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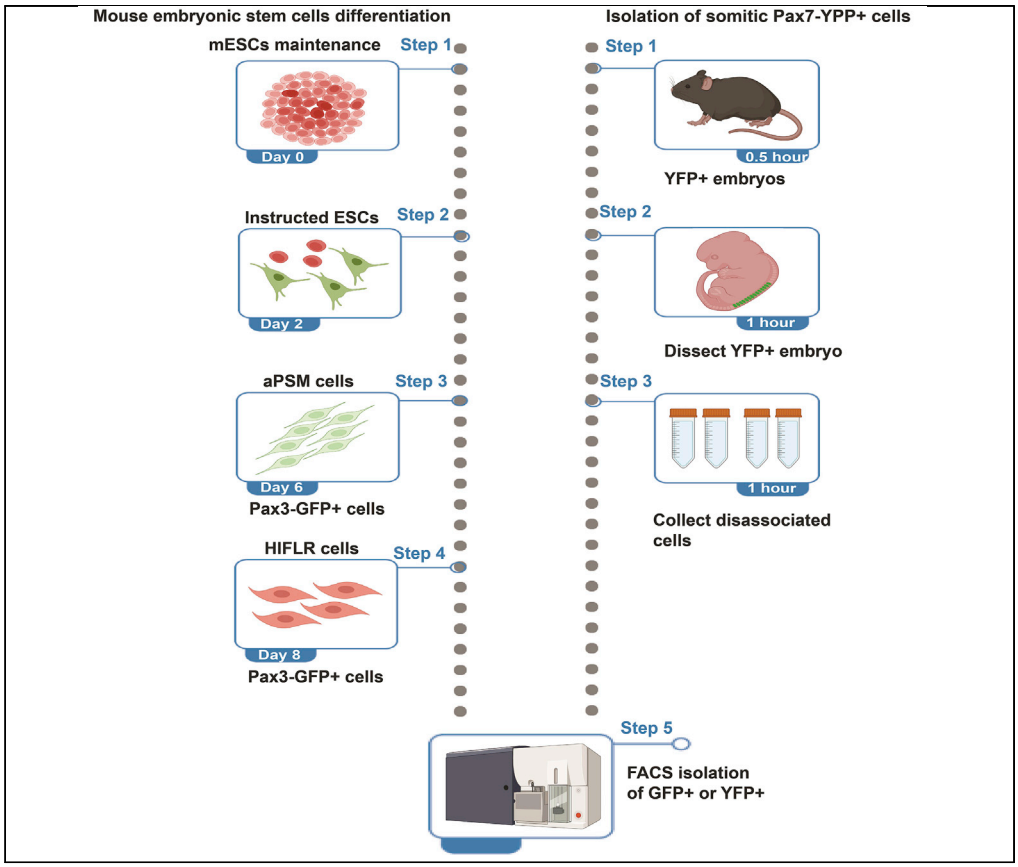
Protocols to generate and isolate mouse myogenic progenitors both *in vitro* and *in vivo*



Mouse embryonic stem cells (mESCs) can be directed to acquire cell-lineage-specific genetic programs and phenotypes by stepwise exposure to defined factors, allowing the development of *in vitro* models for studying disease and tissue generation. In this protocol, we describe the use of cultured mESCs to generate presomitic-like mesoderm cells undergoing further specification towards myogenic progenitors (MPs). Further, we describe here a procedure to obtain, dissect, and fluorescence-activated cell sorting (FACS)-isolate somitic cells from genetically labeled *Pax7*$^{+/Cre}$; *Rosa26*$^{YFP/+}$ embryos.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to induce differentiation of naïve mESCs into presomitic-like mesoderm cells

Protocol to differentiate presomitic-like mesoderm cells into myogenic progenitors

FACS isolation of genetically labeled *Pax7*$^{+/Cre}$; *Rosa26*$^{YFP/+}$ somites

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Protocols to generate and isolate mouse myogenic progenitors both *in vitro* and *in vivo*

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SUMMARY

Mouse embryonic stem cells (mESCs) can be directed to acquire cell-lineage-specific genetic programs and phenotypes by stepwise exposure to defined factors, allowing the development of *in vitro* models for studying disease and tissue generation. In this protocol, we describe the use of cultured mESCs to generate presomitic-like mesoderm cells undergoing further specification towards myogenic progenitors (MPs). Further, we describe here a procedure to obtain, dissect, and fluorescence-activated cell sorting (FACS)-isolate somitic cells from genetically labeled *Pax7*$^{+/Cre}$; *Rosa26*$^{YFP/+}$ embryos.

For complete details on the use and execution of this protocol, please refer to Khateb et al.[1]

BEFORE YOU BEGIN

Cell culture and media preparation

This protocol provides step-by-step instructions for differentiating mESCs towards myogenic progenitors (MPs). To execute this protocol, mESCs were cultured and maintained in mESC maintenance media in a humified 37°C incubator with 5% CO2. mESCs were cultured and differentiated by using different culture media (instructing medium, anterior presomitic mesoderm medium (aPSM medium) and medium supplemented with HGF, IGF-1, FGF-2, LDN and Rspo3 (HIFLR medium). Differentiation media, solutions, and gelatin-coated plates are prepared before initiating the differentiation protocol. Growth factors and small molecules are added to the medium immediately before use.

Note: All media should be equilibrated to 37°C before starting the differentiation procedure.

Gelatin-coated plates preparation

Timing: 1 h

1. Add 1 mL of 0.1% gelatin (EmbryoMax, see key resources table) to each well of 6-well plates and incubate the plates at 37°C for 1 h before starting the differentiation procedure.

Timed mating set up for E12.5 embryo isolation

Timing: 13 days

Obtain *Pax7*$^{+/Cre}$; *Rosa26*$^{YFP/+}$ embryos by setting up timed mating between *Pax7-Cre* mice (*Pax7*$^{+/Cre}$) (JAX: 010530) and *Rosa26-YFP* (*Rosa26*$^{YFP/YFP}$) (JAX: 006148) mice.

2. House 1 male *Pax7*$^{+/Cre}$ and 2 female *Rosa26*$^{YFP/YFP}$ mice in the same cage at around 4 pm the day before plug checking
3. Check the vagina of each female the next morning for the presence of a vaginal plug. The day of vaginal plug was recorded as E0.5. In this protocol, we aimed to obtain E12.5 embryos.

Institutional permissions

All of the animal experiments were performed according to the National Institutes of Health (NIH) Animal Care and Use regulations. All animal protocols have been approved by the NIAMS Animal Care and Use Committee (ACUC).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
EmbryoMax ultrapure water with 0.1% gelatin	Millipore	ES-006-B
Knock-Out ™ Serum Replacement (KSR)	Thermo Fisher Scientific	10828028
Recombinant Protein-Rspo3	R&D Biosystems	4120-RS-025
LIF Recombinant Mouse Protein, embryonic stem cell-qualified (100 µg)	Gibco	A35935
Recombinant Murine BMP4	Peprotech	315-27
BSA fraction V (7.5%)	Gibco	15260-037
LDN193189	Stemgent	04-0074
PD325901 (2 mg, 10 mM)	Stemgent	04-0006-02
CHIR99021 (2 mg, 10 mM)	Stemgent	04-0004-02
Recombinant murine FGF-2	Peprotech	450-33
Recombinant murine IGF-1	Peprotech	250-19
Recombinant mouse HGF	R&D Biosystems	2207-HG-2-025
Neurobasal medium	Gibco	21103-049
DMEM/F12 (1:1)	Gibco	11320-033
B-27™ Supplement (50X), serum free	Gibco	17504044
N-2 Supplement (100X)	Gibco	17502048
Dimethyl sulfoxide	Sigma	276855
2-Mercaptoethanol	Sigma	M6250
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	12430-054
Pen/Strep glutamine (100x)	Gibco	10378-016
GlutaMAX (100x)	Gibco	35050-061
MEM NEAA (100x)	Gibco	11140-050
Sodium pyruvate (100 mM)	Gibco	11360-070
BSA fraction V (7.5%)	Gibco	15260-037
1× PBS	Gibco	10010023
5 mL polystyrene round-bottom tube with cell-strainer cap	Falcon	352235
FBS (fetal bovine serum)	Gibco	10-437-028
TRIzol reagent	Thermo Fisher Scientific	15596018
Phase Lock Gel Heavy	5 PRIME	2302810
Papain from papaya latex	Millipore Sigma	P4762-25MG
Experimental models: Cell lines		
mESC Pax3-GFP	Chal et al.[2]	N/A
Experimental models: Organisms/strains		
Mouse: *Pax7*$^{+/Cre}$, Adult males	The Jackson Laboratory	(JAX: 010530)
Mouse: *Rosa26*$^{YFP/YFP}$, Adult femals	The Jackson Laboratory	(JAX: 006148)

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
BioRender	BioRender.com	N/A
Others		
5 mL polystyrene round-bottom tube with cell-strainer cap	Falcon	352235
6 well plates	Falcon	353224
12 well culture plates	Falcon	353225
Petri dish (100 mm × 15 mm)	Fisher Scientific	FB0875713
Falcon tube (15 mL)	Falcon	352196
Falcon tube (50 mL)	Falcon	352070
40 μm strainer filter	Falcon	352340
FACS tube	Falcon	352063
FACS tube with strainer top	Falcon	352235
Cell culture incubator	Panasonic	N/A
Thermocycler	Applied Biosystem Inc.	N/A
Centrifuge	Eppendorf Inc.	5804 R
Scissors	Electron Microscopy Sciences	77926-5C
Forceps	Electron Microscopy Sciences	72702-D

MATERIALS AND EQUIPMENT

Equipment	Source	Identifier
FACSAria Fusion	BD Bioscience	N/A
Cellometer Vision	Nexcelom Bioscience	N/A
Fluorescence microscope	Leica DMI6000	N/A

ESCs maintenance medium

Reagent	Final concentration	Amount
FBS	15%	75 mL
Pen Strep Glutamine (100x)	1x	5 mL
Sodium pyruvate buffer (100 mM)	1 mM	5 mL
NEAA (100x stock)	1x	5 mL
B-mercaptoethanol	0.1 mM	3.5 μL
LIF (100 μg)	2000 units/mL	50 μL
CHIR99021 (2 mg, 10 mM)	3 μM	75 μL
PD0325901 (2 mg, 10 mM)	1 μM	50 μL
DMEM		409.8 mL
Total	N/A	500 mL

The media should be stored at 4°C for up to 2–3 weeks
The medium should pre-warmed to 37°C.

Instructing medium

Reagent	Final concentration	Amount
Neurobasal Medium	N/A	22.65 mL
DMEM/F12	N/A	22.65 mL
B-27™ Supplement (50X), serum free	1x	1 mL
N-2 Supplement (100X)	1x	0.5 mL
BSA fraction V (7.5%)	0.1%	667 μL
KSR	1%	0.5 mL
GlutaMAX (100x)	1x	0.5 mL
Pen/Strep (100x stock)	1x	0.5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
NEAA (100x stock)	0.1 nM	0.5 mL
Sodium pyruvate (100x stock)	1x	0.5 mL
B-mercaptoethanol	0.1 mM	0.4 μL
BMP4 (10 μg/mL)	10 ng/mL	50 μL
Total	N/A	50 mL

The media should be stored at 4°C.
Fresh medium should be prepared before each differentiation experiment or stored up to 1 week at 4°C.

aPSM differentiation medium		
Reagent	Final concentration	Amount
FBS	1%	500 μL
KSR	14%	7 mL
DMSO	0.5%	250 μL
LDN193189 (10 mM)	0.1 μM	0.5 μL
Rspo3 (200 μg/mL)	10 ng	2.5 μL
7.5% BSA fraction V	0.1%	667 μL
Pen/Strep (100X stock)	1x	0.5 mL
GlutaMAX (100x)	1x	0.5 mL
NEAA (100X stock)	1x	0.5 mL
B-mercaptoethanol	0.1 mM	0.4 μL
Sodium pyruvate (100X stock)	1x	0.5 mL
DMEM	N/A	39.6 mL
Total	N/A	50 mL

The media should be stored at 4°C.
Fresh medium should be prepared before each differentiation experiment or stored up to 1 week at 4°C.

HIFLR differentiation medium		
Reagent	Final concentration	Amount
FBS	1%	500 μL
KSR	14%	7 mL
DMSO	0.5%	250 μL
LDN193189 (10 mM)	0.1 μM	0.5 μL
Rspo3 (200 μg/mL)	10 ng	2.5 μL
HGF (10 μg/mL)	10 ng/mL	50 μL
IGF-2 (20 μg/mL	2 ng/mL	5 μL
FGF-2(20 μg/mL	20 ng/mL	50 μL
7.5% BSA fraction V	0.1%	667 μL
Pen/Strep (100X stock)	1x	0.5 mL
GlutaMAX (100x)	1x	0.5 mL
NEAA (100X stock)	1x	0.5 mL
B-mercaptoethanol	0.1 mM	0.4 μL
Sodium pyruvate (100X stock)	1x	0.5 mL
DMEM	N/A	39.5 mL
Total	N/A	50 mL

The media should be stored at 4°C.
Fesh medium should be prepared before each differentiation experiment or stored up to 1 week at 4°C.

Embryo disassociation medium

2.5 mg/mL Papain in 1× PBS (pH 7.4); store at −20°C. The medium is stable for at least 3 months.

Somite sorting medium

15% FBS in 1× PBS (pH 7.4); store at 4°C. The medium is stable for at least 1 month.

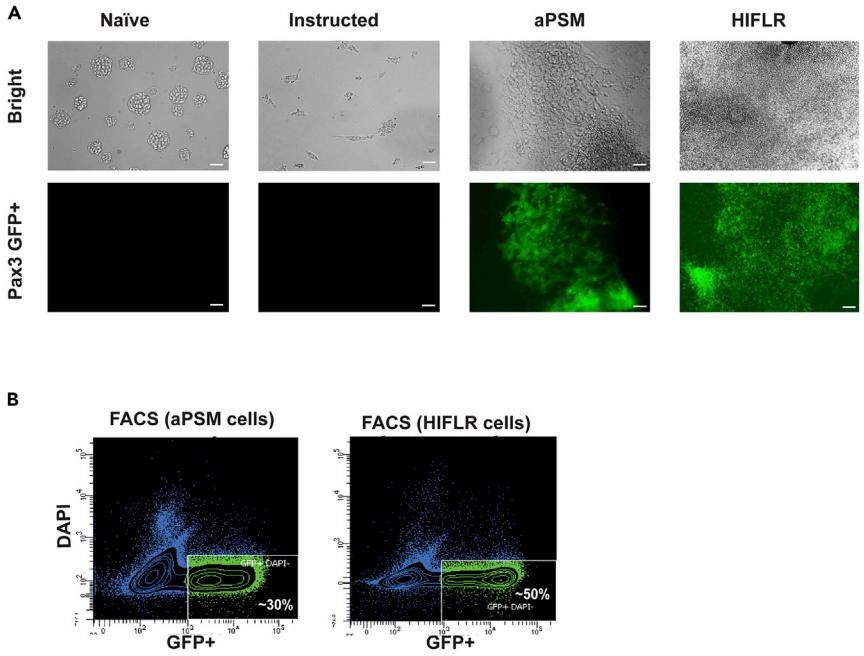

Figure 1. Induction and FACS-isolation of mESCs-derived Pax3-GFP$^+$ cells

(A) Bright-field and fluorescence microscopy images of naïve mESCs and mESCs-derived Pax3-GFP$^+$ cells at different stages of induced differentiation. Bar, 100 μm

(B) FACS plot of mESCs-derived Pax3-GFP$^+$ cells at day 6 (aPSM cells) and day 8 (HIFLR cells) of differentiation.

STEP-BY-STEP METHOD DETAILS

Differentiation of mouse embryonic stem cells (mESCs) into myogenic progenitor cells (MPs) and FACS of Pax3-GFP cells

This section provides a detailed protocol to generate myogenic progenitor cells from engineered mouse Pax3-GFP mESCs described previously.[2] The first step of this protocol is the differentiation of mESCs (Figure 1A) into instructed mESCs by using bone morphogenetic protein 4 (BMP4) and removing LIF and 2i (CHIR99021 and PD0325901). BMP4 is an essential factor for mesoderm formation.[3] The culture medium of instructed mESCs is changed to aPSM differentiation medium to prompt differentiation towards aPSM-like fate (Figure 1A). Both chemicals (LDN193189 and Rspo3) used in aPSM medium can induce the instructed mESCs to activate Pax3 expression. Approximately ~30–40% Pax3-GFP positive cells are obtained by exposure to aPSM medium that can be FACS-sorted based on GFP expression (Figure 1B). Then, the medium is switched to a HIFLR medium supplemented with hepatocyte growth factor (HGF), insulin growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF-2), LDN193189, and Rspo3 for additional 48 h and Pax3-GFP positive can be FACS sorted (Figure 1A and 1B). At this time, cells express Pax7 protein as shown[1]

Differentiation of mESCs into MPs

Timing: ~12 days

Note: To ensure efficient mESCs differentiation, it is essential that mESCs are not cultured for more than 10 passages. mESCs should form round-shaped colonies, and cell confluency should be 50–60%. All differentiation experiments are done in 6-well plates (see key resources table).

1. Thaw mESCs,

a. Plate mESCs on gelatin-coated 6-well plates.
b. Maintain mESCs in maintenance medium (see materials and equipment).
c. Passage the cells twice before starting differentiation.

2. Day 0: Remove the culture medium and wash mESCs once with 1 mL PBS.
3. Remove PBS and add 200–300 μL trypsin for 3–5 min at 37°C.
4. Add 1 mL instructing medium (see materials and equipment) and gently pipette to detach the cells.
5. Collect mESCs into a 15 mL falcon tube with 3 mL instructing medium.
6. Centrifuge cells at 160–185 × *g* for 5 min at 25°C.
7. Resuspend the cells in 1 mL instructing medium and count them.
8. Determine cell number using Cellometer Vision according to the manufacturer's instructions (http://www.nexcelom.com/Literature/Literature_Cellometer_Vision.pdf).
9. Plate 140–150K cells per well of gelatin-coated 6-well plates (total volume for each well is 2 mL of instructing medium).
10. Culture the cells for two days.
11. Day 2: Carefully remove the instructing medium, wash the cells with 1 mL of 1× PBS.

Note: Cells at this stage (day 2) detach easily. Wash the cells with 1 mL 1× PBS carefully and avoid shaking the plate.

12. Remove PBS and add 2 mL aPSM differentiation medium (see materials and equipment) to each well.
13. Continue culturing the cells for four days in aPSM medium.

Day 6: ~30% GFP-positive cells should appear (see Section PAX3-GFP-positive cells isolation using FACS, step 17).

14. Remove aPSM medium and add 2 mL HIFLR medium (see materials and equipment) to each well.
15. Keep HIFLR medium for additional two days.
16. Day 8: GFP-positive cells number should increase to ~50% and cells can be prepared for FACS sorting (Figure 1A and 1B).

PAX3-GFP-positive cells isolation using FACS

Timing: ~1 day

This section provides detailed description for sorting Pax3-GFP-positive cells at day 6 (aPSM cells, step 14) and day 8 (HIFLR cells, step 16) of differentiation for further analyses (RNA-seq, ATAC-seq, single cell RNA-seq, single cell ATAC-seq and single cell multiome analysis).

17. On the sorting day (step 14 or step 16), add 300–500 μL of trypsin to each well for 5–10 min at 37°C.
18. Gently pipette cells every 1–2 min until cells detach.

Note: The differentiated cells at this stage are hard to dissociate, they easily form clumps and aggregates. For better dissociation, keep the trypsin for longer time (~10 min) and gently pipette the cells with the trypsin every 1–2 min until they detach from the plate.

19. Add 1 mL of differentiation medium (aPSM medium if the sort at day 6 or HIFLR medium if at day 8).
20. Transfer the cells to 15 mL falcon tube and add 5 mL medium to the falcon tube.
21. Spin at 160 × *g* for 3 min and discard supernatant carefully.
22. Resuspend the pellet with an appropriate amount (500 μL–2000 μL, depending on the pellet size) of differentiation medium.

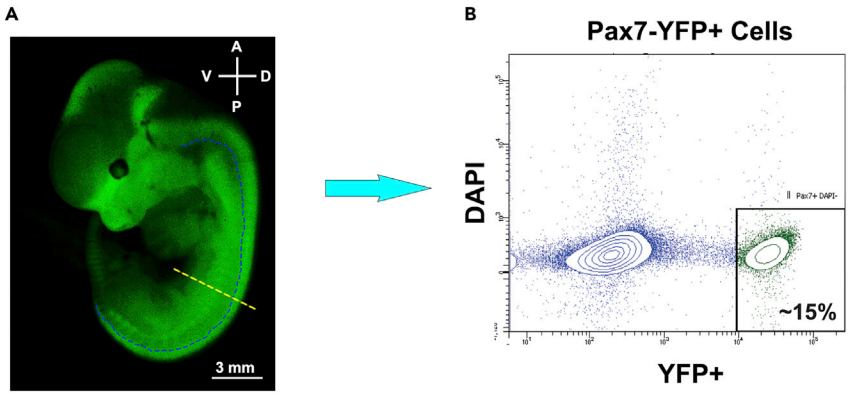

Figure 2. FACS isolation of YFP$^+$ somitic cells

(A) A representative E12.5 *Pax7$^{+/Cre}$; Rosa26$^{YFP/+}$* embryo with orientation index; A: anterior, P: posterior, D: dorsal, V: ventral. The blue dashed line marks the boundary between spinal cord and somites; the yellow dashed line marks the axial level of dissection. Somites ventral to the blue line, posterior (caudal) to the yellow line were dissected out in each embryo.

(B) FACS plot of YFP$^+$ cells isolated from somites of *Pax7$^{+/Cre}$; Rosa26$^{YFP/+}$* embryos.

Note: To avoid clumps during FACS sorting, add 1 mM EDTA to differentiation medium.

23. Transfer the cells through a 40 μm cell strainer sitting on a 15 mL falcon tube or through polystyrene round-bottom tube with cell-strainer cap (see key resources table).
24. Take cells to FACS on ice.
25. Sort by gating for GFP (Figure 1). About 200,000–300,000 GFP positive cells can be obtained from a 6 well plate.
26. After FACS, collect cells by spinning them down at 210–305 × *g* for 5–8 min.
27. Discard supernatant, wash the cells twice with 1 mL of 1× PBS.
28. Store pellets or proceed for further analysis.

Isolation of somitic cells from genetically labeled *Pax7$^{+/Cre}$; Rosa26$^{YFP/+}$* embryos

This section provides a detailed protocol to harvest, dissect, dissociate and FACS-isolate somitic cells, including MPs, from genetically labeled *Pax7$^{+/Cre}$; Rosa26$^{YFP/+}$* embryos, in which somitic cells including MPs are labeled with YFP.

Embryo collection

Timing: ~0.5 h

29. Fill a bucket with ice, fill 4 petri dishes (100 × 15 mm) and one 12-well culture plate with 1× PBS and put them on ice. Sanitize dissection tools (2 scissors, 2 forceps) with 70% ethanol spray and Kimwipes.
30. Euthanize a pregnant mouse (at day 12 after a positive plug) by CO_2 asphyxia according to Animal Care and Use Committee (ACUC) regulations.
31. Position the mouse belly up on a clean paper towel; spray the belly with 70% ethanol and make a 1 cm cut on the belly skin with scissors; pull the skin bidirectionally toward the head and the toe to expose the sac containing a string of embryos.
32. Make a cut on the body wall of abdominal cavity and carefully remove the whole string of embryos and transfer them in a petri dish placed on ice.
33. Peel off each uterus wall to release each embryo into the dish. An E12.5 embryo is covered with a very thin layer of amniotes and vascularized yolk sac. Tear these membranes off to expose the embryo, then cut off the umbilical cord from placenta.
34. Rinse all embryos in a new petri dish with 1× PBS, then allocate each embryo in one well of a 12-well culture plate.

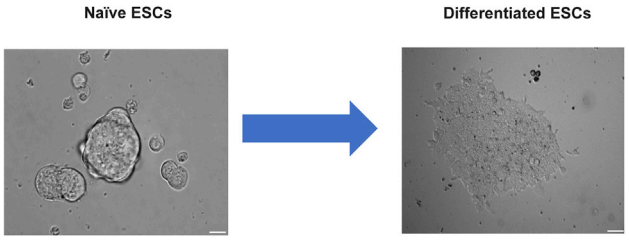

Figure 3. Representative image showing spontaneous differentiation of ESCs

The image illustrates the morphology of naïve (left) and spontaneously differentiating (right) ESC clones. Bar, 100 μm.

35. Screen embryos under the fluorescent dissecting microscope to identify *Pax7*$^{+/Cre}$; *Rosa26*$^{YFP/+}$ embryos; mark the wells with YFP-positive embryos.

Note: At this embryonic stage, Pax7 is also expressed in the dorsal neural tube. Under the fluorescent microscope, one can clearly see the YFP expressing spinal cord and bilaterally situated somites with striped expression pattern of YFP (Figure 2A).

YFP-positive embryo dissection

Timing: ~1 h

36. Take one YFP-positive embryo, place it in a new petri dish with 1× PBS.
37. Eviscerate the embryo's abdominal cavity and thoracic cavity, flatten the embryo in the dish with belly down and back up.
38. Cut the embryo approximately by half through the trunk region, between the two pairs of limbs; take the posterior half for the next step dissection.

Note: In this study, we aimed to use relatively nascent somites harboring undifferentiated MPs. Therefore, we isolated ~ 25 pairs of somites in the posterior (caudal) half of the embryo.

39. On the flattened posture, one can clearly see the boundary between spinal cord and lateral somites (Figure 2A). Use fine tipped forceps to carefully carve out the YFP-positive spinal cord and discard it.
40. Put the rest of the tissue including YFP-positive somites in a 1.5 mL tube with 250 μL cold 1× PBS, place the tube on ice.
41. Repeat steps 36–40 to finish the dissection of all YFP-positive embryos, each embryo in one 1.5 mL tube. Mince the tissue in each tube by scissors one by one.

Note: It takes approximately 10 min to dissect one embryo. Normally one litter has ~ 4–6 YFP-positive embryos, so it takes approximately 1 h to complete this step.

Tissue dissociation

Timing: ~1 h

42. Add 250 μL Embryo dissociation medium (see materials and equipment) to each tube and incubate in a 33°C water bath for 20 min. While incubating, use a 1 mL tip to pipet the tissue up and down to help dissociation.
43. When the tissue is completely dissociated, add 500 μL somite sorting medium (see materials and equipment) to stop the digestion.

44. Move the dissociated tissue into a 15 mL falcon tube and resuspend the tissue with 10 mL of somite sorting medium.
45. Filter each resuspended tissue through a 40 μm cell strainer sitting on a 50 mL falcon tube.
46. Centrifuge (~210 × *g*) 50 mL Falcon tubes to spin down cells. A small pellet should be seen on the bottom of each tube.
47. Carefully remove the supernatant from each tube by aspirating through a 2 mL aspirating pipette.
48. Resuspend the pellet with 500 μL somite sorting medium, then filter through a 5 mL Falcon tube with a strainer top (40 μm).

Note: Coat the strainer top with 100 μL FBS before filtering to help the cells dropping.

Cell sorting by FACS

Timing: ~2 h

49. FACS-isolate cells by gating on GFP channel (Figure 2B).
50. From each embryo, approximately 10–15% sorted cells are YFP-positive (Figure 2B); approximately 100,000 to 150,000 YFP-positive cells were collected per embryo.
51. Store cells in −80°C freezer or use them immediately for RNA extraction.

EXPECTED OUTCOMES

This protocol provides a comprehensive model to investigate lineage commitment towards myogenic specification starting from mESCs. Using defined factors at specific time points, Chal and coworkers[2] have described the transition from mESCs towards myogenic progenitors and further commitment towards muscles formation. Employing the protocol described here, we have provided a comprehensive map of gene regulation and chromatin state change during mESCs differentiation[1]

LIMITATIONS

This protocol has been used to generate myogenic progenitors from mESCs.[2]

From Pax3-GFP ESCs differentiation we obtained 30–40% GFP positive cells at day 6 of differentiation. However, the reproducibility of these numbers could vary between experiments, depending on mESCs passage, cell number plated at the starting point (day 0) and reagents stability (avoid thawing and freezing). The sorted YPF-positive somitic cells contain not only MPs, but also differentiated myocytes.

TROUBLESHOOTING

Problem 1

mESCs morphology changes and spontaneous differentiation (step 4) (Figure 3).

Potential solution

To overcome this problem, do not allow mESCs to overgrow (more than 80%) and do not culture them for high passage numbers. We suggest to aliquot the LIF and 2i inhibitors to avoid freeze and thawing.

Problem 2

Cells at day 2 detach easily, potentially affecting the differentiation process.

Potential solution

Cells number is very critical to achieve efficient differentiation. To avoid detaching cells at day 2 of differentiation, treat the cells very gently (don't shake the plate) and remove medium slowly. When adding new differentiation medium, add the medium to the side of the plate.

Problem 3

Myogenic progenitor cells are difficult to dissociate (steps 14 and 16).

Potential solution

Differentiated myogenic cells were hard to detach or dissociate. To overcome this problem, the cells should be treated with 0.25% trypsin for longer time (8–10 min) at 37°C. During this time gently pipette every 1–2 min.

Problem 4

Cells clumping during FACS sorting causing clogging.

Potential solution

Differentiated cells tends to re-attach again and clump. To overcome this problem, add to sorting medium 1 mM EDTA and dilute the pellet with appropriate medium volume (depends on the pellet size). Medium can be stored at 4°C.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Vittorio Sartorelli (sartorev@mail.nih.gov).

Materials availability

Materials associated and used in this protocol are available upon request from the lead contact.

Data and code availability

We do not report datasets or codes in this protocol.

ACKNOWLEDGMENTS

We thank Jim Simone (Flow Cytometry Section, NIAMS) for assistance with FACS procedures. This work was supported in part by the Intramural Research Program of the NIAMS at the NIH (grants AR041126 and AR041164 to V.S.).

AUTHOR CONTRIBUTIONS

M.K. performed the differentiation of mESCs, imaged the cells, and prepared samples to FACS. X.F. developed the somite isolation protocol and prepared samples to FACS. S.D. reviewed/edited manuscript. V.S. supervised and developed the projects. All the authors contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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