Mesoderm Differentiation: Hematopoiesis

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Title	Mesoderm Differentiation Hematopoiesis
Date Submitted	January 5th 2012
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Contributors -	
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This next section will be on the side and is only relevant for website programmers

Score	=Likes/(Likes+Dislikes)+Likes/Downloads or some other algorithm… Suggestions??? I can also come up with something better in due time.
Downloads	#
"Likes"	#
"Dislikes"	#
Status	[Validated (>=3, In Progress (=1-2), Not Validated (none)]
Validated Core 1	Name / Institute
Validated Core 2	Name / Institute
Validated Core 3	Name / Institute

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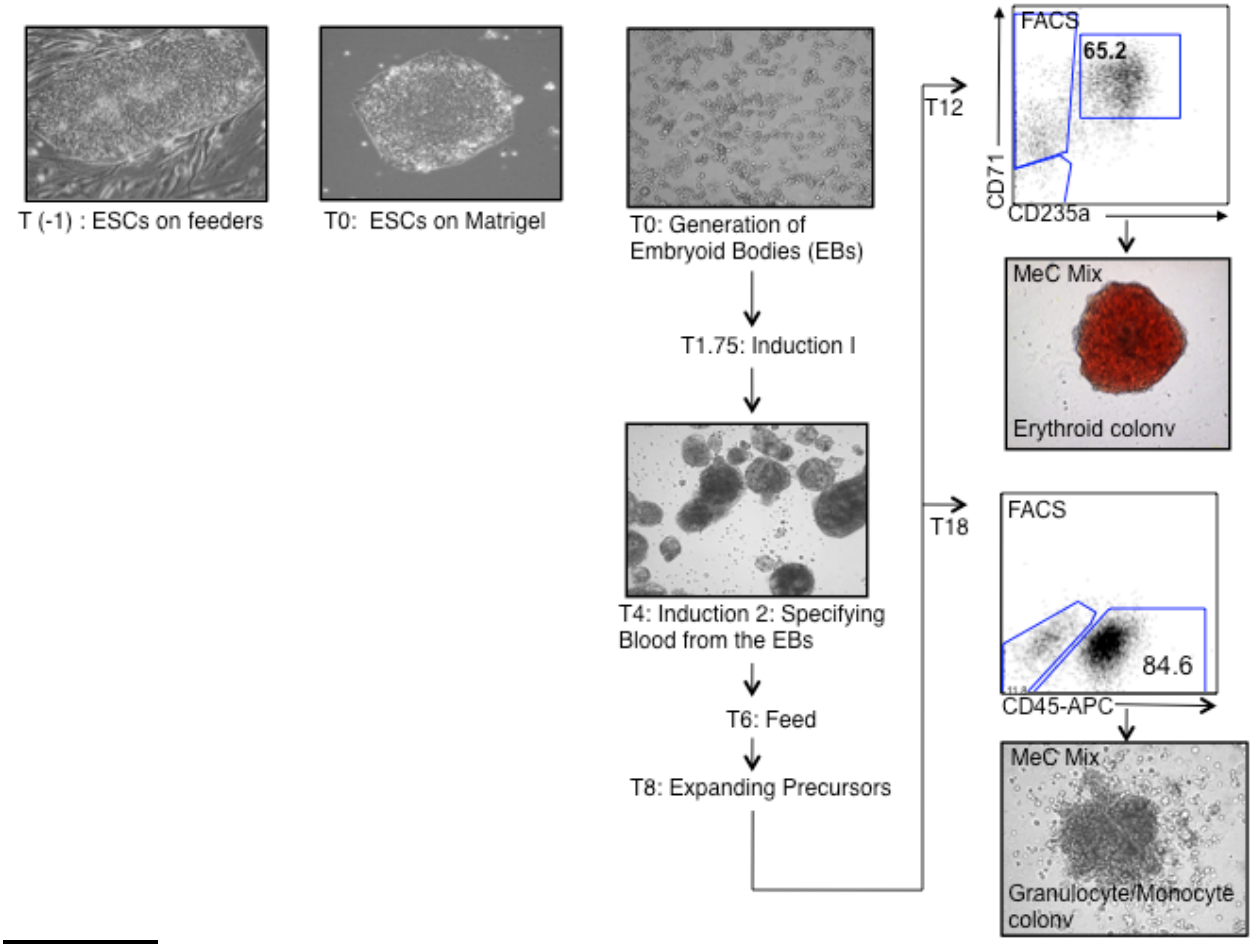
6. Contributors

INTRODUCTION

The protocol outlined below describes the method for generating hematopoiesis from hESCs, specifically H1 cells. Generally the first hematopoietic progenitors start appearing at T5-7 and are mainly primitive erythroid with a few macrophages. We have defined stages by cell surface markers and colony morphology, in our differentiation cultures, that may reflect the emergence of yolk sac, fetal and definitive hematopoiesis

FLOWCHART



The formation of EBs is the first important step in the differentiation of hESC. This is best achieved by culturing small aggregates of hESCs in miminal amounts of BMP-4 for 24 hours. At this stage, BMP-4 functions to promote the survival of the hESCs.

MATERIALS AND PREPARATION

COLLAGENASE (FOR DISSOCIATION)*(SIGMA# C-0130)*

Collagenase Type 1 is mainly used for dissociation of the embryoid bodies.

		Final Conc.	For 1 Liter
Collagenase	*(SIGMA# C-0130)*	0.2%	2g

FCS			200mL
PBS (with Ca^{2+}, Mg^{2}	*(Cellgro# 21-030-CM)*		800mL

1. Weigh 1 g of Collagenase Type 1 in the fumehood (inhalation is hazardous) and dissolve in 400 mL of PBS by gently stirring. Check carefully that the collagenase is completely dissolved.
2. Add 100 mL FCS
3. Filter sterilize (may need more than one filtration apparatus)
4. Store at -20ºC as 12 mL aliquots in 15 mL screw cap tubes
5. One cycle of freeze-thaw is acceptable

Note: final concentration is 0.2% or 2 mg/mL in 20% FCS/

L-ASCORBIC ACID (AA) *(SIGMA # A-4544)*

Prepare a stock solution of 5 mg/mL in cold TC-H_2O. Leave on ice and vortex periodically until completely dissolved. Filter sterilize, aliquot and store at -20ºC. Use once and discard

MONOTHIOGLYCEROL (MTG) *(SIGMA# M-6145)*

The amounts of MTG indicated in our protocols are recommended concentrations. However, it is important to test each new batch of MTG as there is variability between them. MTG should be aliquoted (1 mL) and stored frozen (-20ºC). When aliquots are thawed, they can be used for several experiments and then discarded. Aliquoting of MTG is strongly recommended as it minimizes the amount of oxidation due to repeated opening

TRANSFERRIN *(ROCHE# 10 652 202)*

The amounts of Transferrin indicated in our protocols are recommended concentrations. However, it is important to test each new batch of transferrin as there is variability between them. It should be aliquoted (2 mL) and stored at 4ºC.

CYTOKINES

All cytokines are stored lyophilized, at -20^{o}C, except Erythropoietin which is stored at 4^{o}C

Cytokine		Buffer	Stock Conc.
hBMP-4	(R&D Systems# 314-BP)	H20, 4mM HCL, 0.1%BSA	10ug/mL
hbFGF	(R&D Systems# 234-FSE)	PBS, 0.1%BSA,1mM DTT	10ug/mL
hVEGF	(R&D Systems# 293-VE)	PBS, 0.1%BSA	5ug/mL
hSCF	(R&D Systems# 255-SC)	PBS, 0.1%BSA	50ug/mL
hErythropoietin	Janssen-Ortho Inc (EPREX)	PBS, 0.1%BSA	2000units/mL
hIL-6	(R&D Systems# 206-IL)	PBS, 0.1%BSA	5ug/mL
hIL-11	(R&D Systems# 218-IL)	PBS, 0.1%BSA	5ug/mL

STEMPRO 34 *(Invitrogen# 10639-011)*

Stempro 34 is sold as a kit with 2 components. The supplement is kept at -20^{o}C and the liquid media at 4^{o}C. When combined, the media is unstable, therefore, we use it for a

maximum of 2 weeks. If not used right away, we store the medium as 50mL aliquots and supplement them as needed. The supplement is frozen as 1.3mL aliquots which is the amount required for 50mL. of medium

		Final Conc.	For 500mL
STEMPRO 34 Kit	*(Invitrogen 10639-011)*		500 mL
Penicillin/Streptomycin P/S	*(Gibco# 15070-063)*	1%	5 mL

1. Warm the media, P/S and frozen supplement in a 37^{o}C waterbath for 15 to 20 minutes
2. Mix the supplement well with a pipette and add to the warm media along with the P/S.
3. Warm the mixture for another 30 minutes and then aliquot into 50mL. (Label the tube that supplement has been added, to avoid confusion.)
4. Store for a maximum of 2 weeks
5.

***MATRIGEL (REDUCED FACTOR)** *(BD# 356 230)*

Each batch of matrigel has its own unique levels of endotoxin and protein concentrations. We find that the endotoxin levels should not be higher than 2 endotoxin units/mL and the protein levels should range between 7 to 10 mg/mL. If the protein levels are higher than this you may need to dilute the matrigel more than 1:1. This is determined by observing the hESC colony morphology and the ability of the hESCs to differentiate into the lineage required of them.

Caution: When working with matrigel, all tubes, plates and pipettes should be pre-chilled, as matrigel solidifies at room temperature.

MATRIGEL1:1 PREPARATION

1. Thaw frozen bottles of matrigel on ice overnight in the cold room. We normally thaw 6X5-mL bottles per batch.
2. The next day, make a 50% working stock by adding an equal volume of IMDM+P/S to each bottle. Resuspend gently with a pre-chilled 5 mL pipette.
3. Leave the bottles on ice all day to allow the matrigel to completely equilibrate with IMDM.
4. Pool 3 bottles of 1:1 matrigel (30 mL) into a pre-chilled 50 mL tube. Gently mix with a chilled 10 mL pipette and aliquot.
5. Transfer 2.5 mL into pre-chilled and pre-labelled 4-mL snap cap tubes
6. Store aliquots at -20ºC

The formation of EBs is the first important step in the differentiation of hESC. This is best achieved by culturing small aggregates of hESCs in the presence of BMP-4 for 24 hours. At this stage, BMP-4 functions to promote the survival of the hESCs.

(A) COLLAGENASE B *(Roche# 11 088 831 001)*

Collagenase B is routinely used for dissociation and passaging of undifferentiated hESCs

	Final Conc.	For 1 Liter

Collagenase B	*(Sigma# T-4799)*	0.25%	1 g
DMEM/F12	*(Cellgro# 10-092-CV)*	1 mM	1000 mL

1. Weigh 1 g of Collagenase B in the fume hood as inhalation is hazardous and dissolve in 1 Liter DMEM/F12 +P/S by stirring gently at room temperature for 1 h (or until completely dissolved)
2. Filter sterilize and store at -20ºC in 12 mL aliquots

(B) DNASE I *(*VWR, Cat # 80510-412, 10MU*)*

1. Want final concentration to be 1mg/ml

2. $10\text{MU} \times \frac{1\text{X}10^6\ \text{U}}{1\ \text{MU}} \times \frac{1\text{mg}}{65150\ \text{U}} = 153\text{mg}$

3. In the hood transfer powder to a 125 ml bottle
4. Bring the volume up to 153 ml with ice cold sterile water
5. Let dissolve on ice for 1-2 hours
6. Filter and aliquot 1ml/eppendorf
7. Store at –20.
8. Filter sterilize, aliquot in 1 mL amounts and store frozen at -20ºC
9. Use aliquots once and discard excess

(C) TRYPSIN-EDTA

		Final Conc.	For 1 Liter
Trypsin	*(Sigma# T-4799)*	0.25%	2.5 g
EDTA 0.5 M (pH 8)		1 mM	2 mL
PBS (without Ca^{2+}, Mg^{2+})	*(Cellgro# 21-031-CM)*		1000 mL

1. Warm to dissolve (15 min, 37ºC), filter sterilize, aliquot and store at -20ºC
2. For aggregate formation we use a 1/4 dilution of the above (ie: 10mL of TRYPSIN-EDTA in 30mL of PBS (without Ca^{2+}, Mg^{2+})

(D) STOP MEDIUM

		Final Conc.	For 40 mL
hESC WASH Medium		50%	20 mL
FCS		50%	20 mL
+/- Matrigel (1:1) *	*(BD# 356 230*)	1:800	100 uL

(E) hESC WASH MEDIUM

		Final Conc.	For 500 mL
Supplemented DMEM/F12			475 mL
Knockout™ Serum Replacement	*(Gibco# 10828-028*)	5%	25 mL

SUPPLEMENTED DMEM/F12

		Final Conc.	For 500mL
DMEM/F12	*(Cellgro# 10-092-CV)*		500 mL
Penicillin/Streptomycin	*(Gibco# 15070-063)*	1%	5 mL

Glutamine	*(Gibco# 25030-081)*	1%	5 mL

1. Make 40 mL aliquots of the hESC WASH MEDIUM
2. You can add 100 uL of Matrigel 1:1 to each aliquot when needed

INDUCTION / DIFFERENTIATION MEDIA

(F) AGGREGATION MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 ml
STEMPRO 34				50ml
Glutamine	100x	1%	10uL	500ul
Transferrin	300mg/ml	150ug/mL	5uL	250ul
Ascorbic Acid	5mg/ml	50ng/mL	10uL	500ul
MTG	26λ/2mls		3uL	150ul
BMP4*	10ug/ml	10ng/mL	1uL	50ul

(G) INDUCTION 1 MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 ml
STEMPRO 34				50ml
Glutamine	100x	1%	10uL	500ul
Transferrin	300mg/ml	150ug/ml	5uL	250ul
Ascorbic Acid	5mg/ml	50ng/ml	10uL	500ul
MTG	26λ/2mls		3uL	150ul
BMP4*	10ug/ml	10ng/ml	1uL	50
ActA**	10ug/ml	0.3ng/ml	0.03uL	1.5ul
bFGF	10ug/ml	5ng/ml	0.5uL	25ul

*****Bmp4 and ActA**concentration may vary according to lot# or the hES cell line used…these concentrations are for H1 cells.

(H) IMDM+10%FCS

		Final Conc.	For 500 mL
Iscove's Modified Dulbecco's Medium	*(Cellgro# 15-016-CV)*		450 mL
FCS (batch tested)	*(Gibco# 10828-028)*	10%	50 mL

(I) INDUCTION 2 MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 ml
STEMPRO 34				50ml
Glutamine	100x	1%	10uL	500ul
Transferrin	300mg/ml	150ug/ml	5uL	250ul
Ascorbic Acid	5mg/ml	50ng/ml	10uL	500ul
MTG	26λ/2mls		3uL	150ul
VEGF	5ug/ml	10ng/ml	2uL	100ul
Dkk	50ug/ml	150ng/ml	3ul	150ul
bFGF	10ug/ml	5ng/ml	0.5uL	25ul

(J) INDUCTION 2 MEDIUM (Feed T6)

	Stock Conc.	Final Conc	per mL	For 50 ml
STEMPRO 34				50ml
Glutamine	100x	1%	10uL	250ul
Transferrin	300mg/ml	150ug/ml	5uL	100ul
Ascorbic Acid	5mg/ml	50ng/ml	10uL	500ul
MTG	26λ/2mls		3uL	150ul
VEGF	5ug/ml	10ng/ml	2uL	100ul
bFGF	10ug/ml	5ng/ml	0.5uL	50ul
hIL6	**10ug/ml**	**10ng/ml**	**2ul**	**100ul**
hSCF	**50ug/ml**	**50ng/ml**	**2ul**	**100ul**
hIL-11	**5ug/ml**	**5ng/ml**	**2ul**	**100ul**
hEPO	**2000units/ml**	**2units/ml**	**2ul**	**100ul**
hIGF-1	**25ug/ml**	**25ng/ml**	**2ul**	**100ul**

Bold type indicates a 2x concentration of the cytokine as the media will be diluted by 1/2

(K)HEMATOPOIETIC EXPANSION MEDIUM

	Stock Conc.	Final Conc	per mL	For 50 ml
STEMPRO 34				50ml
Glutamine	100x	1%	10uL	500ul
Transferrin	300mg/ml	150ug/ml	5uL	250ul
Ascorbic Acid	5mg/ml	25ng/ml	5uL	250ul
MTG	26λ/2mls		2uL	100ul
hTPO	10ug/ml	50ng/ml	5ul	250ul
hIL-3	10ug/ml	50ng/ml	5ul	250ul
hFLT3L	10ug/ml	20ng/ml	2ul	100ul
hSCF	50ug/ml	100ng/ml	2ul	100ul
hIL-11	5ug/ml	5ng/ml	1ul	50ul
hEPO	2000units/ml	2units/ml	1ul	50ul
hIGF-1	25ug/ml	25ng/ml	1ul	50ul

HEMATOPOIETIC MEC MIX

Mec Mix								
Reagent	Stock/ml	Final	10ml	14ml	18ml	24ml	32ml	45ml
MEC	100%	55%	5.5ml	7.7ml	10ml	13ml	17.6ml	25ml
PDS	100%	15%	1.5ml	2.1ml	2.7ml	3.6ml	4.8ml	6.8ml
PFHM-11	100%	5%	0.5ml	0.7ml	0.9ml	1.2ml	1.6ml	1.3ml
Glutamine	100x	1x	100ul	140ul	180ul	240ul	320ul	450ul
Transferrin	30mg	150ug/ml	50ul	70ul	90ul	120ul	160ul	275ul
TPO	10ug	50ng/ml	50ul	70ul	90ul	120ul	160ul	225ul
IL-3	10ug	50ng/ml	50ul	70ul	90ul	120ul	160ul	225ul
VEGF	5ug	10ng/ml	20ul	28ul	36ul	48ul	64ul	90ul
SCF	50ug	100ng/ml	20ul	28ul	36ul	48ul	64ul	90ul
EPO	2000units	4units	20ul	28ul	36ul	48ul	64ul	90ul
IL-6	5ug	10ng	20ul	28ul	36ul	48ul	64ul	90ul
IGF-1	25ug	50ng	20ul	28ul	36ul	48ul	64ul	90ul
IL-11	5ug	5ng	10ul	14ul	18ul	24ul	32ul	45ul
GM-CSF	1ug	1ng	10ul	14ul	18ul	24ul	32ul	45ul
IMDM	100%		2.2ml	3ml	3.9ml	5.2ml	7ml	9.7ml

PROTOCOL

T0: Generation of embryoid bodies (EBs)

1. Remove the medium from hES cells that have been feeder depleted on matrigel coated plates for 24-48 hours(see hES cell maintainance protocol)

2. To each well, add 1mL of COLLAGENASE B containing 10uL/mL DNase **(A,B)**, for 20 min. and then aspirate. The cells should still be attached.

3. Add 1mL of 1/4 TRYPSIN-EDTA **(C)** to the wells and then watch carefully for 1-3min. The cells will separate from each other, but should not lift from the plate. (Note; each cell line has a different time requirement so one should continuously monitor the wells.

4. Remove the trypsin from the well and stop the reaction with 1mL of STOP MEDIUM +MATRIGEL**(D)** containing 10uL/mL DNase**.** Scrape gently with a cell scraper. The ES cells should lift as clusters into the medium.

5. Add 1ml of hESC WASH MEDIUM +MATRIGEL**(E)** to each well and resuspend with a 2mL pipette 3-5x, until the clusters are 10-20 cells in size. Transfer to a 15ml tube containing 4ml of hESC WASH MEDIUM +MATRIGEL (usually 3 wells per tube)

6. Also ,at this time, we completely trypsinize one well of the matrigel hESCs to single cells, to get an accurate cell count for the experiment. The average cell count per starting matrigel well, using this protocal, is 5x10^5 to 1x10^6.

7. Spin at 800 rpm, aspirate and resuspend each tube with 2mL of AGGREGATION MEDIUM **(F)** using a 2mL pipette (resuspend gently 2-3 times). To calculate the amount of AGGREGATION MEDIUM required for the experiment you will need 2mls for every 5x10^5 to 1x10^6 cells harvested. Adjust the final volume of each tube appropriately.

8. With a 5mL pipette, evenly distribute 2 mL of aggregates into each well of a 6 well Locluster plate *(Costar#3071)*. **Incubate for 24 hours at 37^{0}C in an 5%CO^2,5%O^2 Incubator.**

T1: Feeding Aggregates and expansion

Add 2mls of AGGREGATION MEDIUM **(F)** supplemented with 10ng/ml bFGF to the cultures. The final concentration of bFGF in the well will be 5ng/ml.

- **Please note low concentration of BMP4 (1-2ng/ml) at D0, followed by 10ng of BMP4 at D1-D4 and 4-5ng of ActA from D1-D4 led to a drastic increase in the amount of erythroid progenitors. All other cytokines are as described.**

- **Whereas 10ng/ml of BMP4 at D0, followed by 10ng of BMP4 from D1-D4 and 0.5 ng of ActA from D1-D4 led to a drastic increase in the amount of myeloid progenitors. All other cytokines are as described.**

T1.75: Induction 1

1. There will be some debris in the cultures. We separate the aggregates from the debris by harvesting 3 wells into one 15ml round bottomed tube and then allowing them to settle for 30min in an 37^{o}C,5%CO^2,5%O^2 incubator

2. While the aggregates are settling, make INDUCTION 1 MEDIUM **(G)** so that there will be 2mL for each well harvested. After the aggregates have settled, aspirate the supernatant and then add INDUCTION 1 MEDIUM . Dispense the aggregates into a new 6 well Locluster plate at 2mL per well. Distribute the aggregates evenly.

3. Incubate as above until T3-4

Notes

Mesoderm induction and hemangioblast specification in the EBs should be evaluated by flow cytometric analysis, monitoring the cells for expression of KDR and CD117 (c-kit). As each hESC line has its own unique kinetics, it is best to define the hemangioblast stage based on the profile seen below, rather than by time in culture.

The hemangioblast stage is defined by the appearance of a population that expresses medium levels of KDR and are CD117^{neg}. EBs at this stage also contain a KDR^{neg}CD117^{pos} population. This profile is detected in H1-derived EBs at day 3 and in

HES2-derived EBs at day 4. The window for hemangioblast development is narrow and occurs before CD31 is expressed on the cells.

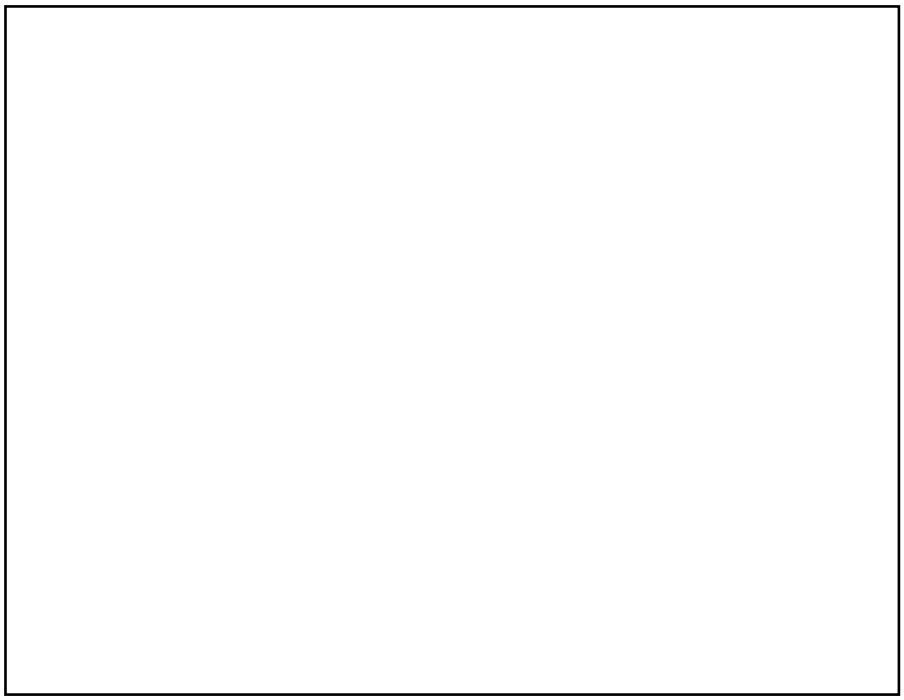
T3-4: Harvest for staining

1. Settle1 well of EBs in a round bottomed tube for 15minutes. Aspirate the medium and add 1ml of TRYPSIN-EDTA. Incubate at 37^0C in a waterbath for 5 minutes and then stop the reaction with 0.5ml of STOP MEDIUM+dnase .

2. Make to single cells by passing the EBs 4-6x through a syringe bearing a 20 Guage needle and wash with IMDM+10%FCS **(H).**

3. Spin for 5min at 1000 RPM, aspirate and resuspend in IMDM+FCS (usually 500ul per well harvested). Pass the cells through a 70micron filter to remove any clumps that are still remaining. You should recover 5x10^5-1x10^6 cells per well of EBs harvested

Facs Profiles



T4-8: Induction 2: Specifying Blood from the EBs

1. Pipette the EBs into a round bottomed 15ml tube (1-3 wells per tube). Spin for 5 min at 500RPM. Carefully aspirate the media and then add 4ml of Stempro media. Let the EBs settle for 20 min and aspirate again. This step allows for 2 washes to remove the previous cytokines, as well as, any debris floating in the supernatant.

2. Resuspend the EBs in INDUCTION 2 MEDIA**(I)** at 2mls per well harvested. This media contains VEGF and FGF at low concentrations which is necessary for expanding the earliest blood precursors. **Incubate at 37^{0}C in an 5%CO^2,5%O^2 Incubator.**

3. Feed the cultures at **T6** with 2ml of the INDUCTION 2 MEDIA **(J)** with supplemental cytokines which are in **BOLD** and added at a 2x concentration.

Notes

If one monitors the cultures at this time with FACS, the KDR/CD117 and KDR/CD31 profiles should be the same as the Post-Hemangioblast profiles above.

T8: Expanding Precursors

Settle and wash the EBs as above at T4

Resuspend and distribute as above in HEMATOPOIETIC EXPANSION MEDIUM **(K)** At this time cocktails of cytokines may vary as well as culture methods according to the lineage one desires. For general expansion of hematopoietic lineages we maintain our cultures in suspension changing the media every 4 days.
The cultures at this time can be grown in ambient oxygen conditions at 37^0 and 5%CO_2

Harvest to single cells for FACs, Sort, Counts or Reaggregations

Dissociation T1-8 (Trypsin-EDTA)

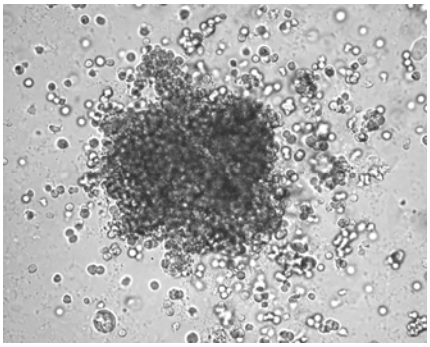
1. Harvest each group of 2 dishes into a 2059 tube and let the EBs settle
2. Remove supernat.and add 2mls of Cellgro Trypsin
3. Incubate for 5-8min in 37degree water bath
4. Stop trypsin with FCS1:1+dnase 30ul/ml
5. Syringe 6x with a 20Guage needle and then add 7mls of wash media
6. Spin for 5min at 1100rpms
7. Resuspend in 1ml of wash media and filter through blue cap of 2035 tube

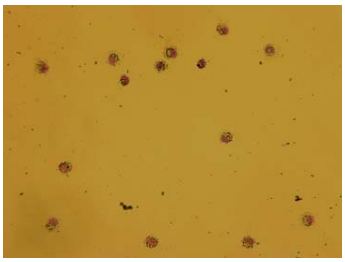
Dissociation T8+(Collagenase)

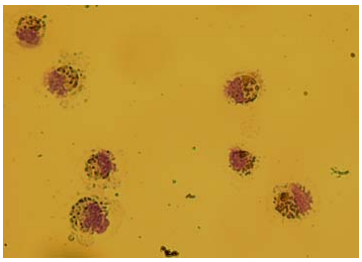
1. Harvest each group of 2 dishes into a 2059 tube and let the EBs settle
2. If there are hematopoietic clusters in your supernatant collect the sup., keep on ice during the dissociation step and add back to your cells at step 9
3. Remove supernat.and add 2mls of Cellgro Trypsin
4. Incubate for 5-8min in 37degree water bath
5. Stop trypsin with FCS1:1+dnase 30ul/ml
6. Syringe 6x with a 20Guage needle and then add 7mls of wash media
7. Spin at 1200rpm and resuspend in 1ml/well Collogenase Type 1***(L)***
8. Incubate at 37 degrees for 60min
9. Syringe 6x with a 20Guage needle, add your supernatant cells and wash media.
10. Spin at 1200rpm and resuspend in 1ml.
11. Count and use in your assays

IMAGES OF HEMATOPOIETIC COLONIES IN METHYLCELLUOSE COLONIES

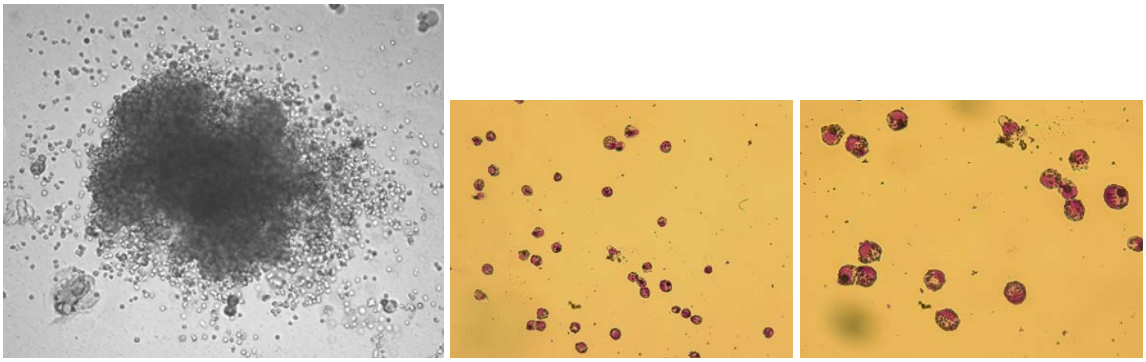
MAST COLONY – 20X



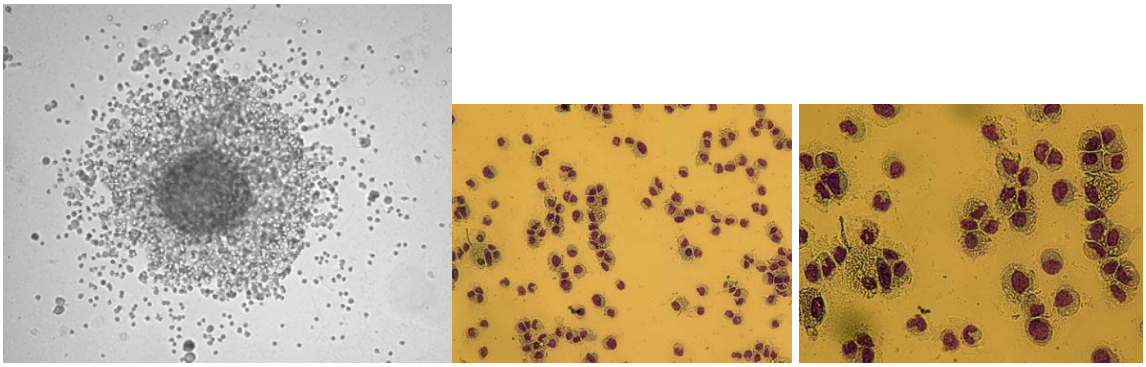




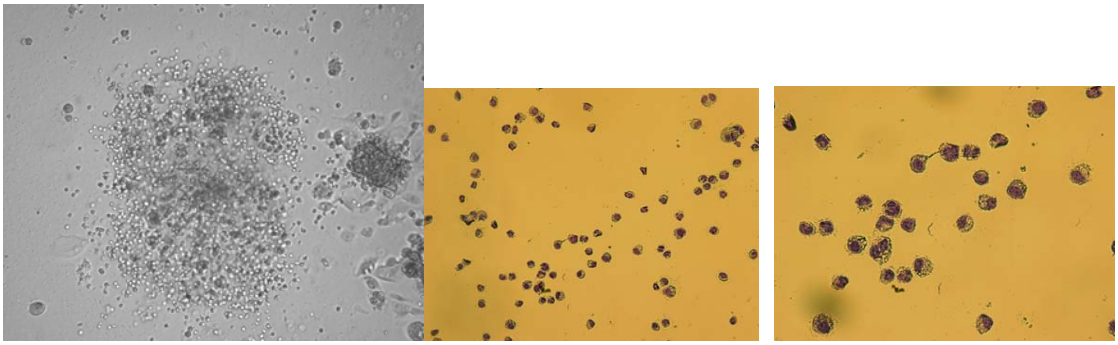
MAST COLONY – 10X



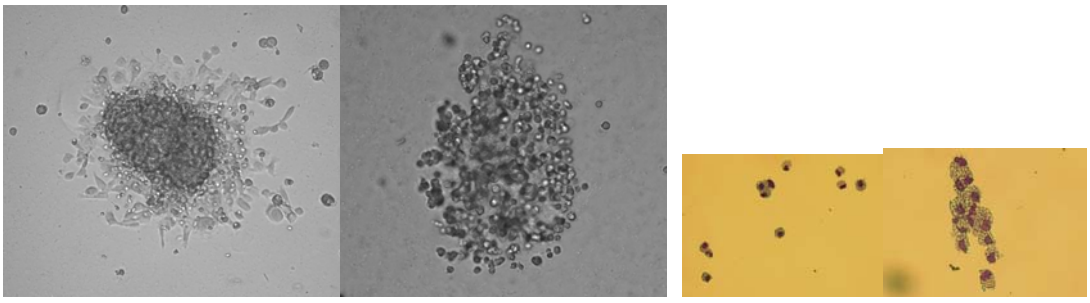
MAC/MONO – 10X



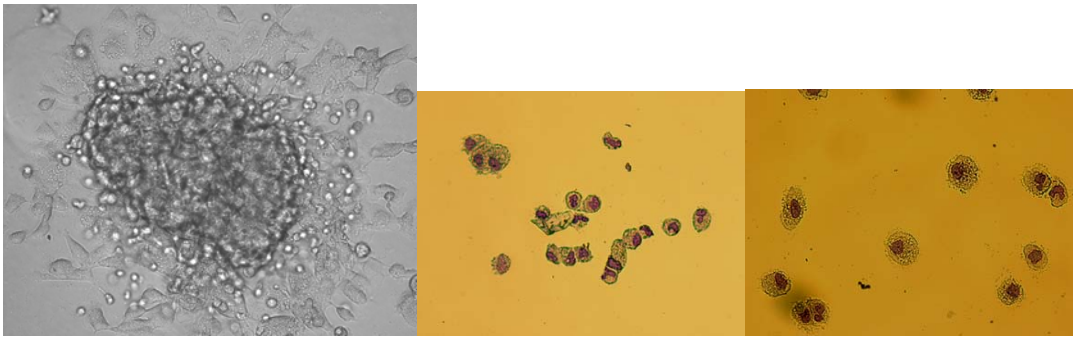
MAC/MONO – 10X



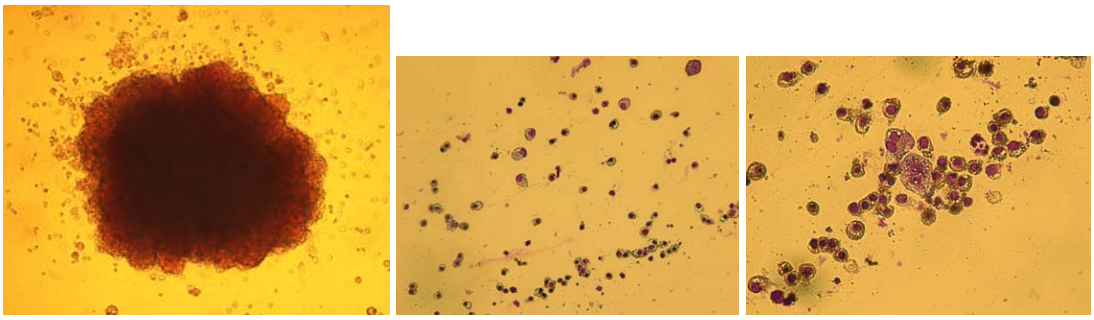
MACROPHAGE – 10X



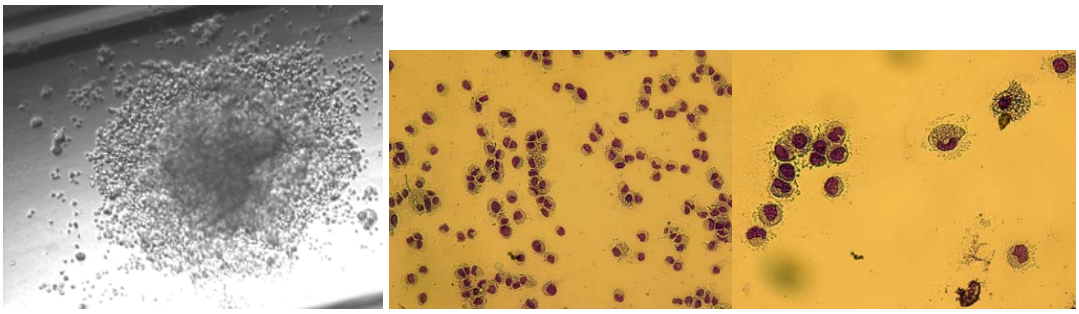
MACROPHAGE – 20X



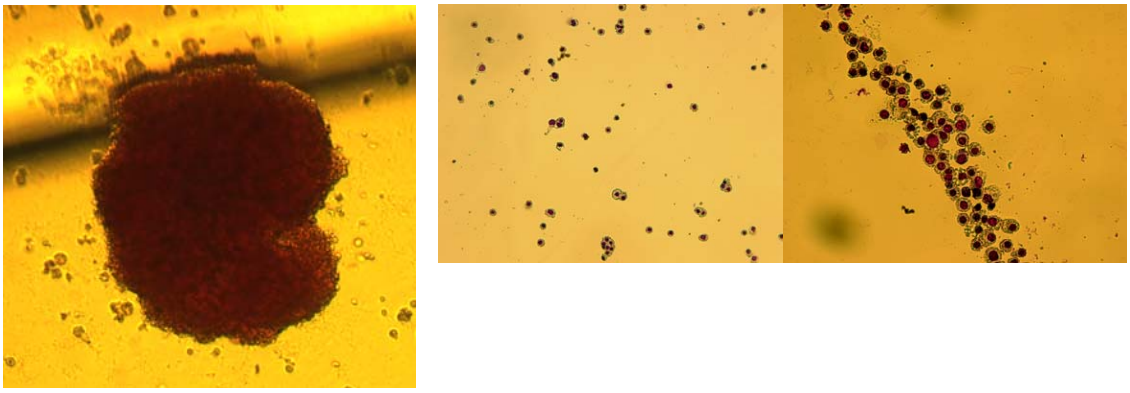
ERY-MYELO – 10X



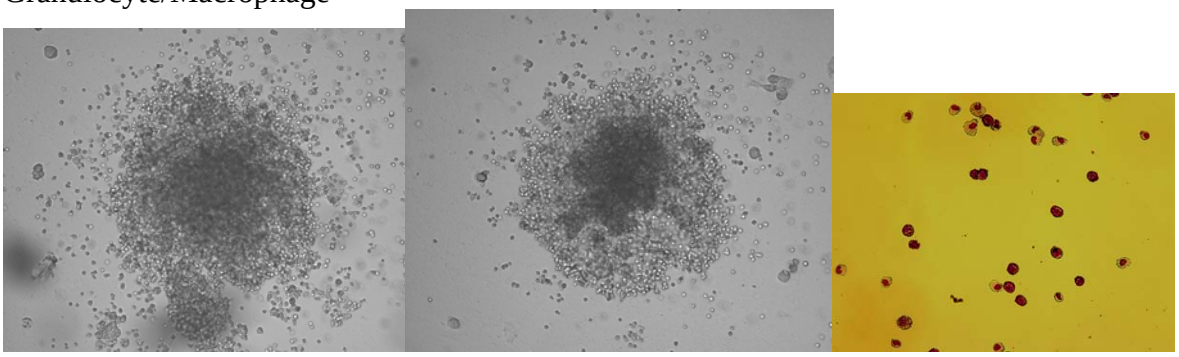
MACROPHAGE – 10X



ERYTHROID – 10X



Granulocyte/Macrophage



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Directed differentiation of hematopoietic precursors and functional osteoclasts from human ES and iPS cells.

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