Direct and indirect coculture of mouse hepatic progenitor cells with mouse embryonic fibroblasts for the generation of hepatocytes and cholangiocytes

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Abstract The widespread use of hepatocytes and cholangiocytes for regenerative medicine and tissue engineering is restricted by the limited number of hepatocytes and cholangiocytes; a simple and effective method for the expansion and differentiation of the hepatic progenitor cells (HPCs) is required. Recent studies demonstrated that mouse embryonic fibroblasts (MEFs) play an important role in supporting the proliferation of the mouse hepatic progenitor cells (mHPCs). However, the effect of direct and indirect coculture of MEFs with mHPCs on the differentiation of mHPCs is poorly studied. Herein, we show that mHPCs rapidly proliferate and form colonies in direct or indirect contact coculture with MEFs in the serum-free medium. Importantly, after direct contact coculture of the mHPCs with MEFs for 6 days, mHPCs expressed the hepatic marker albumin (ALB) and did not express the cholangiocyte marker CK19, indicating their differentiation into hepatocytes. In contrast, after indirect contact coculture of the mHPCs with MEFs for 6 days, mHPCs expressed the cholangiocyte marker CK19 and did not express the hepatic marker

ALB, indicating their differentiation into cholangiocytes. These results indicate that direct and indirect contact cocultures of the mHPCs with MEFs are useful for rapidly producing hepatocytes and cholangiocytes.

Keywords Hepatic progenitor cells · Mouse embryonic fibroblasts · Coculture · Differentiation

Abbreviations

MEFs	Mouse embryonic fibroblasts
mHPCs	Mouse hepatic progenitor cells
HPCs	Hepatic progenitor cells
FACS	Fluorescence-activated cell sorting
DPBS	Dubecco's phosphate buffered saline
Dlk	Delta-like 1 homologue
ALB	Albumin

Introduction

Due to the shortage of donor organs, use of liver transplantation in the treatment of terminal hepatic failure diseases is limited. Hepatic regenerative medicine, such as cell transplantation, bioartificial liver support systems, or organ bioengineering, may offer an alternative form of therapy for these diseases. However, all these treatments are limited by a lack of a sufficient quantity of the organs or cell resources. Therefore, the ability to obtain an adequate amount of hepatocytes and cholangiocytes would improve the

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development of cell-based therapies for liver diseases. (Hu and Li 2015; Nicolas et al. 2017; Alwahsh et al. 2018). Hepatic progenitor cells (HPCs) can proliferate and can differentiate into hepatocytes and cholangiocytes; therefore, HPCs can be used as a source of hepatocytes and cholangiocytes (Kamiya and Inagaki 2015; Oertel 2011). However, the events controlling the bidirectional differentiation of HPCs into either hepatocytes or cholangiocytes remain unclear.

During hepatogenesis, after the liver has budded out of the endoderm, the HPCs quickly expand and differentiate into either hepatocytes or cholangiocytes (Kamiya and Inagaki 2015; Gordillo et al. 2015). HPCs which are located near the portal vein mesenchyme (PVM) differentiate into cholangiocytes. In contrast, hepatoblasts in the liver parenchyma, which are distant from the portal vein, differentiate into hepatocytes (Boulter et al. 2013; Si-Tayeb et al. 2010). Microenvironmental regulation plays a key role in the stem and progenitor cell fate in development. The interactions between hepatoblasts and other non-parenchymal cells are important for the differentiation and proliferation of hepatoblasts (Hoppo et al. 2004; Suzuki et al. 2008; Onitsuka et al. 2010; Ito et al. 2014). Recently, some studies have found that mouse embryonic fibroblasts (MEFs) support the proliferation of the mouse hepatic progenitor cells (mHPCs) isolated from the early or middle-fetal mouse livers (Okada et al. 2012; Kamiya et al. 2015). Moreover, previous studies have shown that fibroblast cells help to preserve hepatocyte viability and functionality (Bhandari et al. 2001; Ito et al. 2012; Liu et al. 2014). However, the effects of direct and indirect coculture of MEFs with mHPCs on the differentiation of mHPCs are not well-known.

In this study, various coculture methods were used to investigate the effect of direct and indirect coculture of MEFs with mHPCs on the differentiation of mHPCs. Importantly, direct coculture of mHPCs with MEFs promotes mHPC directional differentiation into the ALB+/CK19– hepatocytes. Nevertheless, indirect coculture of mHPCs with MEFs promotes mHPC directional differentiation into ALB–/CK19+ cholangiocytes. Thus, we have developed a serum-free protocol for the directional differentiation of mHPCs into hepatocytes and cholangiocytes by using various coculture methods.

Materials and methods

Animal experiments

All animal experiments were performed in compliance with the approved guidelines, and the protocols were approved by the Animal Experimental Ethics Committee of Chongqing Medical University. The animals were mated during the night, and if by noon of the next day the vaginal plug was found, it was assumed to correspond to 0.5 days of gestation. Fetuses at 14.5 days were used in the studies.

Isolation and treatment of MEFs

Mouse embryonic fibroblasts (MEFs) from the E14.5 mouse embryos were isolated under sterile conditions as described previously (Jiang et al. 2016). Briefly, mouse embryos were dissected, and then the head and internal organs were removed. The torso was minced and digested in 0.125% trypsin–EDTA (Sigma-Aldrich, USA) at 37 °C for 20 min. Samples were then suspended in the MEF medium consisting of 90% high-glucose DMEM (Sigma-Aldrich, USA) with 10% FBS (GIBCO, Grand Island, NY) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY). The digested samples were filtered by using a strainer mesh and pelleted by centrifugation at 1000 rpm for 5 min. After washing in PBS, the cells were resuspended in the MEF medium. Isolated MEFs were plated in 75 cm² flasks and grown in the MEF medium. After 1 day of culture, non-adherent cells were removed by changing the culture medium. The medium was then changed every 2–3 days and the cells were passaged. Passage 3 MEFs were treated with mitomycin C (Cayman Chemical, Ann Arbor, MI) at 37 °C for 2.5 h and used as the feeder cells in the subsequent coculture experiments.

Isolation of Dlk1⁺ hepatoblasts from the fetal liver

Delta-like 1 homologue (Dlk1) fetal mHPCs were purified from the E14.5 livers using fluorescence-activated cell sorting (FACS) as previously described, with some modifications (Naoki Tanimizu et al. 2003; Wang et al. 2018). Briefly, the fetal livers were isolated from the E14.5 mice under the aseptic conditions and washed twice with Dulbecco's phosphate buffered saline (DPBS). The fetal livers were

transferred to a 15 ml conical centrifuge tube with 3 ml Accutase (Invitrogen) and incubated at 37 °C for 5 min. Then, a single cell suspension was generated by pipetting up and down using a Pasteur pipette. The digested samples were filtered by using a strainer mesh and pelleted by centrifugation at 600 rpm for 5 min, and then washed with DPBS supplemented with 3% FBS (GIBCO, Grand Island, NY) and incubated with anti-Dlk1 antibody (MBL International, Japan) against cell surface markers at 4 °C for 30 min. After washing with DPBS supplemented with 3% FBS and staining the dead cells with 7-AAD (KeyGEN BioTECH, China), the cells were analyzed and sorted using a flow cytometer FACS Vantage SE cell sorter (Becton Dickinson, San Jose, CA). The results obtained with the isotype control antibodies are shown as a negative control.

Direct and indirect cocultures

Coculture experiments were divided into two groups, namely, the direct contact coculture group and the indirect coculture group. In the direct contact coculture group, mitomycin C-treated MEFs were plated onto 0.1% gelatin-coated 24-well plates (1×10^5 cells per well). After 12 h of the culture, the DLK1⁺ cells were inoculated onto mitomycin-C treated-MEFs at a low density (5×10^3 cells per well in 24-well culture plates). In the indirect contact coculture group, 1×10^5 mitomycin C-treated MEFs were grown on the 3.0- μ m pored transwell membranes (Corning, USA). After 12 h of the culture, a total of 5×10^3 DLK1⁺ cells were plated onto gelatin-coated 24-well plates with 1×10^5 mitomycin-C treated-MEFs grown on the 3.0- μ m pored transwell membranes (Corning, USA). The cells were incubated in the standard culture medium. The standard culture medium is a 1:1 mixture of the H-CFU-C medium (DMEM/Ham's F12 half medium (GIBCO, Grand Island, NY) supplemented with 15% KnockOut™ Serum Replacement (Invitrogen, Carlsbad, CA), $1 \times$ Insulin–Transferrin–Selenium $\times$ Invitrogen, Carlsbad, CA), 10 mM nicotinamide (Sigma-Aldrich, USA), 10^{-7} M dexamethasone (Sigma-Aldrich, USA), 2.5 mM HEPES (Sigma-Aldrich, USA), $1 \times$ penicillin/streptomycin (GIBCO, Grand Island, NY), 2 mM L-glutamine, and $1 \times$ nonessential amino acid solution (GIBCO, Grand Island, NY) and fresh DMEM (GIBCO, Grand Island, NY) supplemented

with 15% KnockOut™ Serum Replacement (Invitrogen, Carlsbad, CA). Cells were cocultured for 6 days in the presence of 40 ng/ml hepatocyte growth factor (Peprotech, London, United Kingdom) and 20 ng/ml epidermal growth factor (Peprotech, London, United Kingdom).

The morphological comparison of the mHPCs in two groups

During the 6-day coculture, the morphological characteristics of the mHPCs in both groups were observed and captured with a microscope (Olympus, Tokyo, Japan).

Immunohistochemistry

The immunohistochemical localization of ALB and CK19 was performed as described previously (Wang et al. 2018). Briefly, FACS-isolated DLK1⁺ cells were sorted onto glass slides, fixed with 2% PFA for 15 min at room temperature and permeabilized with 0.5% Triton X-100 for 10 min. Cultured cells were fixed with 4% PFA for 15 min at room temperature and permeabilized with 0.5% Triton X-100 for 10 min. After blocking, the cells were incubated with the diluted primary antibodies (Table 1) overnight at 4 °C. After washing with PBS, the cells were incubated for 1 h at room temperature with a DyLight 488 goat anti-rabbit IgG antibody, DyLight 488 rabbit anti-goat IgG antibody and DyLight 594 goat anti-mouse IgG antibody (Abbkine, USA). Cell nuclei were stained with DAPI. Then, the cells were imaged under a fluorescence microscope (Olympus, Japan).

Results

Isolation and Identification of DLK1⁺ cells from the E14.5 fetal livers

To enrich and purify the hepatic progenitor cells, the unfractionated E14.5 fetal mouse hepatic cells were selected for Dlk-1 using fluorescence-activated cell sorting (FACS). The DLK1⁺ cells isolated by FACS constituted approximately 10–20% of the total E14.5 fetal hepatic cells (Fig. 1b). The identification of the Dlk-1-selected fetal hepatic cells was based on the immunocytochemistry staining for the AFP and ALB

Table 1 Primary antibodies used in the immunohistochemistry experiments

Antibody	Company	Catalogue number	Host animal	Dilution
AFP	Merck Millipore	MAB1368	Mouse	15 ug/ml
CK19	Abcam	ab52625	Rabbit	1:200
ALB	Santa Cruz	sc-46293	Goat	1:50
ALB	Santa Cruz	sc-374670	Mouse	1:50

expression. The DLK1⁺ cells isolated freshly by FACS coexpressed AFP and ALB according to immunocytochemistry (Fig. 1d, e). These results suggest that the DLK1⁺ cells isolated freshly by FACS were mHPCs.

The morphological comparison of the mHPCs in the direct coculture group and the indirect coculture group

To investigate the effects of the direct and indirect coculture methods on the proliferation and differentiation of mHPCs, the FACS-sorted DLK1⁺ cells were subjected to direct contact coculture or indirect coculture with MEFs. During 6 days of coculture, the morphological changes of mHPCs in the direct group or indirect coculture group were observed under a microscope. As shown in Fig. 2, the DLK1⁺ cells were rapidly proliferating and formed colonies on day 1 and day 2 (Fig. 2a, b, d, e), suggesting that MEFs support mHPC proliferation in these coculture systems. Additionally, in the direct coculture group, the DLK1⁺ cells formed large colonies in a paving stone-like arrangement and the colonies was surrounded by MEFs on day 6 (Fig. 2c, arrow). Nevertheless, in the indirect coculture group, the DLK1⁺ cells adhere to the wall and their morphology begins to change into the spindle cells or triangles (Fig. 2f). The DLK1⁺ cells formed colonies and displayed high proliferation potential. Thus, we analyzed whether these coculture systems can produce sufficient number of the descendant cells. Indeed, a total of 5×10^3 DLK1⁺ cells were expanded to almost $4\text{--}6 \times 10^4$ cells after 6 days of in vitro coculture (Fig. 2g, h), suggesting that these coculture systems can produce a large number of the descendant cells.

Direct coculture with MEFs regulates mHPC differentiation into hepatocytes

To identify the effects of direct coculture with MEFs on the differentiation of mHPCs, we immunocytochemically assessed the expression of ALB and CK19 in the direct coculture group and indirect coculture group after coculture for 6 days. As shown in Fig. 3, the Dlk-1⁺ cell-derived colonies have strong expression of ALB but almost no expression of CK19 (Fig. 3b, c). These results suggest that mHPCs maintained their high proliferative activity and differentiated into the ALB+/CK19− hepatocytes in direct coculture with MEFs.

Indirect coculture with MEFs regulates mHPC differentiation into cholangiocytes

After coculture for 6 days, we immunocytochemically assessed the expression of ALB and CK19 in the indirect coculture group. As shown in Fig. 3, in contrast to the direct coculture group, Dlk-1⁺ cell-derived colonies strongly expressed CK19 but had almost no expression of ALB (Fig. 3f, g). These results indicate that mHPCs differentiate into the ALB−/CK19+ cholangiocytes in indirect coculture with MEFs.

Discussion

Stem cells and progenitors cells are capable of self-renewal and differentiation into specialized cells (Weissman and Gage 2001). Differentiation of hepatoblasts into hepatocytes or cholangiocytes is important for their use in regenerative medicine and tissue engineering (Nikolaos et al. 2015). Therefore, effective culture systems that maintain both proliferative activity and mature functions need to be developed for direct differentiation of hepatoblasts into hepatocytes and cholangiocytes. In this study, directed

Fig. 1 Isolation and identification of DLK1⁺ from the E14.5 fetal hepatic cells. **a, b** FACS was used to select the Dlk-1⁺ cells from the E14.5 hepatic cells. Unfractionated fetal hepatic cells contained approximately 10–20% Dlk-1⁺ cells; **c–f** Dlk-1⁺ cells isolated by FACS were double immunostained for AFP (red)

and ALB (green). DAPI was used for staining of the nuclei (blue). The immunocytochemistry results show that all Dlk-1⁺ cells coexpress AFP and ALB. Scale bars: 50 μ m. (Color figure online)

differentiation of mHPCs into hepatocytes and cholangiocytes was established by using the direct and indirect contact cocultured methods. Interestingly,

mHPCs differentiate into hepatocytes in the direct contact coculture with MEFs, but they differentiate into cholangiocytes in the indirect contact coculture

Fig. 2 Morphological comparison of mHPCs in direct and indirect coculture. **a–f** After enrichment of the Dlk-1⁺ fetal liver cells with FACS, 5×10^3 DLK1⁺ cells were direct contact cocultured or indirectly cocultured with 1×10^5 mitomycin C-treated MEFs for 6 days. **g, h** After 6 days of coculture, in the

direct coculture group, the number of the cells in the colonies was counted. The cells in the indirect coculture group were trypsinized and counted. The results are presented as the mean cell count $\pm$ SD of triplicate culture samples. Scale bars: 50 μ m

Fig. 3 Regulation differentiation of mHPCs in direct coculture or transwell coculture with MEFs. FACS-sorted Dlk-1⁺ cells were directly cocultured or indirectly cocultured with MEFs for 6 days. Expression of ALB (red) and CK19 (green) in the Dlk-

1⁺ cell colonies was detected. Cells were double stained with anti-ALB (red) and anti-CK19 (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bars: 100 μ m. (Color figure online)

with MEFs (Figs. 3, 4). Consistent with previous studies, FACS-Dlk1⁺ cells of the E14.5 fetal liver rapidly proliferate and form colonies (Okada et al. 2012; Kamiya et al. 2015), suggesting that the FACS-Dlk1⁺ cells have a high proliferation potential. Additionally, the FACS-Dlk1⁺ cells can differentiate into hepatocytes and cholangiocytes, indicating that mHPCs isolated by us are the bipotential progenitor cells.

Recent studies have shown that human pluripotent stem cells (hPSCs) can differentiate into cholangiocytes and hepatocytes by various differentiation protocols (Tsuruya et al. 2015; Takayama et al. 2017; Asumda et al. 2018; Dianat et al. 2014; Ogawa et al. 2015). However, these differentiation processes are complex and require a long time. In contrast, our coculture systems are simple and generate hepatocytes and cholangiocytes in 6 days. The interactions between hepatoblasts and mesenchymal cells regulate the differentiation and expansion of hepatoblasts (Hoppo et al. 2004; Onitsuka et al. 2010; Ito et al. 2014; Kamiya et al. 2015). Our coculture systems mimic the interaction between hepatoblasts and mesenchymal cells in the coculture of mHPCs with MEFs. Previous studies have reported that MEFs can function as the niche-like cells supporting mHPC proliferation in the coculture system (Okada et al. 2012; Kamiya et al. 2015). Indeed, our data have shown that the DLK1⁺ cells were rapidly proliferating and formed colonies on day 1 and day 2 in the direct and indirect contract cocultures. Moreover, 5×10^3 DLK1⁺ cells proliferated into almost $4\text{--}6 \times 10^4$ cells in these coculture systems on day 6, indicating that our

coculture systems can produce a large number of the descendant cells.

The effects of direct and indirect coculture of MEFs with mHPCs on the differentiation of mHPCs are not well understood. Previous studies have shown that mesenchymal cells promote the differentiation of mHPCs toward mature hepatocytes in direct cell-to-cell contact coculture (Hoppo et al. 2004; Nagai et al. 2002). Indeed, direct coculture of mHPCs with MEFs promoted mHPC differentiation into the ALB+CK19[−] hepatocytes. However, in indirect coculture of mHPCs with MEFs, mHPCs differentiated into the ALB[−]CK19⁺ cholangiocytes. A previous study has found that fetal HPCs precultured on the gelatin-coated dishes differentiate into the ALB[−]CK19⁺ cholangiocytes (Anzai et al. 2016). However, when mHPCs were cultured alone on the gelatin-coated dishes, most of the cells died within 2–3 days (Wang et al. 2017), suggesting that our indirect coculture system will produced more ALB[−]CK19⁺ cholangiocytes. Thus, our results clearly show that mHPCs differentiate into the ALB+/CK19[−] hepatocytes in the direct contact coculture with MEFs and differentiate into the ALB[−]/CK19⁺ hepatocytes in the indirect contact coculture with MEFs. However, the intimate interactions between mHPCs and MEFs are complex. Several factors can affect the differentiation of the mHPCs including cell attachment, direct cell–cell contact, cell–extracellular matrix interaction and growth factors. The molecular mechanisms of regulation of differentiation of mHPCs with lineage restriction to a desired adult fate are unknown and await further investigation.

Fig. 4 Diagrams of the direct and indirect contract coculture systems used in the study. Direct and indirect cocultures of MEFs with mHPCs. **a** It should be note that direct coculture with MEFs lead to mHPC differentiation into hepatocytes. **b** However, indirect coculture with MEFs leads to mHPC differentiation into cholangiocytes

Conclusion

In conclusion, we have presented a simple and efficient differentiation method to direct the differentiation of mHPCs into hepatocytes and cholangiocytes. These coculture systems not only directs the differentiation of mHPCs into hepatocytes and cholangiocytes but also produce a large number of differentiated cells. These findings may highlight a potential therapeutic role for differentiation and expansion of the hepatic progenitor cells.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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