Hepatic Differentiation of Embryonic Stem Cells by Murine Fetal Liver Mesenchymal Cells.

Running title: Hepatic differentiation of ESCs by murine liver mesenchymal cell.

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i. Abstract

Hepatocytes derived from embryonic stem cells (ESCs) are a potential cell source for regenerative medicine. However, it has been technically difficult to differentiate ESCs into mature hepatocytes because the definitive growth factors and molecular mechanisms governing hepatocyte differentiation have not yet been well defined. The $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells that reside in murine fetal livers induce hepatic progenitor cells to differentiate into mature hepatocytes by direct cell-cell contact. Utilizing these cells, we employ a two-step procedure for hepatic maturation of ESC: first, ESCs are differentiated into endodermal cells or hepatic progenitor cells, and second, ESC-derived endodermal cells are matured into functional hepatocytes by co-culture with the murine fetal liver mesenchymal cells. The ESC-derived hepatocyte-like cells possess hepatic functions, including the ammonia removal activity, albumin secretion ability, glycogen synthesis and storage, and cytochrome P450 enzymatic activity.

ii. Keywords: embryonic stem cell; fetal liver; hepatocyte; hepatic progenitor cell; mesenchymal cell; Thy1; gp38.

1. Introduction

Embryonic stem cells (ESCs) are established from inner cell masses and possess a pluripotency to differentiate into all three germ layers. Hepatocytes derived from ESCs are anticipated as a cell source for cell transplantation, bio-artificial livers, and drug discovery support systems. However, there have been difficulties differentiating ESCs into mature functional hepatocytes because the molecular mechanisms that underlie hepatic development are largely unknown.

Our previous study revealed that the hepatic maturation of fetal hepatic progenitor cells is greatly facilitated by mesenchymal cells that reside in the fetal livers *(1)*. These mesenchymal cells are fractionized as $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ cells *(2)*. In addition, our further experiments demonstrated their ability to mature murine and human ESCs into functional hepatocytes *(3, 4)*. The effect of the $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells on hepatic maturation is achieved by direct cell-cell contact *(5)*. They do not induce hepatic maturation of undifferentiated ESCs, suggesting that mesenchymal cells are effective in hepatic maturation of immature endodermal cells, but are relatively ineffective at hepatic specification and differentiation of

undifferentiated ESCs *(4)*.

In this chapter, we describe a two-step procedure for the hepatic maturation of mouse ESCs utilizing the $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells based on their biological characteristics. First, undifferentiated ESCs are differentiated into endodermal cells of the hepatic lineage using several growth factors and extracellular matrix. Second, the ESC-derived endodermal cells are matured into functional hepatocyte-like cells by co-culture with $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells.

2. Materials

2.1. Culture of mouse ESCs

1. A murine ESC line (*see* **Note 1**).

2. ESC culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS, HyClone, Logan, UT), 0.1 mM 2-mercaptoethanol, nonessential amino acids, 1 mM sodium pyruvate, and 1000 U/ml leukemia inhibitory factor (LIF, ESGRO, Chemicon International Inc., Temecula, CA) (*see* **Note 2**). A stock solution of 1×10^7 U/ml LIF is stored at 4 ºC.

3. A solution of 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA).

4. 35 mm plastic culture dishes with a mouse embryonic fibroblast (MEF) feeder layer treated with 10μg/ml mitomycin C for 2 hours (*see* **Note 3**).

2.2. Differentiation of ESCs into endoderm

1. Serum-free endoderm differentiation medium (SFE medium): DMEM supplemented with 10% Knockout SR (Gibco, Grand Island, NY), 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin (50 units/ml each).

2. All-trans retinoic acid is dissolved at 10 mM in 99.5% ethanol, and stored in aliquots at −80 ºC. LIF is dissolved at 1×10^6 U/ml in a culture medium, and stored at 4 ºC. Basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) are dissolved to a concentration of 20 μM in phosphate-buffered saline (PBS, Ca^{2+}-free) supplemented with 0.5% bovine serum albumin (BSA), and stored in aliquots at −80 ºC. These growth factors are added to culture dishes as required (*see* **Note 4**).

3. 60 mm culture dishes coated with type I collagen (pre-coated dishes purchased from BD Biosciences, Franklin Lakes, NJ).

2.3. Primary culture of murine fetal liver cells

1. A stereomicroscope system.

2. A set of sterilized surgical instruments, including scissors, micro forceps, and a surgical knife.

3. A pair of sterilized surgical gloves.

4. Two pregnant C57/BL6 mice at day 13.5 of gestation (*see* **Note 5**).

5. HBSS-based buffer: Ca^{2+}-free Mg^{+}-free Hank's balanced salt solution with phenol red

(HBSS (−)) with 10 mM HEPES and 0.5 mM EDTA.

6. Irrigation solution 1 (50 ml): HBSS-based buffer (45 ml) supplemented with 10% FBS (5 ml), and 2 U/ml heparin sodium solution (0.1 ml). Heparin sodium solution at 1000 U/ml is readily purchased from several pharmaceutical companies. This solution is prepared as required and kept at 4 ºC (*see* **Note 6**).

7. Irrigation solution 2 (50 ml): HBSS-based buffer (45 ml) supplemented with 50 mg/ml DNase I (1 ml of stock solution), and 2 U/ml heparin sodium (0.1 ml). DNase I is dissolved at 25 mg/ml in distilled water, and stored in single use aliquots at −20 ºC. This solution is prepared as required, and kept at 4 ºC (*see* **Note 7**).

8. Digestion medium (30 ml): 0.5 % (w/v) collagenase type II (Gibco) is dissolved in 30 ml collagenase buffer and 0.1 ml heparin sodium. This medium is kept at 37 ºC prior to use. Collagenase buffer contains 0.2 g $MgSO_4 \cdot 7H_2O$, 0.735 g $CaCl_2 \cdot 2H_2O$, 2.383 g HEPES, and 0.05 g trypsin inhibitor in 1 l HBSS (−). This buffer can be preserved at 4 ºC for a month.

9. Hepatocyte differentiation medium (HD medium): DMEM with 10% FBS, 1 mM sodium pyruvate, penicillin/streptomycin (50 units/ml each), 10 mM nicotinamide, 2

mM L-ascorbic acid phosphate, insulin-transferrin-selenium supplement mixture (Gibco), 1×10^{-7} M dexamethasone, 20 ng/ml HGF, and 10 ng/ml oncostatin M. Oncostatin M is dissolved at 10 mg/ml in 0.5% BSA/PBS, and stored at −80 ºC. This medium can be preserved at 4 ºC for a month, and should be kept at 37 ºC before use.

10. Autoclaved nylon meshes with 50 μm and 100 μm pore size.

11. 100 mm and 35 mm Petri dishes (BD Biosciences).

12. 6-well culture plates coated with type I collagen (pre-coated culture plates purchased from BD Biosciences).

13. A water bath at 37 ºC.

2.4. Isolation of murine fetal liver mesenchymal cells using flow cytometry

1. Solution of 0.25% trypsin-EDTA.

2. PBS (30 ml) with 3% FBS (1ml) (3%FBS/PBS).

3. The following antibodies are used:

Anti-CD45-PE (clone 30-F11, diluted at 1:100, BD Biosciences)

Anti-CD49f-PE (clone GoH3, 3:100, BD Biosciences)

Anti-Thy1-FITC (clone 30-H12, 1:100, BD Biosciences)

Anti-gp38 (Podoplanin, 1:100, Medical and Biological Laboratories, Nagoya, Japan)

The anti-gp38 antibody is conjugated with APC using a conjugation kit (*see* **Note 8**).

4. 5 ml round-bottom tubes with 35 μm nylon meshes.

5. FACSVantage SE (BD Biosciences).

6. HD medium (*see* **subheading 2.3.9**).

7. 24-well culture plates coated with type I collagen (pre-coated plates from BD Biosciences).

2.5. Co-culture of ESC-derived endodermal cells and murine fetal liver mesenchymal cells

1. A 24-well culture plate coated with type I collagen with murine fetal liver mesenchymal cells as a feeder layer.

2. HD medium (*see* **subheading 2.3.9**).

3. Methods

3.1 Culture of ESC

Mouse ESCs are cultured in the undifferentiated state on MEF feeder layers (*6, 7*). They are sub-cultured using 0.25% trypsin/EDTA solution. MEFs are prepared according to the standard protocols.

3.2. Differentiation of ESCs into endodermal cells

1. Undifferentiated mouse ESCs that are maintained on 60mm culture dishes are washed 4 ml PBS twice, added 2ml 0.25% trypsin/EDTA solution at 37 ºC for 2 min, and then added 2 ml ESC medium followed by pipetting well to dissociate. They are centrifuged at 180 g for 3 min, and resuspended with 4ml ESC medium. In order to deplete MEFs, the dissociated cells are transferred onto a plastic culture dish in ESC culture medium, and incubated at 37 ºC for 15 to 30 min.

2. The cell suspension is harvested carefully to avoid removal of the adherent cells and centrifuged at 180 g for 3 min. The cell pellet is suspended and plated at a concentration of 1×10^5 cells/ml on 60 mm culture dishes coated with type I collagen in SFE medium

(*see* **Note 9**).

3. 10 μM ATRA and 1000 U/ml LIF are added to the SFE medium for the first two days (days 0-1), and 20 ng/ml HGF and 20 ng/ml bFGF are added for the next five days (days 2-6). Culture medium should be changed every other day at least.

4. At day 7, the cultured cells are dissociated using a 0.25% trypsin/EDTA solution (*do as* **subheading 3.2.1**) and re-suspend with HD medium for further experiments (*see* **subheading 3.5**) (*see* **Note 10**).

3.2. Primary culture of murine fetal liver cells

1. Two timed-pregnant mice are sacrificed according to institutional guidelines. All uteri are removed and placed into a 100 mm Petri dish with cold irrigation solution 1. Amniotic membranes and placentae are removed, and fetal mice are transferred to a new 100 mm Petri dish with cold irrigation solution 1.

2. The liver tissues are dissected under a stereomicroscope (**Fig. 1**), and placed into a new 100 mm Petri dish with cold irrigation solution 1. The harvested livers are then minced into pieces no larger than 1 mm in diameter with a surgical knife.

3. A nylon mesh (50 μm pore size) is placed on a 50 ml centrifuge tube. The minced liver tissues are filtered through this mesh. The mesh is inverted and carefully transferred onto a new 50 ml centrifuge tube (*see* **Note 11**). The flow-through can be discarded.

4. Warm digestion medium (30 ml) is added through the inverted mesh into the 50 ml centrifuge tube, collecting the liver tissues into the tube together with the digestion medium. The tube is incubated in a water bath at 37 ºC for 12 to 15 min with agitation.

5. Four nylon meshes (100 μm pore size) are placed onto four 15 ml centrifuge tubes. The digested tissues are divided into four equal aliquots, filtered through meshes using a pipette, collected in the centrifuge tubes, and then centrifuged at 10 g for 5 min (*see* **Note 12**).

6. The cell pellet is suspended in 25 ml irrigation solution 2, collected in a 15 ml centrifuge tube, and then centrifuged at 10 g for 5 min. This procedure is repeated three times in total.

7. The cell pellet is suspended in HD medium at a density of 5×10^5 cells/ml to 1×10^6 cells/ml, and inoculated onto 35 mm Petri dishes (*see* **Note 13**).

8. The dissociated cells are incubated at 37 ºC, 5%CO_2 overnight. Cell aggregates are collected in a 15 ml tube and subjected to gravity sedimentation for 10 min (*see* **Note 14**).

9. After the supernatant is removed with a pipette, the sedimented cell aggregates are suspended in new HD media and plated on 6-well culture plates coated with type I collagen (*see* **Note 15**).

10. The cell aggregates are cultured at 37 ºC, 5%CO_2 for one to two days. The culture media are changed every day. The cell aggregates adhere to the culture plates and grow as monolayer colonies.

3.4. Preparation of $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells as a feeder layer

A key step in this procedure is to obtain a pure and viable cell fraction of the mesenchymal cells from murine fetal livers.

1. Following 1-2 days of culture, the adherent cells are washed twice with 500 µl PBS and are then incubated with 200 µl 0.25% trypsin/EDTA at 37 ºC for 10 min. HD medium is added to stop trypsin activity, and all of the cell suspension is collected in a

15 ml tube.

2. The collected cells are centrifuged at 180 g for 3 min, and then washed with 5 ml 3% FBS/PBS twice by centrifuging at 180 g for 3 min.

3. The cell pellet is suspended in 200 μl 3% FBS/PBS, and transferred into a 1.5 ml tube. The dissociated cells are incubated with 2 μl CD45-PE (1:100 dilution), 6 μl CD49f-PE (3:100), 2 μl Thy1-FITC (1:100), and 2 μl gp38-APC (1:100) antibodies on ice in the dark for 30 min.

4. The cells are centrifuged at 630 g for 2 min, and then the supernatant is discarded.

5. The cells are washed with 500 μl 3% FBS/PBS three times.

6. The cells are resuspended in 2 to 4 ml 3% FBS/PBS and collected in a 5 ml round-bottom tube through a nylon mesh (35 μm pore size).

7. The $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ cell fraction is separated using a FACSVantage SE. Dot plots using CD45, CD49f, Thy1, and gp38 antibodies are shown in **Fig. 2** (*see* **Note 16**). The separated $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells are collected in HD medium.

8. The collected cells are suspended in HD medium and seeded in 24-well culture plates

coated with collagen type I at a density of 1×10^4 cells per well (*see* **Note 17**).

9. The $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells are grown to approximately 80% confluency and treated with 10 μg/ml mitomycin C for 2 hours. After washing twice in PBS, fresh HD medium is added. The inactivated cells can be used from the next day.

3.5. Maturation of ESC-derived endodermal cells

1. The dissociated endoderm cells derived from mouse ESCs, generated in section 3.2, are inoculated on a feeder layer of $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal cells at a density of 1×10^4 cells/well (*see* **Note 18**).

2. The ESC-derived endoderm cells are cultured in HD medium on the $CD45^{-}CD49f^{+/-}Thy1^{+}gp38^{+}$ mesenchymal feeder layer for 7 to 14 days. Culture media are changed every day.

3. These ESC-derived mature hepatocyte-like cells can be used for further analyses including drug metabolism and albumin secretion.

4. Notes

1. In this protocol, a murine ESC line derived from C57BL6 mice are used. The passage number is less than 50. Our protocol works with a human ESC line *(4)*.

2. Because ESC culture medium contains LIF, it should be used within a month.

3. Culture dishes with a MEF-feeder layer can be used for one week after mitomycin C treatment.

4. Unless stated otherwise, growth factors are added from stock solutions to culture medium as required.

5. The number of pregnant mice can be increased to four mice per experiment. In this case, the described protocol can be scaled up. However, it might be technically difficult to handle more than five mice at one time since it may be more time-consuming, decreasing the viability of harvested liver cells.

6. Heparin is added in order to prevent clot formation.

7. DNase is added in order to reduce viscosity caused by DNA that is released from damaged cells.

8. The combination of the fluorescent dyes is actually atypical, because PE labels both

anti-CD45 and anti-CD49f antibodies. However, as shown in **Fig. 2**, the CD45-positive cell fraction is clearly distinguishable from the CD49f-positive cell fractions based on their fluorescent intensities *(1)*. This may result from the difference in the expression levels between CD45 and CD49f antigens.

9. Usually two to four 60 mm collagen type I coated dishes can be harvested from undifferentiated ESCs on one confluent 60 mm dish. The concentration of ESCs seeded on culture plates is important. Under this condition, differentiating ESCs can be cultured for seven days without further passages. During this period, culture medium should be changed every other day.

10. This protocol makes it possible to obtain alpha-fetoprotein (AFP)-producing endodermal cells at the efficiency of more than 40% at day 7. However, the optimal condition for endodermal differentiation may vary widely with the type of ESCs. For example, our recent study revealed that ATRA does not induce hepatic differentiation in human ESCs, and that Matrigel (BD Biosciences) is more efficient for hepatic differentiation than Type I Collagen *(8)*. Therefore, culture protocols may require optimization depending on the ESC lines.

11. Because fetal livers act as hematopoietic organs during embryonic stages, the harvested liver tissues contain a large number of hematopoietic cells. This procedure is necessary in order to eliminate hematopoietic cells. The flow-through contains hematopoietic cells.

12. This procedure is performed to eliminate undigested liver tissues.

13. The harvested liver cells obtained from one pregnant mouse can usually be seeded onto three 35 mm Petri dishes, although the yield depends on the number of fetuses.

14. The cell aggregates can be formed in a few hours. The cell aggregates consist of hepatic progenitor cells, hematopoietic cells, and mesenchymal cells. Forming cell aggregates can enrich hepatic progenitor cells and mesenchymal cells, and greatly facilitate the purification of mesenchymal cells using flow cytometry *(9)*.

15. The cell aggregates cultured in one 35 mm Petri dish can be usually transferred to one well of a 6-well culture plate.

16. This protocol can be also used to separate hepatic progenitor cells that reside in fetal murine livers. The $CD45^{-}CD49f^{+/-}Thy1^{-}$ cell fraction corresponds to hepatic progenitor cells. In this case, it is best to set a small gate for the $CD45^{-}CD49f^{+/-}Thy1^{-}$ fraction in

order to obtain a pure fraction of hepatic progenitor cells.

17. Cell viability is remarkably decreased at a lower density of cultured cells. Eventually, 1.5 - 2.0 × 10^4 $CD45^-CD49f^{+/-}Thy1^+gp38^+$ mesenchymal cells can be isolated from one pregnant mouse.

18. The ESC-derived endodermal cells consist of not only endodermal cells, but also ectodermal and mesodermal cells. Therefore, experimental circumstances may require purification of only endodermal cells. In these cases, it may be helpful to generate transgenic ESCs that express fluorescent proteins driven by an endoderm-specific gene (e.g. AFP or albumin) promoter by gene manipulation *(3, 10)*.

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Figure legends

Fig. 1

This photograph shows a mouse fetus after removal of the amniotic membrane and placenta. The fetal liver is a red organ located in the middle of a fetus. Under a stereomicroscope, the liver is dissected using micro forceps. The gallbladder and intestinal tract should be removed from the liver.

Fig. 2

Dot plots following flow cytometric analyses. (A) Cell aggregates derived from murine fetal livers are mainly divided by CD45, CD49f, and Thy1 into three cell fractions. The $CD45^{+}Thy1^{-}$ fraction corresponds to hematopoietic cells, the $CD45^{-}CD49f+^{dim}Thy1^{-}$ cell fraction corresponds to hepatic progenitor cells, and the $CD45^{-}CD49f^{+/-}Thy1^{+}$ cell fraction is mesenchymal cells. (B) The $CD45^{-}CD49f^{+/-}Thy1^{+}$ mesenchymal cells are further fractionated by gp38 into two groups. The gp38-positive cells account for approximately 16% of the $CD45^{-}CD49f^{+/-}Thy1^{+}$ mesenchymal cells.

Figure 1. T. Ishii et al.

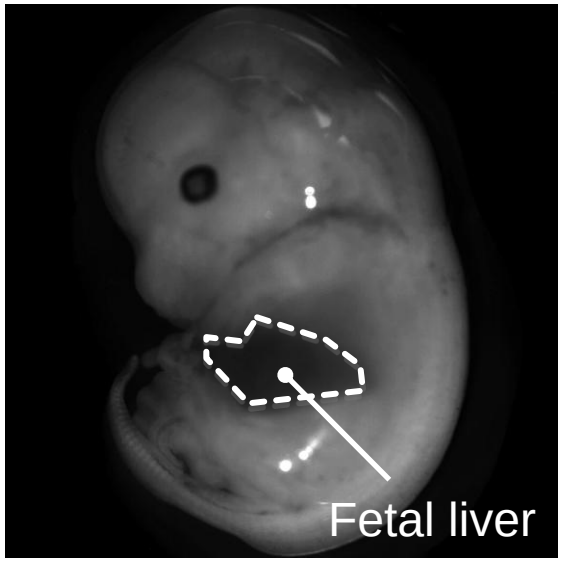
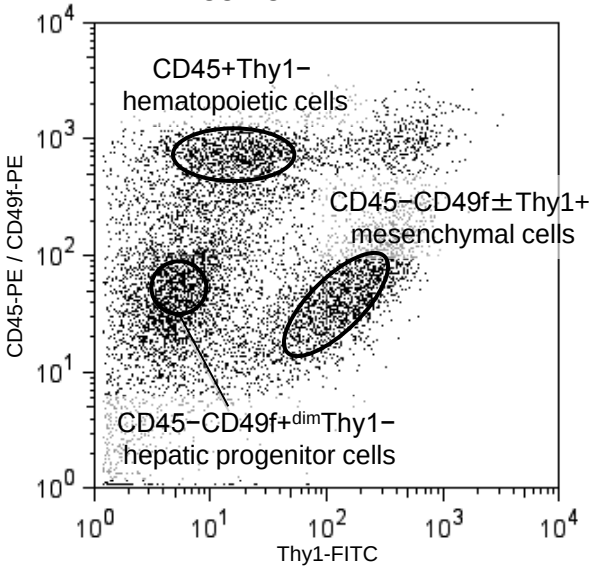
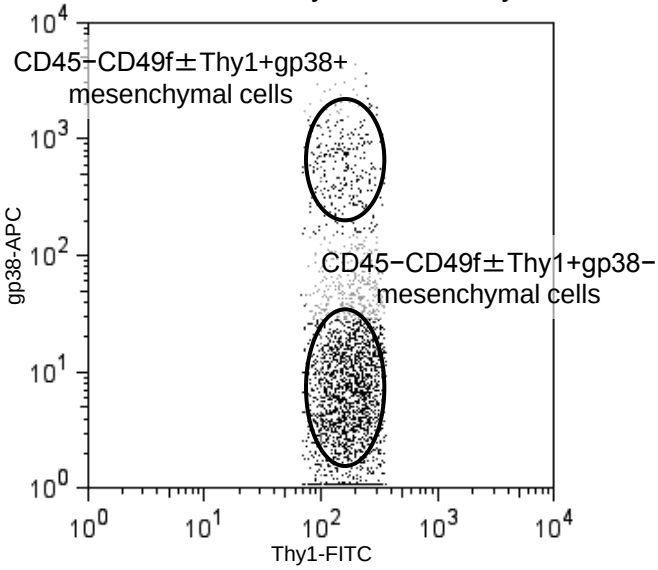


Figure 2. T. Ishii et al.

A. Cell aggregates from fetal livers



B. CD45−CD49f±Thy1− mesenchymal cells



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