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Serum-Free Generation of Multipotent Mesoderm (Kdr-positive) Progenitor Cells in Mouse Embryonic Stem Cells For Functional Genomics Screening

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Abstract

This unit describes a robust protocol for producing multipotent Kdr-expressing mesoderm progenitor cells in serum-free conditions and functional genomics screening using these cells. Kdr-positive cells are known to be able to differentiate into a wide array of mesoderm derivatives including, vascular endothelial cells, cardiomyocytes, hematopoietic progenitors and smooth muscle cells. The efficient generation of such progenitor cells is of particular interest because it permits subsequent steps in cardiovascular development to be analyzed in detail, including deciphering the mechanisms that direct differentiation. The oligonucleotide transfection protocol used to functionally screen siRNA and microRNA libraries is a powerful tool to reveal networks of genes, signaling proteins and microRNAs that control the diversification of cardiovascular lineages from multipotent progenitors. The discussion addresses technical limitations, troubleshooting and potential applications.

Keywords

mouse embryonic stem cells; mesendoderm; mesoderm; endoderm; siRNA transfection; Kdr; Foxa2

INTRODUCTION

Mesoderm is one of the three germ layers that constitute the building blocks of the body plan in most animals. Organs such as the heart, kidney, skeletal muscle, blood as well as the lymphatics and blood vasculature mainly derive from mesoderm cells. Understanding genetic and ageing-associated pathologies in these organ systems and the potential for regeneration motivated our study of their respective developmental programs. To date our understanding of how these different organs are formed and differentiate remains incomplete, primarily because studying these processes directly in embryos of higher vertebrates is inherently difficult. In the past few years, the development of in vitro differentiation assays using embryonic stem cells or induced pluripotent cells has greatly improved our ability to study these genetic programs. Here we describe a chemically-defined protocol that robustly produces multipotent mesoderm (Kdr-positive) cells that can subsequently differentiate into cardiomyocytes, vascular endothelial cells and vascular smooth muscle cells from mouse embryonic stem cells. We describe the use of these cells in

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functional genomics screens of siRNA and microRNA libraries that are potent tools for deciphering the genetic and signaling hierarchies that control development.

BASIC PROTOCOL 1

Culture of Mouse Embryonic Stem Cells and Preparation of Frozen mESC Stock

The purpose of Basic Protocol 1 is to prepare the mESCs for cryopreservation and scale up cultures for the differentiation and screening protocol (Basic Protocol 2 and Alternate Protocol 2).

NOTE: All steps of the protocol should be performed using proper aseptic technique.

NOTE: Before using the mESC Growth Media (mESC GM), chemically defined medium (CDM), Freezing Medium (FM), trypsin, or PBS warm to 37°C.

Materials

0.1% Gelatin solution (Stem Cell Technologies Cat. # 07903)
Kdr-eGFP mouse embryonic stem cells, frozen
mESC growth media (mESC GM; see recipe)
0.25% Trypsin EDTA (Gibco Cat. # 25200)
Freezing Media (FM; see recipe)
Inverted bright field microscope
10-cm tissue culture plates (Corning Cat. # 430167)
Electronic pipet-aid
5 mL, 10 mL, and 25 mL serological pipettes
37°C water bath
15-mL centrifuge tubes
Pasteur Pipettes
10 µL, 200 µL, and 1000 µL pipettes tips
1 mL cryotubes
Cryo 1°C Freezing Container (Nalgene Cat. # 5100-0001)

Protocol steps

- 1 Prepare a gelatinized 10cm tissue culture grade plate by covering the surface of the plate with 3mL of 0.1% gelatin in water solution. Aspirate the remaining gelatin using a Pasteur pipette.
- 2 Thaw one vial of Kdr-eGFP mESCs in the 37°C water bath until the media within the tube has thawed. Using a 1000 µL pipette tip, transfer the thawed cells into 10mL of mouse embryonic stem cell growth media (mESC GM). Spin the cells for 3 minutes at 300×g.
- 3 Aspirate the media using a Pasteur pipette. Resuspend the cells in 10mL of mESC GM, and add all cells to the gelatin-coated tissue culture plate.

After adding the cells to the gelatin-coated plates, ensure that they are evenly distributed by sliding the plate in perpendicular axes. Avoid moving or

swirling the plate in a circular motion since the cells will cluster in the center of the wells.

- 4 Grow the cells for 2 days at 37°C + 5% CO₂

Passage of Cells

- 5 When the cells have reached a confluency of ~70%, the cells should be passaged onto a new gelatin-coated plate and new mESC GM. *When preparing the mESCs for differentiation (described below), the cells are usually passaged twice at 1:6 before suspension in the chemically defined medium (CDM).*

Aspirate the medium from the 10cm plate using a pasteur pipette, and add 3mL of 0.25% Trypsin EDTA. Incubate the cells at 37°C or until all the cells have been lifted from the bottom of the plate.

- 6 Using a 10mL serological pipette, add 10mL of mESC GM to the 10cm plate and pipette up and down to mechanically break up any remaining clusters of cells.
- 7 Transfer the mixture into a 15mL centrifuge tube and spin for 3 minutes at 300×g to pellet the cells.
- 8 Aspirate the media and resuspend the cell pellet in 6 mL of mESC GM. Add 1 mL of cell suspension and 9mL of mESC GM to gelatin coated 10cm plates and grow at 37°C and 5% CO₂ for 2 days.

Freeze Cells

- 9 When the plates at ~70% confluent, aspirate media and dissociate cells as described in steps 6–8 above.
- 10 Resuspend the cells in freezing media (3mL for every confluent 10cm tissue culture dish) and transfer 1mL of the suspension into each cryotube.
- 11 Place all the cryotubes into the Cryo 1°C freezing container for at least four hours.

Step annotations

None

BASIC PROTOCOL 2

Differentiation of Mouse Embryonic Stem Cells into Multi-Potent Mesoderm Progenitor (Kdr+) Cells

The purpose of this protocol is to plate mESC-derived cardiovascular progenitors into cell culture dishes and probe differentiation by functional genomics screening. mESCs cultured according to the Basic Protocol 1 above are differentiated in bulk EB culture until day 4 of differentiation, at which point they are dispersed and plated onto 384-well plates in the presence of oligonucleotide and transfection reagents to induce and probe differentiation.

NOTE: All CDM must be prepared on the day that it is used. CDM cannot be made in bulk and used throughout the differentiation assay.

Materials

Kdr-eGFP mouse ES cells, cultured using Basic Protocol 1

0.25% Trypsin EDTA (Gibco Cat. # 25200)
mESC growth media (mESC GM; see recipe)
1x phosphate-buffered saline (Mediatech Cat. # 21-040-CV)
Chemically defined medium (CDM, see recipe)
Activin A(R&D Systems 338-AC-050)
0.1% Gelatin solution (Stem Cell Technologies Cat. # 07903)
siRNA against Acvr1b (Ambion s61928)
Opti-MEM® Reduced Serum Medium (Invitrogen 31985-070)
Lipofectamine™ RNAiMAX (Invitrogen 13778-100)
8% paraformaldehyde (8% PFA, see recipe)
37°C water bath
Inverted bright field microscope
Pasteur Pipettes
Electronic pipet-aid
5 mL, 10 mL, and 25 mL serological pipettes
15 mL and 50 mL centrifuge tubes
10 µL, 200 µL, and 1000 µL pipettes tips
40 µm nylon cell strainer (BD Falcon Cat. # 352340)
10cm Low attachment tissue culture plates (Fisherbrand Petri Dishes 08-757-13)
16-channel Electronic Pipette, 2.0-125 µL (Thermo Scientific Cat. # 2061)
384-well aerosol-free pipette tips (Thermo Scientific Cat. # 7445)
Reservoirs (VWR Cat. # 89094-678)
384-well optical tissue culture plates, sterile (Greiner Bio-One Cat. # EK-30091)

Protocol steps

1. Four days prior to day 0 of the differentiation protocol (Basic Protocol 2), thaw a vial of Kdr-eGFP mESC cells as described in steps 1–4 of Basic Protocol 1. Passage the cells two days prior according to steps 5–9 of Basic Protocol 1.
2. Day 0: Collect and dissociate cells according to steps 5–8 as described in Basic Protocol 1. Once the cells have been centrifuged, resuspend the cell pellet with 10 mL of 1x PBS and spin for 3 minutes at 300×g.
3. Aspirate the PBS using a Pasteur pipette and resuspend in appropriate amount of CDM (see step 4 and Step Annotation below). Pour the cells through a 40 µm nylon cell strainer and into a 50mL centrifuge tube and perform a viable cell count using a hemocytometer.
4. Add 1×10^6 mESCs in 10 mL of CDM to each 10 cm low attachment tissue culture plate and incubate at 37°C and 5% CO₂ for 44–52 hours to make embryoid bodies (EBs).

After 24 hours, the EBs should look round (about 100 – 200 cells/EB), with few floating (dying) cells present. If greater than 30% of cells are visible as single, floating cells, discard the culture.

5. Day 2: Collect the EBs from the 10 cm low attachment tissue culture plate using a 10 mL serological pipette and place in a centrifuge tube of the appropriate size. Centrifuge the EBs at 200×g for 2 minutes.

When performing the assay in a larger format, combining the cultures from multiple plates of EBs in the same centrifuge tube allows for easier manipulation at later steps.

6. Aspirate the media using a Pasteur pipette and add 3 mL (5 mL in larger formats) of 0.25% trypsin EDTA to each centrifuge tube. Incubate the suspension at 37°C for 4 minutes or until nearly all of the EBs have become dissociated.
7. Add 10mL of mESC GM once dissociated and pipette up and down to homogenize the suspension and mechanically break up any remaining EBs. Centrifuge the cells for 3 minutes at 300×g to pellet the cells.
8. Aspirate the media and resuspend the cell pellet in 10 mL of 1x PBS. Pipette up and down to homogenize the mixture and spin for 3 minutes at 300×g.
9. Prepare CDM containing 30 ng/mL of activin A. Resuspend the cells in CDM with activin A and replate 10 mL of cells in CDM with activin A into each low attachment plate.

The number of plates of EBs made on day 0 dictates the amount of CDM with activin A that must be made and how many low attachment tissue culture plates are used on day 2. The number of plates made on day 0 is the same that the cells are replated into at day 2.

10. Day 3: Coat one 384-well optical tissue culture plate with 0.1% gelatin in water. Aspirate the gelatin and allow plates to dry while preparing the cells.
11. Collect the EBs from the 10 cm low attachment tissue culture plate using a 10 mL serological pipette and place in a centrifuge tube of the appropriate size. Centrifuge the EBs at 200×g for 2 minutes.
12. Aspirate the PBS with a Pasteur pipette and resuspend the cells in an appropriate volume of CDM. Once resuspended, transfer the cells through a 40 µm nylon cell strainer and perform a viable cell count using a hemocytometer.
13. Once the cells have been counted, dilute the siRNA against *Acvr1b* to 500nM and add 5µL per well of the diluted siRNA into the coated 384-well plate using a 16-channel electronic pipette.

Note that microRNAs and other siRNAs can be screened to probe function, see Step Annotation and Alternative Protocol 2, below.

14. Prepare the transfection reagent by mixing 0.2 µL of lipofectamine RNAiMax and 14.8 µL of Opti-MEM per well into an appropriate sized centrifuge tube. Once mixed, incubate the transfection reagent at room temperature for 3–4 minutes while gently inverting three times after 2 minutes.

The scale at which the assay is performed will determine how much Opti-MEM and lipofectamine RNAiMAX is used. The transfection reagent is incubated to equilibrate the mixture before it is mixed with the siRNA.

15. After incubation, add 15 μL of the transfection reagent mixture to each well containing siRNA with the 16-channel electronic pipette. Centrifuge the plates for thirty seconds and $300\times g$. After centrifugation, incubate the plate(s) at room temperature for 20 minutes before adding the cells.
16. While the siRNA and transfection reagent are incubating, dilute the cells to a concentration of 1.25×10^4 cells per 80 μL . After the incubation time has passed, add 80 μL of diluted cells to each well of the plate.

When diluting the cells, be sure to account for the dead volume of the reservoir plus the actual volume of cells required for plating.
17. After plating the cells, allow the cells to settle to the bottom of each well by incubating at room temperature for 30 minutes. Place the cells in a 37°C incubator with 5% CO_2 for 44–52 hours.
18. Day 5: Remove the plate(s) from the incubator and aspirate gently and slowly 50 μL of media from each well using a 16-channel electronic pipette. Add 50 μL of fresh CDM to each well. Repeat twice such that 100 μL total has been aspirated and 100 μL total has been replaced in each well. Continue incubation at 37°C and 5% CO_2 for another 22–26 hours.
19. Day 6: The cells are fixed to the 384 well plate according to the protocol described in Support Protocol 2.

Step annotations

Step 3 – In order to achieve the proper cell density for counting, dilute the cells such that the cell density is approximately 2.0×10^6 cells/mL. From this stock, a 1:5 dilution of cells to media can be made in order to accurately calculate the cell density. Assuming that the cells divide 5 times between day 0 and day 3, then each 10cm low attachment plate should have about 1×10^6 cells. Add 5mL of CDM for every 10cm low attachment plate combined during the day 3 protocol.

Step 13 - Transfection of siRNA against *Acvr1b* is used as a robust positive control for generation of a large population of Kdr-eGFP+ cells. However, for the purpose of screening siRNA or miRNA libraries, this experiment can be scaled up as described in Alternative Protocol 2.

ALTERNATE PROTOCOL 2

High-Throughput Functional Screening of siRNA/miRNA Libraries

This protocol describes the modifications to the Basic Protocol 2 that permit the use of Kdr+ progenitor cells in functional genomics screens. The protocol was developed for screening of libraries of microRNAs and mouse siRNAs that are now available from several commercial vendors.

Materials

Kdr-eGFP mouse ES cells, cultured using Basic Protocol 1
0.25% Trypsin EDTA (Gibco Cat. # 25200)
mESC growth media (mESC GM; see recipe)
1x phosphate-buffered saline (Mediatech Cat. # 21-040-CV)
Chemically defined medium (CDM, see recipe)

Activin A(R&D Systems 338-AC-050)
 0.1% Gelatin solution (Stem Cell Technologies Cat. # 07903)
 siRNA or miRNA library, such as provided by Ambion/Life Technologies or Dharmacon/Thermo Scientific.
 Opti-MEM® Reduced Serum Medium (Invitrogen 31985-070)
 Lipofectamine™ RNAiMAX (Invitrogen 13778-100)
 8% paraformaldehyde (8% PFA, see recipe)
 37°C water bath
 Inverted bright field microscope
 Pasteur Pipettes
 Electronic pipet-aid
 5 mL, 10 mL, and 25 mL serological pipettes
 15 mL and 50 mL centrifuge tubes
 10 µL, 200 µL, and 1000 µL pipettes tips
 40 µm nylon cell strainer (BD Falcon Cat. # 352340)
 10cm Low attachment tissue culture plates (Fisherbrand Petri Dishes 08-757-13)
 16-channel Electronic Pipette, 2.0-125 µL (Thermo Scientific Cat. # 2061)
 384-well aerosol-free pipette tips (Thermo Scientific Cat. # 7445)
 Reservoirs (VWR Cat. # 89094-678)
 384-well optical tissue culture plates, sterile (Greiner Bio-One Cat. # EK-30091)
 Hamilton Star Liquid Handling Robot (or equivalent)
 Anti-Pecam1 (V-16) (Santa Cruz SC-31045)
 Anti-Smooth muscle actin (B4) (Santa Cruz SC-53142)
 Donkey anti-mouse Alexa Fluor 568 (Molecular Probes A-10037)
 Donkey anti-Goat Alexa Fluor 647 (Molecular Probes A-21447)

Protocol steps

1. To perform a functional screen of the siRNA/miRNA library, cells must be prepared using steps 1–9 of Basic Protocol 2. However, when scaling the assay to perform a screen, the size of the screen should be taken into consideration and the proper quantity of cells should be differentiated.

A single 10cm low attachment plate prepared at day 0 according to Basic Protocol 2 will generate $\approx 1 \times 10^7$ cells at day 3 of Basic Protocol 2. Since 1×10^5 cells are added to each well of the 384 well plate, a single 10cm low attachment plate will produce enough cells to fill two 384 well plates. Depending on the size of the screen, half as many 10cm low attachment plates should be prepared at day 0 to achieve the proper cell number at day 3 of Basic Protocol 2, the day of transfection.
2. Spotting of the siRNA/miRNA library is performed using a fluid handling robot (Hamilton Star or equivalent), and should be performed on the same day as the

transfection. Transfection of the cells at day 3 will proceed the same way as steps 10–17 of Basic Protocol 2 with the exception of step 13.

Step 13 is the dilution and addition of siAcvr1b, and in alternative protocol 2 a siRNA or miRNA library is substituted. The concentration of the library should be taken into consideration so that the final concentration of siRNA/miRNA is 25nM. If the library is at a concentration greater than 500nM, the spare volume of the 5 μ L used to spot the siAcvr1b should be replaced with the equivalent volume of OptiMEM.

3. Day 5: the CDM should be replaced according to step 18 of Basic Protocol 2. If the screen required the use of more than four 384 well plates, then the Hamilton Star (or equivalent) should be used to aspirate and replace media from the plates. The cells should be placed in a 37°C incubator with 5% CO₂ for 44–52 hours.

The fluid handling robot ensures that the volumes of CDM are accurately aspirated and replaced such that every well receives a consistent volume of fresh media.

4. Day 7: 50 μ L of CDM should be removed and replaced using a fluid handling robot. Place the cells in a 37°C incubator with 5% CO₂ for 44–52 hours
5. Day 9: Media should be replaced according to step 4 of Alternate Protocol 2 using the fluid handling robot.
6. Day 11: Media should be replaced according to step 4 of Alternate Protocol 2 using the fluid handling robot.
7. Day 12: Cells should be fixed and blocked according to steps 1–4 of support protocol 2.
8. Immunostaining is performed according to steps 5–9 of support protocol 2.

The primary antibody against HNF3 β is substituted with anti-Pecam1 and anti-SMA to stain for differentiated vascular endothelial cells and vascular smooth muscle, respectively. Donkey anti-goat 647 and donkey anti-mouse 568 are substituted for donkey anti-goat 568.

9. Imaging and quantification of fluorescence is performed according to Basic Protocol 3.

Step annotations

None

SUPPORT PROTOCOL 2

Fixation of mESCs in a 384-well Plate using Paraformaldehyde and Immunostaining

The purpose of this protocol is to prepare the 384 plates for automated microscopy.

Materials

mESCs differentiated according to Basic Protocol 2 and/or Alternate Protocol 2

16-channel Electronic Pipette, 2.0–125 μ L (Thermo Scientific Cat. # 2061)

384-well aerosol-free pipette tips (Thermo Scientific Cat. # 7445)

Reservoirs (VWR Cat. # 89094-678)

8% Paraformaldehyde (PFA; see recipe)

PBS 1X (Without calcium and magnesium) (Mediatech 21-040-CV)

5L plastic beaker

Blocking buffer (see recipe)

anti-Foxa2 (M-20) (Santa Cruz SC-6554)

Donkey anti-Goat Alexa Fluor 568 (Molecular Probes A-11057)

DAPI solution (Invitrogen D3571)

Storage solution (see recipe)

Protocol steps

1. Remove the plate(s) from the incubator and aspirate gently and slowly 50 μ L of media from each well using a 16-channel electronic pipette. Add 50 μ L of 8% Paraformaldehyde to each well and incubate at room temperature for one hour.
2. After one hour, aspirate 50 μ L of the PFA/CDM mixture using a 16-channel electronic pipette, and gently add 50 μ L of 1x PBS to each well. Repeat this process five successive times.

3. After the PFA has been sufficiently diluted, all liquid should be aspirated from each well.

One technique to aspirate the media is to “flick” the plate into a safety approved container and dispose of the waste in a chemical waste container. This same technique will be helpful in other steps throughout the protocol.

4. Add 20 μ L of blocking buffer to each well and incubate at room temperature while gently shaking.
5. After 30 minutes, “flick” the plate into a safety approved container and add 20 μ L of primary antibody solution to each well and incubate for one hour at room temperature while shaking gently.

The primary antibody solution is a 1:100 dilution of the anti-Foxa2 primary antibody in blocking buffer.

6. After one hour, “flick” the plate and submerge the empty plate into a 5L plastic beaker containing 1x PBS and incubate at room temperature for five minutes while gently shaking. Repeat this step for 3 total washes of five minutes per wash.
7. When washing has concluded, “flick” the plate into a safety approved container and add 20 μ L of secondary antibody solution and incubate at room temperature for one hour while gently shaking.

To prepare the secondary antibody solution, make a 1:1000 dilution of the Donkey anti-Goat Alexa Fluor 568 and a 1:10000 dilution of the DAPI solution in blocking buffer.

8. Following the one hour incubation, wash the plate(s) as described in step 6 of support protocol 2.
9. After washing, “flick” the plate one final time to remove all media and replace with 50 μ L of storage solution. The plates are now ready for Basic Protocol 3.

Step annotations

None

BASIC PROTOCOL 3

High-Throughput Imaging and Quantification of Kdr and Foxa2 Expression

The purpose of this protocol is to quantify the Kdr and Foxa2 fluorescent signals to determine the proportion of cardiovascular and endodermal progenitors in the cultures, respectively. The protocol is for the InCell1000 (GE Healthcare), but is modifiable for other platforms.

Materials

Automated inverted fluorescence microscopy workstation (GE Healthcare, In Cell Analyzer 1000)

Nikon Plan Apo VC objective (10X, 0.45 N.A.)

Filter Sets as in Table 1

CyteSeer® automated image analysis program

Protocol steps

- 1 *Prepare the InCell 1000 for imaging:* Turn on the InCell 1000, and ignite the light source before acquiring images.
- 2 Install the Nikon Plan Apo VC 10X objective (10x objective, numerical aperture, 0.45 N.A.)
- 3 Open the InCell 1000 Analyzer software and set imaging parameters by clicking on "Acquisition protocol manager" icon. Open a protocol to image the plate and define the following parameters:
 - Well definition: Select wells to be imaged in the 384 well plate
 - Camera: select binning parameters. In this protocol we use 4x4 binning.
 - Filter selection and display: Select proper excitation and emission filters (see table 1). Define exposure time for each color (see table 1). Define imaging height for each colors (HWAF offset for our parameters see table 1)
 - Acquisition options: Define location and number of fields to be imaged per well. In this protocol we imaged 9 fields per well. Hit "Run" and enter plate name
- 5 Open images in CyteSeer
 - Select source folder
 - Select algorithm to run. We typically run a custom algorithm in CyteSeer based on the principles described in (Bushway and Mercola, 2006), see Step Annotation below.
 - Adjust sensitivity (90% for eGFP, 225% for AlexaFluor-568)
 - Select thresholding method – Savitzky and Golay (Savitzky and Golay, 1964)
 - Define the number of images per well to be processed. In this protocol we capture 9 images and run the analysis the 3x3 cluster (3 images across and 3 images down).
 - Hit "Run"

- 6 To access data, go to View/Data
 - Export Data to desired location
 - Open folder in Excel
- 7 Mesoderm (green) or endoderm (red) quantification

We use Total Integrated Pixel Intensity (average pixel intensity within the mask×the area of the mask) as a metric to quantify Green and Red signals and therefore deduce how much mesoderm (green) or endoderm (red) is present in each well.

Step annotations

Step 5 - Automated Imaging Algorithm in CyteSeer:

The image analysis routine was designed to quantify the phenotypic output - fluorescent protein expression associated with gene activity of lineage markers and immunofluorescent staining of proteins of interest. Images are routinely collected in 3 channels (e.g. blue, green and red fluorescent spectra). The algorithm can be used on any of three spectra by simply adjusting the image capture sequence on the imaging instrument. If a channel is unused, we have found it advantageous to use it as a non-specific measure of broadband fluorescence and to subtract this from data quantified from a channel with relevant fluorescence data. The image processing and analysis routine described here is specific to the CyteSeer software package, but we have implemented the basic principle of image thresholding on other commercially available image processing and quantification packages. A pipeline of CyteSeer image processing plugins used in the algorithm is outlined below in the order executed in the routine:

NB: The spelling is as displayed in CyteSeer. As an example “GrayImage” (sic).

1. Get GrayImage Info (Channel 1).
 - i. Collects specific channel image dimensions, binning, etc.
2. Create Constant LabeledMask (Channel 1).
 - i. Fixes the label to 1 for all areas of the image above threshold.
 - ii. If omitted, non-contiguous area is quantified independently.
3. Median Filter GrayImage (Channel 1).
 - i. Removes bright punctate signal (salt and pepper noise) in the image.
 - ii. User enables/disables input and range prior to starting analysis.
4. Compute Threshold and Mask (Channel 1).
 - i. Thresholding can be performed with various methods.
 - ii. User provides input prior to starting analysis.
 - iii. In this protocol Savitsky-Golay (Savitzky and Golay, 1964) was used.
5. Apply BinaryMask to LabeledMask (Channel 1).
 - i. LabeledMask is modified in-place.
 - ii. Applies a common label to all areas of the image above threshold
6. Measure Area on a LabeledMask (Channel 1).

- i. Appends area to measurement list; otherwise not included.
 - ii. Provides a measure of area independent of pixel value.
7. Apply LabeledMask to GrayImage (Channel 1).
 - i. Overlay mask allowing quantification of areas above threshold.
8. Measure Basics on GrayImage (Channel 1).
 - i. Total Integrated Intensity.
 - ii. Average Pixel Intensity.
 - iii. Median Pixel Intensity.
 - iv. Standard Deviation of Pixel Intensity.
9. Repeat Step 7 (Channel 1 Mask is applied to Channel 2).
10. Repeat Step 8 (Channel 2).
11. Repeat Step 7 (Channel 1 Mask is applied to Channel 0).
12. Repeat Step 8 (Channel 0).
13. Invert a BinaryMask.
 - i. BinaryMask is modified in-place.
 - ii. This is the inversion of the mask created from Channel 1.
 - iii. This mask allows quantification of “non-specific” or “background” area.
14. Apply BinaryMask to LabeledMask (Channel 1 inverted mask; see Step 5 for details).
 - i. LabeledMask is modified in-place.
15. Measure Area on LabeledMask (Channel 1 inverted mask; see Step 6 for details).
 - i. Appends area to measures list; otherwise not included.
16. Apply LabeledMask to GrayImage (Channel 1 inverted mask; see Step 7 for details).
 - i. GrayImage is modified in-place.
17. Measure Basics on GrayImage (Channel 1 inverted mask).
 - i. Total Integrated Intensity.
 - ii. Average Pixel Intensity.
 - iii. Median Pixel Intensity.
 - iv. Standard Deviation of Pixel Intensity.
18. Repeat Step 16 (Channel 1 inverted mask is applied to Channel 2).
19. Repeat Step 17(Channel 2).
20. Repeat Step 16 (Channel 1 inverted mask is applied to Channel 0).
21. Repeat Step 17 (Channel 0).

REAGENTS AND SOLUTIONS

Mouse Embryonic Stem Cell Growth Medium (mESC GM)

DMEM High Glucose (Thermo Scientific Cat. # SH30081.01)
Fetal Bovine Serum, US origin (Gibco Cat. # 16000-044)
L-glutamine (Gibco Cat. # 25030)
Sodium Pyruvate (Sigma-Aldrich Cat. # S8636)
Penicillin-Streptomycin (Thermo Scientific Cat. # SV30010)
Non-essential Amino Acids (Gibco Cat. # 11140)
2-Mercaptoethanol (Sigma-Aldrich Cat. # M7522)
Leukaemia inhibitory factor (Millipore Cat. # ESG1107)

Freezing Media (FM)

DMEM High Glucose (Thermo Scientific Cat. # SH30081.01)
Characterized Fetal Bovine Serum (Thermo Scientific Cat. # SH3039603)
Dimethyl sulfoxide (Sigma-Aldrich Cat. # D2650)

Chemically Defined Medium (CDM)

IMDM (Gibco Cat. # 12440-053)
Ham's F-12 (Gibco Cat. #31765)
Albumin, Bovine Serum (Calbiochem Cat. # 12659)
L-glutamine (Gibco Cat. # 25030)
Penicillin-Streptomycin (Thermo Scientific Cat. # SV30010)
B-27 Serum-free Supplement (Gibco Cat. # 17504-044)
N-2 Supplement Ascorbic Acid (Gibco Cat. # 17502-048)
1-thioglycerol (Sigma-Aldrich Cat. # M1753)

Blocking Buffer

Horse Serum, New Zealand Origin, heat-inactivated (Life Technologies Cat. # 16050-122)
PBS 1X (Without calcium and magnesium) (Mediatech Cat. # 21-040-CV)
Triton X-100 (Sigma Cat. # T8787)
Gelatin (Stem Cell Technologies Cat. # 07903)

8% Paraformaldehyde

Cell Storage Solution—

PBS 1X (Without calcium and magnesium) (Mediatech Cat. # 21-040-CV)
Gelatin (Stem Cell Technologies Cat. # 07903)

COMMENTARY

Background Information

Critical Parameters

Culture of Mouse Embryonic Stem Cells and Preparation of Frozen mESC Stock: To ensure consistent results from one differentiation to another, it is essential that an ample frozen stock is generated, and that the cells are passaged the same number of times before beginning the differentiation protocol. Moreover, one vial of stock should be used to generate a single differentiation and then be discarded. mESCs should not be kept in culture when not planning an experiment. To increase the recovery rate when thawing cells, the vial should be placed immediately into a 37°C water bath and transferred to serum containing media as quickly as possible. When passaging the cells, the critical step is the disassociation. The cells should be suspended in trypsin for the least amount of time necessary to obtain a single cell suspension, and gently resuspended in serum containing media. The quality of the culture is dependent the disassociation step.

Differentiation of Mouse Embryonic Stem Cells into Multi-Potent Mesoderm

Progenitor (Kdr+) Cells: Obtaining consistent results from one differentiation to the next is the most difficult aspect of the protocol. However, consistent results can be achieved by meticulously performing a few critical steps of the differentiation protocol (Basic Protocol 2). When growing the mESCs for differentiation, ensure that the cells never become >80% confluent as passaging then only a single time two days prior to day 0 of the protocol is crucial. Over-trypsinizing the cells can greatly decrease viability, and is the most critical step in executing this protocol efficiently. Additionally, the potency of each lot of Activin A should be assayed to ensure the proper activation of the pathway by transfecting an activin response element expressing construct with varying doses of Activin A. Without proper activation of the Activin/Nodal signaling pathway, the assay will not work properly. We have noted as much as a 10-fold variation in potency between suppliers, and individual suppliers can have several-fold lot to lot variation in potency. When both aspirating and replacing media on the cells, the speed of aspiration and dispensation should be slow and gentle to avoid lifting the cells from the plate.

High-Throughput Imaging and Quantification of Kdr and Foxa2 Expression: One should evaluate the intensity of each signal being quantified so to avoid under/over-exposure of the signal. Saturation can compromise assay dynamic range and can be avoided by looking for saturated pixels in the specific channel or positive values too close to background signal (e.g. within 2-fold) and adjusting the exposure time appropriately. A second issue to consider is that the threshold for the channel masks in the routine we developed for Cyteseer is determined relative to the global image average pixel intensity. This means that the algorithm functions with greatest accuracy when the number of cells expressing the signal of interest comprises a minor proportion of the total image field. This approach is appropriate for cardiovascular differentiation from Kdr+ progenitors under our screen conditions. However, If this condition is dramatically altered (e.g. 90% of cells are producing positive signal), then the algorithm will score this condition very conservatively since a high proportion of responding cells in a well will skew the global average higher and, consequently, only the highest pixel values in the population will be scored. Thus, method used for setting the threshold for the channel mask is critical and might need to be altered if a high proportion of cells will produce the signal of interest.

Troubleshooting

Anticipated Results—During days 0–2 of the differentiation protocol (Basic Protocol 2), the mESCs should appear as compact spherical structures, and proliferate rapidly during the

time between day 2 and day 3 after Activin A has been added to the media. After day 3 of the differentiation protocol has been completed and the cells have settled to the bottom of the plate, the cells should be evenly spaced and aggregate into EB structures that are attached to the plate about 12 hours later. On day 5 of the differentiation assay (Basic Protocol 2), eGFP fluorescence should begin to appear in cells that surround the EBs and are migrating away from the EB. eGFP fluorescence will peak on day 6 of the differentiation protocol (Basic Protocol 2) when the cells are to be fixed. After the immunofluorescence has been performed, the Kdr positive cells should be flat, broad, and non-overlapping with the Foxa2 positive cells, which appear as tightly clustered groups of cells. The Foxa2 staining is nuclear while the eGFP is cytoplasmic. The ratio of Kdr positive cells to Foxa2 positive cells is much greater in the siAcvr1b condition versus the control condition.

Time Considerations

Basic Protocol 1—The time required to generate a proper stock of frozen cells is dependent on the initial confluency of the 10cm tissue culture dish. As long as the stock vial used for the expansion recovers properly, then the following time considerations will be valid. Once the cells are plated, the media should be changed two days post-plating, and then allowed to expand for another two days before being trypsinized and frozen in cryotubs. After one week, the cells should be 70%–80% confluent and ready for cryopreservation although only three days are necessary to complete the protocol steps.

Basic Protocol 2—The time considerations for Basic Protocol 2 should take into account the time required for the cells to recover after being thawed, which usually takes two passages (the same time requirement for Basic Protocol 1). Once the cells are ready for differentiation, the assay takes an additional six days to reach the peak of Kdr-eGFP expression. Throughout the protocol, there are certain days (days 1 and 4) that the cells incubate for the full day. Therefore, eight days are required to complete the protocol steps necessary for successful generation of Kdr-eGFP positive cells.

Alternate Protocol 2—The purpose of Alternate Protocol 2 is to generate fully differentiated mesoderm derivatives. In order to allow the cells to fully differentiate, seventeen days total will be necessary to complete the protocol. However, since there are days when the cells are left to incubate (days 1,4,6,8,10) and CDM is changed every other day after day 5 of Alternate Protocol 2, a total of nine days is necessary to complete all steps of the protocol.

Support Protocol 2—The fixation and immunostaining requires four hours of incubation in addition to the time necessary to prepare the reagents for the protocol. Support Protocol 2 can be completed in a single day.

Basic Protocol 3—The time required to image and quantify a single assay depends on the size of the assay. Using the exposure times described in Basic Protocol 3, a single plate can be imaged in two hours with another hour necessary to adjust the Cyteseer algorithm to quantify true signal and eliminate background fluorescence.

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LITERATURE CITED

- Bushway PJ, Mercola M. High-throughput screening for modulators of stem cell differentiation. *Methods Enzymol.* 2006; 414:300–316. [PubMed: 17110199]
- Savitzky A, Golay B. Smoothing and Differentiation of Data by Simplified Least Squares Procedures. *Analytical chemistry.* 1964; 36:1627–1639.

Table 1

Color	Green	Red
Dye	eeGFP	Alexa-Fluor 568
Excitation λ (nm)	488	578
Emission λ (nm)	509	603
Excitation Filter Emission Filter	480/20 535/25	535/25 620/30
Exposure Time	60 ms	300 ms
HWAF offset	−6 μ m	−6 μ m