# TITLE:

**Isolation of Primary Murine Skeletal Muscle Microvascular Endothelial Cells**

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**SUMMARY:**

Microvascular endothelial cells of skeletal muscles (MMECs) shape the inner wall of muscle capillaries and regulate both the exchange of fluids/molecules and the migration of (immune) cells between muscle tissue and blood. The isolation of primary murine MMEC, as described here, enables comprehensive *in vitro* investigations of the “myovascular unit”.

**ABSTRACT:**

The endothelial cells of skeletal muscle capillaries (muscle microvascular endothelial cells [MMECs]) build up the barrier between the bloodstream and skeletal muscles regulating the exchange of fluids and nutrients, as well as the immune response against infectious agents by controlling immune cell migration. For these functions, MMECs form a functional “myovascular unit” (MVU) with further cell types, such as fibroblasts, pericytes, and skeletal muscle cells. Consequently, dysfunctions of MMECs and, therefore, the MVU contribute to a vast variety of myopathies. However, the regulatory mechanisms of MMECs in health and disease remain insufficiently understood and their elucidation precedes more specific treatments for myopathies. The isolation and in-depth investigation of primary MMEC functions in the context of the MVU might facilitate a better understanding of these processes. This article provides a protocol to isolate primary murine MMECs of the skeletal muscle by mechanical and enzymatic dissociation, including purification and culture maintenance steps.

**INTRODUCTION:**

*Via* the bloodstream, cells and organs are supplied with oxygen, substrates, and other necessary molecules. This interchange takes place in capillaries, the smallest vessels. Capillaries are formed by an inner endothelial cell (EC) layer whose integrity remains a prerequisite to the successful regulation of muscle homeostasis between the intravascular and interstitial space. To ensure a selective transition of soluble factors and cells, ECs constitute a monolayer interconnected by tight and adherens junctions1. Besides their role as a barrier for nutrients or metabolic products, ECs regulate the recruitment of leukocytes in inflammatory processes. Inflammation or tissue damage leads to an upregulation of adhesion molecules on the EC surface and the production of chemokines facilitating leukocyte attachment and transmigration into the target tissue2. Consequently, ECs are critically involved in the regulation of inflammatory processes, such as the defense against pathogens or tissue repair.

Dysfunctions of ECs are directly associated with vascular diseases, chronic kidney failure, and venous thrombosis severe pathogen infections. Furthermore, ECs are virtually always involved in organ-specific autoimmunity, such as diabetes mellitus or multiple sclerosis3. The barrier function between the bloodstream and organs is, therefore, controlled by a concerted interplay of different cell types. In the skeletal MMECs, together with muscle cells, fibroblasts and pericytes form a functional unit, the MVU. Therefore, dysfunctions of the MVU might play a critical role in the pathophysiology of myopathies. However, a deeper understanding of these regulatory mechanisms is still missing and currently precludes the identification of new, urgently needed, therapeutic targets in myopathies.

To investigate the complex physiological and pathophysiological mechanisms, animal models are commonly used. However, *in vitro* models offer the advantage of focusing on the subject of interest by excluding a variety of confounding factors. To investigate processes *in vitro*,it is necessary to isolate pure and viable primary cells. In contrast to cell lines, primary cells isolated from transgenic animals enable researchers to investigate the consequences of genetic modifications *in vitro*.

Here, a method to isolate primary murine MMECs is described by using mechanical and enzymatic dissociation followed by magnetic-activated cell sorting (MCS) techniques for purification. For this purpose, magnetic beads against specific surface markers are used. Platelet endothelial cell adhesion molecule-1 (PECAM1, CD31) is mainly expressed on ECs and can be used to enrich this cell type. To warrant a high cell purity, cells of hematopoietic origin are excluded by a negative selection for protein tyrosine phosphatase receptor type C (PTPRC, CD45). Further, quality controls, the cultivation of primary murine MMECs, potential applications, and limitations, as well as special considerations, are presented.

**PROTOCOL:**

All animal experiments were approved by the local authorities and conducted according to the German animal welfare act (84-02.05.20.13.097).

1. **General Remarks on Animal Experiments**
   1. Perform all mouse experiments in accordance with the guidelines of the respective institutional animal care and use committee.
   2. Keep the mice under standardized conditions and according to international guidelines, such as the Federation for Laboratory Animal Science Associations (FELASA).

NOTE: In general, this isolation technique can be used for mice independent of age, gender, or genetic background. To obtain a sufficient cell number, 4- to 10-week-old males are preferred, because biological properties can vary with age and gender.

1. **Preparation of the Solutions, Media, and Coating**
   1. Prepare digestion solution (DS) by mixing 2.2 mL of Dulbecco’s modified Eagle’s medium (DMEM) with 200 μL of collagenase-dispase and 45 μL of deoxyribonuclease (DNase).
   2. Prepare 500 mL of endothelial cell medium (ECM) by mixing 450 mL of DMEM with 50 mL of fetal calf serum (FCS; approximately 10%), 0.25 mL of basic fibroblast growth factor (bFGF; 20 μg/mL; approximately 0.05%), and 5 mL of penicillin-streptomycin (approximately 1%). Perform a sterile filtration in a glass tube (with filter pores of 0.2 µm in diameter). Afterward, store the solution at 4 °C.
   3. Coat cell culture plates as described below.
      1. For the cultivation of freshly isolated primary murine MECs, cover the whole surface of a 6-well cell culture plate with 1 mL per well of a gelatin-based speed coating solution (see **Table of Materials**) according to the manufacturer’s instructions, for 3 - 5 min at room temperature (RT).
      2. Aspirate and discard the coating solution. Rinse each well with 2 mL of sterile phosphate-buffered saline (PBS), pH 7.1 - 7.5, in two consecutive washing steps.
      3. Aspirate and discard the PBS. Fill up the wells with a 1 mL volume of ECM.

1. **Isolation of Primary Murine MMECs**

Euthanize one adult, 4- to 12-week-old male mouse by cervical dislocation without any anesthesia. The aim is to quickly separate the spinal cord from the brain so as to provide the animal with a fast and painless death.

* 1. Sever the extremities.
     1. Use surgical sharp/blunt (with a cutting edge of 42 mm, straight) and sharp/sharp (with a cutting edge of 23 mm, straight) scissors and straight (serrated, 2 mm x 1.25 mm x 12 cm) and curved (serrated, 1.3 mm x 1 mm x 13 cm) forceps.
     2. Disinfect all surgical instruments with 70% ethanol.
     3. Position the animal on its back and moisten the legs with 70% ethanol. Sever the whole leg by cutting at the hip joint with the sharp/blunt surgical scissors. Place the extremities in a closed cell culture dish (35 mm x 10 mm).

NOTE: From now on, perform every step under a sterile laminar flow hood and work with sterilized instruments.

* 1. Isolate the muscle tissue (preferentially, use the musculus quadriceps femoris or musculus triceps surae from both legs).
     1. Cut the skin open from the hip to the tip of the toe by using sharp/sharp scissors and curved forceps. Hold the toe or footpad with the straight forceps. Peel off the skin with the curved forceps from the toe to the hip.
     2. Isolate the musculus quadriceps femoris by cutting the tendon from the knee and sever the muscle along the femur to the hip. Isolate the musculus triceps surae by severing the Achilles tendon. Hereafter, cut along the tibia to the popliteal fossa and remove the muscle.

NOTE: If large vessels (*Arteria femoralis*, *Arteria tibialis anterior*, *Arteria tibialis posterior*, *Arteria fibularis*) are visible in the isolated muscles, remove the vessels to avoid contamination by macrovascular endothelium.

* + 1. To remove large vessels, hold the part of muscle not containing large vessels with curved forceps and cut it off next to the vessel. For this protocol, 1 g or less muscle tissue equal to both quadriceps femoris and triceps surae is recommended.
    2. Add 2445 µL of DS (from step 2.1) to a cell culture dish (35 mm x 10 mm) and determine the weight.
    3. Transfer all muscle pieces to this cell culture dish containing the 2445 µL of DS and determine the weight. The difference of both measured values provides the dry weight of the muscle tissue, which must not exceed 1 g.
    4. Cut the whole muscle tissue into small pieces (≤2 mm cubes, about 100 pieces) by using the sharp/sharp scissors.
  1. Dissociate the muscle tissue.

NOTE: This step takes about 120 min.

* + 1. Store the muscle/DS suspension at 37 °C (in an incubator with 5% CO2) for 1.5 h. Mix the suspension carefully every 20 min for about 5 min, using a 1 mL insulin syringe.
    2. Transfer the suspension to a 70 μm nylon cell strainer placed on a 50 mL tube and collect the flow through. Wash the cell strainer with 8 mL of DMEM and collect the flow through.
    3. Discard the cell strainer and centrifuge the suspension for 10 min at 300 x *g* at 20 °C. Carefully remove the supernatant.

CAUTION: The pellet is easy to lose.

* + 1. Resuspend the cell pellet in 1 mL of an ammonium-chloride-potassium (ACK) lysing buffer (see **Table of Materials**) for the lysis of red blood cells and incubate for 30 s at RT. Add 9 mL of DMEM + 10% FCS to stop the reaction and transfer the cell suspension to a 15 mL tube.
  1. Deplete CD45+ cells, following the instructions of the CD45 microbeads’ manufacturer. For all following steps of CD45+ depletion, use MCS buffer (see **Table of Materials**).

NOTE: This step takes about 60 min.

* + 1. Determine the cell numbers using a Neubauer cell counting chamber (expect about 5 x 106 cells per gram of muscle tissue).
    2. Centrifuge the suspension at 300 x *g* for 10 min at 4 °C. Take off the supernatant completely and resuspend the cell pellet in 90 µL of MCS buffer (per 107 cells or less). Add 10 μL of CD45 microbeads (per 107 cells or less).
    3. Mix the cell suspension and incubate it for 15 min in the fridge at 4 - 8 °C.
    4. Add 1 mL of MCS buffer (per 107 cells or less). Centrifuge at 300 x *g* for 10 min at 4 °C. Remove the supernatant completely and resuspend the cells in 500 μL of MCS buffer.
    5. For magnetic cell separation, use a large magnetic column (LMC) with a reservoir volume of 8 mL and a capacity of up to 2 x 109 total cells. Position the LMC in the magnetic field of the separator.
    6. Rinse the reservoir of the LMC with 3 mL of MCS buffer. After rinsing, place a 15 mL conical tube below the column to collect the flow through. Apply the whole cell suspension (500 µL) onto the column and let it flow through completely.
    7. Wash the column 3x by adding 3 mL of MCS buffer into the reservoir, and wait until the column’s reservoir is empty before performing the next washing step.
    8. Collect the unlabeled cells passing the column to use them for further separation steps (representing the CD45- fraction). Labeled cells (representing the CD45+ fraction) that have accumulated in the column can be discarded.
  1. Accumulate CD31+ cells, following the instructions of the CD31 microbeads’ manufacturer. For all following steps of CD31+ accumulation, use MCS buffer.

NOTE: This step takes about 60 min.

* + 1. Determine the cell number, using a Neubauer cell counting chamber (expect about 4 x 106 cells per gram of muscle tissue).
    2. Centrifuge the obtained unlabeled cell suspension from step 3.5 for 10 min at 300 x *g* at 4 °C.
    3. Remove the supernatant completely. Resuspend the pellet in 90 μL of MCS buffer and add 10 μL of CD31 microbeads (per 107 total cells or less). Mix the whole suspension and incubate for 15 min in the fridge at 4 - 8 °C.
    4. Add 1 mL of MCS buffer and centrifuge at 300 x *g* for 10 min at 4 °C. Take off the supernatant and resuspend the cells in 500 µL of MCS buffer.
    5. For the magnetic cell separation, use a medium magnetic column (MMC) with a reservoir volume of 3.5 mL and a capacity of up to 2 x 108 total cells.
    6. Position the MMC in the magnetic field of the separator. Rinse the MMC with 500 µL of MCS buffer. After rinsing, place a 15 mL conical tube below the column to collect the flow through.
    7. Apply the whole cell suspension (500 µL) onto the column and let it completely flow through.
    8. Wash the column 3x by adding 500 µL of MCS buffer, and wait until the column’s reservoir is empty before performing the next washing step. Unlabeled cells passing the column represent the CD45- CD31- fraction. Store them for further quality controls.
    9. Remove the column from the separator and place it on a suitable collection tube (15 mL tube). Pipette 2 mL of MCS buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. This CD45- CD31+ fraction represents the enriched primary murine ECs.
    10. Centrifuge the cell suspension for 5 min at 350 x *g* at 20 °C. Remove the supernatant completely and resuspend the cells (expect around 0.6 x 106 cells per gram of muscle tissue) in 1 mL of ECM (see step 2.2). Transfer the cells (0.5 x 106 to 0.7 x 106 cells) to a single well of a coated 6-well culture plate (from step 2.3) containing 1 mL of ECM.
  1. For cultivation, incubate the cells at 37 °C and 5% CO2 in a sterile incubator. Refresh the ECM every 2 - 3 days.

1. **Primary Murine MMEC Purification**
   1. Perform a second cycle of CD31+ accumulation, following the manufacturer’s instructions (CD31 microbeads protocol; see step 3.6).
      1. Detach the cells with trypsin/EDTA solution when they are at 80% - 90% confluence (usually after 7 days). For this, rinse each well with 2 mL of sterile PBS, in two consecutive washing steps. Use 800 μL of trypsin/EDTA solution per well of a 6-well plate and incubate at 37 °C and 5% CO2 in a sterile incubator for 3 - 5 min. Stop enzymatic activity by using 1,200 μL of DMEM containing a minimum of 10% FCS.
      2. Centrifuge the cell suspension for 5 min at 350 x *g* at 20 °C.
      3. Perform the second CD31-MCS step to increase the purity (according to the instructions of the CD31 microbeads’ manufacturer; see step 3.6).
      4. Observe cell confluence *via* bright-field or standard phase-contrast microscopy using a 20X magnification and 0.35 lens numerical aperture. See **Figure 1**.

NOTE: The cells can be used for respective experiments or kept in culture by passaging. Use the cells for experiments promptly, in lower passages. It is not recommended to use the cells after passages 8 - 10.

1. **Quality Control**
   1. Perform a flow cytometry of primary murine MMECs after the second CD31-MCS step.
      1. Prepare a fluorescence-activated cell sorting (FACS) tube by adding 1 mL of flow cytometry (FC) buffer (see **Table of Materials**).
      2. Detach the cells with trypsin/EDTA solution when they are at 100% confluence (usually after 14 days). For this, rinse each well with 2 mL of sterile PBS, in two consecutive washing steps. Use 800 μL of trypsin/EDTA solution per well of a 6-well plate and incubate at 37 °C and 5% CO2 in a sterile incubator for 3 - 5 min. Stop the enzymatic activity by using 1,200 μL of DMEM containing 10% FCS.
      3. Determine the cell number, using a Neubauer cell counting chamber, and add a minimum of 1 x 105 cells to the prepared FACS tube.
      4. Centrifuge the cell suspension for 10 min at 300 x *g* at 4 °C. Perform two washing steps by removing the supernatant, resuspending the cells in 1 mL of FC buffer, and centrifuging for 10 min at 300 x *g* at 4 °C.
      5. Prepare a staining mix by adding anti-mouse CD31-FITC antibody (1:100) and anti-mouse CD45-PE (1:100) with FC buffer.
      6. Resuspend the cells in 100 μL of staining mix in the FACS tubes. Incubate for 30 min at 4 °C. Add 1 mL of FC buffer. Centrifuge the cell suspension for 5 min at 300 x *g* at 4 °C. Carefully remove the supernatant and resuspend the cells in 200 μL of FC buffer.
      7. Measure the cells in a flow cytometer, following the gating strategy as shown in **Figure 2A**.

* 1. Alternatively, perform immunofluorescence staining of primary murine MMECs after the second CD31-MCS step.
     1. Coat the coverslips.
        1. Transfer a sterile 13 mm-diameter round glass coverslip into a well of a 24-well plate. Coat the coverslip by adding 300 µL of speed-coating solution per well. Incubate for 3 - 5 min at RT.
        2. Aspirate and discard the coating solution. Rinse each well with 2 mL of sterile PBS in two consecutive washing steps. Aspirate and discard the PBS.
        3. Seed 1 x 105 cells in 1 mL of ECM on the coated coverslip and incubate at 37 °C and 5% CO2 in a sterile incubator until the cells are 99% confluent.
     2. Perform fixation and immunofluorescence staining.
        1. Remove the ECM medium of the cultivated cells from step 5.2.1.3.
        2. Fix the cultured cells by adding 300 µL of 4% paraformaldehyde (PFA) with a pH of 7.4 per well, for 10 min at 4 °C.

CAUTION: PFA is toxic and must be handled with care.

* + - 1. Perform three washing steps by adding 1 mL of PBS per well, and remove the supernatant after 5 min at RT.
      2. Prepare a blocking solution containing 5% bovine serum albumin (BSA), 0.2% Triton-X, and 1% goat serum in PBS.
      3. Add 300 µL of blocking solution to each well and incubate for 1 h at RT.
      4. Remove the supernatant and add 200 µL per well of an anti-PECAM-1 (CD31) antibody (1:200) in 5% BSA and 1% goat serum in PBS, and incubate at 4 °C overnight.
      5. Perform three washing steps by adding 1 mL of PBS per well and removing the supernatant after 5 min at RT in each step.
      6. Prepare a secondary antibody solution containing Cy3-conjugated anti-rat IgG antibody (1:500) in 1% BSA and PBS. Add 300 µL per well of the secondary antibody solution and incubate for 1 h at RT in the dark.
      7. Perform three washing steps by adding 1 mL of PBS per well and removing the supernatant after 5 min at RT in each step.
    1. Carefully take out the round glass coverslips with forceps and transfer them to a microscopy slide. Add an appropriate amount of mounting medium containing 4′,6-diamidino-2-phenylindol (DAPI) on the cell layer and carefully put a cover glass on it (see **Table of Materials**).
    2. Observe the cells with an appropriate fluorescence microscope equipped with a fluorescence light source (120 W) and two filter sets: an excitation/emission filter set with 550/25 nm and 605/70 nm wavelengths to detect Cy3 540/562 nm.
    3. Use a second filter set with an excitation/emission wavelength of 365/50 nm and 445/50 nm to detect DAPI 350/440 nm. Use a magnification of 40X and 80% intensity of 14.5 mW/cm2 for DAPI with an acquisition duration of 30 ms. For the detection of Cy3, use 80% of 67.5 mW/cm2 with an acquisition duration of 60 ms.
  1. Perform quantitative PCR.
     1. Isolate mRNA.
        1. Follow standard procedures, using an acid guanidinium thiocyanate-phenol (AGTP) reagentto isolate mRNA. Use a minimum of 0.5 x 106 cells from the CD45- CD31- (step 3.6.8) or CD45- CD31+ (step 3.6.9) fractions and cultivated primary murine MMECs (step 4.1.3).
        2. Centrifuge the cell suspension for 10 min at 300 x *g* at 20 °C. Take off the supernatant completely. Resuspend the cell pellet in 500 µL of AGTP reagent and transfer the cell suspension to a 2 mL reaction tube. Incubate for 5 min at RT.
        3. Add 100 µL of chloroform and shake vigorously for 15 s. Incubate for 3 min at RT.
        4. Centrifuge the suspension for 15 min at 12,000 x *g* at 4 °C.
        5. Carefully take off the upper aqueous phase (containing RNA) and transfer it to a 1.5 mL reaction tube. Add 250 µL of isopropanol and incubate for 10 min at RT.
        6. Perform a centrifugation step at 12,000 x *g* for 10 min at 4 °C.
        7. Take off the supernatant and add 1 mL of 75% ethanol carefully. Centrifuge at 7,500 x *g* for 5 min at 4 °C.

NOTE: The pellet is easy to lose.

* + - 1. Remove the supernatant completely. Let the pellet dry on air for 5 - 10 min.

NOTE: Ethanol needs to be removed, but do not dry the pellet too much.

* + - 1. Dissolve the pellet in 30 µL of diethylpyrocarbonate-treated water and heat it for 10 min at 55 °C.

NOTE: The RNA concentration and purity can be measured by a spectral photometer.

* + 1. Perform cDNA synthesis (reverse transcriptase PCR).
       1. Prepare a master mix containing 10 µL of PCR buffer (5x), 2.5 µL of deoxyribonucleoside-triphosphate (dNTP [10 mM]), 0.5 µL of a random mixture of single-stranded primer (0.2 µg/µL), 1 µL of RNase inhibitor (40 U/µL), 0.4 reverse transcriptase (200 U/µL), and 5.6 µL of diethylpyrocarbonate-treated water (see **Table of Materials)**.
       2. Dilute 500 ng of RNA in 30 µL of diethylpyrocarbonate-treated water in 0.2 mL PCR tubes and add the whole 20 µL of the master mix.
       3. Use a PCR cycler to perform the following steps: 10 min at 25 °C, 30 min at 50 °C, 5 min at 85 °C, and hold at 4 °C.
  1. Perform quantitative PCR (qPCR).

NOTE: Perform the qPCR of the samples in duplicates or triplicates.

* 1. Use a primer with two different fluorescence dyes. Use a primer with an absorption of 495 nm and an emission of 517 nm for the gene of interest.
  2. For the detection of the satellite cell marker gene expression, use primers for paired box protein 7 (*Pax7*) and M-cadherin (*Cdh15*) (see **Table of Materials**).
  3. For the detection of the endothelial cell marker gene expression, use primers for claudin-5 (*Cld5*), occludin (*Ocln*), and zonula occludens-1 (*Tjp1* or *ZO1*) (see **Table of Materials)**.
  4. As a reference gene for 18S rRNA, use a primer with an absorption of 538 nm and an emission of 554 nm in wavelength.
  5. Prepare a qPCR master mix for each gene of interest with the respective primer. The master mix contains 10 µL of qPCR buffer (2x), 1 µL of the primer for the gene of interest (10 µM), 1 µL of the primer for 18S rRNA, and 4 µL of nuclease-free water.
  6. Add 16 µL of the master mix to a single well of a 96-well reaction plate. Add 4 µL of cDNA (obtained from step 5.3.2) per well containing the master mix.
  7. Use a real-time PCR cycler, performing the following steps: the holding stage with 50 °C for 2 min and 95 °C for 10 min and the cycling stage with 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

**REPRESENTATIVE RESULTS:**

One day after isolation, primary murine MMECs and residual other cells form conglomerates and adhere to the bottom of culture dishes (**Figure 1A**, day 1). From day 7 onward, flat and elongated cells can be observed. However, contamination of other, mostly spheroid cells, is still visible (**Figure 1A**,day 7). Thus, another cycle of CD31 positive selection *via* MCS is required. Hereafter, primary murine MMECs proliferate to a density of approximately 80% - 90%. Upon confluence, they typically form a nonoverlapping monolayer of longitudinally aligned cells (**Figure 1A**, day 14). Proliferation stops upon confluence due to contact inhibition. After 14 days, about 5 x 105 to 10 x 105 cells can be used for further investigations.

Quality control *via* flow cytometry, using fixable viability dye FVD780 (to stain for dead cells), PECAM1 (to stain for CD31), and PTPRC (to stain for CD45, as a marker for cells of hematopoietic origin), showed values for both viability and purity ranging around 70% each for cells immediately after the isolation (**Figure 2A**). Cells cultivated after another CD31 positive selection *via* MCS showed satisfying values for purity, as well as for viability, ranging up to 95% each (**Figure 2B**).

To evaluate the accuracy of the selection steps, obtained cells were further investigated for the gene expression of the muscle satellite cell marker genes paired box protein 7 (*Pax7*) and M-cadherin (*Cdh15*) on mRNA level by quantitative PCR (qPCR). Primary murine MMECs (pmMMEC) and differentiated primary murine muscle cells (pmMC) were used as negative and positive control, respectively. Murine muscle cells were commercially purchased. As expected, only the CD45- CD31- fraction, as well as the pmMC, expressed *Pax7* and *Cdh15*, whereas CD45- CD31+ and the primary murine MMECs were negative for these markers (**Figure 2C**).

ECs derived from capillaries in the endomysium of skeletal muscle express tight junction proteins4,5. By using qPCR, the expression of claudin-5 (*Cld5*), occludin (*Ocln*), and zonula occludens-1 (*Tjp1* or *ZO1*) of confluent primary murine MMECs after the second CD31-MCS step was evaluated. PmMC were used as controls (**Figure 2D**). Primary murine MMECs expressed high levels of *Cld5*, *Ocln*, and *Tjp1*, whereas pmMC only show a low expression of *Tjp1*. Furthermore, immunofluorescence staining as quality control confirmed the surface expression of the endothelium-specific marker PECAM1 in primary murine MMECs (**Figure 2E**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Morphology of primary murine MMECs.** (**A**) Representative image of cultured primary murine MMECs by phase-contrast microscopy from days 1 to 14. d1 = day 1, d7= day 7, d14 = day 14.

**Figure 2: Quality control of primary murine MMECs.** Gating strategy for flow cytometric analysis of primary murine MMECs (**A**) immediately after isolation and (**B**) on day 15, using (I) FSC/SSC, (II) FVD780, and (IIIa) CD45 and (IIIb) CD31 (dashed lines = isotype control). (**C**) Expression level of *Pax7* and M-cadherin (*Cdh15*) in CD45- CD31- and CD45- CD31+ fractions after MCS isolation, as well as in primary murine MMECs (pmMMEC) and primary murine muscle cells (pmMC). Expression levels are shown as ΔCt values (sample‒18S rRNA); n.d. = not determined. (**D**) Expression levels of tight junction proteins claudin-5 (*Cldn5*), occludin (*Ocln*), or zonula occludens-1 (*Tjp1* or *ZO-1*) of pmMMEC and pmMC, shown as ΔCt values. (**E**) Immunofluorescence staining for PECAM1 (red) in cultivated primary murine MMECs, 24 h after the second CD31 positive selection. Nuclear staining with DAPI-containing (blue) mounting medium; left: anti-PECAM1/DAPI, right: negative control/DAPI.

**DISCUSSION:**

Microvascular ECs provide barrier functions in all tissues, and their dysfunction results in the disease of the associated organs3. Moreover, organ-specific studies of microvascular ECs could pave the way for new therapeutic strategies. Therefore, a deeper understanding of microvascular EC function under physiological and pathophysiological conditions is of great scientific interest. The modulation of leukocyte/endothelium interaction is successfully used to treat multiple sclerosis patients with natalizumab, an antibody which prevents lymphocyte adhesion and, thereby, transmigration6. However, various myopathies, including the inflammatory subtypes, still remain only poorly treatable to date. Therefore, the elucidation of EC properties, specifically in skeletal muscle endothelium, could help to expand available treatments to myopathy or result in novel therapeutic approaches.

Immortalized endothelial cell lines (IECLs) have been established and used for several *in vitro* models. These cell lines offer some advantages compared to primary microvascular ECs due to immortalization and fast proliferation. Furthermore, primary cultured cells can undergo senescence and lose or change their morphology or physiological function after some time or passages. However, IECLs only maintain some endothelial cell properties and cannot resemble the variety of different organ-borne primary microvascular ECs. Of note, they are rarely derived from skeletal muscles. To date, only a single publication describes a human skeletal microvascular endothelial cell line, named TSM155. In contrast, murine microvascular ECs have not been described so far. Finally, primary cells from transgenic animals provide the opportunity to study genetic modifications *in vitro*. However, primary cell cultures also hold certain pitfalls. First, contamination with cells of no interest can interfere with the validity of experimental findings. Therefore, the accuracy of several selection steps and a high purity of the isolated cells must be guaranteed. Cell suspension after dissociation of muscle tissue contains erythrocytes and different mononuclear cell types, such as immune cells, satellite cells, pericytes, fibroblasts, and ECs7. Therefore, cell fractions after the CD31-MCS step were tested for markers exclusively expressed by muscle satellite cells. As expected, CD45- CD31- fraction and primary muscle cells showed *Pax-7* and *Cdh15* expression, whereas CD45- CD31+ and cultivated primary murine MMECs were negative for these markers after a second CD31-MCS step. Furthermore, a quality control of isolated or cultured cells is inevitable, to guarantee reliable and reproducible experiments. Several methods for quality control of primary murine MMECs are available: besides the microscopy of morphological properties, flow cytometry, PCR, and immunofluorescence staining for characteristic EC markers (*e.g.*, CD31, Sca-1) can be used. The purity assessment of freshly isolated primary murine MMECs was only about 60% - 70%, whereas a viability of about 70% could be detected. The microscopy of these cells partially shows cell conglomerates, which might consist of contaminating cells such as fibroblasts or pericytes attached to ECs. After 7 days, mainly flat and elongated cells and spheroid cells can be observed. However, two CD31-MCS passages resulted in a high viability and purity. Alternative approaches to increase purity, such as puromycin-containing media, failed in primary murine MMECs while being effective in murine brain microvascular ECs8.

Since microvascular ECs derived from capillaries express tight junction proteins, the gene expression of *Cld5*, *Ocln*, and *Tjp1* in primary murine MMECs compared to differentiated muscle cells was examined. As expected, all tight junction molecules were detected in primary murine MMECs. In comparison, muscle cells demonstrated only a low expression of *Tjp1*, whereas no expression of *Cld5* and *Ocln* could be determined. Similar expression patterns were previously demonstrated in whole murine skeletal muscle. Here, an expression on gene and protein level could be detected for *Tjp1* but not for occludin9. Further functional features, such as transendothelial resistance, could be measured.

The herein described protocol features only the isolation of microvascular ECs from murine muscle tissue. However, it might be transferred to various other species as, for example, a similar protocol was published for rat microvascular ECs derived from epididymal fat pads. Here, a CD31 positive selection *via* MCS was preceded by density gradient centrifugation10. Similar approaches were successful in macrovascular EC isolation from rat femoral arteries11. A recently published method to isolate microvascular ECs of heart and lung tissue uses CD31 and endoglin (CD105) as antigens for MCS12. However, the purity of ECs could not be increased further by using additional CD105 magnetic bead separation. Another possibility to isolate and purify primary murine MMECs is multicolor FACS. A distinct population of Sca-1+, CD31+, CD34dim, and CD45- cells from murine muscles could be isolated and was characterized as primary murine MMECs13. An advantage of this method is a pure EC population which can be directly used or cultured for further experiments (note that FACS leads to increased cell death due to higher cell stress). Further, dead cells or conglomerates of different cell types can significantly reduce the isolated cell numbers by FACS. Additionally, it is reported that primary murine MMECs isolated by FACS were unsuccessfully cultured at commonly used culture conditions of 21% O2 and 5% CO2. Here, the oxygen level had to be adjusted to 5% for a sufficient cultivation13. Moreover, the FACS technique is more complex and time-consuming, and the infrastructural prerequisites are expensive.

The musculus quadriceps femoris and the musculus triceps surae of male mice in the age of 4 - 12 weeks are most suitable for the isolation of primary murine MMECs as they are easily accessible and large enough for the isolation of sufficient cell numbers (5 x 105 to 10 x 105 per gram of muscle). Therefore, it must be ensured that conditions are comparable in independent experiments. As described above, primary cells can undergo senescence. Hence, use cells for respective experiments promptly, in lower passages. After passages 8 - 10, primary murine MMECs change their morphology, demonstrate slow proliferation rates, and lose their contact inhibition as signs of dedifferentiation and senescence.

Further, there are some notes about troubleshooting for this protocol. Sterile working is essential to avoid contaminations. Quality controls are necessary to monitor the purity and viability of isolated cells. Used materials in this protocol must be stored and applied according to the manufacturer’s instructions. Additionally, the age and sex of the used animals, as well as different muscle groups, might influence the quality and comparability of the isolated cells in experiments.

The described protocol should be considered as a platform method to isolate the primary microvascular ECs of skeletal muscles. Isolated cells can be used for several applications to gain further insights into blood-muscle-barrier function. The cells are suitable for both protein and RNA expression studies, as well as functional assays (including transmigration and adhesion studies). However, this protocol was optimized for studies focusing on inflammatory processes, and hence, slight modifications might be necessary with respect to different research areas.

To model the complex spatial and functional cellular interactions within the MVU, primary murine MMECs can be cultivated in more complex three-dimensional cell culture systems together with other cell types. Prospectively, it should also be possible to generate MVU organoids as it already has been described for other tissues14. However, the successful isolation of primary murine MMECs is vital when aiming for this next step.

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The authors have nothing to disclose.

**REFERENCES:**

1. Rodrigues, S.F., Granger, D.N. Blood cells and endothelial barrier function. *Tissue Barriers*. **3** (1-2), e978720 (2015).

2. Michiels, C. Endothelial cell functions. *Journal of Cellular Physiology*. **196** (3), 430-443 (2003).

3. Pierce, R.W., Giuliano Jr., J.S., Pober, J.S. Endothelial cell function and dysfunction in critically ill children. *Pediatrics*. **140** (1), 20170355 (2017).

4. Nasdala, I. *et al.* A transmembrane tight junction protein selectively expressed on endothelial cells and platelets. *Journal of Biological Chemistry*. **277** (18), 16294-16303 (2002).

5. Sano. H. *et al.* Establishment of a new conditionally immortalized human skeletal muscle microvascular endothelial cell line. *Journal of Cellular Physiology*. **232** (12), 3286-3295 (2017).

6. Kappos, L. *et al.* Natalizumab treatment for multiple sclerosis: Updated recommendations for patient selection and monitoring. *Lancet Neurology*. **10** (8), 745-758 (2011).

7. Birbrair, A. *et al.* Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Research*. **10** (1), 67-84 (2013).

8. Ruck, T., Bittner, S., Epping, L., Herrmann, A.M., Meuth, S.G. Isolation of primary murine brain microvascular endothelial cells. *Journal of Visualized Experiments*. (93), e52204 (2014).

9. Hwang, I. *et al*. Tissue-specific expression of occludin, zona occludens-1, and junction adhesion molecule A in the duodenum, ileum, colon, kidney, liver, lung, brain, and skeletal muscle of C57BL mice. *Journal of Physiology and Pharmacology*. **64** (1), 11-18 (2013).

10. Frye, C.A., Patrick Jr., C.W. Isolation and culture of rat microvascular endothelial cells. *In Vitro Cellular & Developmental Biology - Animal*. **38** (4), 208-212 (2002).

11. Le, G.Y., Essackjee, H.C., Ballard, H.J. Intracellular adenosine formation and release by freshly-isolated vascular endothelial cells from rat skeletal muscle: Effects of hypoxia and/or acidosis. *Biochemical and Biophysical Research Communications*. **450** (1), 93-98 (2014).

12. Cheung, K.C., Marelli-Berg, F.M. Isolation of microvascular endothelial cells. *Bio-protocol*. **8** (12) (2018).

13. Ieronimakis, N., Balasundaram, G., Reyes, M. Direct isolation, culture and transplant of mouse skeletal muscle derived endothelial cells with angiogenic potential. *PLoS One*. **3** (3), e0001753 (2008).

14. Pham, M.T. *et al.* Generation of human vascularized brain organoids. *Neuroreport*. **29** (7), 588-593 (2018).