

Video Article

Generation of Myospheres From hESCs by Epigenetic Reprogramming

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Abstract

Generation of a homogeneous and abundant population of skeletal muscle cells from human embryonic stem cells (hESCs) is a requirement for cell-based therapies and for a "disease in a dish" model of human neuromuscular diseases. Major hurdles, such as low abundance and heterogeneity of the population of interest, as well as a lack of protocols for the formation of three-dimensional contractile structures, have limited the applications of stem cells for neuromuscular disorders. We have designed a protocol that overcomes these limits by ectopic introduction of defined factors in hESCs - the muscle determination factor MyoD and SWI/SNF chromatin remodeling complex component BAF60C - that are able to reprogram hESCs into skeletal muscle cells. Here we describe the protocol established to generate hESC-derived myoblasts and promote their clustering into tridimensional miniaturized structures (myospheres) that functionally mimic miniaturized skeletal muscles⁷.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51243/>

Introduction

Because of their unique ability to self-renew while retaining pluripotency, human embryonic stem cells (hESCs) are considered an invaluable resource in regenerative medicine. Stem cell mediated repopulation of diseased muscles and hESC- or induced pluripotent stem cells (iPSC)-derived generation of skeletal muscles for *in vitro* disease modeling are key goals of studies aimed at identifying treatments and elucidating the pathogenesis of many neuromuscular diseases. However, these studies have been challenged by the resistance of hESCs to convert into skeletal muscle cells and by the paucity of information regarding the molecular regulation of hESC-commitment toward skeletal myogenesis. Indeed, previous attempts to generate skeletal muscle progenitors from hESCs revealed that only embryoid body (EB)-derived progenies or mesenchymal cells are competent to activate skeletal myogenesis following ectopic expression of transcriptional activators (e.g. Pax3 or Myf5)¹⁻³ or by exposure to specific culture conditions⁴⁻⁵.

We have previously shown that the SWI/SNF component, BAF60C (encoded by *SMARCD3*), is an essential component of the transcriptional machinery that allows MyoD-mediated activation of muscle-specific loci⁶. We have recently discovered that the selective absence of BAF60C confers onto hESCs the resistance to MyoD-mediated activation of skeletal myogenesis⁷ that is otherwise observed in BAF60C expressing somatic cells⁸⁻¹⁰, a process commonly referred to as myogenic conversion. Forced expression of BAF60C enables MyoD to directly activate skeletal myogenesis in hESCs, upon specific culture conditions, with BAF60C and MyoD imposing an epigenetic signature that commits hESCs towards the myogenic lineage⁷. Of note, previous proteomic analysis revealed the selective absence of BAF60C, among the SWI/SNF components, in ESCs¹¹. We used this knowledge to impose an epigenetic commitment of hESCs onto the myogenic lineage, leading to the generation of a homogeneous population of skeletal myoblasts that could be aggregated to form three-dimensional contractile structures (myospheres) that functionally mimic miniaturized skeletal muscles⁷. Indeed, our method of generating skeletal muscle progenitors from hESCs relies on the epigenetic commitment of hESCs to the myogenic lineage, which is phenotypically latent until cells are exposed to differentiation signals, such as cell aggregation and culture in differentiation medium (see specific protocol). This strategy permits the expansion of a homogeneous population of hESCs that are epigenetically committed to skeletal muscle lineage and suitable to formation of three-dimensional contractile myospheres that recapitulate histological and functional properties of skeletal muscles. The myospheres provide the first evidence of miniaturized muscles exploitable for a "disease in a dish" model of muscular diseases. When generated from patient derived iPSCs, these myospheres have the potential to elucidate longstanding developmental questions and the pathogenesis of rare diseases, in addition to offering the tremendous potential as a tool for high throughput screening of therapeutic compounds. We also note that one immediate readout of myosphere analysis could be provided by immunohistochemistry on sections, as described in a JoVE protocol by Gomes *et al.*¹²

Protocol

1. Infection of hESCs

This protocol requires high titer lentiviruses encoding BAF60C and MyoD¹³. These constructs are available upon request.

1. Recommendations before starting
 1. In order to ensure high efficiency of infection, plate hESCs for infection on feeder-free conditions (**Figure 2A**) to eliminate cellular competitors during the viral uptake. Indeed, MEFs are notoriously easier to infect as compared to hESCs and are competent for MyoD-mediated conversion. Thus, eliminating MEFs from hESC cultures increases the infection rate.
 2. It is recommended to have high titer lentiviruses to ensure high efficiency of infection. Options to improve efficiency of infection are discussed in the section "**checking infection**".
 3. Prepare matrigel-coated plates by adding 1 ml of 1 mg/ml Matrigel in each well and leave at least 1 hr at RT (or O/N sealed at 4 °C if prepared the day before).
2. Infection procedure
 1. Day before the infection
 1. Add hES Cell Cloning & Recovery Supplement in the medium at 1,000X dilution (2 mM final concentration).
 2. Day 0 - Infection with BAF60C

Note: Perform the infection of hESCs with BAF60C first followed by infection with MyoD.

 1. Dissociate a well of a 6-well plate of hESCs grown as colonies¹⁴ in a single-cell suspension by incubation with 1 ml of TrypLE for 5 min at 37 °C.
 2. Transfer suspension to a 15-ml tube, add 9 ml of hESC medium and centrifuge for 5 min at 1,200 rpm.
 3. During the centrifugation, prepare the infection mix in a clean tube by adding to 1 ml of mTeSR1, polybrene (6 mg/ml) and hES Cell Cloning & Recovery Supplement (2 mM).
 4. Re-suspend the cell pellet (approximately 5×10^5 - 1×10^6 cells from one confluent well of hESCs) with 1 ml of infection mix and plate onto a 6-well low attachment plate, which prevents the cells from adhering to the plastic.
 5. Add BAF60C-iRES-GFP virus at 10^8 MOI and incubate 3 hr in the incubator
 6. Collect the cell suspension containing the viruses and divide equally in two matrigel-coated wells. Then add 2 ml of mTeSR1+hES Cell Cloning & Recovery Supplement to each well and leave O/N in the incubator.
 3. Day 1
 1. Carefully remove the medium containing the virus and replace with 2 ml of mTeSR1 and hES Cell Cloning & Recovery Supplement. Cells will start clumping as shown in **Figure 2B**.
 4. Day2 - Infection with MyoD
 1. Before proceeding check the fluorescence status of the cells under a fluorescence microscope to make sure to have a high efficiency of infection. If not, see the section "*checking infection*".
 2. If the cells have reached at least 80% of confluence, proceed as follows; otherwise wait for an additional day prior to proceeding.
 3. Remove the medium and add 1 ml of TrypLE reagent to dissociate the cells. Incubate 5 min at 37 °C.
 4. Repeat as described from 1.2.2.2 - 1.2.2.6 adding MyoD virus at 10^8 MOI.
 5. Day 3
 1. Carefully remove the medium containing the virus and replace with 2 ml of mTeSR1 and hES Cell Cloning & Recovery Supplement.
 2. Plate MEFs on matrigel coated plates at 2.5×10^5 /cm² for the next day to allow propagation of the infected cells.
 6. Day 4
 1. Remove the medium and add 1 ml of TrypLE reagent to dissociate the cells. Incubate 5 min at 37 °C.
 2. Transfer the cells in a 15-ml falcon tube, add 9 ml of hESC medium and centrifuge for 5 min at 1,200 rpm.
 3. Remove the supernatant and re-suspend in 6 ml of hESC medium.
 4. Remove the medium from the MEFs-containing 6-well plate and plate hESCs at 1:2 split ratio, dispensing 3 ml for each MEFs-containing well.

TIP! When the colonies reach confluence, some of the wells of MyoD/BAF60C-infected cells can be frozen in liquid nitrogen as a reserve stock. Freezing medium includes 90% of KOSR and 10% DMSO plus 2 mM hES Cell Cloning & Recovery Supplement. The remaining wells can be then exposed to the differentiation protocol - see below for description. If more cells are needed to produce more myospheres, infected hESCs can be propagated further as follows:
 5. Remove the medium and incubate with 1 ml of 1 mg/ml collagenase IV for 5 min at 37 °C.
 6. Remove collagenase IV and add 1 ml of hESC medium.
 7. Under a dissection microscope scrape the colonies with a 10 ml tip.
 8. Collect the chunks of colonies in a 15-ml tube and allow to sediment for 3 min. Then remove the supernatant, resuspend in hESC medium and plate on MEFs plates using a ratio 1:4.
3. Checking infection
 1. While propagating the cells, it is recommended to plate the infected cells in a smaller scale to harvest for RNA analysis and perform protein expression analysis by immunofluorescence in order to check for biomarkers that reflect the correct biological status at each stage (**see Representative results**). For high titer lentiviruses the percentage of expected infected cells should be at least 80%.

2. To ensure high efficiency, the lentiviral vectors can be modified, introducing a fluorescent selection marker and/or an antibiotic resistance. Our lentiviral plasmid expressing BAF60C also carries GFP fluorescence.
3. Sorting hESCs for a fluorescent marker. In the case of low efficiency of infection is recommended to use fluorescence activated cells sorting (FACS-sorting) to enrich for cells expressing BAF60C virus and then infect the cells with high titer MyoD virus.
 1. Add hES Cell Cloning & Recovery Supplement in the medium the day before the sorting.
 2. The day of the sorting, dissociate the cells by TrypLE (as described at day 0) and resuspend the cells in KO replacement serum plus hES Cell Cloning & Recovery Supplement in FACS-tubes.
 3. At the end of the sorting, collect the cells in KO replacement serum and plate on MEFs plates adding hES Cell Cloning & Recovery Supplement.

2. EB-like Induction and Differentiation

1. Induction and differentiation procedure
 1. Day 0 - EB induction
 1. Make sure, before starting differentiation from BAF60C/MyoD-infected hESCs, to have high number of colonies in the well (**Figure 2C**).
 2. For each well remove the medium, wash with PBS and add 1 ml of 1 mg/ml collagenase IV. Incubate for 5 min at 37 °C.
 3. Remove collagenase and add 1 ml of EB medium.
 4. Under a dissection microscope quickly mechanically dissociate the colonies into 400-800 cell aggregates by scraping with a 10-ml tip.
 5. Collect the chunks of colonies in a 15-ml tube and allow to sediment for 3 min. Remove the supernatant, re-suspend in 3 ml of EB medium and transfer the cell suspension to one well of a 6-well low attachment plate.
 2. Day 1
 1. Check the size of colonies and the cell death before proceeding. If big chunks are present, break them by gently pipetting up and down with a 5-ml pipette, 3-4 times. If the cell death is very high (many floating cells and debris) replace the medium by collecting the aggregates in a 15-ml tube by gravity and plate them with 3 ml of fresh EB medium, otherwise proceed as follows.
 2. Add 1.5 ml of fresh EB medium in the wells
 3. Day 2
 1. Change the medium by collecting the aggregates from the wells in a 15-ml tube. Let them settle down for 2 min.
 2. Remove the supernatant, replace with 3 ml of fresh EB medium and redistribute on the same wells.
 4. Day 3
 1. Add 1.5 ml of fresh EB medium in the wells.
 5. Day 4
 1. Proceed as described in "Day 2".
 6. Day 5 - Myogenic differentiation of EB-like clusters (**Figure 2D**).
 1. Collect the aggregates as described in 2.1.3.1 and wash once with 2 ml of PBS; allow the aggregated to sediment.
 2. Remove PBS and add 3 ml of DM medium; plate on the same wells.
 7. Day 6 - Day 20
 1. From d6 to d20 replace the medium with fresh medium every 3 days. Representative myospheres are shown in **Figure 2E**.
2. Checking myosphere differentiation
 1. Before proceeding further, it is recommended to check the efficiency of myogenic differentiation within the myospheres by making sections of the myospheres embedded in O.C.T. compound, as described in a previous JoVE protocol¹². Perform an immunofluorescence staining for myogenic markers such as myogenin (clone F5D, DSHB) and myosin heavy chain (clone MF20, DSHB) (**see Representative results**).

TIP! To be successful with the staining for myogenin on EB sections it is critical to perform antigen retrieval by treatment with sodium dodecyl sulfate (SDS) 0.5% for 5 min. Additionally, it is possible to use this protocol for the double staining using F5D and MF20 antibodies.

3. Media Recipes

1. ESC medium
 - DMEM-F12
 - 1 mM L-glutamine
 - 20% Knockout Serum Replacement medium (KOSR)
 - 0.1 mM nonessential amino acids (NEAA)
 - 50 U/ml penicillin/streptomycin (Pen/Strep)
 - 0.1 mM beta-mercaptoethanol
 - 8 ng/ml basic fibroblast growth factor (bFGF)
2. EB medium

- DMEM F12
- 1 mM L-glutamine
- 15% FBS
- 5% Horse Serum
- 3. DM medium
- DMEF F12
- 1X ITS Liquid Media Supplement
- 1X N-2 Supplement

Representative Results

Figure 1A shows a schematic of the protocol proposed to generate myospheres from hESCs. The critical steps to control (indicated by the arrows) are the efficiency of the infection in hESCs and the myogenic conversion within the myospheres.

In our experiments we take advantage of a lentiviral vector encoding BAF60C that carries GFP fluorescence. We usually infect the cells with BAF60C and FACS-sort 72 hr after the infection to enrich for the population expressing BAF60C. When the cells reach around 70-80% of confluence, we infect with MyoD lentivirus. After the first infection it normally takes two or three days for the cells to reach that confluence.

Figure 1B shows expression levels of the exogenous genes as fold induction relative to hESCs not infected. **Figure 1C** shows the expression and distribution at the protein level of the factors introduced. BAF60C expression is shown as GFP fluorescence, while MyoD expression is detected by immunofluorescence with a MyoD antibody. We consider Day 0 the day we start the differentiation of infected hESCs into EB-like aggregates. After 15 days in DM medium, a portion of the myospheres (usually one entire well) is embedded in O.C.T. compound¹² and sectioned to perform immunofluorescence for myogenic markers to check myogenic conversion efficiency. **Figure 1D** shows representative staining for myogenin and myosin heavy chain in an optimal myogenic differentiation. Myospheres can have different or unusual shapes, perhaps as a result of fusion with smaller myospheres. Starting from Day 10, it is possible to observe sporadic contraction in the myospheres (see movie 1 and 2).

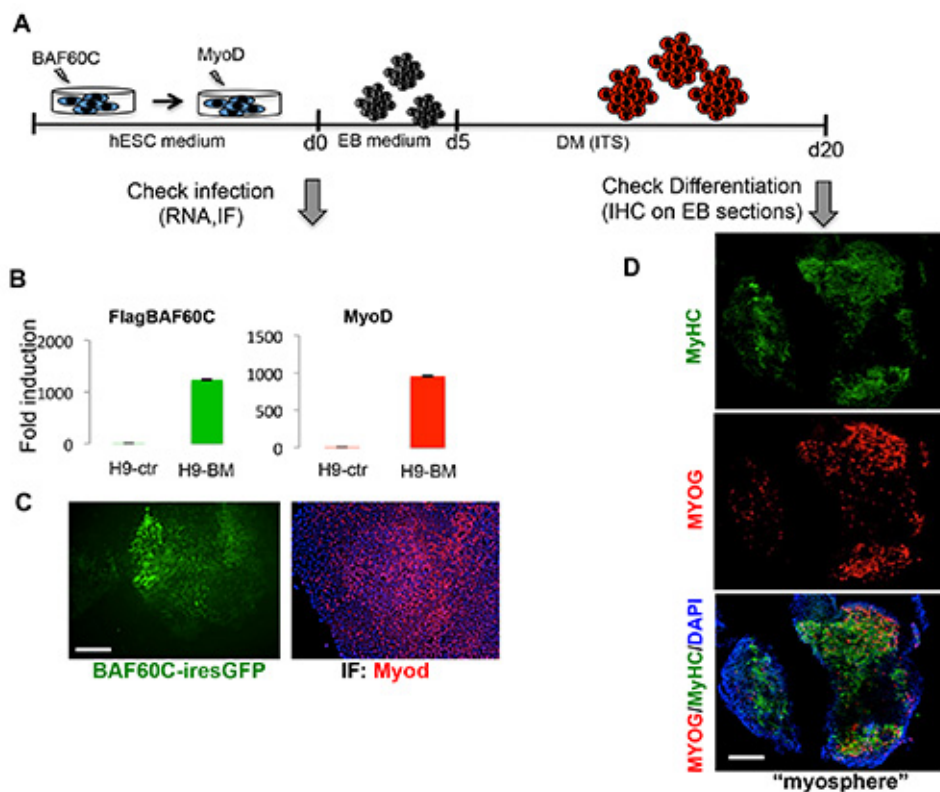


Figure 1. **A)** Schematic of the protocol used for the generation of myospheres from hESCs. **B)** mRNA levels of the exogenous genes in hESCs infected with BAF60C and MyoD (H9-BM) monitored by qRT-PCR and expressed as fold induction, as compared to mock infected hESCs (H9-ctr). **C)** Immunofluorescence images showing MyoD staining (red) and BAF60C fluorescence (green) due to the IRES-GFP element in the vector. **D)** Immunofluorescence performed on myosphere sections stained for Myogenin (MYOG), Myosin Heavy Chain (MyHC) and counterstained for DAPI (see Immunofluorescence details). Scale bar is 100 μ m.

Part I- Preparation of hESC expressing BAF60C and MyoD



Part II- EB-like differentiation and generation of myspheres

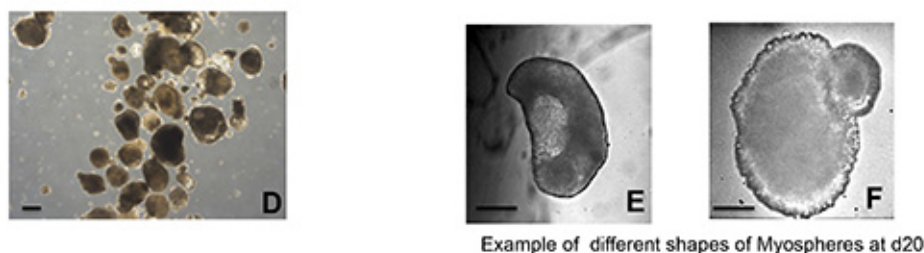


Figure 2. Representative pictures of cell appearance at different stages of the protocol from the infection (part I) to the differentiation (part II). Scale bar is 100 μ m. Whereas EB-like structures from d1 to d5 are mostly circular in shape (D), myspheres grown over 15 days according the scheme in Figure 1A present an unpredictable shape and are heterogeneous in culture; see two examples in E and F.

Discussion

The protocol proposed here describes how to generate three-dimensional clusters of contractile myofibers (myspheres) directly from hESCs. The strategy proposed has the unprecedented and extraordinary potential to produce mini muscles in suspension that can be suitable as a "disease in a dish" model for both screening assays and developmental studies. Moreover, the method to generate myspheres from hESCs is straightforward and does not require any FACS-sorting step during the differentiation, which typically has a negative impact on the yield of cells recovered. Besides, a sorting procedure during the differentiation would interfere with the aggregation since it implies a dissociation of the EB into single cells.

The method proposed is based on the epigenetic reprogramming of hESCs with specific factors, MyoD and BaAF60C, which are not expressed in pluripotent embryonic stem cells. MyoD and BAF60C provide the "core" protein complex that marks the genomic loci from which transcription proceeds to activate the skeletal myogenic program. The two factors are delivered to the cells by means of lentiviral infections and it is therefore critical to obtain a high efficiency of infection of both factors in all cells. This can be achieved by using high titer viruses or viruses endowed with selection markers. Culture conditions also play an important role in optimizing the extent of the myogenic differentiation. We propose a serum-based differentiation protocol for the differentiation of MyoD/BAF60C expressing hESCs into clusters of myogenic precursors, followed by incubation in serum-free defined medium (containing insulin transferrin - ITS) to achieve conversion of myogenic precursors into skeletal myotubes. However, it is possible that other defined media can achieve an equal or better myogenic differentiation. One current limitation of the protocol is that it relies on the use of fetal bovine serum, which, besides containing many animal proteins and substances, shows lot-to-lot variations. This may result in lower efficiency or heterogeneity of myogenic conversion within myspheres. Of note, not all the EB-like structures derived from BAF60/MyoD-expressing hESCs are myspheres; namely, some are EB-like aggregates fully composed of myofibers. The number of myspheres normally derived from hESCs expressing BAF60C and MyoD can vary from 30 to 60% as estimated by immunostaining on EB sections performed at the end of the protocol (see Results). A potential approach to enrich a culture dish for only myspheres would be to use a myogenic reporter. This would facilitate discarding the partially or not differentiated EB-like clusters and selecting only the myspheres.

Lastly, a better understanding of the mechanisms underlying the temporal requirements of the factors introduced would be useful to improve the method of delivery. For example, if BAF60C and MyoD are required only for short time to "kick" the cells in the right direction, methods based on modified mRNA delivery might be applied. By contrast, if the factors are required for a longer time before the cells exploit their endogenous proteins, an episomal approach would be recommended to eliminate variability and uncontrolled effects due to integration in the genome. Finally, we note that it is mandatory to express BAF60C prior to or at the same time as MyoD (never after) in order to achieve hESC conversion into skeletal muscles. The requirement for prior expression of BAF60C presumably relies on its role in pre-setting the epigenetic landscape for proper MyoD chromatin distribution through the genome^{6,15}. As such, future protocols might be improved by compounds or other manipulations that promote BAF60C expression in hESCs.

Disclosures

We have nothing to disclose.

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