Video Article

Isolating Stem Cells from Soft Musculoskeletal Tissues

Yong Li[1,2,3,4], Haiying Pan[1], Johnny Huard[1,2,3,4,5]

[1]Stem Cell Research Center, Childrens Hospital of Pittsburgh of UPMC

[2]Department of Bioengineering, University of Pittsburgh

[3]Department of Orthopedic Surgery, University of Pittsburgh

[4]Department of Pathology, University of Pittsburgh

[5]Department of Molecular Genetics & Biochemistry, University of Pittsburgh

Correspondence to: Yong Li at yongli@pitt.edu

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Abstract

Adult stem cells have long been discussed in regards to their application in regenerative medicine. Adult stem cells have generated a great deal of excitement for treating injured and diseased tissues due to their impressive capabilities to undergo multi-lineage cell differentiation and their self-renewal ability. Most importantly, these qualities have made them advantageous for use in autologous cell transplantation therapies. The current protocol will introduce the readers to the modified preplate technique where soft tissues of the musculoskeletal system, e.g. tendon and muscle, are 1st enzymatically dissociated and then placed in collagen coated flasks with medium. The supernatant, which is composed of medium and the remaining floating cells, is serially transferred daily to new flasks. The stem cells are the slowest to adhere to the flasks which is usually takes 5-7 days (serial transfers or preplates) . By using this technique, adult stem cells present in these tissues can be easily harvested through fairly non-invasive procedures.

Video Link

The video component of this article can be found at https://www.jove.com/video/2011/

Protocol

In general, the complete modified preplate procedure requires 1-2 days of preparation, 1 week of performing the technical procedure, 2-3 days of cell identification with immunocytochemistry, and an additional 2-3 weeks of stem cell population expansion. The equipment required for stem cell culture are similar to that of other cell culture systems which includes a bench-top centrifuge, CO_2 incubator, Laminar flow tissue culture hood and an inverted microscope equipped with fluorescence and a digital camera. The isolated stem cells also can be cloned and can be stored long-term in liquid nitrogen for the current or future studies [1,2]. All reagents and materials used for this procedure must be sterile and handled aseptically.

Step 1: Preparation

1. Collagen coat tissue culture dishes and flasks: Type I collagen derived from calf skin (0.1 g in 1 liter; Sigma-Aldrich). The plasticware to coat includes: T-25 flasks, 6 or 12 well plates, dishes (60 and 100 mm, BD Falcon) as described before 1,2. Gently shake the plasticware every half hour, for 4 hours to ensure even coating of the collagen. The collagen solution is then removed. And the coated plasticware is then allowed to dry in the tissue culture hood with the UV light and the hood both on, to ensure sterility, and finally recapped or covered for later use.
2. Prepare cell culture medium : 400mL DMEM (DMEM, high glucose; Invitrogen), 50mL Heat Inactivated Fetal Bovine Serum (FBS, Invitrogen), 50mL Horse Serum (HS, Invitrogen), and 5 mL Chick Embryo Extract (CEE; Accurate Chemical Co.) will be vacuum filtered using a disposable 0.22-µm 500-ml sterile filter system (Corning). Sterile penicillin/streptomycin solution (Invitrogen) will be added finally 100 Units/mL. The prepared solutions can be stored at 4°C for at least one month. This solution is used to detach the cells from the flask when splitting cultures or preparing the cells for transplantation.
3. Warm up solutions for cell isolation: The following reagents need to be warmed up to 37°C prior to their use for cell isolation: Hank's Buffered Salt Solution (HBSS, Invitrogen); 0.2% (wt/vol) collagenase-type XI (Sigma-Aldrich), with an average of 3,200 collagen digestion units per mL HBSS; Dispase 2.4 units/1mL (Invitrogen); 0.5% (wt/vol) trypsin (Invitrogen).
4. Sterilize surgical equipment in preparation: Surgical procedure require sterile surgical instruments including: different-sized forceps, micro-scissors, iris scissors and/or scalpel blades. Additionally, 15 and 50-mL polypropylene centrifuge tubes (BD Falcon); Cell strainer (pore size 70 m) (BD Falcon); 18-, 23- and 27-gauge needles (BD PrecisionGlide); and 10- or 20-ml sterile syringes (BD).

Step 2: Tissue biopsies, isolation and mechanical dissociation[1-4]

The tissue biopsies are performed under a sterile culture hood and include freshly harvested tendons and skeletal muscles are obtained from the tibia or soleus muscles of the hindlimb of wild-type mice (Female C57BL/6J, 4-5 weeks of age or younger, Jackson Laboratory). Any visible connective tissue, blood vessels, and adipose tissue are then removed from the biopsy samples. The tissues of tendon and muscle are then carefully identified under a surgical dissecting microscope to remove remnants of skin and bone and placed in a dish containing cold (4°C) HBSS (supplemented with add 5 % of FBS). The isolated tissues are then separately placed on collagen coated dishes containing cold HBSS and will be minced (<1x1 mm^3) into a coarse slurry using micro-scissors and/or scalpel blades.

Step 3: Enzymatic digestion of the tissues:

The minced tissue is then enzymatically dissociated through a series of steps[2,4,5]

1. Transfer the minced tissue slurries into separate 15-ml tubes and centrifuge at ~3500 rpm at 4 °C for 5 mins.
2. Remove the supernatant, wash with HBSS and repeat the centrifugation again.
3. Remove the supernatant, and digest these slurries by adding 10 ml of prewarmed 0.2% collagenase-type XI (Sigma). Incubate for 60 mins at 37 °C while continuously gently rocking the tubes. Alternatively, shake by hand every 10 min.
4. Centrifuge and resuspend these slurries in 10 ml dispase (2.4 Units/mL, Gibco) solution. Incubate for 45 mins at 37 °C, shaking with hands or gently rocking every 10 min.
5. Centrifuge and re-suspend these slurries in 10 ml of 0.2% trypsin HBSS solution. Incubation will vary based on the extent to which the single cells are released from the tissues which can be performed by observing the suspension under microscope. It is not recommend to exceed 30 mins at 37 °C with inverting the tubes or gently rocking every 10 min.
6. Centrifuge the resulting cells and re-suspend the cell pellet in 10 ml of Proliferation Medium (PM).
7. Dissociate the cell suspension by passing the extract through a series of needles. The cells are then passed 2 times through an 18G, then a 23G, and finally a 27G needle.
8. Pass the cell extract through a 70-µm cell strainer.

Step 4: Preplating to isolate different cell populations based on cell adhesion rate[1-3]

1. Centrifuge and resuspend the cell pellets in Proliferation Medium.
2. Plate the cell mixtures onto a collagen-coated T-25 flask (or 60 mm dish) and mark it as PP1. The cell suspension should be observed as possessing large numbers of refractive nuclei and other debris under the microscope.
3. Incubate at 37 °C in a humidified, 5% CO_2 incubator for 2 hours.
4. Transfer nonadherent cells to a new collagen-coated T-25 flask (or 60 mm dish) and mark is as PP2. Add 5 ml of PM into the plate labeled PP1. The early adhering cells which attach to the flask are mostly myofibroblasts[3].
5. Return the flasks to the incubator for another 24 hours, transfer nonadherent cells to a new collagen-coated T-25 flask (or dish) and mark it as PP3. Add 5 ml of Proliferation Medium into the plate labeled PP2. These adhering cells which attach to this flask are mostly fibroblasts[2,3].
6. Return flasks to the incubator and repeat the procedure in another 24 hours until population PP6 is created. Maintain each group of suspended cells and allow them to proliferate and examining them regularly using an inverted microscope. PP3-PP4 are mostly myoblasts while muscle satellite cells are often seen mostly in PP5 [2].
7. Most of the slowly adhering cells typically attach by PP6. At this stage, the cells appear small, round, light refractive and are very sparse in number and are considered to be a stem cell population 1,2.

Step 5: Identification and expansion of isolated stem cells

1. The isolated PP6 cells are very few in number and need to be maintained in FBS rich Proliferation Medium (20% FBS in DMEM) that is changed daily. Most of the cells present in the PP6 culture die during the following 1-2 weeks of culturing, but a large, healthy PP6 population is usually created after an additional 1-2 weeks of expansion. The stem cells isolated from mice highly express stem cell antigen 1 (Sca1), CD34, fetal liver kinase 1 (Flk1), and other stem markers[1,2,6,7] which can be visualized via immunocytochemical staining. These stem cells also exhibit multiple differentiation capacities[3,6,8].
2. The isolated cells are maintained and expanded at a low cell density in culture (12-well dishes at 50-100 cells/well or <20-30% confluence in the flask or dish) to avoid differentiation [1,2]. Very few cells form clones during expansion, and these cloned stem cells can undergo long time proliferation (LTP) for any other competence studies. The cloned stem cells also can be store in liquid nitrogen using general cell storage procedures e.g. PM medium with 10% DMSO. Make a number of low passage stocks of the stem cells for any other back up studies.

Discussion

It has been recognized that some adult tissues contain multiple stem cells. Over the past few years of study, stem cells have been detected in the soft musculoskeletal tissues, including skeletal muscle and tendon. This current protocol details a procedure, known as the modified preplate technique, that has been successfully used in our laboratory to isolate adult stem cells from tendons and skeletal muscle. Using the modified preplate technique described above the cells can be divided into several populations based on their adhesion characteristics to collagen coated flasks (Figure 1). Fibroblasts and myofibroblasts found within the tissues quickly adhere to the collagen coated flasks within 24-48 hours and are initially separated from other cells in PP1-PP2. The myogenic or endothelial cells adhere to collagen coated flasks after a longer time period, within 48-96 hours (PP3-PP5), and can be harvested and identified by their cell surface markers. The later preplated cells, 96 hours or later (PP6), are regarded as adult stem cells (Figure 2). The late preplate cells appear small, round, and translucent at the beginning of their isolation and can be further characterized by flow cytometry or immunocytochemistry for their expression of stem cell antigen 1 (Sca1), CD34,

fetal liver kinase 1 (Flk1), and other stem cell markers (Figure 3). The isolated stem cells have the capacity for self-renewal and experimental studies have demonstrated their multipotency through their differentiation into the three germ layers: mesoderm, ectoderm, and endoderm [9]. Multiple investigations have also revealed that these stem cells differentiate into cells of the mesoderm lineage including osteocytes, adipocytes, chondrocytes, and hematopoietic cells [6,8]. Other studies have demonstrated that the adult stem cells differentiate into ectoderm cell lineages through their expression of neuronal and glial cell markers and their ability to improve limb function after the peripheral nerve has been damaged [10]. These isolated adult stem cells have been beneficial for repairing injured and diseased musculoskeletal tissues, and other related *in vivo* studies have been performed on other tissues including heart, liver, and spinal cord as well as specific medical conditions like small intestinal submucosa and bladder to treat stress urinary incontinence [9].

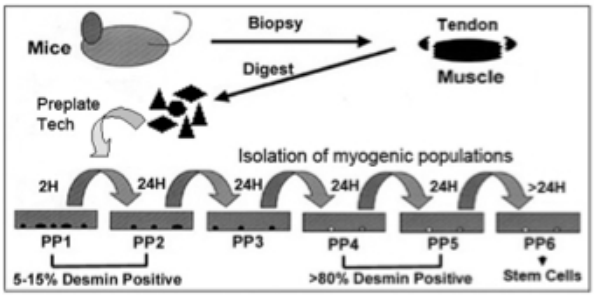

Figure 1.

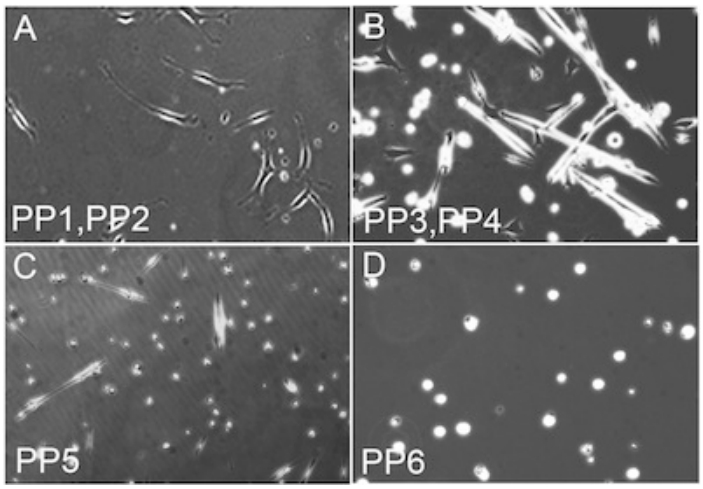

Figure 2.

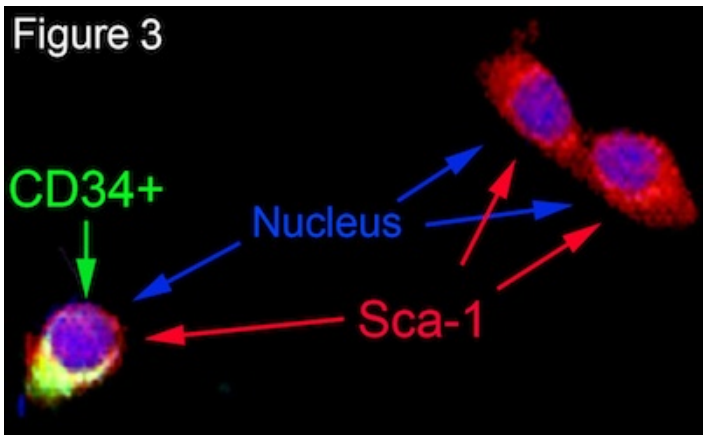

Figure 3.

Disclosures

No conflicts of interest declared.

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