

CINC-2 and miR-199a-5p in exosomes secreted by transplanted Thy1+ cells activate hepatocytic progenitor cell growth in rat liver regeneration

Norihisa Ichinohe (Inchi@sapmed.ac.jp)

Sapporo Medical University School of Medicine Graduate School of Medicine: Sapporo Ika Daigaku Igakubu Daigakuin Igaku Kenkyuka https://orcid.org/0000-0003-3723-7418

Naoki Tanimizu

University of Tokyo Institute of Medical Science: Tokyo Daigaku Ikagaku Kenkyujo

Keisuke Ishigami

Sapporo Medical University School of Medicine Graduate School of Medicine: Sapporo Ika Daigaku Igakubu Daigakuin Igaku Kenkyuka

Yusuke Yoshioka

Tokyo Medical University: Tokyo Ika Daigaku

Naoki Fujitani

Sapporo Medical University School of Medicine Graduate School of Medicine: Sapporo Ika Daigaku Igakubu Daigakuin Igaku Kenkyuka

Takahiro Ochiya

Tokyo Medical University: Tokyo Ika Daigaku

Motoko Takahashi

Sapporo Medical University School of Medicine Graduate School of Medicine: Sapporo Ika Daigaku Igakubu Daigakuin Igaku Kenkyuka

Toshihiro Mitaka

Sapporo Medical University School of Medicine Graduate School of Medicine: Sapporo Ika Daigaku Igakubu Daigakuin Igaku Kenkyuka

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Abstract Background

Small hepatocyte-like progenitor cells (SHPCs) are hepatocytic progenitor cells that transiently form clusters in rat livers treated with retrorsine and with 70% partial hepatectomy (PH). We previously reported that transplantation of Thy1⁺ cells derived from d-galactosamine-treated livers promotes SHPC expansion, resulting in the acceleration of liver regeneration. Extracellular vesicles (EVs) produced by Thy1⁺ cells act on sinusoidal endothelial cells (SECs) and Kupffer cells to secrete IL17B and IL25, respectively, resulting in SHPC activation through IL17 receptor B (RB) signaling. Our aim is to identify factors in Thy1-EVs that activate IL17RB signaling.

Methods

Thy1⁺ cells isolated from rats with d-galactosamine-induced liver injury were cultured for one week. Although some liver stem/progenitor cells proliferated into colonies, others maintained as mesenchymal cells (MCs). Thy1-MCs or Thy1-liver stem/progenitor cells were transplanted into retrorsine/PH-treated livers to examine their effects on SHPCs. SHs isolated from adult rat livers were used to validate factors regulating growth induction.

Results

The number and size of SHPCs remarkably increased in livers transplanted with Thy1-MCs. Comprehensive analysis of Thy1-MC-EVs revealed that *miR-199a-5p*, CINC-2, and MCP-1 are candidates for stimulating SHPC growth. Administration of the *miR-199a-5p* mimic, and not CINC-2, promoted SH growth. SECs treated with CINC-2 induced *IL17b* expression and their conditioned medium promoted SH growth.

Conclusion

Thy1-MC transplantation may accelerate liver regeneration due to SHPCs expansion, which is stimulated by CINC-2/IL17RB signaling and *miR-199a-5p*.

Background

Thy1 (CD90) was reported as a lymphocyte marker more than 50 years ago [1]; followingly, it has been demonstrated that Thy1 is expressed on the surface of diverse cell types [2], especially, bone marrow-derived stem cells [3]. Oval cells are liver stem/progenitor cells (LSPCs) that emerge in regenerating livers of rats treated with hepatotoxins, such as 2-acetylaminofluorene, d-galactosamine (GalN), 3-

diethoxycarbonyl-1, 4-dihydrocollidine, and dipin, combined with or without partial hepatectomy (PH) [4–7]. Petersen *et al.* reported that oval cells express Thy1 on their cell membranes [8] and proposed that oval cells are derived from the bone marrow [9]. However, whether Thy1 is a specific marker of oval cells is controversial [10, 11].

Small hepatocytes (SHs) are a subpopulation of hepatocytes identified in rats, mice, and humans. SHs isolated from healthy adult rat [12, 13], mouse [14], and human [15] livers clonally proliferate to form colonies and differentiate into mature hepatocytes (MHs) in vitro. Previously, we identified CD44 as a marker of SHs [16]. In GalN-induced rat liver injury, oval cells were reported to appear in the periportal area and then differentiate into MHs through basophilic small-sized cells [17, 18]. Moreover, we found that Thy1⁺ cells appeared in the periportal area 2 day after GalN-treatment. Soon after the emergence of Thy1⁺ cells, CD44⁺ cells transiently appeared near the periportal area between Thy1⁺ cells and resident hepatocytes. CD44⁺ cells then differentiated into MHs and lost CD44 expression [16, 19, 20]. The sequential histological alterations in GalN-treated livers were validated using sorted cells. When Thy1⁺ cells isolated from the liver at 2 d after GalN-treatment were plated on dishes, most cells showed fibroblast-like features and proliferated. Subsequently, epithelial cell colonies (LSPCs) appeared and expanded. Although very few number colonies appeared, the cells expressed CD44 as well as Thy1, which were morphologically similar to SHs, as hepatic progenitor cells [19, 20]. The number of the Thy1⁺ cells that could form epithelial colonies increased in the Thy1⁺ cell population isolated from the livers 3 d after GalN-treatment. Thy1⁺ cells consisted of epithelial colonies that sequentially converted their phenotypes from Thy1⁺/CD44⁻ to Thy1⁻/CD44⁺ through Thy1⁺/CD44⁺ and then differentiated into MHs [19, 20]. The other Thy1⁺ cells maintain fibroblast-like appearance and can grow. The fibroblastic cells expressed desmin in their cytoplasm, which have been reported to be hepatic myofibroblasts [10, 11]. We named these fibroblastic cells mesenchymal cells (MCs).

Retrorsine (Ret) severely inhibits the replicative capacity of MHs. Therefore, administration of Ret before PH inhibits liver regeneration. In the Ret/PH-treated rat liver, small hepatocyte-like progenitor cells (SHPCs) emerge and contribute to the recovery of lost tissue [21, 22]. Although SHPCs rapidly expand to grow, it takes approximately one month to recover the original liver volume [21]. The morphology of SHPCs is similar to that of MHs, whereas their size is much smaller than MHs. They share some phenotypic characteristics with fetal hepatoblasts, oval cells, and MHs, though their origin is still debatable [23–25]

We previously reported that transplantation of Thy1⁺ cells isolated from GalN-treated liver increased the number and the size of SHPC clusters in Ret/PH-treated rat livers [26]. Comprehensive analysis of gene expression revealed that Thy1⁺ cell transplantation upregulated the expression of interleukin 17 receptor b (II17rb) in SHPCs. On the other hand, IL17B and IL25, ligands of IL17RB, were expressed by sinusoidal endothelial cells (SECs) and Kupffer cells (KCs), respectively. In addition, we demonstrated that the growth of SHPCs was stimulated by the extracellular vesicles (EVs or exosomes) secreted by cultured Thy1-MCs via IL17RB signaling. However, it is still unclear how Thy1-EVs activate IL17RB signaling to

stimulate SHPC growth in the local environment of recipient livers. In this study, we revealed that *miR-199a-5p*, *miR-125b-5p*, CINC-2, and MCP-1 are abundant in Thy1-EVs. CINC-2 stimulates IL17B expression in SECs. In addition, Thy1⁺ cells trigger the secretion of EVs, containing *miR-199a-5p* and CINC-2, in KCs to promote SHPC proliferation and IL17B production in SECs, respectively. Thus, the acceleration of liver regeneration by transplanting Thy-MCs may result from the expansion of SHPCs stimulated by CINC-2/IL17RB signaling and *miR-199a-5p*.

Methods

Animals

Male F344 rats (dipeptidyl-peptidase IV (DPPIV)⁺ strain; Sankyo Lab Service Corporation, Inc., Tokyo, Japan) and female F344 rats (DPPIV⁻ strain; Charles River Japan, Yokohama, Japan) were used. All animals received proper care, and the Committee of Laboratory Animals approved the experimental protocol following guidelines stipulated by Sapporo Medical University (Approval No.: 17–032, 17–033, 17-034, 19-055, and 20-058). For GalN-induced injury in livers, GalN (Acros, Geel, Belgium, http://www.acros.com; 75 mg/100 g body weight dissolved in PBS) was intraperitoneally administered. For the transplantation experiment, female F344 rats (DPPIV⁺ strain; Charles River Japan, Yokohama, Japan, http://www.crj.co.jp) were administered two intraperitoneal injections of Ret (30 mg/kg body weight; Sigma-Aldrich, Co., St. Louis, MO, www.sigma-aldrich.com) two weeks apart [21, 27]. Two weeks after the second injection, 70% PH was performed. Sorted Thy1⁺ cells (DPPIV⁺ donor cells, 5×10^5 cells) and isolated EVs derived from Thy1⁺ cells were transplanted into Ret/PH livers (DPPIV⁻ rat) through the spleen. Forty rats were randomly divided into control and 7 target groups (5 rats for each). Twenty-seven rats were used for the experiments of cell culture and transplantation. Therefore, the total number of rats used in this study was 67. The humane endpoints were established and monitored following guidelines stipulated by Sapporo Medical University. However, since any rats did not show the signs of the established endpoint in this study, all rats were euthanized by cutting inferior vena cava at PBS perfusion under anesthesia and the livers were resected for histological analyses. In all procedures of the treatments, rats were anesthetized using a mixture of O_2/N_2 (1:1) and isoflurane.

Sorting and culture of cells isolated from the liver

The detail of the isolation and subculture methods have been reported [20, 26, 28, 29]. Briefly, cells were isolated from DPPIV⁺ rats using the two-step collagenase-perfusion method [16]. After perfusion, the cell suspension was centrifuged at $50 \times g$ for 1 min. The supernatant and precipitate were used for isolating Thy1⁺ cells and MHs, respectively. Thy1⁺ cells were isolated from injured livers 2 d after GalN-treatment (GalN-D3) [26]. SHs, MHs, SECs, and KCs were isolated from the liver of a healthy rat (8–12-week-old). The cells were cultured as described [20]. For cell sorting, mouse anti-rat Thy1 (Serotec, Raleigh, NC), mouse anti-rat SE-1 (Immuno-Biological Lab., Takasaki, Japan), and mouse anti-rat CD68 (Serotec) antibodies were used as primary antibodies (**Supplemental Table 1**). The cells were then sorted using

magnetic cell sorting. Thy1⁺ cells were plated on 10-cm culture dishes. SECs and KCs were plated on a 24-well plate coated with rat-tail collagen. The number of viable cells was counted using the trypan blue exclusion test, and 1 × 10⁵ cells/mL were plated on hyaluronic acid-coated dishes (Sigma-Aldrich Co., St. Louis, MO, 1 mg/35-mm dish). SHs were cultured for 10 d and then subcultured on Matrigel-coated 12-well plates as described [28, 29]. The cells were cultured in DMEM/F12 medium (Sigma-Aldrich) supplemented with 20 mM HEPES (Dojindo Chemical Laboratories, Kumamoto, Japan), 25 mM NaHCO₃ (Kanto Chemical Co. Inc., Tokyo, Japan), 30 mg/L l-proline (Sigma-Aldrich), 0.1% bovine serum albumin (Serologicals Proteins Inc., Kankakee, IL), 10 mM nicotinamide (Sigma-Aldrich), 1 mM ascorbic acid 2-phosphate (Fujifilm Wako Pure Chem., Osaka, Japan), 10 ng/mL epidermal growth factor (EGF, BD Biosciences, Bedford, MA), ITS-X (BD Biosciences), 10⁻⁷ M dexamethasone, and antibiotics. The medium was replaced every other day.

Separation of Thy1-MCs

Thy1⁺ cells derived from GaIN-D3 were plated onto 10-cm culture dishes and cultured for 7 d. The proliferated cells consisted of epithelial cells and MCs (Fig. 1A). The epithelial cells form colonies resembling normal SHs and possess characteristics of LSPCs. These colonies were detached from the dish using the cell dissociation buffer at day 7 and collected. The MCs remaining on the dish were detached using 2% Trypsin/0.02% EDTA/PBS. Then, the number of viable cells was counted and epithelial cells and MCs (5×10^5 cells/mL) were transplanted to the Ret/PH model rat liver through the spleen. For isolating EVs, separated epithelial cells and MCs (1×10^6 cells) were seeded on 10-cm dishes and cultured in serum-free medium for 2 d. EVs were isolated from conditioned medium (CM).

Flow cytometry

Thy1⁺ cells were cultured for 7 d, collected by trypsinization, washed with PBS, and centrifuged at $150 \times g$ for 5 min. The cells were then incubated with mouse anti-rat antibodies against CD90, CD73, and CD44 in DMEM containing 10% FBS for 30 min at 4°C; washed with PBS containing 2% FBS (wash buffer); centrifuged at $150 \times g$ for 5 min; and incubated with rabbit anti-mouse IgG (H + L) antibodies conjugated with Alexa Fluor 488 in DMEM containing 10% FBS for 30 min at 4°C (**Supplemental Table 1**). Then, the cells were washed and centrifuged at $150 \times g$ for 5 min. The pellet was suspended in a wash buffer containing propidium iodide solution and passed through a 35-µm cell strainer (Falcon, Corning Inc). The cells were analyzed on the FACSCanto flow cytometer (BD Biosciences, San Jose, USA). All antibodies used in this study are listed in **Supplemental Table 1**. The data were analyzed using the Kaluza Flow Cytometry Software version 1.1 (Beckman Coulter, Inc., Brea, USA).

Immunohistochemistry

The antibodies used in for immunohistochemistry are listed in **Supplemental Table 1**. Recipient rats were euthanized at 14 d after transplantation and their livers were immediately harvested and sliced on ice. Five-mm-thin sections were embedded in Tissue-Tek (Sakura Finetechnical Co., Tokyo, Japan), frozen in isopentane/liquid nitrogen, and stored at – 80°C until use. Some slices were fixed in 10%

paraformaldehyde/buffered PBS. Enzyme- and immuno-histochemistry for DPPIV were performed to identify donor cells [16, 26]. SHPCs in Ret/PH-treated rat livers were identified as clusters comprising more than 10 small-sized hepatocytes, and the areas of SHPCs in the livers were measured using the cellSens Dimension software (OLYMPUS Corp., Tokyo, Japan).

Laser microdissection and gene expression analysis

Clusters of SHPCs in recipient livers were collected by using laser microdissection, following the manufacturer's protocol [22, 30]. Total RNA was isolated from the captured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, 7-µm-thin frozen sections were prepared from liver tissues and stained with hematoxylin. SHPC clusters were dissected under a microscope using an ultraviolet laser (MMI CellCut; Molecular Machines & Industries, Glattbrugg, Switzerland). The gene expression patterns were analyzed using quantitative real-time PCR (qRT-PCR).

miRNA extraction, miRNA microarray, and qRT-PCR

MicroRNAs (miRNAs) were extracted from EVs using the Qiazol lysis reagent and miRNeasy mini kit (Qiagen). A comprehensive analysis of miRNA expression was performed using the 3D-Gene miRNA Labeling kit and the 3D-Gene miRNA Oligo Chip (Toray Industries, Inc. Tokyo, Japan), which was designed to detect 727 miRNA sequences registered in miRBase release 20. For miRNA expression analysis, miRNAs were transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, USA) and RT primers provided with the TaqMan miRNA assay (Applied Biosystems). The cDNA products were analyzed using TaqMan miRNA sequence-specific probes for U6 small nuclear 2 *(U6), miR-125b-5p, miR-145-5p, miR-199a-5p, miR-451-5p, miR-3473-5p*, and *let-7f*, Premix Ex Taq (Takara), and the ABI Prism 7500 sequence detection system (Applied Biosystems) [30, 31]. The primers used are listed in **Supplemental Table 2.** All miRNA microarray data are registered in the GEO database (Accession No.

GSE222517). Quantitative real-time PCR

For qRT-PCR, RNA was reverse-transcribed using the OmniScript RT Kit (Qiagen) and random hexamers as primers. qRT-PCR analyses were performed using TaqMan RNA sequence-specific probes and Premix Ex Taq (Takara). All qRT-PCR reactions were performed in triplicate in 96-well optical plates for all samples using the ABI Prism 7500 cycler (Applied Biosystems). The relative expression of each gene was normalized to the expression of *Gapdh* as a control. The primers used are listed in **Supplemental Table 2**. **Morphological analyses of cultured cells**

The cultured cells were photographed using a phase-contrast microscope equipped with a CCD camera (Olympus Corp., Tokyo, Japan) to count the colonies and cells per colony. Ten fields per dish or well were selected randomly, at least three dishes or wells were examined per experiment, and at least two

independent experiments were performed. All captured images were analyzed using the cellSens Dimension software (OLYMPUS Corp.).

Measurement of the labeling index

Cultured cells were treated with 40 µM 5-bromo-2 -deoxyuridine (BrdU) for 18 h before fixation. The cells were fixed with absolute cold ethanol for 15 min and incubated first with 2 N HCl for 30 min at RT and then with 0.6% hydrogen peroxide in absolute methanol for 30 min at RT. The cells were blocked with BlockAce for 30 min at RT and incubated with a mouse anti-BrdU antibody for 60 min. The dishes were rinsed with PBS and subsequently incubated with a biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 min at RT. Next, the cells were incubated with an avidin-biotin complex solution (VECTASTAIN ABC kit; Vector Laboratories) and treated with 3,3-diaminobenzidine for color development. The number of cells with BrdU-positive nuclei was counted to determine the labeling index.

Isolation of EVs

EVs were separated from CM as reported [30]. Thy1⁺ cells were cultured for 5 d and washed with PBS and the medium was replaced with serum-free DMEM (Sigma-Aldrich Co.). After 48 h, CM was collected and centrifuged at 2,000 × g for 10 min at 4°C. The supernatant was filtered through a 0.22-µm filter (Millipore, Billerica, USA) to remove cellular debris. To prepare EVs, CM was ultracentrifuged at 110,000 × g for 70 min at 4°C [30, 31]. The supernatant was used as CM without EVs (CM-EVs) and the precipitate was resuspended in 200 µL saline. The concentration of EVs was measured using the NanoDrop 1000 spectrometer (Thermo Fisher Scientific, Inc, Waltham, MA), and protein concentration was determined using a BCA assay kit (Thermo Fisher Scientific, Inc.).

Mass spectrometry

To evaluate the characterization of EVs, proteome analysis was performed by mass spectrometry. Aliquots containing 15 µg of total protein extracted from EVs were reductively alkylated with 10 mM dithiothreitol (Fujifilm Wako, Japan) followed by treatment with 20 mM iodoacetamide (Fujifilm Wako). The reductively alkylated proteins were digested with 0.75 µg of sequence grade trypsin/Lys-C (Promega, WI, USA) for 16 h at 37°C. The resultant peptides were desalted with a styrene divinylbenzene polymer tip column (GL Science, Tokyo, Japan). The desalted peptides were completely evaporated on a centrifugal evaporator. Finally, the obtained peptides were redissolved with 10 µL of ultrapure water containing 0.1% formic acid. This sample solution was subjected to Orbitrap Q Exactive Plus (Thermo Fisher Scientific, Inc.) through the EASY-nLC system equipped with an C18 column (0.075 mm × 125 mm, Nikkyo Technos, Tokyo, Japan). The sample was eluted with acetonitrile gradient from 0–30% in 90 min at a flow rate of 300 nL/min. All data were acquired in data-dependent mode and tandem MS (MS/MS) was performed by higher energy collision-induced dissociation. The MS/MS data were processed using the MaxQuant software 1.6.3.3 [32]. Peptide searches were performed with reference to the proteomic data of *Rattus norvegicus* obtained from UniProtKB (https://www.uniprot.org/). The mass spectrometry proteomics data are registered in the Proteome Xchange Consortium database (Accession No: PXD039384).

Transplantation of EVs into Ret/PH livers

Thy1⁺ cells were cultured for 7 d. Then, CM and the cells were used for isolating EVs and transplantation, respectively. The amount of EVs secreted by 5×10^5 donor Thy1⁺ cells in 48 h was quantified. The pellet obtained after ultracentrifugation of CM was resuspended in 200 µL saline and administered to the recipient liver through the spleen using a low dead-space syringe with a 21-gauge needle (NIPRO, Tokyo, Japan).

Transfection of cultured SHs with mimics

SHs cultured at 5×10^4 cells/well on Matrigel-coated 12-well plates were transfected with TaqMan miRNA mimics corresponding to *miR-125b-5p* (Applied Biosystems, MC), *miR-145-5p* (MC), *miR-199a-5p* (MC), *miR-451-5p* (MC), *miR-3473-5p* (MC), and *miR-let-7f* and the negative controls (Applied Biosystems, Cat No. 4464058) (Final concentration: 50 nM). The transfections were performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, USA) following the manufacturer's instructions [30]. After 2 d, the medium was replaced, the cells were cultured for 5 d, and colony growth was evaluated.

Analysis of cytokine content

Isolated EVs were lysed in RIPA buffer containing 1 mM protease inhibitors (Sigma-Aldrich). The resulting lysates were assayed to detect Thy1-MCs-derived proteins using a custom-designed Quantibody ratspecific protein array (RayBiotech, Peachtree Corners, USA, cat. No. QAR-CAA-67) at Cosmo Bio, Ltd. (Tokyo, Japan) [30].

Overexpression of miR-199a-5p in EVs derived from Thy1⁺ cells using lentivirus

The transfections were performed using the XMIRXpress vector (SBI System Biosciences) according to the manufacturer's instructions [26, 29]. Briefly, 293TN cells (3×10^6 cells) were plated on 75-cm² culture flasks. Two µg of transfer plasmid (*miR-125b-5p, miR-199a-5p*, or non-target miRNA [NT]) and 20 µL pPACKH1-plasmid were mixed with 800 µL of serum-free DMEM in tubes and mixed by pipetting. Next, 24 µL of PureFection reagent (SBI System Biosciences) was added to tubes, vigorously vortexed, and incubated at room temperature for 15 min. The mixtures were added drop-wise into the flask and swirled to disperse evenly. After 2 d, media were collected into 12-mL tubes and centrifuged at 3,000 × *g* for 15 min to pellet cell debris. The viral supernatant was added to Thy1⁺ cell culture medium. EVs produced using transfected MCs were collected and evaluated for the expression of *miR-125b-5p* and *miR-199a-5p* by qRT-PCR. Their abilities to stimulate SHPC growth in Ret/PH model rats were examined as described. **Statistical analysis**

Array data were analyzed using the MultiExperiment Viewer software. Microarray data were analyzed using Student's *t* test. All other data were analyzed using Tukey's multiple comparison test. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Statistical

significance was accepted at p < 0.05. The experimental results are expressed as mean ± standard error (SE).

Results

Thy1-MCs accelerated SHPC growth through IL17RB signaling

Recipient livers were histologically evaluated 14 d after transplantation for Thy1-positive cells. Many SHPC clusters comprising small cells were observed in livers transplanted with Thy1-MCs compared with those transplanted with Thy1-LSPCs and nontransplanted liver controls. SHPC clusters were randomly distributed in liver lobules, and not limited to a specific area (Fig. 1B). The number of clusters in livers with Thy1-MCs was approximately two-fold larger than that of clusters in the control (Fig. 1C). The number of cells per cluster increased significantly more than that of cells in Thy1-LSPCs and controls (Fig. 1D).

Thy1-EVs were reported to stimulate SHPC growth through IL17RB signaling in the livers of Ret/PH model rats [26]. To confirm IL17RB expression in SHPCs, first we performed qRT-PCR. *IL17rb* expression was significantly increased in SHPCs with Thy1-MCs (Fig. 1E). Next, we performed double immunohistochemistry for IL17RB and HNF4α. Many SHPCs in the livers transplanted with Thy1-MCs were strongly stained with the anti-IL17RB antibody, although the intensity of expression varied between the clusters (Fig. 2A). Although most HNF4α⁺ hepatocytes, including SHPCs, in livers with or without Thy1-LSPCs express IL17RB, the level of IL17RB expression in SHPCs with Thy1-MCs is much higher than that in other hepatocytes. In addition, we evaluated the expression of the IL17RB ligand IL17B using immunohistochemistry and found that IL17B was expressed in SE-1⁺ cells, especially those within the SHPC cluster in the liver with Thy1-MCs, and not expressed in livers with LSPCs and controls (Fig. 2B). These results indicate that Thy1-MCs can induce SHPC proliferation through IL17RB signaling.

Characterization of Thy1-MCs and secreted EVs

We examined whether Thy1-MCs express MSC-positive markers such as CD90, CD73, CD44, and CD271 [33] and found that 89.2% and 90.4% of Thy1-MCs expressed CD90 and CD73, respectively, whereas some cells expressed CD44 (3.8%) (Fig. 3A). Thy1-MCs expressed neither CD31⁺ endothelial nor CD68⁺ macrophage markers. EVs collected from CM of Thy1-MCs cultured for 48 h had an average particle size of ~ 175 nm (Fig. 3B **and C**). Thy1-MCs (5×10^5 cells) produced approximately 4.9×10^9 particles in 48 h, which corresponds to 44.0 µg of protein. Proteome analysis revealed that EVs produced by Thy1-MCs contained CD63, CD81, CD82, CD47, actin, and GAPDH (**Supplemental information**) as well as THY1 (CD90) and NT5E (CD73) as cell membrane protein of the original cells.

Thy1-EVs were reported to stimulate SHPC growth [26]. We confirmed whether EVs secreted by Thy1-MCs induce SHPC growth. EVs derived from Thy1-LSPCs and -MCs (1.4 μ g) were added to the SH culture

medium at 3 h after plating, and the medium was replaced with fresh medium without EVs at 48 h after plating. Immunocytochemistry for BrdU shows that the addition of Thy1-MC-EVs accelerated SH growth (Fig. 4A). Seven days after plating, the number of SH colonies treated with Thy1-MC-EVs became larger than control and Thy1-LSPCs (Fig. 4B), and the size of the colonies treated with Thy1-MC-EVs were approximately 2-fold and 1.5-fold larger than that of control and Thy1-LSPCs, respectively (Fig. 4C). Labeling index (LI) of SH colonies treated with Thy1-MC-EVs, Thy1-LSPCs-EVs, and control was approximately 40%, 35%, and 24%, respectively (Fig. 4D). These results suggest that Thy1-MC-EVs contain factors that induce proliferation of hepatic progenitor cells.

Identification of miRNA as growth-related agents contained in EVs secreted by Thy1-MCs

EVs are enriched with many bioactive molecules, such as proteins, lipids, RNAs, and mitochondrial DNAs [33]. We examined miRNAs present in EVs derived from cultured Thy1-MCs and compared them with those from SHs and MHs. We used the miRNA Oligo Chip to perform a comprehensive analysis of miRNAs and selected miRNAs (*miR-125b-5p, miR-145-5p, miR-199a-5p, miR-451-5p, let-7f*, and *miR-3473*) that were expressed at more than 5-fold higher levels in Thy1-MC-EVs than SH- and MH-EVs (Fig. 5A). The expression of the selected miRNAs was confirmed by qRT-PCR. The expression of *miR-125b-5p, miR-145-5p, miR-199a-5p*, and *miR-451-5p* was relatively higher in Thy1-MC-EVs than SH- and MH-EVs (Fig. 5B). Therefore, the miRNAs were further analyzed.

Mimics of the selected miRNAs were administered to cultured SHs, and their effect on SH growth was evaluated (Fig. 6A). Seven days after treatment, the number of colonies per dish and cells per colony significantly increased in cells treated with *miR-125b-5p* and *miR-199a-5p* mimics (Fig. 6B–D). Moreover, the ratio of BrdU⁺ cells was significantly increased in cells treated with *miR-125b-5p* and *miR-125b-5p* and *miR-199a-5p* may enhance (Fig. 6E). These results indicate that mimics of *miR-125b-5p* and *miR-199a-5p* may enhance hepatic progenitor cell growth. Next, we examined whether mimics of *miR-125b-5p* or *miR-199a-5p* induced the expression of *IL17rb* and *IL17b* in SHs and SECs, respectively. However, either mimic could not induce *II17rb* and *II17b* expression in SHs and SECs, respectively (data not shown).

Administration of Thy1-MC-EVs transfected with miR-199a-5p to Ret/PH-treated rat livers

We examined the efficiency of *miR-125b-5p* and *miR-199a-5p* overexpression in Thy1-MC-EVs. The amount of *miR-199a-5p* in EVs from Thy1-MCs was ~ 20-fold higher than that from control and negative target control (NT) (Fig. 7A). Although several sequences of *miR-125b-5p* mimics were examined, we could not induce *miR-125b-5p* overexpression in Thy1-MCs. Therefore, we examined the effect of EVs overexpressing *miR-199a-5p* on SHPC growth. NT-EVs and *miR-199a-5p*-overexpressing EVs (miR199a-EVs) were administered to Ret/PH-treated rat livers through the spleen. miR199a-EVs could neither induce the emergence nor enhance SHPC proliferation (Fig. 7B). However, transplantation of *miR-199a-5p*-

overexpressing Thy1-MCs could induce SHPC proliferation, but could not increase the number of SHPC clusters (Fig. 7C **and D**).

Identification of cytokines present in EVs secreted by Thy1-MCs

In addition to miRNAs, Thy1-MC-EVs contain cytokines that could stimulate SH growth (Fig. 2). We examined the major cytokines produced by Thy1-MCs using a cytokine array. We then selected CINC-2 (Cxcl3) and MCP-1 (Ccl2) because they were expressed at two-fold higher levels in Thy1-MCs than MHs (Fig. 8A). We then quantified both cytokines produced by Thy1-MC EVs or MH EVs (Fig. 8B). Levels of CINC-2 and MCP-1 were much higher in Thy1-MC-EVs than MH-EVs. To examine whether CINC-2 and MCP-1 could stimulate SH growth, we treated SHs with CINC-2 and MCP-1 individually. Neither CINC-2 nor MCP-1 could stimulate SH growth (Fig. 8C).

Next, we examined whether either cytokine could induce *ll17b* expression in SECs and KCs. CINC-2 induced *lL17b* expression in SECs and KCs, whereas MCP-1 could not induce *lL17b* expression in SECs or KCs (Fig. 9A). To validate that *ll17b* expression was induced by CINC-2/CXCR2 signaling, we added neutralizing antibodies against CINC-2 or CXCR2 inhibitor (SB225001) to the culture medium of SECs treated with Thy1-EVs or CINC-2 (20 ng/mL). Thy1-EVs induced *ll17b* expression in SECs (Fig. 9B), which was suppressed by the neutralizing antibody or SB225001. Although CINC-2 induced *ll17b* expression in SECs, the degree of induction was much less than that by Thy1-EVs. The neutralizing antibodies against CINC-2 and SB225001 significantly inhibited the induction of *ll17b* expression in SECs. These results indicate that CINC-2 in Thy1-EVs play a major role in inducing IL17B production in SECs through CXCR2 signaling.

Next, we examined whether SECs treated with CINC-2 (CINC-2-SEC) can stimulate hepatic progenitor cell growth. SEC-CM and CINC2-SEC-CM were added to SH culture medium. CINC-2-SEC-CM apparently accelerated SH growth (Fig. 9C). Seven days after plating, the number of SH colonies increased approximately two-fold compared with the control (Fig. 9D). The size of the colonies was significantly larger than that of the control (Fig. 9E). Furthermore, SHs treated with CINC-2-SECs-CM showed higher LI than control (Fig. 9F). Considering that IL17B administration accelerated SH growth, these results indicate that CINC-2 within Thy1-EVs activate SECs to produce IL17B, resulting in the acceleration of hepatic progenitor cell growth.

Activation of KCs by Thy1-EVs

This study and other studies [22] show that IL17B, IL25, CINC-2, *miR-125b-5p*, and *miR-199a-5p* play important roles in hepatic progenitor cell growth. Therefore, we examined whether Thy1-EVs could increase the expressions of these inducers in KCs. Administration of Thy1-EVs to cultured KCs upregulated the expression of CINC-2, IL25, and *miR-199a-5p* compared with the control (Fig. 10). However, *IL17b* expression was not induced by Thy1-EVs, notwithstanding that CINC-2 could induce *IL17b* expression in KCs (Fig. 9A).

Discussion

In this study, we showed that EVs secreted by Thy1-MCs, and not Thy1-LSPCs, stimulated SECs and SHPCs to express IL17B and IL17RB, respectively, which resulted in the expansion of SHPCs and the acceleration of liver regeneration. EVs are enriched with many bioactive molecules, such as protein, lipid, RNA, and mitochondrial DNA [33]. Previously, we found that *miR-146a-5p* included in EVs secreted by BM-MCs played an important role in SHPC activation [26]. Therefore, we hypothesized that miRNAs within EVs secreted by Thy1-MCs are involved in SHPC growth. Analysis of miRNAs in Thy1-MC-EVs revealed that *miR-125b-5p* and *miR-199a-5p* stimulate SHPC growth. *miR-199a* has been correlated with hepatocyte proliferation. Chen *et al.* reported that monocytes or macrophages expressed *miR-199a-5p* for their differentiation [34]. However, *miR-199a-5p* was reported to affect hepatocyte proliferation both negatively and positively. Liu *et al.* reported that *miR-199a-5p* negatively affected the proliferation of hepatocellular carcinoma by targeting CDC25A [35]. According to Zhang *et al., miR-199a-5p* positively affected hepatocyte proliferation by targeting TNFa/TRADD/caspase3 signaling and accelerating liver regeneration in PH model rats [36]. Our findings revealed that *miR-199a-5p* is involved in regulating hepatocyte growth. However, *miR-199a-5p* activates SHPCs is unclear.

In contrast to growth promotion by *miR-199a-5p*, when *miR-199a-5p*-overexpressing EVs (miR199a-EVs) were administered to Ret/PH-treated rat livers through the spleen, miR199a-EVs could neither induce the emergence nor enhance SHPC proliferation. This result suggests that transient exposure to *miR-199a-5p* alone may not be enough for inducing SHPC proliferation *in vivo*. Because Thy1-EVs increase some factors, including CINC2 and miR199a, in KCs (Fig. 10), the contents of Thy1-EVs, including *miR-199a-5p*, cooperatively act on KCs, thereby promoting SHPC proliferation. Although transplantation of *miR-199a-5p*-overexpressing Thy1-MCs induced SHPC proliferation, EVs containing *miR-199a-5p* cannot directly act on SHPCs. We previously reported that the suppression of phagocytosis by KCs with gadolinium inhibited the expansion of SHPCs induced by the transplantation of Thy1⁺ cells [26]. Thus, we hypothesized that phagocytosis of Thy1⁺ cells by KCs might be a trigger for the expansion of SHPC clusters. Taking these results into consideration, phagocytosis of Thy1-MCs by KCs may play a significant role in the growth regulation of SHPCs. However, commercial *miR-125b-5p* mimics could not increase the synthesis of *miR-125b-5p* in Thy1-MCs. It remains to be validated whether *miR-125b-5p* is involved in IL17RB signaling. In addition, it may be necessary to clarify why *miR-125b-5p* overexpression was hard to induce in Thy1-MCs.

CINC-1, CINC-2, and CINC-3 belong to the CXC chemokine family and are potent chemotactic factors for neutrophils [37, 38]. CINCs recruit neutrophils by signaling through the CXCR2 receptor [39]. In this study, we found that CINC-2 could induce *IL17b* expression in SECs and KCs. The induction was suppressed with neutralizing antibodies against CINC-2 and SB225001. These results suggest that IL17B production in SECs and KCs is regulated through CINC-2/CXCR2 signaling. Lung-resident mesenchymal stem cells (LR-MSCs) contain a population that can differentiate into alveolar epithelial type II cells [40]. Similar to BM-MSCs, LR-MSCs express Thy1 as well as Sca-1, CD29, CD44, and CD106. Rat alveolar epithelial type II

cells were reported to secrete CINC-2 and MCP-1 in response to a combination of IL-1β, TNF-α, and IFN-y [41]. Therefore, it is feasible to consider that Thy1⁺ LR-MSCs can produce EVs, which contain CINC-2 and MCP-1. In this study, we found that Thy1-MCs, which emerged in the lobules after GalN-treatment, also expressed CINC-2 and MCP-1 and could secrete EVs. In healthy rat livers, Thy1⁺ cells are rarely observed in the lobule and localize within Glisson's sheath. Although portal Thy1⁺ cells isolated from healthy livers do not express CINC-2, the cells that were cultured in the presence of TNF-α can express *Cinc-2* (data not shown). These results suggest that Thy1-MCs can synthesize CINC-2 as an inflammatory response. In this study, we found that CINC-2 could act as a factor for inducing IL17B other than a chemotactic factor for neutrophils as functions of CINC-2. Moreover, CINC-2 can induce *IL17b* expression in SECs, although CINC-2 cannot directly stimulate SH growth. However, CM of CINC-2-treated SECs could promote SH proliferation. In addition, because SH growth was suppressed by the administration of the neutralizing antibody against IL17B and CXCR2 inhibitor, it appears that SECs produce IL17B. In livers transplanted with Thy1-MCs, SECs in SHPC clusters stained intensively for IL17B and SHPCs distinctly expressed IL17RB. However, why Thy1-MC treatment induces stronger *IL17b* expression than CINC-2 expression in SECs is unclear. By contrast, EVs produced by Thy1-MCs contained not only CINC-2 but also MCP-1. In this study, although we examined not a few experiments to elucidate the role of MCP-1 in SHPC growth, MCP-1 was not involved in the indirect effect to IL17RB signal transduction as well as the direct effect to the proliferation of hepatocytic progenitor cells. Further experiments are necessary to clarify the effects of MCP-1 in livers transplanted with Thy1-MC.

Conclusion

In this study, we identified IL17B, IL25, CINC-2, *miR-125b-5p*, and *miR-199a-5p* as growth inducers of hepatocytic progenitor cells in liver regeneration induced by Thy1-MC transplantation. The interactions between SHPCs, SECs, KCs, and the inducers may be summarized as follows:

- 1. Transplanted Thy1-MCs produce EVs containing *miR-125b-5p*, *miR-199a-5p*, and CINC-2.
- 2. EVs directly affect SHPCs, resulting in the induction of IL17RB expression and stimulation of SHPC growth by miRNAs.
- 3. EVs directly affect SECs, resulting in IL17B production due to CINC-2.
- 4. KCs engulf Thy1-MCs to be activated, resulting in the secretion of EVs containing *miR-199a-5p* and CINC-2 as well as IL25.
- 5. CINC-2 and *miR-199a-5p*, contained in EVs secreted by KCs, induce IL17B production in SECs and stimulate SHPC growth, respectively.
- 6. Stimulation of SHPC growth by SECs and KCs continues for at least 2 weeks after transplantation.

Abbreviations

LSPCs, liver stem/progenitor cells; GalN, D-galactosamine; PH, partial hepatectomy; SHs, small hepatocytes; MHs, mature hepatocytes; MCs, mesenchymal cells; SHPCs, small hepatocyte-like progenitor

cells; Ret, retrorsine; IL, interleukin; RB, receptor B; SECs, sinusoidal endothelial cells; KCs, Kupffer cells; EVs, extracellular vesicles; CINC, ; DPPIV; dipeptidyl-peptidase IV; CM; conditioned medium

Declarations

Ethics approval and consent to participate

All animals received proper care, and the Committee of Laboratory Animals approved the experimental protocol following guidelines stipulated by Sapporo Medical University. "Analysis about the behavior of donor cells and the reaction of recipient cells in the livers by hepatic stem/progenitor cells transplantation. Approval No.: 17-032, Approval Date: March, 6, 2017." "Isolation and analysis of hepatic stem/progenitor cells. Approval No.: 17-033, Approval Date: March, 6, 2017." "Study of proliferation and differentiation of hepatic stem cells and bone marrow derived cells derived from GFP transgenic rats. Approval No.: 17-034, Approval Date: March, 6, 2017." "In vivo analysis of liver regeneration mechanism by miRNA derived from tissue stem cells. Approval No.: 19-055, Approval Date: February, 4, 2020." And "In vitro analysis of liver regeneration mechanism by miRNA derived from tissue stem cells. Approval No.: 20-058, Approval Date: May, 20, 2020."

Consent for publication

Not applicable in individual person's data.

All the co-authors have approved the manuscript and agree with its submission to *Stem cell research & therapy*.

Competing interests

The authors indicate no potential conflicts of interest.

Availability of data and materials

All miRNA microarray data are registered in the GEO database (Accession No.

GSE222517). The mass spectrometry proteomics data are registered in the Proteome Xchange Consortium database (Accession No: PXD039384).

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Author Contributions

N. I.: Conception and experimental design, financial support, collection and assembly of data, data analysis and interpretation, and manuscript writing.

N. T.: Collection and assembly of data, data analysis and interpretation.

K. I.: Collection and assembly of data.

- Y. Y.: Collection and assembly of data, data analysis and interpretation.
- T. O.: Collection and assembly of data, data analysis and interpretation.
- N. F.: Collection and assembly of data, data analysis and interpretation.
- M. T.: Collection and assembly of data, data analysis and interpretation.

T. M.: Conception and experimental design, data analysis and interpretation, financial support, and final approval of the manuscript.

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Figures

Figure 1



Figure 1

Transplantation of Thy1-LSPCs and Thy1-MCs to Ret/PH-treated rat livers.

(A) Schematic showing the cell transplantation method. (B) Photos of SHPC clusters (black dotted lines) in hematoxylin–eosin (H-E)-stained livers transplanted with control (left), Thy1-LSPC (center), and Thy1-MC (right) (A). Inset shows enlarged typical SHPCs (yellow square) in livers with and without Thy1⁺ cell

transplantation. Fine fat droplets (empty vesicles) are observed in the cytoplasm of SHPCs. Scale bars, 500 mm. The number of SHPC clusters per area (C) and area of the cluster (D) were measured 14 d after transplantation. (E) *IL17rb* expression in SHPCs of livers that received Thy1⁺ cell transplantation was confirmed by qRT-PCR. Asterisks indicate statistically significant differences, p < 0.05



IL17rb and *IL17b* expression in SHPCs with and without Thy1⁺ cell transplantation.

Double immunohistochemistry for IL17RB/HNF-4a (A) and IL17B/SE-1 are shown in Ret/PH-treated rat livers transplanted with control, Thy1-LSPCs, and Thy1-MCs.

SHPC clusters are enclosed by dotted lines. Scale bars, 100 mm.





Characterization of Thy1-MCs and EVs.

(A) Flow-cytometry analysis of cell surface markers (CD90, CD73, CD44, CD31, CD68, and CD271) present on BM-MCs. (B) Sample video frame of EVs derived from cultured Thy1-MCs after 48 h, analyzed using the NanoSight particle tracking system. (C) Overall size distribution (histograms) and mode (nm). (D) Heatmap of miRNA expression pattern in Thy1-MCs, BM-MCs, SHs, and MHs.



Figure 4

Effect of EVs derived from Thy1-LSPCs and Thy1-MCs.

Panel A shows photos of typical SH colonies treated with EVs derived from Thy1-LSPCs and Thy1-MCs at day 7 after treatment. Immunocytochemistry for BrdU and hematoxylin staining can be visualized. Scale bars, 100 μ m. The number of SH colonies (B), number of cells per colony (C), and percentage of BrdU⁺ cells per colony (D) are evaluated. Bars show SEs. Asterisks indicate statistically significant differences; *p* < 0.05.

Figure 5

Α



В



Figure 5

Identification of miRNAs contained in EVs secreted by Thy1-MCs.

(A) Six miRNAs were 5-fold upregulated compared with hepatic Thy1-MCs and are shown as a heatmap.

(B) Quantification of *miR-125b-5p, miR-145-5p, miR-199a-5p, miR-451-5p, miR-let-7f,* and *miR-*

*3473*expression in the EVs secreted by MHs, SHs, and hepatic Thy1-MCs using qRT-PCR. Four of six

miRNAs were more abundant in the EVs secreted by Thy1-MCs than in other cells. Bars show SEs and asterisks indicate statistically significant differences, p < 0.05.

Figure 6



milerias miR-1998

250

miRASI

Figure 6

Effect of selected miRNAs in Thy1-MC EVs on SH proliferation.

142 mile-1998 P. niR-145

250

miRASI

nieths

250

miR-1992

Photos show SH colonies, transfected with *miR-125b-5p*, *miR-145-5p*, *miR-199a-5p*, and *miR-451-5p* mimics added to the culture medium for 2 d (A). The cells were cultured until day 7 and BrdU was added to the culture medium 24 h before fixation. BrdU was immunostained. Scale bars, 100 μ m (B). Total number of SH colonies per well (C), number of cells per colony (D), and percentage of BrdU⁺ cells per colony (E). Asterisk indicates statistically significant differences compared with control, *p* < 0.05



Figure 7



Overexpression of *miR-199a-5p* in Thy1-MC EVs.

Administration of Thy1-MC EVs and cells transfected with *miR-199a-5p* to Ret/PH-treated rat livers. EVs derived from the conditioned medium of cultured Thy1-MCs transfected with *miR-125b-5p* and *miR-199a-5p* for 48 h were administered, and the livers were examined 14 d later. (A) Quantification of *miR125b-5p* and *miR-199a-5p* in Thy1-MC EVs with and without transfection using qRT-PCR. Bars show SEs. Asterisks indicate statistically significant differences; p < 0.05. (B) Photos of SHPC clusters in the livers of control, EVs secreted by Thy1-MCs transfected with negative control (NT), or *miR-199a-5p*(miR-199a). The samples were stained with H–E. SHPC clusters are enclosed by dotted lines: scale bar, 200 µm. (C) The number of SHPC clusters per liver area and (D) number of cells per cluster is shown. Bars show SEs and asterisks indicate statistically significant differences, p < 0.05.





Figure 8

Effects of cytokines present in EVs derived from Thy1-MCs.

Volcano plot of cytokines expressed by Thy1-MCs and MHs (A). The amount of cytokines present in EVs secreted from hepatic Thy1-MCs and MHs were evaluated using a custom-designed Quantibody ratspecific protein array (QAR-CAA-67) (B). Higher concentrations of CINC-2 and MCP-1 were present in Thy1-MC-EVs than MH-EVs. CINC-2 and MCP-1 were added to the culture medium 3 h after plating and refreshed every other day. Panel C shows the photos of typical SH colonies treated with each cytokine. BrdU was added to the culture medium 24 h before fixation, and immunocytochemistry for BrdU was performed 7 d after plating. Scale bars, 100 μ m. The total number of SH colonies per well, number of cells per colony, and percentage of cells with BrdU⁺ nucleus per colony are shown. Asterisk indicates statistically significant differences, *p* < 0.05.



Figure 9

Figure 9

Induction of IL17B expression in SECs by cytokines contained in Thy1-MC EVs.

Administration of CINC-2 and MCP-1 to SECs and Kupffer cells (A). EVs were prepared from the conditioned medium of 2-d cultured Thy1-MCs and administered to SECs and Kupffer cells isolated from a normal rat liver. Thy1-EVs were administered 3 h after plating, and the cells were cultured for 48 h. Effects of CINC-2/CXCR2 signaling, using CINC-2 neutralizing antibody or CXCR2 inhibitor (SB225001), were examined for the induction of IL17b expression in SECs by CINC-2 and Thy1-EVs, respectively. *IL17b* expression was measured by real time quantitative PCR (RT-qPCR) (B). SHs were cultured in serum-free media on Matrigel-coated dishes. SECs-CM with and without CINC-2 treatment were administered to SHs 3 h after plating and cultured for 7 d. *IL17rb* expression was measured by RT-qPCR (C). Asterisk indicates statistically significant differences, p < 0.05. Abbreviations: EVs, extracellular vesicles; MHs, mature hepatocytes; SECs, sinusoidal endothelial cells; SHs, small hepatocytes. Panel C shows the photos of typical SH colonies treated with each cytokine. BrdU was added to the culture medium 24 h before fixation, and immunocytochemistry for BrdU was performed at 7 d after plating. Scale bars, 100 µm. The total number of SH colonies per well (D), number of cells per colony (E), and percentage of cells with BrdU⁺ nucleus per colony (F). Asterisk indicates statistically significant differences, p < 0.05.



Figure 10

Activation of KCs by Thy1-EVs.

Thy1-EVs were administered 3 h after plating, and the cells were cultured for 48 h. Expression of *CINC-2, IL17b, IL25, miR-125b-5p,* and *miR-199a-5p* was measured using real time quantitative PCR. Asterisk indicates statistically significant differences, p < 0.05.

Supplementary Files

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