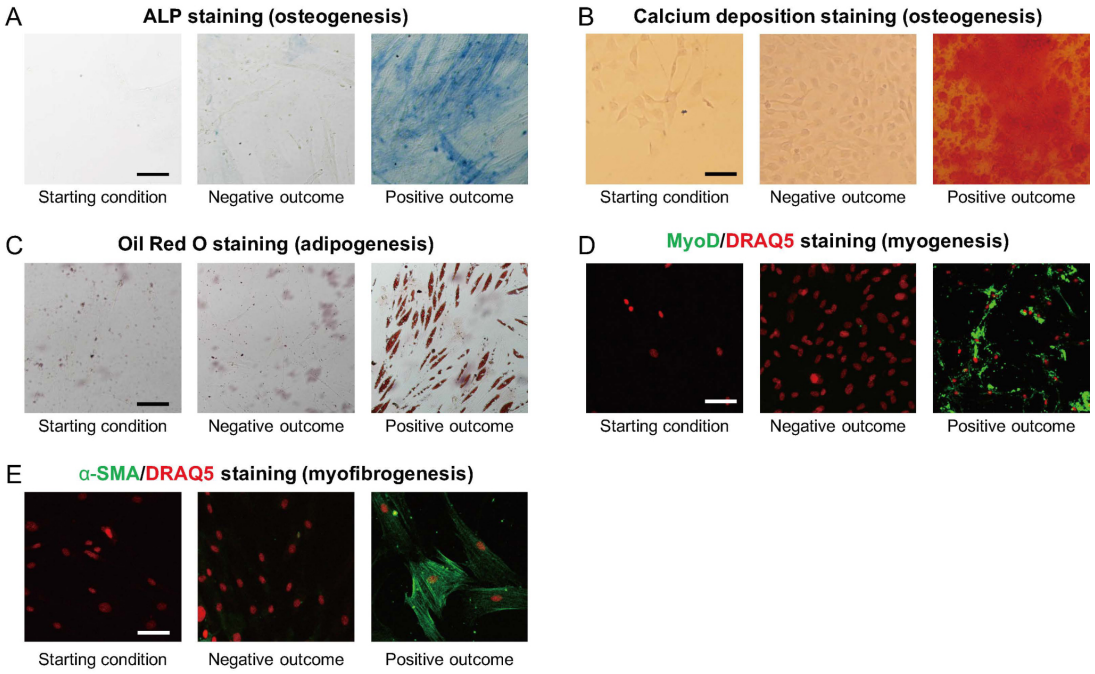

Figure 3. Expected results of multilineage induction from CiDAs. A. Expected ALP staining to test osteogenesis of CiDAs, at starting points, negative outcome, and positive outcome. Scale bar, 100 μm. (Step E). B. Expected calcium deposition staining to test osteogenesis of CiDAs, at starting points, negative outcome, and positive outcome. Scale bar, 50 μm. (Step E). C. Expected Oil Red O staining to test adipogenesis of CiDAs, at starting points, negative outcome, and positive outcome. Scale bar, 100 μm. (Step F). D. Expected MyoD staining to test

myogenesis of CiDAs, at starting points, negative outcome, and positive outcome. Scale bar, 50 μm. (Step G). E. Expected α-SMA staining to test myofibrogenesis of CiDAs, at starting points, negative outcome, and positive outcome. Scale bar, 50 μm. (Step H)

Trouble-shooting

1. Low efficiency of adipogenesis of mesenchymal stem cells or adipose progenitors
   Possible cause: Low cell density before induction of adipogenesis.
   Possible repair: Increasing expansion time of MSCs/adipocyte progenitors culturing before exchanging to adipogenesis induction medium. MSCs/adipocyte progenitors should reach 90% confluency before exchanging to the adipogenesis induction medium.
2. Contamination with non-adipocytes after ceiling culturing
   Possible cause: Insufficient digestion of adipocytes before density sorting.
   Possible repair: Increasing digestion time to trypsin the cells, and gently pipette the cells to sufficiently break down cell-to-cell contact and generate monodispersed cells.
3. A limited number of adherent adipocytes after ceiling culturing
   Possible cause: Flow shear or harsh pipetting damages adipocytes during preparation.
   Possible repair: Be gentle when digest and pipette the cells; keep the cells at 4 °C during density sorting; avoiding flow shearing during fulfilling the culture flask, and be sure to remove all the air bubbles before turning the flask upside down.
4. Adipocytes dying and detaching during culturing under high osmotic compression
   Possible cause: Quick exchange of hypertonic medium and quick osmotic shock.
   Possible repair: Exchange only half of the medium when changing medium, let the hypertonic medium slowly diffuse into the remaining culture medium, and reach the final concentration.

Future direction

There is significant heterogeneity in primary adipose tissue, which makes adipocytes isolated from different original sites behave much differently from each other. Thus, future works could involve optimizing the protocol to generate CiDAs from adipocytes of different origins. Secondly, mesenchymal stem cells or progenitor cells from different tissue usually exhibit different lineage potentials. Thus, for CiDAs generated from different origins of adipocytes, we also need to test their lineage potentials, which will help to define their practical applications in stem cell therapy, tissue engineering, and regenerative medicine. Another direction is further improving the efficiency of generating CiDAs and shortening the time required for the dedifferentiation of adipocytes. This could be done by combining mechanical stimulations and biochemical treatment. Current work from our group revealed that Wnt/β-catenin signaling plays an important role in adipocytes differentiation (Li *et al.*, 2020b), which is also supported by another work (Gustafson and Smith, 2010). Another study from Kuang's group revealed that Notch activation drove adipocyte dedifferentiation (Bi *et al.*, 2016). Based on these understandings, a dedifferentiation cocktail medium, that not only mimics native physical stresses but also contains growth factors regulating Wnt/β-catenin signaling or Notch

signaling, could more efficiently induce dedifferentiation of adipocytes and generate CiDAs. Overall, we hope that further development of this method may make CiDAs more accessible for many groups, and more stable for applications in regenerative medicine.

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Competing interests

The authors declare no conflict of interests.

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