Video Article

Isolation and Immortalization of Patient-derived Cell Lines from Muscle Biopsy for Disease Modeling

Jerome D. Robin[1], Woody E. Wright[1], Yaqun Zou[2], Stacy A. Cossette[3], Michael W. Lawlor[3], Emanuela Gussoni[4]

[1]Department of Cell Biology, UT Southwestern Medical Center

[2]National Institute of Neurological Disorders and Stroke, National Institute of Health

[3]Division of Pediatric Pathology, Department of Pathology and Laboratory Medicine, Medical College of Wisconsin

[4]Division of Genetics and Genomics, Boston Children's Hospital

Correspondence to: Emanuela Gussoni at gussoni@enders.tch.harvard.edu

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Abstract

The generation of patient-specific cell lines represents an invaluable tool for diagnostic or translational research, and these cells can be collected from skin or muscle biopsy tissue available during the patient's diagnostic workup. In this protocol, we describe a technique for live cell isolation from small amounts of muscle or skin tissue for primary cell culture. Additionally, we provide a technique for the immortalization of myogenic cell lines and fibroblast cell lines from primary cells. Once cell lines are immortalized, substantial expansion of patient-derived cells can be achieved. Immortalized cells are amenable to many downstream applications, including drug screening and *in vitro* correction of the genetic mutation. Altogether, these protocols provide a reliable tool to generate and preserve patient-derived cells for downstream applications.

Video Link

The video component of this article can be found at https://www.jove.com/video/52307/

Introduction

Molecular diagnostics has dramatically evolved in the past 20 years. Genomic DNA is now routinely isolated from sputum or cheek swab, while in the past it required a blood draw. With the current fast turnaround time and ease of gene sequencing, many disease mutations are routinely identified with no need of additional testing. In the case of muscle disease diagnostics, identification of dozens of new genes in the past decade responsible for either muscular dystrophy or myopathy have dramatically changed the ways these diseases are diagnosed [1,2]. Currently, there are dozens of genes that have been identified as causes of muscular dystrophy and congenital myopathy, although the mechanisms by which many of these genes produce disease remain unclear. In particular, rare diseases constitute a challenge due to the small size of the patient populations. For these cases, as well as for more common diseases, the generation of stable tools that facilitate studies on the mechanism of pathogenesis and screening of therapeutic drugs is highly desirable.

Despite dramatic progress in DNA diagnostics, muscle biopsies are still performed to establish the primary diagnosis in many patients in whom primary metabolic or muscle disease is suspected. When a muscle biopsy is necessary, it offers the opportunity for additional diagnostic and research tissue collection with minimal additional morbidity risk for the patient. As there are a number of uses for each tissue specimen, it is highly desirable to establish techniques for primary cell culture using surgical tissue that are straightforward, efficient, and require minimal amounts of tissue. Proper triage of muscle or skin biopsies is required to maximize isolation of primary cells from tissue and long-term storage of live material. Additionally, stem cell research and drug screening holds great promise for developing therapies for many diseases using cell-based assays [3,4].

We herein describe methods for primary cell isolation from human muscle or skin biopsies. Additionally, we include a protocol for immortalization of myogenic cells, which is useful for generating large numbers of cells from an individual. These cells can be used for downstream applications, such as custom drug-screenings, which are otherwise unachievable with the overall low number of cells obtained from primary tissue.

Protocol

NOTE: Protocols for collection of human tissue must be reviewed and approved by the Institutional IRB committee. Collection of discarded, de-identified human tissue has been approved by the Boston Children's Hospital and Brigham and Women's Hospital IRB Committees. The methods described below have been applied for the isolation of myogenic cells from de-identified, discarded tissue. The described methods are applicable to tissue collected from consented patient material.

1. Cell Isolation

1. Dissociation of muscle biopsy and purification of myogenic cells
    1. Pre-weigh a 10 cm tissue culture plate in a tissue culture biosafety hood, and then re-weigh the plate containing the muscle biopsy to calculate the amount of tissue to be dissociated.
    2. Using sterile scalpels, mince muscle tissue finely and add a few drops of sterile 1x HBSS to prevent tissue from drying out.
    3. Add 3.5 ml each of dispase II and collagenase D per gram of muscle tissue to be digested. Pipette the minced tissue and enzyme solution through a sterile 25 ml pipette a few times. Incubate the plate in a tissue culture incubator at 37 °C with 5% CO_2 for 15 min and digest tissue until the slurry easily passes though a sterile 5 ml pipette.
    NOTE: Tissue dissociation is usually achieved by enzymatic digestion within 45-90 min. Please refer to the 'Representative Results' section for additional details.
    4. Add 2 volumes of sterile growth medium to dissociated tissue, filter through a 100 µm cell strainer over a 50 ml conical tube and pellet cells for 10 min at 329 x g (~1,100 rpm), room temperature. Please refer to the Materials Table for media composition.
    5. Resuspend the pellet in 1 volume of sterile growth medium and add 7 volumes red blood cell lysis solution. Filter the solution through a 40 µm cell strainer, then pellet the cells for 10 min at 329 x g, room temperature.
    6. Count the cells in a hemocytometer and resuspend the cells in 1x HBSS 0.5% BSA at a concentration of 1 x 10^6 cells/100 µl. Set aside ~250,000 cells in a single tube that will be used as a negative (unstained) control. Set aside additional single-color stained control tubes for propidium iodide and for CD56, which are required for proper gating of CD56 positive cells by FACS sorting. Please refer to cell sorting manuals or consult with FACS sorting core facility experts to ensure appropriate controls are included.
    7. Stain the cells to be sorted with 5µl/10^6 cells of anti CD56 antibody. Incubate all samples (including controls) on ice for 30 min.
    8. Wash samples in 10 ml 1x HBSS and pellet cells for 10 min at 329 x g (~1,100 rpm) in a refrigerated centrifuge, 4°C temperature.
    9. Add propidium iodide at a final concentration of 1µg/ml to the sample to be sorted for exclusion of dead cells. Purify myogenic CD56 positive cells from non-myogenic cells using the fluorescence activated cell sorter.

2. Dissociation of skin biopsy
NOTE: Dermal fibroblasts can be isolated from a skin punch from patients when muscle biopsies are not available. Dermal fibroblasts can be used as biomaterial for many studies, including transduction with MyoD to generate myogenic cells. Additionally, dermal fibroblasts can be used to generate iPS cells, which can be differentiated into various cell types for further study.
    1. Transport the skin biopsy to the laboratory in transportation medium. Once the sample is received, perform the primary culture as soon as possible. If the primary culture cannot be established on the same day, store the sample at room temperature overnight.
    2. Transfer the skin biopsy to a sterile 35 mm petri dish in the laminar flow hood.
    3. Rinse the skin biopsy in a petri dish with sterile 1x PBS to remove blood and debris. Remove the adipose tissue with a sterile scalpel.
    4. Add 2 ml collagenase solution and mince the tissue with scalpel, incubate at 37 °C for 45 min to 1 hr, depending on the size of the tissue.
    5. Transfer the digested tissue to a 15 ml conical tube, rinse the petri dish with 2 ml of fibroblast culture medium twice, and collect the medium in the same tube.
    6. Pellet cells at 200 x g for 5 min at room temperature.
    7. Discard the supernatant and wash the pellet with 3 ml of fibroblast medium to remove the collagenase, then pellet cells again. Please refer to the Materials Table for media composition.
    8. Repeat the step 1.2.7 one more time.
    9. Re-suspend the pellet in 5 ml fibroblast medium and plate cells onto a T25 sterile tissue culture flask. Incubate the flask at 37 °C with 5% CO_2.
    10. Evaluate the culture for fibroblast attachment and growth over the next 1-3 days.
    NOTE: Some small tissue pieces may also attach to the plate and fibroblasts migrate out of these tissue pieces.
    11. Maintain the culture under the same conditions until fibroblasts are grown to approximately 80% confluence.
    12. Collect fibroblasts by trypsinization and transfer onto fresh culture flasks for additional expansion. Perform trypsinization by washing the cultures 3 times in 1x PBS free of Ca^{++} and Mg^{++}. Add Trypsin-EDTA (see Materials Table) to the cells (2ml/ T25 flask) for 2 min at 37 °C.
    13. Split detached cells into additional flasks. If some tissue pieces remain attached to the original T25 flasks, add 5 ml fresh culture medium to this flask and more fibroblasts will migrate out continually.
    NOTE: The expanded fibroblast culture can be frozen down as P1 and stored in liquid nitrogen for future experiments.

2. Immortalization of Myogenic Cells

1. Plate 5 million Phoenix Ecotropic packaging cells (PE) overnight in a 10 cm sterile tissue culture plate in DMEM and Medium 199 in a ratio of 4:1, supplemented with 10% calf serum.
2. Feed cells 30 min prior to transfection with 5 ml of fresh media supplemented with 10 mM caffeine.
3. Homogenize a mixture of 2 µg of plasmid DNA from a midi prep (CDK4 or hTERT plasmid) and polyjet and incubate at room temperature for 15 min, as recommended by the manufacturer.
4. Add the plasmid/polyjet mixture to the PE cells overnight.
5. Feed cells with fresh medium (DMEM and Medium 199 in a ratio of 4:1, supplemented with 10% calf serum). Twelve hours later, collect the virus-containing supernatant and filter it through a 0.45 µm pore size filter.
6. Use 1 ml of collected supernatant to infect overnight the amphotropic packaging cell line PA317 [5] and obtain a stable virus-producing cell line after selection with either 0.5 mg/ml neomycin (G418) for CDK4 or 0.5 mg/ml hygromycin for hTERT.
7. Prepare working viral supernatants by growing the stable packaging cells to near confluency, then harvesting the supernatant each morning, evening and morning for three harvests.

8. Filter the viral supernatants and either directly use or divide them into 1 ml aliquots and store at -80 °C for later use. Note that viral supernatant loses 50% infection efficiency with each freeze-thaw. Remember to bleach-wash before discarding everything that has touched the viral particles.
   NOTE: The stable PA317 virus-producing cell line can be frozen and maintained at -150 °C for permanent storage (freezing medium is 10% DMSO; 90% Serum).
9. Plate FACS-purified myogenic cells (described in steps 1.1-1.9) at a density of 5 x 10^4 cells/well in 6-well plates coated with 0.1% gelatin. Ensure that cells are attached to the plate before proceeding with the viral infection.
10. Add 400 µl filtered, freshly produced viral supernatant or frozen aliquots to each six-well plate overnight (keep 2 wells as controls).
11. Change medium by feeding cells with 2.5 ml/well of fresh muscle media (4:1 Dulbecco's modified Eagle medium (DMEM) and Medium 199 supplemented with 15% fetal bovine serum; 0.02 M HEPES buffer; 1.4 mg/L vitamin B12; 0.03 mg/L ZnSO4, 0.055 mg/L dexamethasone, 2.5 µg/l hepatocyte growth factor and 10 µg/L basic fibroblast growth factor). Discard the media and pipettes containing the viral particles in a bleach container. Leave cells in the same medium for 3 days to recover from infection, then treat for selection using either 400 µg/ml neomycin (CDK4 infection) or 300 µg/ml hygromycin (hTERT infection).
12. Maintain cells under drug selection until the cells in the control dish die (1-2 weeks).
13. Passage cells before they become confluent (60-80% confluency; using 0.05% trypsin EDTA), even during the selection period. Replate cells in multiple 10cm dishes with fresh myoblast medium (as described in 2.11) supplemented with the selection drug. Maintain immortalized selected cells as a heterogeneous population or clone to obtain a completely homogeneous genetic background (same insertion of the transgene in every cell).
14. Perform clonal selection using the following steps:
    1. Seed the cells at low density (*e.g.* 300 to 500 cells in 10 cm dishes) and maintain them for approximately two weeks, until small colonies are formed (10-20 cells).
    2. Remove most of the medium at this point, leaving only a thin film to prevent the cells from drying out.
    3. Place a cloning ring (with one end dipped in silicone vacuum grease) over each desired clone and add a few drops of trypsin/EDTA.
    4. Harvest cells once they become rounded by carefully aspirating the cells using a 1 ml tip or a Pasteur pipet and transferring them to the smallest available multiwell plate (96 or 48, 24 or 12 well plates).
    5. Expand clones as needed to prevent local confluency.

Representative Results

Figure 1 illustrates some of the key steps involved in the primary tissue dissociation: the exact amount of tissue is weighed in a sterile tissue culture petri dish (**Figure 1A, B**). The tissue is then finely minced using sterile scalpels, until a tissue slurry is obtained (**Figure 1C, D**). Following addition of the digestion enzymes, primary muscle tissue dissociation is usually achieved by enzymatic digestion within 45-90 min. The progression of tissue digestion is typically monitored every 15-20 min to prevent overdigestion and cell death. The enzymatic digestion can be gently pipetted and mixed a few times every 15-20 min. At the end of the digestion step, the cells are filtered through a 100 µm (**Figure 1E, F**) and then a 40 µm filter. Depending on the amount of starting muscle tissue and whether the sample is obtained from mildly or severely affected muscle, the dissociated primary cells will be heterogeneous. **Figure 1G** shows an example of mononuclear cells in suspension immediately following the digestion, while **Figure 1H** shows an example of dissociated primary cells 3-6 hr following digestion and plating in tissue culture dishes. Myogenic cells will usually be mixed with fibroblasts and adipogenic cells. Immune cells may also be present in cases where inflammation is associated with the patient's disease. Therefore, prospective separation of the different cell types might be desirable. For prospective isolation of human myogenic cells, CD56 or N-CAM has been widely used as a reliable marker [6-9]. This purification might be beneficial shortly after isolation for enrichment of myogenic progenitors and prior to the cell immortalization process. Alternatively, immortalization of primary cells can be performed at first, then immortalized myogenic cells can be selected based on expression of CD56 by FACS. A schematic on the steps required prior to FACS sorting and an example of a FACS profile is illustrated in **Figure 2**. Briefly, primary cells are counted and resuspended at high concentration as indicated, then the primary antibody (*i.e.* anti CD56) is added to the cells and incubated for 30 minutes on ice. Following a wash, cells are analyzed through a fluorescence-activated cell sorter and purified (**Figure 2**).The approximate yield of CD56 positive cells varies from sample to sample, ranging from 40-70% of the total mononuclear cells. The yield varies depending on the age of the individual and whether myogenic cells are extracted from unaffected or diseased muscle. Unaffected muscle usually has high percentage of myogenic cells, while dystrophic muscle often has lower percentages of CD56 positive cells. The purified cells are plated in complete myogenic growth medium (see **Materials Table**). Cells can be passaged by trypsinization when they reach 60-70% confluence. CD56 expressing cells are myogenic and capable of forming myotubes *in vitro* [10,11].The immortalization of myogenic cells requires the sequential transfection of two genes [12,13] (as schematized in **Figure 3**). Cyclin dependent Kinase 4 (CDK4) is used to overcome the stress of tissue culture due to inadequate culture conditions, and human telomerase reverse transcriptase (hTERT) prevents telomere shortening due to the end replication phenomenon [12]. Both pre-viral plasmids are in the pBAbe backbone vector. The mouse CDK4 cDNA construct contains the neomycin resistance gene and hTERT cDNA construct contains the hygromycin resistance gene. The latter also contains a Herpes Simplex Virus thymidine kinase (TK) cassette and loxP sites placed in both ends of the hTERT/TK cassette. This allows the excision of the entire expression unit by the Cre recombinase [14] and counter-selection using gancyclovir.

For immortalization, working viral supernatants are prepared by growing the stable packaging cells to near confluency, then the supernatant is harvested in the morning, evening, and morning for three harvests. The viral supernatants are filtered and either directly used or divided into 1 ml aliquots and stored at -80 °C for later use. Viral supernatants can lose 50% of infection efficiency with each freeze-thaw.

Newly immortalized cells can be maintained as a population where the viruses are integrated in different sites, or cloned, to obtain a completely homogenic genetic background (single insertion in the cell genome and its progeny). In most cases, isolating clones from one main population will results in slight differences among clones depending on the insertion of the cassette in the cell genome. In addition, extensive tissue culture will favor selection of cells with growth advantage rather than the ones that differentiate. Isolation of single clones is done simply by seeding cells at low density (*e.g.*: 300 to 500 cells in 10 cm dishes) and cultivating for about two weeks until small colonies are formed (10-20 cells). Most of the medium is then removed, leaving only a thin film to prevent the cells from drying out. Clones are trypsinized using cloning rings and then expanded as needed. Expanded immortalized clones can be tested for expression of common myogenic cell markers, such as CD56 by FACS,

desmin and MyoD expression by immunofluorescence, or myotube formation upon differentiation. Myotubes are most apparent when confluent myogenic cells (90% confluency) are placed in differentiation medium (DMEM and Medium 199 in a ratio of 4:1, supplemented with 2% horse serum) after 3-5 days (**Figure 4**). No differences were seen in myotube formation and in myotube contraction ability between non-immortalized control cells and immortalized cells (Videos 1 and 2).

Verification of the immortalization steps can be assayed by several methodologies, including cell growth curves, measurement of telomere length and assessment of telomerase activity (**Figure 5**). Examples of growth curves are shown in **Figure 5A**, where telomere shortening was observed as cells were expanded for extended number of passages. Conversely, successful immortalization is associated with increased telomerase activity (**Figure 5**)

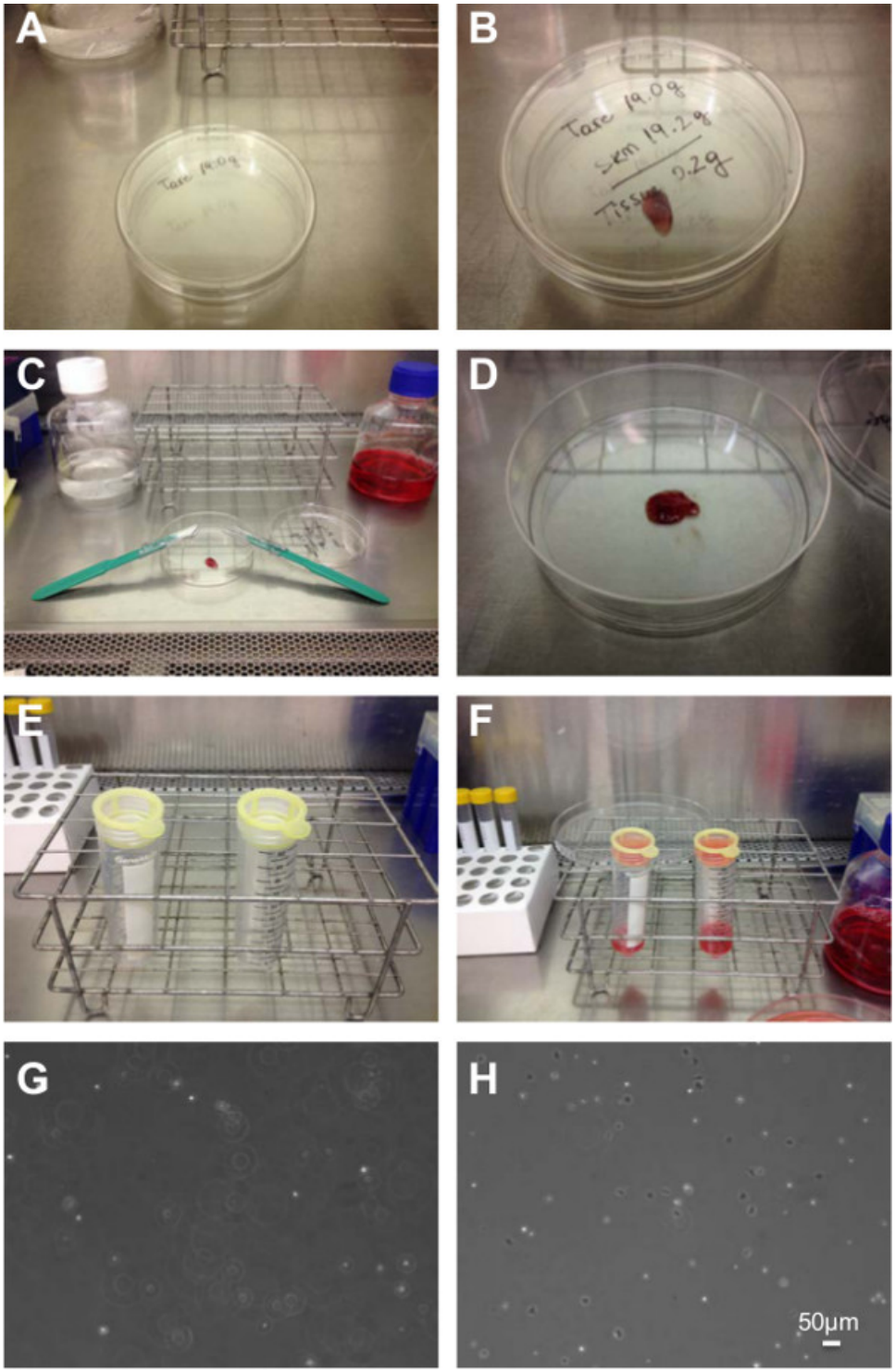

Figure 1: Illustrations of critical steps for the dissociation of primary human muscle tissue. (A) Weighing of the empty culture plate, prior to tissue placement and **(B)** following placement of the tissue. **(C)** Sterile scalpels are used to mince the tissue, until **(D)** the tissue itself is reduced to a slurry. Collagenase and dispase are added and tissue is digested for 45-90 min, then the digestion is filtered through a nylon mesh filter to discard debris **(E, F)**. **(G, H)** examples of freshly dissociated cells plated immediately after dissociation **(G)** or 3 hr after dissociation, when primary cells begin to settle **(H)**.

1. Count cells, resuspend at a concentration of $1x10^6$ cells/100 µl

2. Add anti-CD56 antibody (0.25µg/$1x10^6$ cells)

3. Incubate on ice for 30 min, wash

4. FACS sorting

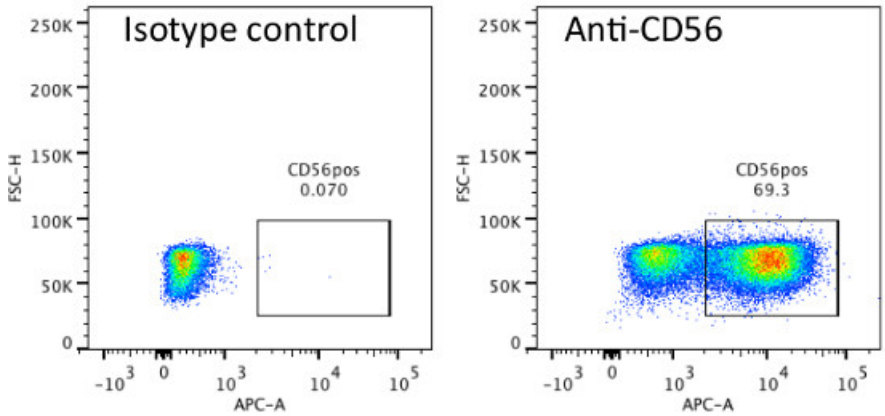

Figure 2: Workflow of procedures involved in human myoblast staining prior to purification via fluorescence activated cell sorter. The critical steps for staining of human cells before FACS analysis are highlighted. Examples of FACS plots are also shown. The negative control (left panel) is used to establish the sorting gate. In the right panels, positive cells expressing CD56 are gated and the percentage of positive cells is shown. This strategy can be used to purify myogenic cells either prior to immortalization or following the immortalization procedure.

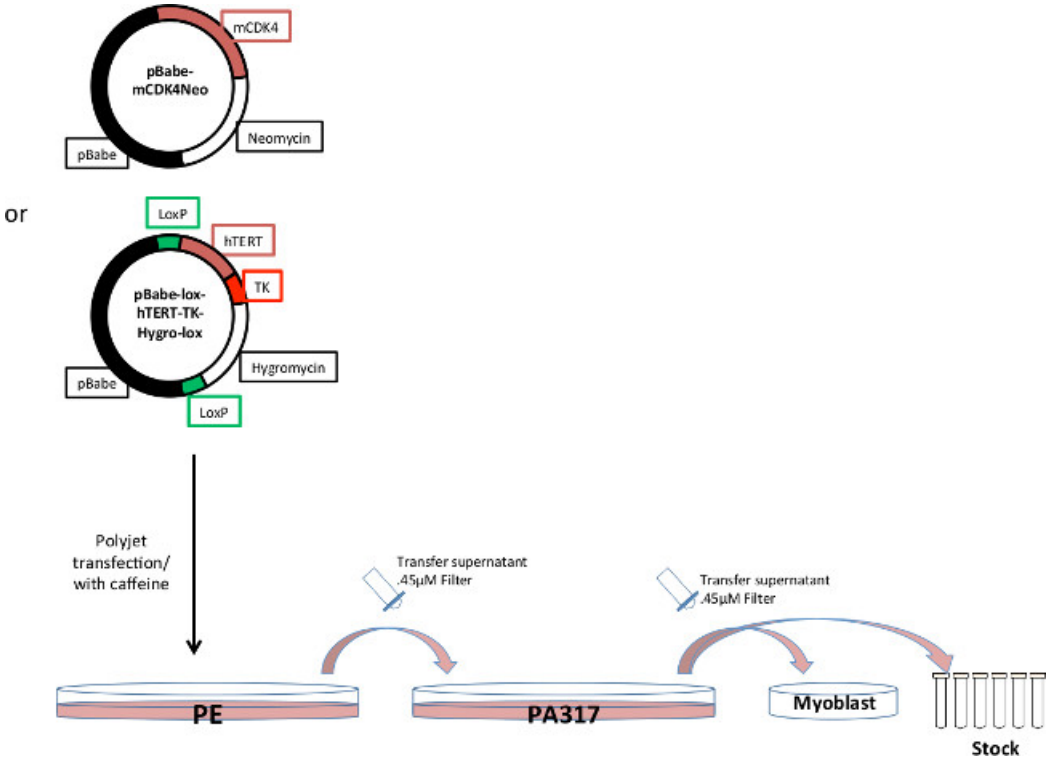

Figure 3: Schematic ofvirus production for myoblast immortalization. Workflow of the retrovirus production used to immortalize myoblasts. A plasmid construct containing either CDK4 or the floxed hTERT-TK cassette in a pBabe backbone (left) is transfected into the Phoenix ecotropic (PE) packaging cell line overnight (8-12 hr) using polyjet. The supernatant is then transferred after filtration onto the Phoenix amphotropic packaging cell line (PA317). The supernatant from these cells can either be used directly or the cells can be selected with either neomycin or hygromycin to generate a stable cell line for future use. Aliquots of the filtered supernatant can be stored at -80 °C for later use (with a lost of 50% efficiency). Bleach is used to clean every consumable used at all points of the procedure.

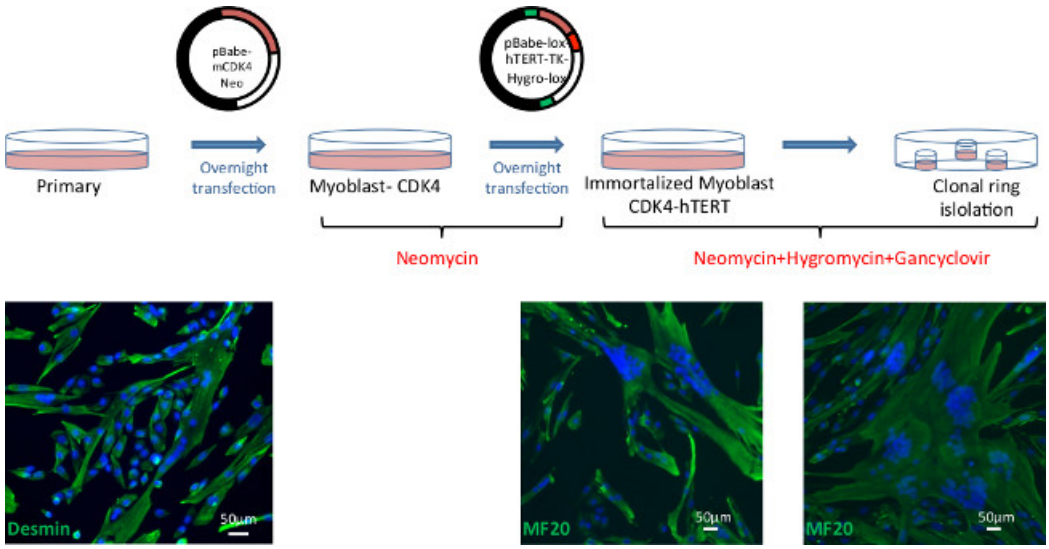

Figure 4: Schematic of reversible myoblast immortalization. Workflow of the immortalization procedure (top): Primary cells are first transfected with CDK4. After Neomycin selection, the myoblasts are transfected with a floxed hTERT cassette containing two selection markers; HSV-TK and Hygromycin. If desired at a later time, the hTERT cassette can be "floxed" out by expressing Cre recombinase and selecting for excision with gancyclovir. This allows the "re-mortalization" of the myoblasts and the re-initiation of telomere shortening. If the telomeres are 20 kb long, this still permits hundreds of doublings, and avoids the complication of the over-expression of hTERT. Finally, isogenic clones from the immortalized population can be isolated. The myogenicity of the newly immortalized cells can be shown using specific markers of muscle cells such as desmin (bottom left panel, cells in growth medium stained with anti-desmin, clone D33, green). This clone is so myogenic that differentiated cells are seen even if cells are maintained in proliferation conditions. When cells are differentiated in differentiation medium (with 2% horse serum, middle and right panels) myotube formation becomes very extensive. Almost all the cells stained with the MF20 antibody to embryonic myosin heavy chain (green) and myotubes have multiple nuclei by DAPI staining (blue, all images). Please click here to view a larger version of this figure.

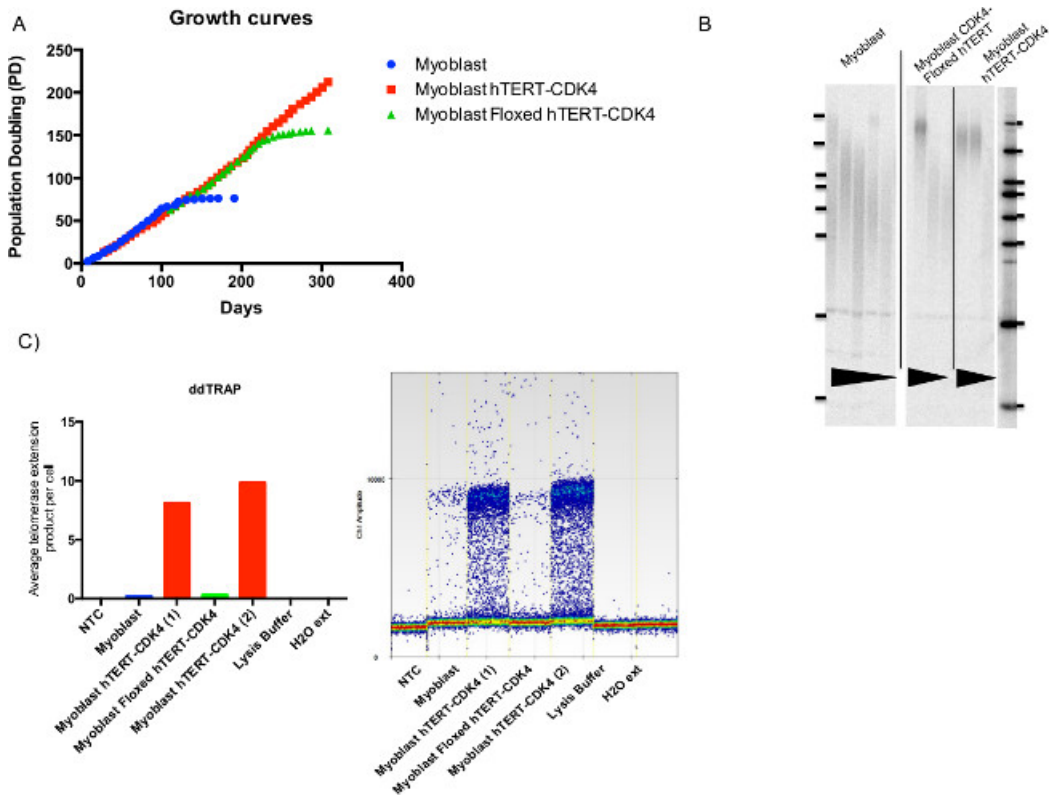

Figure 5: Verification of the immortalization procedure. (A) Growth curves of Isogenic clones from primary myoblast, immortal population (myoblast hTERT-CDK4) and re-mortalized clone (Myoblast floxed hTERT-CDK4) are shown as examples. No differences in growth rates were detectable before the cells reach senescence. Telomere shortening was monitored as a function of population doublings and telomere length was measured by TRF. **(B)** TRF shown are examples of early time points and late replication in myoblasts (5 time points from PD 13 to 75), re-mortalized myoblasts after hTERT excision (3 time points from PD 62 to 152) and immortalized myoblasts (PD 75 and 200). **(C)** Successful hTERT infection translates into an increase in telomerase activity. Telomerase activity was assessed by ddTRAP (ddTRAP readout, right; quantification of the assay, left) [15] in cells before and after hTERT infection. hTERT excision abolished telomerase activity to the original threshold seen in myoblasts. Results are shown as total telomerase products generated per cell equivalent (background corrected). Please click here to view a larger version of this figure.

Please click here to view this video.
Please click here to view this video.

Videos 1 and 2. Myotubes were generated from cells before and after the immortalization process. Cells were grown to confluence in growth media and switched to differentiation media for 10 days. In both videos, functional contractions of one or more myotubes are detectable. Recording of the spontaneous twitching was done using a 20X lens. No differences were observed between immortalized and non-immortalized cells.

Discussion

Cells as a Useful Resource

The isolation and culture of myogenic cell populations is extremely useful when establishing disease phenotypes or *in vitro* models of disease. The myogenic cell isolation procedure described here allows the isolation of myoblasts and fibroblasts from skeletal muscle specimens, which can then be propagated, differentiated, or immediately analyzed. Myoblast structure and function can be assessed through microscopic examination, evaluation of cell survival, evaluation of cell fusion, or through molecular studies of RNA or protein expression. Additionally, myoblasts can be differentiated into myotubes that share many features of immature myofibers, which allows the direct assessment of myoblast function in the context of muscle development and recovery following tissue damage. While all of these phenotypes tend to be fairly reproducible in early passages of myogenic cells, the behavior of many primary myogenic cell lines can be altered over the course of many passages. As a result, it is sometimes desirable to immortalize a given myogenic cell line (as described here), which can facilitate the ease of culture establishment and the generation of reproducible results over a longer period of time.

Selection of Specific Cell Populations

This protocol describes the isolation of myogenic cells from skeletal muscle tissue using FACS as a purification scheme. The proposed strategy of selecting cells includes using propidium iodide (an indicator of dead cells) and CD56 (a marker of myogenic cells) to differentiate live, myogenic cells from dead cells, debris, and non-myogenic cells. Cells contained in the CD56 positive population will include myogenic progenitors capable of forming myotubes, while the CD56-negative population will include non-myogenic cells including fibroblasts and possibly adipogenic cells. Each of these fractions can be independently cultured to produce cell cultures of myoblasts or fibroblasts, and the potential

to establish parallel fibroblast cell cultures can be useful for independent cell or DNA banking exercises or as additional control conditions for assays being performed on the myogenic cell cultures. There are several other options for the selection of specific myoblastic and fibroblastic cell cultures from digested muscle tissue, in cases where FACS sorting is undesirable or unavailable, which are described elsewhere [16,17]. One alternative method to separate myoblastic and fibroblastic cells involves the use of differential adhesion properties between these two cell types to enrich successive passages of cells for a given cell type. As fibroblasts adhere to plastic much more readily than myoblasts, allowing the cell suspensions to incubate on plastic for approximately 1 hr when establishing or passaging the cells and then plating the supernatant onto a different dish will result in the removal of many fibroblasts from the cell suspension [18].

Utility of Fibroblasts vs. Myoblasts

Muscle biopsies from patients are typically used for the purification of primary muscle cells, however diseased myoblasts may not proliferate effectively and can only undergo a few number of divisions *in vitro*, thus requiring cell immortalization to reach the high number of divisions necessary. Given that both fibroblasts and myoblasts can be isolated from muscle biopsies, both cell fractions should be saved from patient samples, as they can both be useful and offer much versatility for downstream applications. For example, fibroblasts can be used for the generation of induced pluripotent stem cells (iPSCs), which hold much promise to generate unlimited cell numbers and the potential to be induced to differentiate into multiple cell lineages. These features are highly desirable for cells obtained from patients with rare diseases, which can potentially be corrected *in vitro* or used in experimental drug screenings in search for therapeutic compounds. Immortalized myoblasts can also be used in drug-based screening. Fibroblasts can also be effectively converted towards a myogenic fate by infection with a virus expressing MyoD [19-21]. This method has been effectively tested using fibroblasts extracted from patients with muscle diseases and confirmed that MyoD-expressing fibroblasts can effectively form myotubes that express mature muscle proteins. Thus, both cell types can be effectively used in several downstream applications and provide invaluable material for diagnostic and therapeutic studies.

Impact of Immortalization

The immortalization of normal diploid cells allows one to prepare a stable human cell line that can be fully characterized and studied by many different laboratories. Primary cultures from independent isolations can vary and their overall proliferative capacity can be variable when damage is introduced in the dissociation process or by overdigestion, which can distort the culture behavior. The immortalization of cells using the expression of the catalytic subunit of telomerase (hTERT) has the advantage to maintain a stable cellular phenotype and cells remain diploid. There have been reports in mice that telomerase can modulate the Wnt pathway [22]. We have not observed this phenomenon in human cells. However, to avoid such potential complication, the hTERT cassette has been flanked with lox sites, so that it can be removed after telomeres have been elongated. Long telomeres confer hundreds of extra divisions, usually sufficient to carry out most experiments.

Because continuous cell expansion always provides a selective advantage for rapid growth, the cellular phenotype can occasionally become biased due to overgrowth of the most rapidly dividing cells, rather than the best differentiating cells. While this has not yet proved to be a significant problem using the described method, it can be remedied by thawing cells that have been frozen early in their culture history or by clonal selection for the cells exhibiting the desired phenotype.

Lastly, while expansion of myogenic cells to near unlimited numbers has advantages for drug screening and possible disease modeling, the current techniques also have intrinsic disadvantages that prevent the use of these cells as therapeutic vehicles in cell-based therapy, or as primary diagnostic tools. Both the extensive *in vitro* culture and immortalization steps change the phenotype of muscle cells compared to their native state. Importantly, these cells are expanded in an environment that is very different from the natural milieu of primary satellite cells within their niche. Therefore, immortalized cells require intense safety testing and possibly development of new techniques if they are to be re-introduced into human patient's muscle.

Disclosures

The authors have nothing to disclose.

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