Video Article

# Isolation and Animal Serum Free Expansion of Human Umbilical Cord Derived Mesenchymal Stromal Cells (MSCs) and Endothelial Colony Forming Progenitor Cells (ECFCs)

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## Abstract

The umbilical cord is a rich source for progenitor cells with high proliferative potential including mesenchymal stromal cells (also termed mesenchymal stem cells, MSCs) and endothelial colony forming progenitor cells (ECFCs). Both cell types are key players in maintaining the integrity of tissue and are probably also involved in regenerative processes and tumor formation.

To study their biology and function in a comparative manner it is important to have both cells types available from the same donor. It may also be beneficial for regenerative purposes to derive MSCs and ECFCs from the same tissue.

Because cellular therapeutics should eventually find their way from bench to bedside we established a new method to isolate and further expand progenitor cells without the use of animal protein. Pooled human platelet lysate (pHPL) replaced fetal bovine serum in all steps of our protocol to completely avoid contact of the cells to xenogeneic proteins.

This video demonstrates a methodology for the isolation and expansion of progenitor cells from one umbilical cord.

All materials and procedures will be described.

## Video Link

The video component of this article can be found at https://www.jove.com/video/1525/

## Protocol

## Part 1: Setting up

### 1. Preparation of cell culture medium

Before starting the medium preparation collect all the materials and tools you will need.
Thaw 2 x 56 ml aliquots of pooled human platelet lysate (pHPL, self-made: reference 1 & JoVE #1523), 10 ml of a 100x Penicillin/Streptomycin solution, 2 x 5 mL of L-Glutamine (both Sigma).

Thaw the cytokine and growth factor aliquots (VEGF, bFGF, EGF, IGF, hydrocortisone, ascorbic acid, Heparin, Amphotericin provided as 'single quots') adjusted for supplementing 1 bottle (500 mL) of EGM-2 medium (Lonza).

From the fridge take one 500 ml bottle each of (i) alpha-modified minimal essential medium (a-MEM) and (ii) endothelial basal medium (EBM).

All of the following steps are performed in a laminar flow tissue culture hood under sterile conditions.

Prepare preservative-free heparin (e.g., Biochrom) by dissolving the powder to a final concentration of 1000 IU/mL with sterile water.

**MSC-Medium:**

Use 500 mL of a-MEM, add 56 mL of thawed pHPL (see also reference 1 for further details) and 2 IU/mL (=224 µl of stock solution) of preservative-free Heparin (avoids coagulation of the medium through clumping of the fibrinogen in the plasma) to reach a final concentration of 10% pHPL. Additionally add Penicillin (100U/mL) /Streptomycin (100µg/mL) solution and 2mM of L-Glutamin (both Sigma).

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Filter the medium through a 20 µm-pore size vacuum filter (Millipore). Label the bottle appropriately (content, date).

**ECFC-Medium:**

Use one bottle (500 mL) of EBM, add the cytokine-aliquots, 56 mL of pHPL, 10 IU/ml (=1120µl of stock solution) of preservative-free Heparin, Penicillin (100U/mL) /Streptomycin (100 g/mL) solution and 2mM of L-Glutamin to the basal medium and filter with a 20 µl-pore size vacuum filter (Millipore). Label the bottle appropriately (content, date).

**2. Sterilization of surgical instruments**

Forceps, sharp pair of surgical scissors and scalpel holder must be heat sterilized ore sterile disposables.

**3. Preparation of tubes for cord collection**

Add 500 IU of preservative - free heparin to 50 ml polypropylene tubes (Falcon) and adjust to a final volume of 20 mL with PBS. Transfer the tubes into the delivery room.

**4. Informed consent (confirmed by your local ethical committee or IRB).**

Ask the parents prior to delivery if they would like to donate a piece of the cord and adequately inform them of the purposes it will serve.

**5. Cord donation**

After delivery and inspection of placenta and cord cut off a 10 cm piece and immediately transfer it to your tube prefilled with heparin to avoid coagulation of the remaining blood inside the cord vessels. Continue on with cell isolation as soon as possible.

## Part 2: Cell isolation

1. Prepare laminar flow tissue culture hood by cleaning surfaces with Incidin or 70% EtOH. Place sterile 150 $cm^2$ cell culture plates, 75 cm cell culture flasks (both Corning), PBS, sterilized surgical instruments, cell scrapers, tube holder, 25 mL stripettes and 5 ml syringes equipped with blunt end needles appropriately in your cleaned hood.
2. Pre-warm your prepared α-MEM and EGM-2 cell culture medium to 37°C in a water bath.
3. Bring the cord from the delivery room to your lab equipped with the cell culture hood. After transferring the cord into the laminar flow wash it twice in PBS (by transferring to a new plate) to get rid of contaminating blood cells. Rinse the umbilical vein after canulating it on one side with a sterile 5mL syringe equipped with a blunt end needle with PBS until the fluid coming out the other end of the cord becomes completely transparent (reddish indicates blood contamination).

**ECFCs:**

4. Prepare a 75 $cm^2$ flask (Corning), label it and fill in 15-20 mL of pre-warmed EGM-2 medium.
5. Place the cord into a new plate filled with sterile PBS.
   Take the surgical scissors and cut the umbilical cord into two pieces of about 5 cm in length (short pieces are easier to process, because the umbilical vessels are twisted along their axis).
6. Try to find the umbilical vein (large vessel), insert one blade of your surgical scissors and cut the vein longitudinally. At this point it could be helpful if an assistant can hold the already cut vein with two forceps while you cut further.
7. Place the cord piece with the cut vein in a new plate without PBS, take the cell scraper and scrape of the inner (endothelial) surface of the umbilical vein by rubbings from one end to the other. Transfer the cells to your prepared flask by cleaning the tip of the scraper in the medium. Repeat this procedure at least 10 times.
8. Close your flask and put it into an incubator (37°C, 5% $CO_2$, 95% Humidity).

**MSCs:**

4. Prepare a 150 $cm^2$ culture plate (Corning) and label it.
5. Take the piece of cord (after scraping off the endothelial layer) and cut the whole cord into very small pieces by using sterile scissors and sterile scalpel. Maximum size of the cord pieces should be 1-2 mm.
6. Transfer your cord pieces with the sterile forceps into your pre-labeled culture plate. By leaving the plate open for 5 minutes before filling up with medium, the attachment of the pieces to the plastic surface is strengthened.
7. Once the cord pieces are connected appropriately to the plastic, gently add in 30-35 mL of pre-warmed alpha modified MEM. Try to use the lowest speed to be sure that all pieces remain attached.
8. Close the plate and put it into an incubator (37°C, 5% $CO_2$, 95% Humidity).

## Part 3: Cell expansion and confirmation of immune-phenotype by flow cytometry

## Cell Expansion

**EFCFs:**

1. Check the attachment of endothelial cells the next day on an inverted microscope and exchange whole EGM-2 medium to get rid of all non-attached cells and particles.

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2. Expand the cells until confluence and exchange one third of the medium twice a week. When confluence is reached, detach the cells with 0.05% Trypsin/0.7 mM EDTA and transfer them to new culture vessels. Depending on the number of cells within your T75 flask, you should choose the size and number of your new flasks to guarantee low seeding density for further expansion.
3. Exchange one third of the medium twice a week during the expansion of the cells.

**MSCs:**

1. The outgrowth of MSCs from the cord will take more time, so you can wait at least 3-4 days till you check on an inverted microscope for the appearance of spindle-shaped cells in the surroundings of the attached tissue pieces. When there is sufficient cell out-growth, the pieces tend to detach because they lose contact with the plastic.
2. After 10-12 days remove the tissue pieces, detach the MSCs from the plastic with 0.05% Trypsin/0.7 mM EDTA and transfer them to new culture vessels. Depending on the amount of cells within your plate you should decide the size and number of your new flasks to guarantee low seeding density for further expansion.
3. Exchange one third of the medium twice a week during the expansion of the cells.

**Flow cytometry**

Equipment needed:

Flow cytometer (BD FACSCalibur), micronic tubes (Corning), tube holder, sheep serum (blocking reagent), Eppifuge (Eppendorf), monoclonal antibodies
MSCs: CD45, CD14, CD19, HLA-DR, CD31, CD73, CD90, CD105, isotype controls
ECFCs: CD45, CD14, CD19, HLA-DR, CD31, CD34, CD90, CD105, CD144, CD146, isotype controls

1. After reaching confluence, detach the cell with 0.05% Trypsin/0.7 mM EDTA and centrifuge at 300g for 7 min at 4°C.
2. Resuspend the cells to a concentration of $1x10^6$ cells/mL in modified PBS and block unspecific antibody binding with 10% v/v sheep serum for 20 min at 4°C.
3. Label the micronic tubes and add the appropriate concentration of fluorescent labeled antibodies.
4. Add 50 µl of cell suspension (blocked) to each tube and incubate for 30 min at 4°C.
5. Wash the cells by adding modified PBS to remove non-binding antibodies.
6. Go with your labeled cell to the flow cytometer and perform the analysis.

## Part 4: ECFC colony growth, fixation, staining and hierarchy analysis

1. Seed the virtually pure harvested ECFCs at very low plating density of 3 or 10 cells per square centimeter in cell culture plates to allow for single colony growth.
2. Exchange one third of the medium twice a week.
3. After 14 days (end of culture) remove medium, wash twice with PBS and fix the colonies with ice cold fixation solution (Acetone : Methanol = 3 : 2) for 15 minutes in the refrigerator.
4. Discard the fixation solution and leave the plates open to dry for 10 minutes.
5. Rehydrate the cells with distilled water for 10 minutes.
6. Add Harris Hematoxylin solution and stain the fixed colonies for 12 minutes.
7. Remove H matoxylin solution and rinse the plates with tap water to get rid of the remaining staining solution.
8. Take photos of each colony by using a stereomicroscope and import *.jpg or *.tif format files onto your computer.
9. Open the photos with ImageJ software and analyze the accurate cell number of each colony as described in the animation.
10. Open the ImageJ software and import a colony image using the ‘File\Open’ function in the pull down menu.
11. Proceed by using the ‘Process\Subtract Background’ function
12. Use the ‘*elliptical* or brush selection’ function in the ImageJ menu to mark the colony margin.
13. Clear the surrounding image using the ‘Edit\Clear Outside’ function and crop the picture by selecting ‘Image\Crop’.
14. Adjust the threshold manually using the ‘Image\Adjust\Threshold’ function, so that all cells are completely colored in red.
15. Count the cell number of the colony by selecting ‘Analyze\Analyze Particles’. Choose appropriate settings (optimized for each cell type) and select ‘OK’. The results box, including cell count and a new image containing the corresponding outlines of the counted cells will appear.

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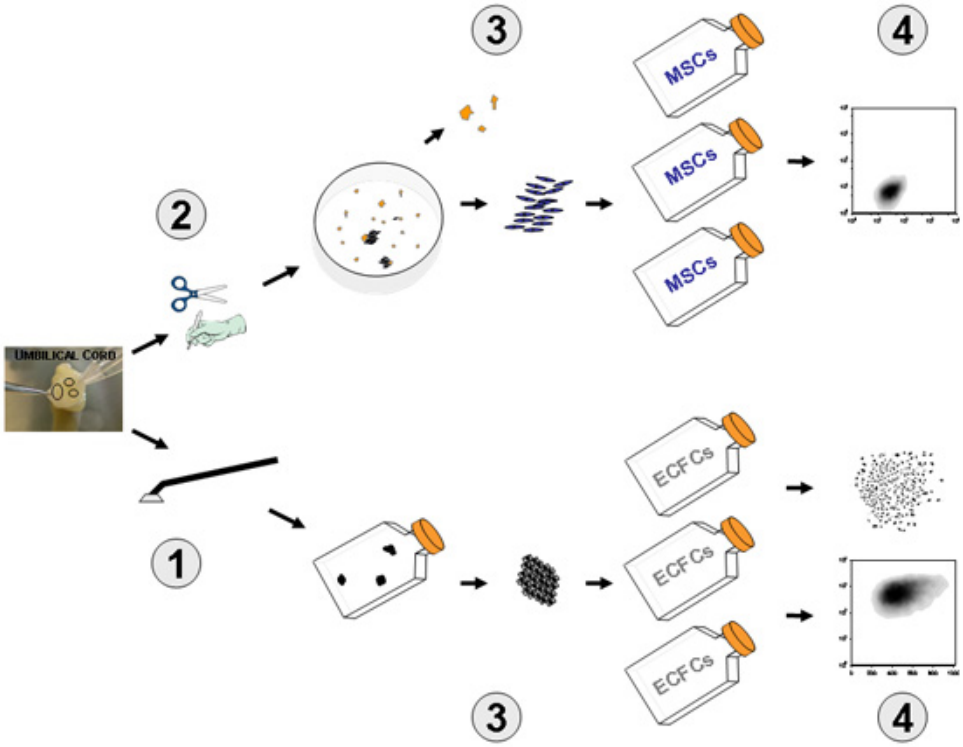


## Discussion

There is a variety of sources from which to get MSCs or ECFCs including bone marrow, adipose tissue, cord blood, etc.

It is necessary to get both types of progenitors from the same source to directly compare the involvement of the different cell types in processes like tissue and vessel regeneration or contribution to tumor progression.

The ease with which to isolate and subsequently study autologous pairs of ECFCs and MSCs from the same donor makes this method advantageous for excluding donor variations within individual experiments.

Possible contamination with hematopoietic cells is minimized through a passaging step and confirmed with flow cytometry.

If done correctly, the isolated cells should (i) share features of progenitor cells, (ii) be available for experimental transplantation in sufficient amounts and (iii) be pure as confirmed by flow cytometry.

## Disclosures

The authors don't have any financial conflicts of interest to declare.

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