Hepatoblast-Like Progenitor Cells Derived From Embryonic Stem Cells Can Repopulate Livers of Mice

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BACKGROUND & AIMS: Hepatocyte-like cells can be derived from pluripotent stem cells such as embryonic stem (ES) cells, but ES cell-derived hepatic cells with extensive capacity to repopulate liver have not been identified. We aimed to identify and purify ES cell-derived hepatoblast-like progenitor cells and to explore their capacity for liver repopulation in mice after in vitro expansion. **METHODS:** Unmanipulated mouse ES cells were cultured under defined conditions and allowed to undergo stepwise hepatic differentiation. The derived hepatic cells were examined by morphologic, fluorescence-activated cell sorting, gene expression, and clonal expansion analyses. The capacities of ES cell-derived hepatic progenitor cells to repopulate liver were investigated in mice that were deficient in fumarylacetoacetate hydrolase (Fah) (a model of liver injury). **RESULTS:** Mouse ES cells were induced to differentiate into a population that contained hepatic progenitor cells; this population included cells that expressed epithelial cell adhesion molecule (EpCAM) but did not express c-Kit. Clonal hepatic progenitors that arose from single c-Kit$^-$EpCAM$^+$ cells could undergo long-term expansion and maintain hepatoblast-like characteristics. Enriched c-Kit$^-$EpCAM$^+$ cells and clonally expanded hepatic progenitor cells repopulated the livers of Fah-deficient mice without inducing tumorigenesis. **CONCLUSIONS: ES cell-derived c-Kit$^-$EpCAM$^+$ cells contain a population of hepatoblast-like progenitor cells that can repopulate livers of mice.**

Keywords: Hepatic Differentiation; Bipotency; Cell Transplantation; Liver Injury.

Hepatic cells differentiated from embryonic stem (ES) cells or induced pluripotent stem cells would provide an unlimited cell source for cell replacement therapy, liver tissue engineering, and models of molecular pathogenesis for liver diseases.[1–3] Although hepatic differentiation from mouse and human ES cells, and recently from human induced pluripotent stem cells, has been successful,[4–15] the derived hepatic cells were heterogeneous populations at different stages of differentiation. More importantly, hepatic cells with extensive liver repopulation capacity are yet to be identified. Without identification of pure lines of hepatic cells capable of therapeutic liver repopulation, the functionality of derived hepatic cells cannot be proven, nor will the nature of pluripotent stem cell-derived hepatic cells be fully understood. Furthermore, derived hepatic cells could be useful in clinical therapeutic cell transplantation.[1,3,16] Thus, the methods to purify and expand stem cell-derived functional hepatic cells may have important and practical utility.

Isolation of ES cell-derived hepatic cells was previously based on expression of selective markers controlled by promoters of hepatic genes such as α-fetoprotein (Afp),[4] Foxa2,[5] albumin (Alb),[6,7] and α1-antitrypsin (Aat).[8] However, it will be necessary to derive genetically unmanipulated hepatic cells for therapeutic applications in the future.[1,3,16] Asialoglycoprotein-receptor (ASGPR) was studied as a selection marker, but it could be only be used to isolate mature hepatocyte-like cells,[11] instead of hepatic progenitor cells. Until now, there was no specific surface marker useful in identifying and enriching ES cell-derived hepatic progenitor cells. It was also unknown whether ES cell-derived hepatic progenitor cells retain differentiation potential and liver repopulation capacity after extensive proliferation in vitro.

In this study, a novel method of stepwise cell differentiation from mouse ES cells was established to derive

Abbreviations used in this paper: Aat, α1-antitrypsin; Afp, α-fetoprotein; Alb, albumin; BSA, bovine serum albumin; CDM, chemically defined medium; DE, definitive endoderm; DMEM, Dulbecco's modified Eagle medium; ES cells, embryonic stem cells; EpCAM, epithelial cell adhesion molecule; FACS, fluorescence activated cell sorting; Fah, fumarylacetoacetate hydrolase; GFP, green fluorescent protein; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; VE, visceral endoderm.

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hepatic cells, which mostly recapitulated essential stages of embryonic hepatogenesis. In our method, the derived hepatic cells characteristic of mouse embryonic hepatoblasts could be obtained as a dominant cell population in culture at day 13 of induced differentiation. This cell population could be identified and enriched in a c-Kit$^-$epithelial cell adhesion molecule (EpCAM)$^+$ cell population, which proved to be ES cell-derived hepatoblast-like progenitor cells by fluorescence activated cell sorting (FACS) analysis, morphologic characters, and gene expression patterns. Furthermore, single hepatic progenitor cells in the c-Kit$^-$EpCAM$^+$ population could be clonally selected and expanded with sustained bipotency. In cell transplantation assays, ES cell-derived hepatic progenitor cells were able to therapeutically repopulate the injured livers of fumarylacetoacetate hydrolase (Fah)-deficient mice. To our knowledge, this is the first identification and enrichment of ES-derived hepatoblast-like progenitor cells capable of therapeutic liver repopulation.

Materials and Methods

ES Cell Culture and Hepatic Differentiation

Based on and slightly modified from N2B27 medium,[17] the chemically defined medium (CDM) consists of a 1:1 mixture of neurobasal medium and Dulbecco's modified Eagle medium (DMEM)/F12 medium supplemented with 0.5X N2 and 0.5X B27 supplements, 0.1% bovine serum albumin (BSA), 2 mmol/L glutamax, and 0.1 mmol/L 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA). Mouse ES cells (E14; American Type Culture Collection, Manassas, VA) were cultured on gelatin-coated dishes in CDM supplemented with 10 ng/mL recombinant human bone morphogenetic protein 4 (BMP4) and 1000 U/mL leukemia inhibitory factor (LIF). To drive hepatic differentiation, ES cells digested with Cell Disassociation Buffer (Sigma–Aldrich, St. Louis, MO) were seeded (2.5×10^3 cells/cm^2) on type IV Collagen (Becton Dickinson, San Jose, CA)-coated dishes in CDM supplemented with 20 ng/mL recombinant Activin A and 1 mmol/L sodium butyrate (Sigma–Aldrich) for 2 days then in CDM with 20 ng/mL Activin A for 3 days. Later, day 5 cells were replated (5×10^4 cells/cm^2) on gelatin-coated dishes in hepatic progenitor specification medium (CDM supplemented with 20 ng/mL Activin A, 20 ng/mL BMP4, and 20 ng/mL fibroblast growth factor 2 [FGF2]) for 2 days, followed in hepatic progenitor expansion medium (CDM supplemented with 10 ng/mL BMP4, 10 ng/mL FGF2, 20 ng/mL hepatocyte growth factor [HGF], 20 ng/mL transforming growth factor-α [TGFα], 10 ng/mL epidermal growth factor [EGF], and 10^{-7} mol/L dexamethasone σ) for 6 days. The medium was changed every other day. All growth factors except LIF (ESGRO, Chemicon, Temecula, CA) were purchased from R&D (R&D Systems Inc, Minneapolis, MN).

Antibodies

Details about antibodies are available in Supplementary Table 2.

Flow Cytometry

Cells were harvested and washed twice in washing buffer (phosphate-buffered saline [PBS] containing 0.2% BSA). One $\times 10^5$ cells were stained in 100 μL washing buffer with chromophore-conjugated antibodies at 4°C for 30 minutes, followed by washing twice and analysis. Alternatively, primary antibody-bound cells were further incubated with chromophore-conjugated secondary antibodies at 4°C for 30 minutes, washed twice, and analyzed. Isotope antibodies were used as negative controls. Intracellular staining of Afp and Alb for FACS analysis was performed as described.[5] Cells were acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and data analyzed with FlowJo software (Tree Star, Ashland, OR). Cell sorting was performed as above except that cells were acquired using a FACSAria II flow cytometer (Becton Dickinson).

In Vitro Colony Formation Assays

Sorted cell populations from mouse ES cell-derived day 13 hepatic cultures were replated (5×10^4 cells/cm^2) on type I collagen (Becton Dickinson)-coated 35-mm dishes in hepatic progenitor expansion medium for 6 to 8 days, numbers of epithelial colonies containing more than 50 cells were quantified, and morphologies were examined.

Establishment of Single c-Kit$^-$EpCAM$^+$/Cell-Cloned Cell Lines

The serum-containing medium consists of DMEM/F12 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin/streptomycin, and 0.1 mmol/L 2-mercaptoethanol (Invitrogen). Day 13 c-Kit$^-$EpCAM$^+$cells were sorted on FACSAria II to seed a single cell into individual wells in 96-well plates precultured with mitomycin C-treated mouse embryonic fibroblasts (MEF) feeder layers. Cells were maintained in serum-containing medium supplemented with 10 ng/mL HGF, 10 ng/mL EGF, 1X insulin-transferrin-selenium X (ITS), 10^{-7} mol/L dexamethasone, 10 ng/mL nicotinamide, and 50 mg/mL gentamicin (all from Sigma–Aldrich). After 6 to 10 days, colonies from single cells were expanded by serial passage. Cloned cell lines were routinely cultured (5×10^4 cells/cm^2) on MEF feeder layers in 6-well plates, with a split ratio of 1:3 every 2 days.

Immunohistochemistry and Immunocytochemistry

Immunohistologic detection of Fah, as well as hematoxylin and eosin (H&E) staining, has been de-