

Comparison of the Biological and Functional Characteristics of Mesenchymal Stem Cells From Intrahepatic and Identical Bone Marrow

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Research

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Abstract

Background: We previously isolated a novel mesenchymal stem cell (MSC)-like neuroglial antigen 2-expressing stem cell population (MLpvNG2) from an uninjured liver by using a “Porcoll-Plate-Wait” method and determined that MLpvNG2 possesses hepatic stem/progenitor cell characteristics.

Methods: In this study, we compared the biological and functional characteristics of the intrahepatic (MSC)-like MLpvNG2 with identical bone marrow-derived MSCs (niBM-MSCs), which are a well-accepted stem cell population in the field. We performed an *in vitro* study using conditioned medium and *in vivo* study using our well-set diethylnitrosamine (DEN)-induced liver fibrotic/cirrhotic murine model as well.

Results: We found that in a fibrotic liver environment, MLpvNG2 survived better than niBM-MSCs obtained through different mechanisms of action. MLpvNG2 mainly differentiates into albumin (ALB(+)) hepatocytes, while niBM-MSCs mainly differentiate into CK/KRT19(+) cholangiocytes. Of note, we identified for the first time that C/EBP α / β is expressed on the cell surface of donor and host hepatic cells. As such, we used anti-C/EBPs neutralizing antibodies to determine the functional characteristics both *in vitro* (conditioned medium) and in the DEN-induced animal model.

Conclusions: Based on our findings, it can be concluded that native-source (liver) stem cells (MLpvNG2) are more efficient than nonnative-sourced stem cells (niBM-MSCs) in the treatment of native (liver)-sourced diseases such as end-stage liver disease.

Background

End-stage liver disease (ESLD) due to liver fibrosis or cirrhosis is an important cause of death worldwide [1]. Such disease results from progressive, extensive fibrosis and impaired hepatic regeneration, the loss and destruction of functional hepatocytes is one of the major features of liver fibrosis [2]. Interest in stem cell therapy is currently increasing [1]. As bone marrow-derived MSC (BM-MSC) transplantation, a well-accepted cells in the field, is not an effective treatment for the disease due to the potential major drawbacks [3-5], a novel cell therapeutic approach needs to be formulated. MLpvNG2⁺ cells might be an alternative hepatic stem progenitor cells (HSPs) for the disease [6].

MLpvNG2⁺ cells have been demonstrated to have a potential role in functionalities including hepatic differentiation, immunomodulation and treatment for liver fibrosis [7, 8]. Although these effective roles seem likely similar to extrahepatic BM-MSCs [9, 10], whether the *in vivo* capacity after transplantation of the two types of cells could exhibit similar role, and which cell type is more interesting from a “cell therapy perspective” in regarding of functional differentiation and endogenous regeneration in response to fibrotic/cirrhotic liver signals remain still unclear [11]. Thus, based on the questions behind [12, 13] and our previous studies [6] we propose that the transplantation of the MLpvNG2 may be more beneficial to treating intrahepatic disease (liver fibrosis/cirrhosis) compared to the transplantation of extrahepatic-sourced BM-MSCs, particularly in mature hepatic cell differentiation and controlled by different

mechanism of action. Therefore, in the present study, we sought to compare the functional characteristics and mechanisms of action of the MLpvNG2 with those of identical extrahepatic-sourced BM-MSCs (niBM-MSCs) in a well-set DEN-induced liver fibrosis/cirrhosis murine model ^[14, 15], as well as the feasibility of the clinical application of the MLpvNG2 cells.

Materials And Methods

This study used methodologies for *in vivo* and *in vitro* experiments. In the *in vitro* study, we used CMs generated from bCM, naïve (nL-CM) and cirrhotic (Cir-CM) liver homogenates. In the *in vivo* study, we used a DEN-induced liver cirrhosis murine model.

Animals

Male inbred C57BL/6 mice (8-10 weeks old) were purchased from the Laboratory Animal Center of the Army Medical University (Chongqing, China). The Institutional Animal Care and Use Committee of the Army Medical University (#SYXK-PLA-2012-00120031, Chongqing, China) approved all experimental protocols, and all methods were carried out in accordance with relevant guidelines and regulations.

Isolation of MLpvNG2⁺ cells and niBM-MSCs

MLpvNG2⁺ cells were isolated according to our previously published protocol ^[6]. Briefly, the mice were euthanized in CO₂ euthanasia chambers. For each isolation, the portal triads from two livers of adult (6-8 weeks old) C57BL/6J mice were quickly cut and dissected in cold minimum essential medium without Ca²⁺ and Mg²⁺ (wMEM) (HyClone Laboratories, Inc., Logan, UT, Cat. No. SH30023.0). The liver tissue blocks were dissociated in trypsin/EDTA (1:1) for 30 min at 37°C under an atmosphere of 5% CO₂. After incubation, the tissue was mixed with 0.6 mL of 0.5% DNase in wMEM for 10 min and centrifuged at 800 × g for 5 min. The cells were washed three times before being layered onto a Percoll gradient (GE Healthcare, Cleveland, OH, USA, Cat. No. 17-0891-02). The gradient was prepared from stock isotonic Percoll (SIP) at 70% in 1× PBS and 30% in 1× wMEM. The tissue was homogenized in 7.5 mL of 30%-SIP and layered slowly onto the top of the 70%-SIP. After centrifugation at 2000 rpm for 30 min, removed the lipid layer on the top of the tube carefully, then transferred 2.0-3.0 mL of the 70-30% interface into a clean conical tube and centrifuged at 1200 rpm for 5 min. The cells were plated at 1×10⁶ cells/mL in 75 cm² flasks coated with poly-L-lysine (PLL) (Sigma Aldrich, St. Louis, MO, USA Cat. No. P1274-100 MG) at 37°C under an atmosphere of 5% CO₂ in DMEM/F12 supplemented with 10% FBS (Complete culture media, CCM) for at least 3 weeks, with 2 mL of DMEM/F12 replenished once per week. After ~3 weeks, colonies appeared and developed, and half of the CCM was replaced every 3 days until 5 weeks, when the cells reached 85% confluence. Subsequently, the cultures were harvested with trypsin, passaged 1:4 into fresh poly-L-lysine (PLL)-coated 75 cm² flasks, grown to confluence and passaged again as described. All used cells were seeded onto poly-L-lysine PLL-coated coverslips allowed to grow for the required number of days, followed by immunocytochemical analyses. The cell purity was determined by anti-Ng2 antibody (Ab) staining (Millipore, Massachusetts, USA Cat. No. AB5320).

niBM-MSCs were harvested from the same C57BL/6 mice according to a previously described protocol [29]. Briefly, the cells were cultured in BGJb medium (Gibco, Paislay, UK Cat. No. 12591038). The mice were euthanized in CO₂ euthanasia chambers, and their tibias and femurs were dissected and cleaned of all surrounding soft tissues. Marrows were slowly flushed out of the bones and suspended in 15% FCS (fetal calf serum)-BGJb (Gibco, Germany 10270-106), 100 U/mL penicillin and 100 mg/mL streptomycin (Beyotime, Biotechnology, China C0222). Mononuclear cell fractions were isolated by gradient density centrifugation, plated in 25-cm² culture flasks, and incubated under a humidified atmosphere at 37°C 5% CO₂ for 3 weeks. Subsequently, the cells were expanded through several passages, and we used the same passage (passage 3) of the two cell types for further comparison experiments.

Flow Cytometry (FCM)

We used FCM to analyze the expression of cell surface markers [44]. MLpvNG2 and niBM-MSCs at passages 2-3 were digested, washed, suspended in PBS, and then incubated with phycoerythrin (PE)-conjugated anti-mouse CD105 (120414), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD90 (202506), CD31 (102407), CD45 (147709) (Biolegend, USA) and PGFR- β antibodies (Abcam, Becton, USA ab32570) for 1 h at 4°C. The isotype controls used as substitutes for the primary Abs consisted of murine FITC-conjugated IgG1 and PE-conjugated IgG2b (Biolegend, Cat. No. 405409). Data from all samples were collected with a BD FACSaria II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using Flowing version 2.5.

Lineage source of MLpvNG2⁺ cells

To determine the mesodermal source of the MLpvNG2⁺ cells, both cell types were seeded into 6-well culture plates, and the ability of the cells to express mesodermal markers (vimentin, N-cadherin, Col1a1, Prrx1 and Snail) and differentiate into the mesodermal lineage of interest (e.g., osteoblasts, adipocytes, and chondrocytes) was assessed. Osteogenic differentiation was induced in osteogenic culture medium [DMEM supplemented with 10% FBS, 10 mM β -glycerophosphate, 0.2 mM ascorbic acid, and 1 nM dexamethasone (Yagen Biosciences, Guangzhou, China Cat. No. MUBMD-90021)] for 3 weeks. Osteogenesis was examined using 1% Alizarin Red S (ARS) (Cyagen Biosciences, Guangzhou, China T181030G001). Adipocyte differentiation was induced with adipogenic induction medium [DMEM with 10% FBS, 0.5 mM indomethacin (Cyagen Biosciences, Guangzhou, China Cat. No. MUBMD-90031), 1 mM ascorbic acid (Cyagen Biosciences, Guangzhou, China MUBMD-90031), and 1 μ M dexamethasone] for 3 weeks. Lipid droplets in the cells were visualized with a 0.4% oil red O staining solution (Cyagen Biosciences, Guangzhou, China T181109G001) [6]. A micromass culture system was used to induce chondrogenic differentiation [45]. Briefly, the same number of cells of both cell types (approximately 2.5×10^5) were centrifuged at $1200 \times g$ for 5 min. The cell pellets were cultured in chondrogenic medium (Cyagen Biosciences, MUBMD-90041, Guangzhou, China) for 21 days at 37°C under an atmosphere of 5% CO₂, with two medium changes performed per week. Chondrogenic differentiation was assessed by

Alcian blue staining (Cyagen Biosciences, Guangzhou, China Cat. No. T181016G001) of the pellet sections.

Immunocytochemistry and microscopy

We used immunofluorescence staining to assess the expression of vimentin, ALB, CK19, C/EBP α (1:100, bs-1630R) and C/EBP β (1:100, bs-1630R) (Bioss, Beijing, China) as mesodermal and immature stem cell markers and hepatic cell markers. Coverslips were coated with poly-L lysine (PLL, 1 mg/mL) overnight, and after rinsing several times with sterile water, they were allowed to dry. A monolayer of cells was generated by plating 1×10^5 cells in 60 μ L of DMEM-F-12 on the center of the coverslip and incubating for 30 min at 37°C to allow the cells to adhere, after which 200 μ L of culture medium was added to initiate the culture. The cell cultures were fixed in 5% acid methanol (-20°C) for 12 min and then washed 2 $\times$ for 5 min in room-temperature DMEM with 5% normal goat serum. The cultures were incubated with the above primary Abs (diluted 1:100 in DMEM) for 25 min at 37°C in a humidified chamber and washed 6 $\times$ for 5 min each in DMEM supplemented with 5% normal goat serum (NGS). Then, the samples were incubated with secondary Abs conjugated to the fluorescent molecule Alexa Fluor 488 or Alexa Fluor 568 and diluted 1:200 in DMEM with 5% NGS for 30 min at RT. The cultures were then washed briefly, and the nuclei were counterstained (mounted) in Vectashield with DAPI (Sigma), followed by a rinse with PBS^[46] and subsequent analysis by fluorescence microscopy (Olympus BX51, Japan).

For tissue sections, the animals were perfused with 4% paraformaldehyde, and selected tissues were cryoprotected in 30% sucrose overnight, frozen in OCT mounting medium, and sectioned (5-20 μ m) on a Leica CM1950 cryostat (Leica, Frankfurt, Germany). Tissue sections were then stained with anti-Ki67 (1:100, BD Pharmingen, New Jersey, USA 550609), anti-F4/80 (1:100, bs-11182R, Bioss, Beijing, China), anti-alpha smooth muscle actin (α SMA, 1:50, sc-130617, Santa Cruz, California, USA), anti-ALB (1:100, sc-271605, Santa Cruz, California, United States), or anti-Krt19 (1:100, Santa Cruz, California, USA sc-374192), incubated with Alexa Fluor 405-, 488-, or 594-conjugated secondary Abs (1:500, JACKSON, Philadelphia, USA) and imaged on a fluorescence microscope (Nikon, Tokyo, Japan).

DEN-induced liver cirrhosis and experimental design

CFSE-labeled donor cells: Liver cirrhosis was induced with DEN in C57BL/6 mice according to published methods^[47]. Briefly, male mice drank water containing 0.014% DEN (Sigma Aldrich, 99.5% purity, N0756) daily for six consecutive weeks, and control animals were fed normal food and water. In this model, the mortality was approximately 10-15%, and *in vivo* studies were performed using this model with at least 15 animals per group. Liver fibrosis was assessed using Masson's trichrome (for collagen fibers), PicroSirius (for elastin fibers), hematoxylin-eosin (for mononuclear cells)^[6], and [alpha-smooth muscle actin](#) (α -SMA)^[48] staining. For histopathological and immunohistochemical analyses, livers from both cell-treated and control animals with the best fibrosis loads were selected. After six weeks of DEN administration, the mice were randomly assigned to the (1) DEN plus PBS, (2) DEN plus MLpvNG2⁺ cells, (3) DEN plus niBM-MSCs, and n, (4) naïve groups. Cells were labeled with CFSE (Invitrogen, USA

1948067)^[49] and injected through the tail vein ($1-5 \times 10^5$ cells/mouse/200 μ L). Control animals received the same volume of PBS. The control and cell-injected groups received DEN continuously for an additional four weeks. At least 3 animals were sacrificed for cell tracking on days 1, 2, and 3 using labeled therapeutic cells.

In the experiments using the neutralizing antibody, MLpvNG2⁺ cell- and niBM-MSC-treated DEN-induced liver cirrhosis mice were intravenously injected with anti-c/EBP α (100 ng/mL, Cat. No. bs-1630R) and anti-C/EBP β (100 ng/mL, Cat. No. bs-1396R) neutralizing antibodies (Bioss Shanghai, China) 24 h prior to cell injection, and non-Ab-treated mice were included as controls. Four weeks after cell treatment, the animals were perfused with 4% paraformaldehyde, and the livers were collected. Then, the livers were (a) snap frozen at -80°C in OCT mounting medium for sectioning (5-20 μ m) on a Leica cryostat after incubation in 30% sucrose overnight, (b) examined by immunohistochemistry and western blotting for hepatic cells (ALB, CK/KRT19, etc.), and (c) used for RNA isolation. Sera samples were obtained for measurements of the levels of proteins related to hepatic function (CYP1a1, LDL and urea) 4 weeks after cell transplantation, at which time the experiment was terminated.

Generation of conditioned mediums (CMs)

Homogenized fibrosis/cirrhotic liver tissues were prepared with a tissue homogenizer, frozen (-80°C)-dissolved (37°C) 3 times and then washed thrice with ice-cold PBS at 4°C (pH 7.4, one liver/2 mL). After centrifugation at $170 \times g$ for 10 min, the supernatant was collected and diluted with DMEM/F12 media at 1:4 - 1:8 (one portion of homogenate to four portions of DMEM/F12) before being passed through a 0.45- μ m filter to yield cirrhotic conditioned media (Cir-CM). The same procedure applied for Cir-CM was used for the preparation of the control CM from naïve liver tissue homogenate exact (nL-CM). The CMs were either used fresh or stored at -4°C for a maximum of 1 month. The cells (1×10^5) were cultured in maintenance medium (DMEM/F12, 10% FBS) for at least 1 week before transiting them to either nL-CM or Cir-CM.

Cytokinetics of MLpvNG2 and niBM-MSCs with CCK8 assay

Cultures were first transferred from their own culture medium into bCM containing DMEM/F12 medium plus 10% FBS and grown for at least 3 days. Then, the cells were transferred into experimental CMs containing nL-CM and Cir-CM generated from naïve and cirrhotic livers, respectively, for different times. Finally, the cells in nL-CM and Cir-CM were removed into a 96-well plate as (1) the blank control group, containing only nL-CM and Cir-CM, and (2) the text group. Both cells were grown in nL-CM and Cir-CM at a density of 1×10^4 cells/100 μ L. The cells were cultured in an incubator at 37°C with 5% CO₂. 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-sulfophenyl)-2H-tetrazole monosodium salt (WST-8)^[50] (Thermo Scientific, Varioskan Flash, USA) was added to the plate (10 μ L/well, once/24 h) for 3 days. A microplate reader detected the values of the optical density (OD) at 450 nm, and the cytokinetics of the cells were analyzed.

Cell counts

For cell counts, at least 3-day cultures in bCM were used, according to the CFU assay ^[51]. Approximately 1000 cells were plated in 60-mm dishes allowed to grow for two weeks. Then, fixed and stained with trypan blue for 10 min. The colonies were counted under an inverted phase contrast microscope (Olympus, CKX41, Japan).

Hepatic protein secretion

The levels of Cyp4501a1 and LDL in sera were quantified 4 weeks after cell transplantation to assess their secretion using Cyp4501a1 and LDL ELISA quantitative kits (Immunology Consultant Lab, OR, USA) ^[52], which provide a reliable assay for evaluating hepatic function, according to the manufacturer's instructions. Urea levels were measured using a urea quantitative colorimetric assay kit (Sigma Aldrich, Cat. No. MAK006-1, St. Louis, MO, USA) ^[50]. The measurements were performed in triplicate.

Western blot analysis

The total proteins were extracted from liver tissues at 4 weeks after cell transplantation for ALB, CK/KRT19, C/EBP α and C/EBP β (Santa Cruz Biotechnology, Dallas, TX, 1:1000, cat#sc-32931). 30-70 μ g of the lysates were separated by SDS-PAGE. The proteins were then transferred to an Immobilon-P PVDF (polyvinylidene difluoride) membrane (Millipore, ON) for determination of the specific protein expression levels using anti-rabbit ALB, CK/KRT19, C/EBP α and C/EBP β antibodies via chemiluminescence. The blots were incubated with an enhanced chemiluminescence solution (Pierce, Bonn, Germany), the intensity of the signals was visualized with ChemiDoc XRS+ System/Image Lab Software (Bio-Rad, California, USA), and protein expression levels were determined as the ratio to either GAPDH (1:5000, Proteintech, Wuhan, China 6000-1-1 g) or actin. A prestained nonexposure western blot marker (KD: 10-180, Fermentas 26616) was used as the molecular size marker.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted using an RNeasy Mini kit (Cat. No. 74194; QIAGEN, Hilden, Germany), and the RNA concentration and purity were determined via spectrophotometry at 260 and 280 nm. The RNA (1 μ g) was reverse-transcribed using a PrimeScriptTM RT reagent kit (RR047A, TaKaRa Biotech Inc., Shiga, Japan), and 25 ng of cDNA was amplified with an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA, USA) with appropriate specific primers. Specific primers targeting genes were designed using Primer Express (Applied Biosystems). The DNA-intercalating SYBR Master Mix reagent (Applied Biosystems) was used for RT-qPCR, which was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). The primer pairs used to detect the mRNA levels of target genes are listed in **Table 1**. All values were normalized to either the Actb or Gapdh housekeeping genes and are expressed as the relative expression or fold change using the formulas $2^{-\Delta CT}$ and $2^{-\Delta\Delta Ct}$, respectively.

Statistical data analysis

We conducted all experiments in triplicate and repeated each experiment at least twice. To quantify the cellular compositions of the cultures, four experiments were performed in duplicate within each experiment at each passage. Statistical analyses were carried out on datasets that consisted of at least three independent experiments using an unpaired Student's t-test when comparing two groups. The results were expressed as the means $\pm$ standard error of the mean (SEM) and compared with two-way analysis of variance (ANOVA) with the Bonferroni post hoc test. Statistical analyses were conducted with SSPS 9.0 for Windows (IBM Corp., Armonk, NY, USA). Two-tailed p values less than 0.05 were considered significant.

Experimental Results

Characteristics of MLpvNG2⁺ cells and niBM-MSCs under physiological conditions

The MLpvNG2⁺ cells were positive for the mesodermal markers CD105, CD90 and platelet-derived growth factor receptor beta (PDGFR- β) and negative for CD31 and CD45, as identified by flow cytometry (FCM) (**Fig. 1Aa**). Immunofluorescence identified the expressions of vimentin (approximately 93%, similar to that of niBM-MSCs, approximately 90%) (**Fig. 1Ab**), *N-cadherin*, *Col1a1*, *Prrxl* and *Snail* (higher than those of niBM-MSCs) detected by RT-qPCR (**Fig. 1Ac**), and the similar induction of trilineage differentiation (**Fig. 1Ad**) suggest MSC-like properties and mesodermal origins of MLpvNG2⁺ cells. According to optical microscopy (OM) from low to high magnification (100X - 400X) (**Fig. 1Ba-c**) and scanning electron microscopy (SEM) observations (**Fig. 1C**), MLpvNG2⁺ cells showed a rhomboid shape with two prominent nucleoli (red arrows), while niBM-MSCs showed a fibroblast-like appearance. Upon using a microscopic micrometer to measure the cell size, under the same magnification, a significantly ($p < 0.0001$) larger size (length: $90.37 \pm 19.49 \mu\text{M}$, range: 59-135 μM ; width: $28.53 \pm 13.01 \mu\text{M}$, range: 13-60 μM) was observed for MLpvNG2⁺ cells than for niBM-MSCs (length: $16.89 \pm 2.72 \mu\text{M}$, range: 13-20 μM ; width: $6.11 \pm 1.33 \mu\text{M}$, range: 3-9 μM) (**Fig. 1Bd**). After transitioning from cultures in basic conditioned medium (bCM, MLpvNG2 in DMEM/F12 medium plus 10% FCS; niBM-MSCs in BGjb medium plus FCS) (Mat/Mat) to nL-CM, which was generated from naïve liver homogenate. The better cell densities (**Fig. 1D**) and colony-forming units (CFU) (**Fig. 1E, F**) in MLpvNG2 cultures compared to niBM-MSC cultures might suggest that extrahepatic-sourced niBM-MSCs adapted to the new (liver) environment.

Differences in hepatic differentiation between MLpvNG2 and niBM-MSCs in response to injured liver signals

To compare the proliferative capacities in response to injured liver signals, we transferred MLpvNG2⁺ and niBM-MSCs from bCM, in which the growth curves of both types of cells were similar from day 1 to day 3 (**Fig. 2Aa**), into nL-CM (**Fig. 2Ab**) and Cir-CM (**Fig. 2Ba-c**). Assessment using the Cell Counting Kit-8 (CCK-8) assay indicated a better growth rate of MLpvNG2 compared to niBM-MSCs in nL-CM (**Fig. 2Ab**), further confirming that the extrahepatic-sourced niBM-MSCs adapt to the new (liver) environment. Interestingly, at 24 h although both MLpvNG2 (**Fig. 2Ba**) and niBM-MSCs (**Fig. 2Bb**) significantly proliferated in Cir-CM (generated from DEN-induced cirrhotic liver extract) compared to nL-CM, MLpvNG2 showed a better

survival than did niBM-MSCs (**Fig. 2Bc**). Immunofluorescence revealed that both cells could differentiate into ALB- and CK/KRT19-positive cells compared to their in nL-CM (**Fig. 2Ca, c, e, g**), but a significant higher number of ALB(+) and CK/KRT19(+) cells showed differentiated from MLpvNG2 (**Fig. 2Cb, d, arrows**) and niBM-MSC (**Fig. 2Cf, h, arrows**) cells respectively (**Fig. 2Da, b; Ea, b**). Similar trend was observed for the mRNA levels at 24 h (**Fig. 2F**). As the culture continued from 24 to 72 h (**Fig. S1**), both MLpvNG2 and niBM-MSCs maintained their own higher numbers of ALB(+) (**A, arrows**) and CK/KRT19(+) (**B, arrows**) at both the protein (**Fig. S1C**) and mRNA levels (**Fig. S1D**). The number of ALB(+) cells were 2.29-fold higher among MLpvNG2 than among niBM-MSCs (**Fig. 2Dc**) and CK/KRT19(+) cells were 2.52-fold higher among niBM-MSCs than among MLpvNG2⁺ cells (**Fig. 2Ec**) 72 h vs. 24 h. These findings suggest that in response to fibrotic/cirrhotic signals, the two types of cells prone to differentiation processes are different.

Differences in the gene expression of MLpvNG2 and niBM-MSCs in conditioned medium

To determine whether the differences in hepatic differentiation of the two cells were reflected by the mRNA levels, we cultured the cells in both nL-CM and Cir-CM and assessed a group of liver development-related genes associated with functional differentiation using RT-qPCR and normalization to the expression of beta-actin. After 24 h, significantly different gene levels (not shown), particularly higher levels of *oct3/4* were found in MLpvNG2 (**Fig. 3Aa**) and *klf4* in niBM-MSCs (**Fig. 3Ba**). For the MLpvNG2 cultures, a 2.65-fold higher *oct3/4* amount was found in nL-CM cultures, and a 2.64-fold higher in Cir-CM (**Fig. 3Ab**). For the niBM-MSC cultures, a 5.37-fold higher amount of *klf4* was found in nL-CM cultures, and a 1.61-fold higher in Cir-CM (**Fig. 3Bb**). The *oct3/4* and *klf4* genes are involved in regulating hepatocyte and cholangiocyte differentiation, respectively [^{11, 12}], during liver development; thus, these findings support the results above (**Fig. 2C-F** and **S1**). The mRNA levels of a group of other hepatocyte- (*hnf4a*, *gata4*, *hnf1β/Tcf2*) [¹⁶⁻¹⁸], cholangiocyte (*hnf1α/Tcf1*, *Hnf3α/Foxa1*, *Hnf6*) [¹⁹⁻²¹]-mediated genes were also analyzed. We found that in nL-CM, there were few changes in the expression of these genes in both cell types, while Cir-CM enhanced expression (**Fig. 3Ca, b; Da, b**). When comparing both cultures in Cir-CM, higher *hnf4a*, *gata4*, *hnf1β/Tcf2* gene expression was higher in MLpvNG2⁺ cells (**Fig. 3Cc**), and *hnf1α/Tcf1*, *Hnf3α/Foxa1*, and *Hnf6* expression was observed in niBM-MSCs (**Fig. 3Dc**). Overall, in MLpvNG2 cultures, there were 10.53 and 12.11-fold increases in the expression of *hnf4a* and *hnf1β/Tcf2* compared to niBM-MSCs, with particularly substantial increases observed in the expression of *gata4*, a red arrow, 47.76-fold, a transcription factor gene for controlling hepatocyte differentiation [²²](**Fig. 3E**). In niBM-MSC cultures, 2.59 and 2.25-fold increases were observed for *hnf1α/Tcf1* and *hnf6* compared to MLpvNG2⁺ cells, with a particularly substantial increase in *hnf3α/foxa1*, a red arrow, 5.23-fold, a gene that regulates cholangiocyte differentiation during development [²³](**Fig. 3F**). In addition, in Cir-CM cultures, a 47.45-fold increase in the alb of MLpvNG2⁺ cells over niBM-MSCs and a 2.31-fold increase in the ck/krt19 of niBM-MSCs over MLpvNG2⁺ cells were observed (not shown), suggesting that the differences in hepatic differentiation between the two cell types may be controlled by these genes.

Functional effect of transplanted cells in a well-set DEN-induced liver fibrotic/cirrhotic model

To compare the capacity of tissue repair, we labeled donor cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) and injected them into a well-set DEN-induced liver fibrotic/cirrhotic murine model^[15]. Observed both cells migrated into the injured livers around blood vessels, but significantly higher numbers of MLpvNG2⁺ cells than that of niBM-MSCs at all-time points (**Fig. 4A**). Further identified by immunofluorescence that a 2.85-fold increase (**Fig. S2D**) in the number of transplanted MLpvNG2⁺ cells with Ki-67 (+) nuclei at 72 h in the livers (**Fig. S2A**, arrows, **C**), exhibited improved hepatic protein Cyp4501a1 and low-density lipoprotein (LDL) (**Fig. 4Ba, b**, black lines) examined with Enzyme-linked immunosorbent and colorimetric assays (ELISAs), and urea (**Fig. 4Bc**, black lines), but dramatic ($p<0.05$) improvements were observed in the MLpvNG2⁺ cell group compared to the niBM-MSC group (**Fig. 4Ba-c**, red lines), compared with transplanted niBM-MSCs (**Fig. S2B**, arrows, **C**). These findings suggest that MLpvNG2⁺ cells have a better capacity to improve hepatic functions than niBM-MSCs. Furthermore, a series of experiments including **Masson's trichrome** staining to mark collagen fibers (blue, counted in circles, **Fig. 4Ca**), PicroSirius staining to mark elastin (red, counted in circles, **Fig. 4Cb**) fibers and hematoxylin-eosin (H&E) to mark infiltrating mononuclear cells (**Fig. 4Da**, counted in circles) exhibited similar trends. Both the MLpvNG2⁺ cell- and niBM-MSC-treated groups improved fibrotic loads and inflammation compared to the phosphate-buffered saline (PBS)-treated group (**Fig. 4Cc, d; Db**, black lines), which showed much heavy fibrotic loads than those of the normal liver (**Fig. 4Cc, d; Db**). However, the MLpvNG2-treated group exhibited significantly ($p<0.05$) better amelioration of liver damage than the niBM-MSC-treated group (**Fig. 4Cc, d; Db**, red lines). Smooth muscle actin (α -SMA) staining showed similar results (**Fig. 4Ea, b**, arrows indicate HSCs). In addition, using an anti-F4/80 antibody to mark macrophages revealed a significantly reduced number of F4/80(+) cells in the MLpvNG2⁺ cell-treated livers than in the niBM-MSC-treated livers (**Fig. 4Fa**, arrows, **b**), suggesting that MLpvNG2⁺ cells are better than niBM-MSCs in regulating initial immunity after transplantation.

Direct functional differentiation and endogenous functionality of transplanted donor cells in DEN-induced fibrotic/cirrhotic livers

To determine whether the hepatic differentiation behavior observed *in vitro* could occur in a real fibrotic/cirrhotic environment, the DEN-induced mice were intravenously injected two types of donor cells. Analysis revealed a greater number of ALB(+) cells directly differentiated from CFSE (green)-labeled transplanted MLpvNG2⁺ cells (**Fig. 5Aa**, arrows) than from the identically labeled niBM-MSCs (**Fig. 5Ab**, arrows; **B**) 4 weeks after transplantation. An 18.74-fold increase in the number of ALB(+) hepatocytes^[24] differentiated from MLpvNG2⁺ cells was observed compared with the number of ALB(+) cells differentiated from niBM-MSCs (**Fig. 5C**). In contrast, transplanted niBM-MSCs exhibited a greater ability to differentiate into CK/KRT19(+) cholangiocytes^[25] (**Fig. 5Db**) than did MLpvNG2⁺ cells (**Fig. 5Da**, arrows; **E**), with a 3.13-fold increase in the number of CK/KRT19(+) cells differentiated from niBM-MSCs compared with that differentiated from MLpvNG2⁺ cells (**Fig. 5F**). Furthermore, the fibrotic livers received MLpvNG2 showed a greater number of host ALB(+) cells (**Fig. S3Aa**) than did those treated with niBM-MSCs (**Fig. S3Ab; Ba**, counted in squares). A 5.77-fold increase of host ALB(+) cells in MLpvNG2⁺ cell-

treated livers versus those treated with niBM-MSCs (**Fig. S3Bb**). The mice that received niBM-MSCs revealed a significantly higher number of host CK/KRT19(+) cells (**Fig. S3Cb**) than those treated with MLpvNG2⁺ cells (**Fig. S3Ca, Da**, counted in squares). There was a 2.93-fold increase of host CK/KRT19(+) cells versus those treated with MLpvNG2 (**Fig. S3Db**). Similar results were obtained at the protein level also by western blotting (**Fig. S3Ea, b**) and mRNA level by RT-qPCR (**Fig. S3Fa, b**), a 5.77-fold increase for *alb* in the MLpvNG2⁺ cell-treated livers in mRNA levels was observed versus the niBM-MSC-treated livers (**Fig. S3Fc**), as well as a 3.93-fold increase for *ck/krt19* in the niBM-MSC-treated livers versus the MLpvNG2-treated livers (**Fig. S3Fd**), suggesting similar trends due to the indirect and bystander effects of MLpvNG2 and niBM-MSCs 4 weeks after transplantation.

Transcriptional CCAAT enhancer binding protein C/EBP β signaling mediates hepatocyte regeneration of transplanted MLpvNG2⁺ cells in DEN-induced liver fibrosis/cirrhosis

To understand the functional differentiation mechanism, using RT-qPCR, we found differences in the expression of *c/ebps*. Although enhanced *c/ebp α / β* mRNA levels in both MLpvNG2 and niBM-MSCs in Cir-CM over nL-CM (**Fig. 6Aa, b**), dramatically higher *c/ebp α* in niBM-MSCs than in MLpvNG2 (**Fig. 6Aa**). In contrast, *c/ebp β* was dramatically higher for MLpvNG2 than for niBM-MSCs (**Fig. 6Ab**) in Cir-CM. DEN-induced fibrotic/cirrhotic livers that received donor cells showed similar trends (**Fig. 6Ac, d**), suggesting that different *c/ebp* signaling were involved in different hepatic differentiation forward. As anti-c/EBP neutralizing antibody (+ α -Ab-Ng2, + α - β -Ab-Ng2) injections blocked c/EBP α / β levels both in donor cells (not shown) and in DEN-induced fibrotic/cirrhotic livers compared to non-Ab-injection treated donor cells (+Ng2 or +niMSCs) (**Fig. 6Ba, b**), might suggest the possibility that these nuclear proteins^[26] might also be expressed on the cell surface. To prove this, through immunofluorescence, we determined that under physiological conditions, c/EBP α / β is indeed expressed not only on donor cells (MLpvNG2, niBM-MSCs) (**Fig. 6Ca, b**, arrows) but also on hepatic cells from naïve liver tissues (**Fig. 6Cd, e**, arrows), which has not been previously reported. Expression of c/EBPs was observed in both donor niBM-MSCs and hepatic tissues, while high expression of c/EBP β showed in donor MLpvNG2⁺ cells than in donor niBM-MSCs, c/EBP α showed in donor niBM-MSCs than in donor MLpvNG2 (**Fig. 6Cc**) and in hepatic tissues (**Fig. 6Cf**).

Given the evidence of c/EBP α / β expression on the cell surface, we first determined how c/EBP β affects the bystander effect of donor MLpvNG2⁺ cells. We intravenously injected α -c/EBP β -Ab (100 ng/mL) into mice 24 h before MLpvNG2⁺ cell transplantation (+ α - β -Ab-Ng2) and compared these mice to non-Ab-treated mice (+Ng2). A significantly decreased level of *c/ebp β* was found, along with an increased *c/ebp α* level 4 weeks after cell transplantation (**Fig. 6Da**). Interestingly, such changed mRNA levels were combined with significantly ($p < 0.05$) decreased *alb* and increased *ck/krt19* mRNA levels (**Fig. 6Db**). Tracing whether such changes correspondingly affect protein levels, through immunofluorescence, we demonstrated that 24 h α -c/EBP β -Ab before donor MLpvNG2⁺ cell injection could reduce the numbers of ALB(+) cells in the injured livers (**Fig. 6Ea**, cycles) but increased numbers of CK/KRT19(+) cells (**Fig. 6Eb**, cycles) compared to the non-Ab-treated livers (**Fig. 6Ec, d**). A 12.6-fold decrease in the number of ALB(+)

cells and a 1.44-fold increase in the number of CK/KRT19(+) cells were observed compared to the non-Ab-treated livers (**Fig. 6Ee**). The decreased ALB and increased CK/KRT19 expression were also confirmed by western blotting (**Fig. 6F**).

C/EBP α signaling mediates cholangiocyte regeneration of transplanted niBM-MSCs in DEN-induced liver fibrosis/cirrhosis

Injection of anti-c/EBP α neutralizing Ab (100 ng/mL, α -Ab) 24 h before niBM-MSCs resulted in decreased c/EBP α expression at the mRNA level (**Fig. 7A**), but surprisingly, the Ab did not substantially increase c/EBP β (**Fig. 7A**, $p>0.05$), not as expected. Interestingly, the α -Ab-treated mice exhibited dramatically decreased mRNA levels of *ck/krt19* compared to non-Ab-treated mice (**Fig. 7B**) 4 weeks after cell transplantation while did not substantially affect the *alb* gene (**Fig. 7B**, $p>0.05$). These results were consistent with the results at protein levels that anti-C/EBP α antibody pre-treated cirrhotic mice that received niBM-MSCs (α -Ab-niMSCs) exhibited a decreased number of host CK/KRT19(+) cells (**Fig. 7Cb**, cycles) compared to non-Ab-treated mice (niMSCs) (**Fig. 7Ca, Da**, counted in cycles) as determined by immunofluorescence, approximately 3-fold decreased the number of CK/KRT19(+) cells in the Ab-treated group (**Fig. 7 Db**). However, the Ab was significantly unaffected the ALB expression (**Fig. 7 Eb**) compared to non-Ab treated groups (**Fig. 7 Ea, Da**, counted in cycles, $p>0.05$). Western blotting identified similar results that decreased CK/KRT19 (**Fig. 7Fa**) and no changes of ALB in Ab groups (**Fig. 7Fb**), suggesting poor hepatocyte regeneration *in vivo* of transplanted niBM-MSCs in a fibrotic/cirrhotic liver environment.

Discussion

End-stage liver disease (ESLD) is the result of an imbalance of extracellular matrix synthesis and degradation that is mediated by portal and parenchymal fibrogenesis. Cell-based therapy has been suggested as an effective alternative therapy for the ESLD [27]. For example, transplantation of, BM-MSCs, well-accepted adult stem cells in the field Although BM-MSCs have several advantages, such as their easy acquisition, *ex vivo* expansion, immunomodulatory properties, and ability to migrate to damaged tissues [28], their capacity to differentiate into functional hepatic lineages especially functional characteristics in injured liver environment remains controversial [29, 30]. We have previously isolated a novel MSC-like stem/progenitor cells from the adult liver portal triad (MLpvNG2⁺ cells) and demonstrated their HSP-like potential [6]. In the present study, we further compared these intrahepatic-sourced MLpvNG2⁺ cells with identical extrahepatic-sourced BM-MSCs (niBM-MSCs) in terms of biological features, particularly the capacities for functional characteristics and mechanisms of action, to evaluate the advantages of MLpvNG2⁺ cells as treatment donor cells over donor niBM-MSCs for ESLD.

We performed both *in vitro* and *in vivo* studies. The *in vitro* study involved the use of conditioned mediums (CMs) including nL-CM and Cir-CM. The *in vivo* study involved the use of a DEN-induced liver fibrosis/cirrhotic murine model. The successful isolation of donor cells was performed using a novel “Percoll-Plate-Wait” protocol [6] for MLpvNG2⁺ cells and a classical protocol for niBM-MSCs [31]. Experiments to determine biological characteristics, which measured the adhesion to plastic, morphology

and expression of specific cell surface markers, were also performed. An FCM measurement revealed positive for CD90, CD105, and PDGFR- β and negative for CD31 and CD45 indicating that the majority of MLpvNG2⁺ cells have similar expression patterns to niBM-MSCs^[32]. The expressions of mesodermal markers of vimentin, *N-cadherin*, *Col1a1*, *Prrxl*, *Snail*^[33] and the ability to differentiate into mesodermal lineages that are consistent with those of other studies that characterized MSCs derived from multiple sources^[34], indicating that MLpvNG2⁺ cells originate from a mesenchymal source^[35, 36]. As *oct3/4* is a main marker for embryonic stem cells^[37] and its higher expression in MLpvNG2⁺ cells than in niBM-MSCs might suggest that MLpvNG2⁺ cells may be more “stem” than niBM-MSCs, needs to be further investigated. Moreover, the evidence of differences in morphologies might suggest different populations of MLpvNG2⁺ cells and niBM-MSCs.

Other studies comparing MSCs derived from various sources including bone marrow in terms of hepatic differentiation and functionalities have still yielded conflicting outcomes in response to injured liver signals. Some studies have reported positive results^[38, 39], while others have reported negative results [add-1x]^[40]. In our study, we performed head-to-head comparisons for both cell types from the same host but different sources (liver, BM) and observed that both liver-derived MLpvNG2 and BM-derived niBM-MSCs are able to differentiate into hepatic lineages in response to DEN-induced fibrosis liver signals, but spuriously found that they had different commitment directions. In Cir-CM cultures, MLpvNG2⁺ cells differentiated mainly toward into ALB(+) hepatocytes, whereas niBM-MSCs revealed CK/KRT19(+) cholangiocyte dominant differentiation. Based on the *in vitro* Cir-CM experiments we further traced donor cells *in vivo* and found the similar trend as *in vitro*. The DEN-induced fibrotic mice that received MLpvNG2 had more ALB(+) hepatocyte generations than those that received niBM-MSCs and the mice that received niBM-MSCs had more CK/KRT19(+) cholangiocytes than those that received MLpvNG2⁺ cells, from both transplanted donor cells and host injured livers four weeks after cell transplantation. Regarding superior improvement functional hepatic proteins CYP1a1, LDL and urea better in fibrotic mice that received MLpvNG2 over those that received niBM-MSCs, such might suggest the differences of transplanted MLpvNG2 and niBM-MSCs in direct and indirect (bystander) effect, MLpvNG2 for hepatocyte regeneration and niBM-MSCs for cholangiocyte regeneration.

Of note, in this study, we found for the first time that CCAAT/enhancer-binding proteins c/EBPs (α/β) signaling, the pathways controls both proliferation and differentiation fate during development^[41], were involved in the mechanism of action for different hepatic lineage differentiations from two types of cells. As our results indicated that the anti-C/EBP α/β neutralizing antibodies could block the corresponding protein expression both in donor cells (not shown) and liver tissues, leading to our proved for the first time that C/EBP α/β was not only intracellular expression^[42], cell surface as well, expresses on stem cells (MLpvNG2, niBM-MSCs) and hepatic cells in our case. Moreover,, the alteration of the mRNA levels of *alb* and *ck/Krt19* along with different hepatic lineage differentiations at protein levels after anti-C/EBP α or β antibody pre-treatment before donor cell usage suggest that C/EBPs signaling may control the functionalities of transplanted cells. In addition, other interesting evidence was also found in this study for example, the decreased fibrotic loads, anti-inflammatory effects and hepatic growth factor (HGF)

secretion, an anti-apoptotic factor^[43] (not shown) may further support the intrahepatic HSPs (MLpvNG2⁺ cells) as donor cells is more beneficial than extrahepatic stem cells (niBM-MSCs) for treatment of ESLD..

Conclusions

In summary, under physiological conditions, the intrahepatic MLpvNG2⁺ cells sourced mesodermal and exhibited some biological characteristics similar to those of extrahepatic niBM-MSCs. Under pathological conditions, such as in response to fibrotic/cirrhotic liver signals, MLpvNG2⁺ cells showed favor differentiation into hepatocytes that seems likely regulated by C/EBP β transcriptional signaling, while niBM-MSCs favor differentiation into CK/KRT19(+) cholangiocytes, which is likely controlled by C/EBP α transcriptional signaling. However, we are aware of the limitations of this study. For instance, the *in vitro* study was based on cell cultures obtained from different sets, which may partly explain the observed diversity and/or similarities because the culture conditions may affect gene expression profiles. In addition, the molecular mechanism behind how C/EBPs signaling controls different hepatic lineage differentiations by different cell types needs additional studies using genetically modified animal models or engineered cells. Such investigations could provide a better understanding of the MLpvNG2⁺ cell-stimulated chronic liver regeneration process and proximal digit tip amputation.

Abbreviations

MSCs: mesenchymal stem cells

MLpvNG2⁺ cells: mouse liver portal triad-derived neuroglia antigen 2-expressing cells

BM: bone marrow

BM-MSCs: BM-derived MSCs

niBM-MSCs: naïve identical bone marrow-derived MSCs

DEN: diethylnitrosamine

ALB: albumin

HSPs: hepatic stem/progenitor cells

FCM: flow cytometry

OM: optical microscopy

SEM: scanning electron microscopy

CM: conditioned medium

bCM: basic conditioned medium

CFU: colony-forming unit

nL-CM: naïve liver homogenate-derived CM

Cir-CM: diethylnitrosamine (DEN)-induced cirrhotic liver homogenate-derived CM

HNFs: hepatic nuclear factors

CFSE: carboxyfluorescein diacetate succinimidyl ester

ELISA: Enzyme-Linked Immunosorbent Assay

LDL: low-density lipoprotein

H&E: Hematoxylin-eosin *staining*

PBS: phosphate buffer saline

α -SMA: alpha smooth muscle actin

C/EBPs: CCAAT/enhancer binding proteins

ESLD: end-stage liver disease

HGF: hepatic growth factor

PLL: poly-L-lysine

Declarations

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Authors' contributions

Zhang, SF. Jiang, and JJ. Lai: Performed most experiments, the induction of the liver in the cirrhotic model, and cell injection and analyzed the data.

Xia, Yujun Zhang, QY. Chen and Ling Shuai: Analyzed the data.

Bai: Conceived and designed the experiments, provided financial support, wrote the manuscript, performed data analysis and interpretation, and provided final approval of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors have declared that no competing interests exist.

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Tables

Table 1. Primer pairs used for RT-qPCR.

Genes	Sequences
<i>N-cadherin</i>	Forward: 5'-AGGCTTCTGGTGAAATTGCAT-3' Reverse: 5'-GTCCACCTTGAAATCTGCTGG-3'
<i>col1a1</i>	Forward: 5'-GCTCCTCTTAGGGGCCACT-3' Reverse: 5'-ATTGGGGACCCTTAGGCCAT-3'
<i>prrx1</i>	Forward: 5'-AAAGAACTTCTCCGTCAGTCACC-3' Reverse: 5'-CCCACACTTTCGTCTGCTTGT-3'
<i>snail</i>	Forward: 5'-CACACGCTGCCTTGTGTCT-3' Reverse: 5'-GGTCAGCAAAAGCACGGTT-3'
<i>oct3/4</i>	Forward: 5'-CACCATCTGTCGCTTCGAGG-3' Reverse: 5'-AGGGTCTCCGATTTGCATATCT-3'
<i>hnf1β/Tcf2</i>	Forward: 5'-AGCCAGTCGGTTTTACAGCA-3' Reverse: 5'-TCCTCCCGACACTGTGATCT-3'
<i>hnf3β/Foxa2</i>	Forward: 5'-CATGGGACCTCACCTGAGTC-3' Reverse: 5'-CATCGAGTTCATGTTGGCGTA-3'
<i>gata4</i>	Forward: 5'-TGGAAGACACCCCAATCTCG-3' Reverse: 5'-AGGTAGTGTCCCGTCCCATC-3'
<i>hnf4α</i>	Forward: 5'-ATGCGACTCTCTAAAACCCTTG-3' Reverse: 5'-ACCTTCAGATGGGGACGTGT-3'
<i>alb</i>	Forward: 5'-GATCAGAGACTGCCTTGTGT-3' Reverse: 5'-ACAAGGTTTGGACCCTCAGT-3'
<i>hnf1α/tcf1</i>	Forward: 5'-CTG TAC ACC TGG TAC GTC CG-3' Reverse: 5'-TAC GCC CCT TCT TAG TTG GC-3'
<i>hnf3α/foxa1</i>	Forward: 5'-AAGGGCATGAGAGCAACGAC-3' Reverse: 5'-AGTTCATGTTGCTGACAGGGA-3'
<i>hnf6</i>	Forward: 5'-AGC AAA CTC AAG TCG GGT CG-3' Reverse: 5'-CTT GCT CTT TCC GTT TGC AGG-3'
<i>ck19krt19</i>	Forward: 5'-CGGACCCTCCCGAGATTACA-3' Reverse: 5'-TGGAGTTGTCAATGGTGGCA-3'

<i>c/ebpa</i>	Forward: 5'-AATGGCAGTGTGCACGTCTA -3'
	Reverse: 5'-CCCCAGCCGTTAGTGAAGAG-3'
<i>c/ebpβ</i>	Forward: 5'-CAATCCGGATCAAACGTGGC-3'
	Reverse: 5'-TACACGTGTGTTGCGTCAGT -3'

N-cadherin: Mus musculus cadherin 2; **Col1a1:** Mus musculus collagen, type I, alpha 1 (Col1a1); **prx1:** Mus musculus paired related homeobox 1; **snail:** Mus musculus snail homolog 1; **oct3/4:** POU domain, class 5, transcription factor 1; **hnf1β/tcf2:** HNF1 homeobox B; **hnf3β/foxa2:** forkhead box A2, **gata4:** Gata binding protein 4; **hnf4α:** hepatic nuclear factor 4 alpha; **alb:** albumin; **hnf1α/tcf1:** *hnf1* homeobox A (*hnf1a*); **hnf3α/foxa1:** forkhead box A1; **hnf6:** hepatocyte nuclear factor 6; **ck19/krt19:** keratin 19; **c/ebpa:** CCAAT/enhancer binding protein-alpha, c/pβ: CCAAT/enhancer binding protein-beta

Figures

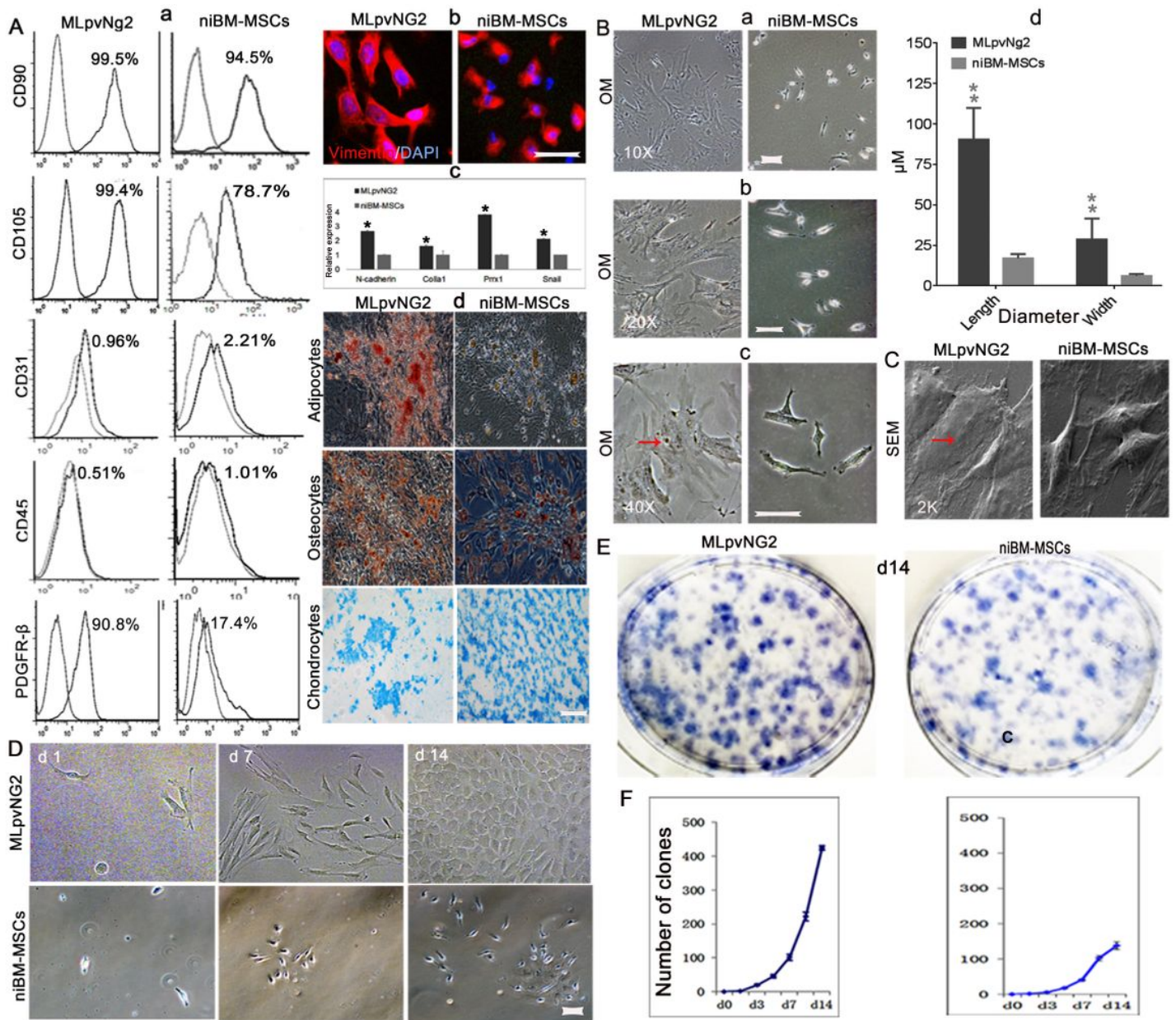


Figure 1

Characteristics of MLpvNG2⁺ cells and niBM-MSCs under physiological conditions. (Aa-d) FCM analysis of the cell surface markers CD90, CD105, CD31, CD45 and PDGFR- β , $n=7$ (a); Immunofluorescence for vimentin, $n=5$ (b); RT-qPCR for N-cadherin, Col1a1, Prrx1, and Snail, $n=5$ (c); and tri-differentiation monitored by the formation of lipid droplets, osteocalcin and chondrocytes, $n=5$ (d). Scale bar = 100 μm . (Ba-d) Cells were visualized using an optical microscope (OM) (a-c, from 100x-400x, scale bar = 50 - 200 μm), $n=5$; quantification of the size (μm) (MLpvNG2⁺ cells: $n=30$; niBM-MSCs: $n=27$) (d); and SEM, 2000 $\times$ (C) $n=5$. (D) Growing colonies of cells in nL-CM at days 1, 7 and 14, $n=5$ /each time point. (E) Colonies of cells visualized by CFU assay with trypan blue at 14 days and quantification from days 1 to 14 (F), $n=5$. The data shown are representative of multiple replicates. * $P \leq 0.05$, ** $P \leq 0.001$ vs. niBM-MSCs.

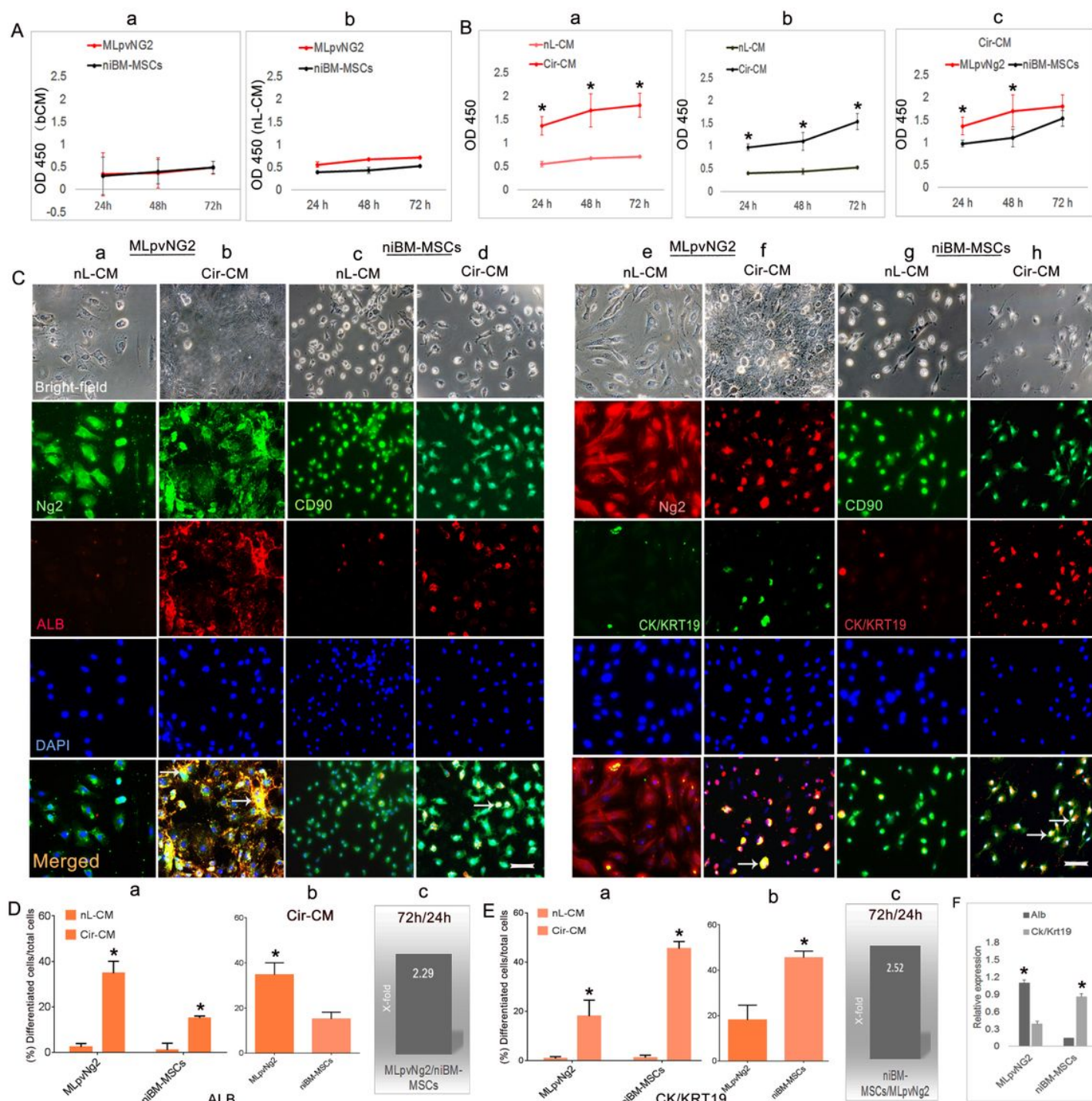


Figure 2

Differences in the hepatic differentiation of MLpvNG2 and niBM-MSCs in conditioned media. (Aa, b) Cultured in bCM (a) and nL-CM (b), then with cells subjected to a CCK8 assay, n=5. (Ba-c) Cultures of MLpvNG2 (a, n=5) and niBM-MSCs (b, n=4) in nL-CM and Cir-CM, and both cells in Cir-CM (c, n=6), then with cells subjected to a CCK8 assay, CCK8 assays above were analyzed by a microplate reader under an optical density (OD) of 450 nm. (Ca-h) Immunofluorescence identified for differentiated ALB(+) (a-d, arrows) and CK/KRT19(+) (e-h, arrows) from both MLpvNG2 and niBM-MSCs in nL-CM and Cir-CM

cultures, n=5, Scale bar = 100 μ m. (Da-c) Quantification of the differentiated ALB(+) cells in Cir-CM vs. nL-CM (a), analysis of differentiated ALB(+) cells of MLpvNG2 and niBM-MSCs in Cir-CM (b), and calculation of the fold changes at 72 h versus 24 h (c), n=5. (Ea-c) Quantification of differentiated CK/KRT19(+) cells (a), analysis of differentiated CK/KRT19(+) cells of MLpvNG2 and niBM-MSCs in Cir-CM (b) and calculation of the fold change at 72 h versus 24 h (c). (F) RT-qPCR analysis of the mRNA expression levels of alb and ck/krt19 in Cir-CM at 24 h. At least three independent experiments were performed, *P < 0.05 vs. niBM-MSCs.

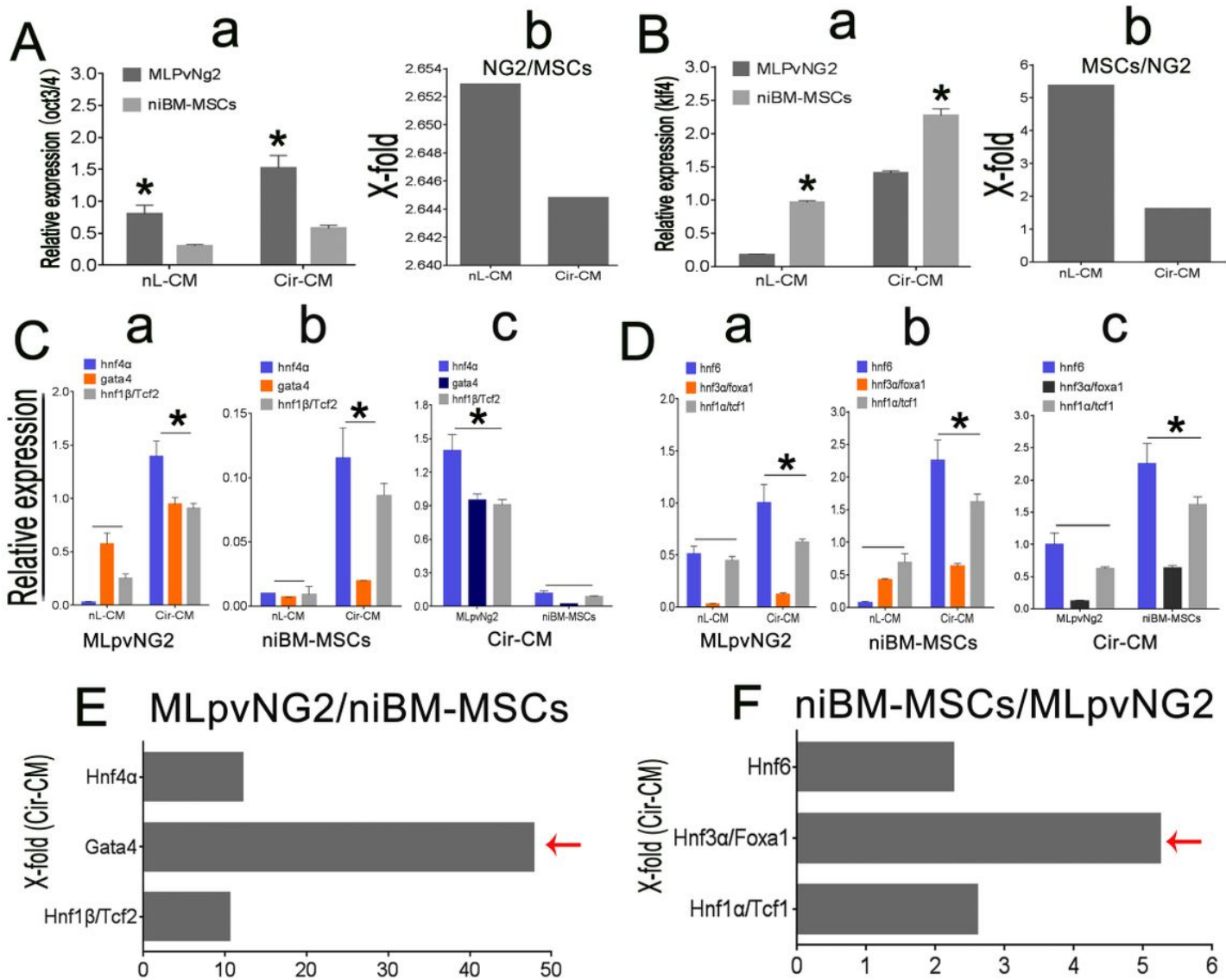


Figure 3

Differences in the gene expression of two types of cells in conditioned media (CMs). (Aa, b) oct3/4 mRNA levels (a) and x-fold changes (b) in nL-CM and Cir-CM cultures of MLpvNG2 and niBM-MSCs, n=9. (Ba, b) Klf4 mRNA levels (a) and x-fold changes (b) in nL-CM and Cir-CM cultures of MLpvNG2 and niBM-MSCs, (Ca-c) mRNA levels of hnf4 α , gata4 and hnf1 β /Tcf2 of MLpvNG2 (a) and niBM-MSCs (b) in nL-CM and Cir-CM (b, c), n=9. (Da-c) mRNA levels of hnf6, hnf3 α /Foxa1 and hnf1 α /Tcf1 of MLpvNG2 (a) and niBM-

MSCs (b) in nL-CM and Cir-CM (b, c), n=9. (E) Fold changes in the expression of hnf4a, gata4 and hnf1β/Tcf2 mRNA in MLpvNG2+ cells vs. niBM-MSCs. (F) Fold changes in the expression of hnf3a/Foxa1 and hnf1α/Tcf1 mRNAs in niBM-MSCs vs. MLpvNG2+ cells; arrows indicate strong expression of genes. Three independent experiments were performed. *P < 0.05 vs. either nL-CM or Cir-CM.

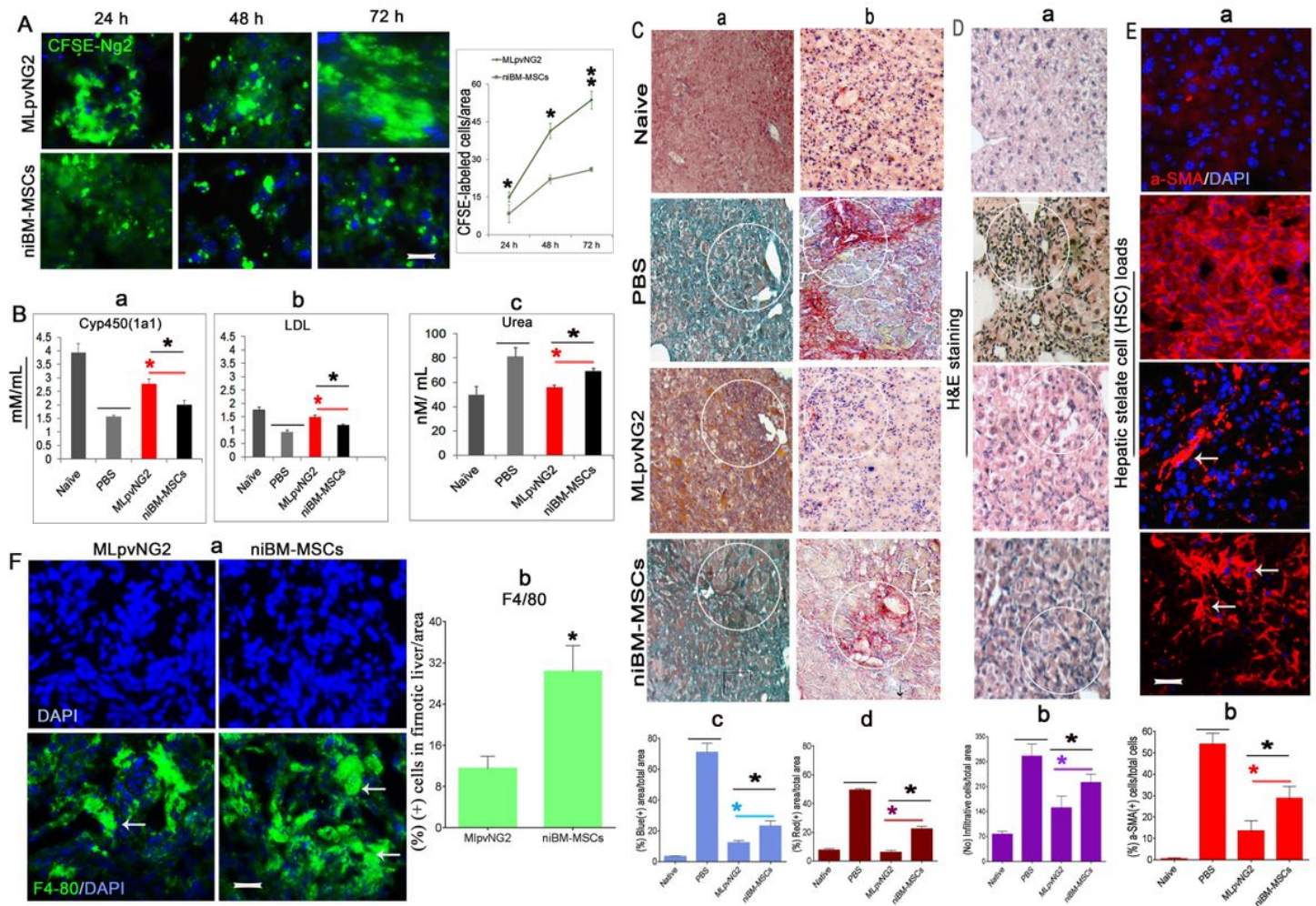


Figure 4

Functional effect of donor cells in a DEN-induced liver fibrotic/cirrhotic model. (A) CFSE-labeled cells homed to the DEN-induced fibrotic livers at 24, 48 and 72 h, n=5. scale bar = 100 μm. (Ba-c) Measurements of the hepatic proteins Cyp4501a1 (a) and LDL (b) in sera using ELISA, n=5, and urea levels using a colorimetric assay (c), n=5. (Ca-d) Masson's trichrome staining for collagen fibers (a, indicated by blue, counted in circles), picosirius staining for elastin fibers (b, indicated by red, counted in circles), and quantification of blue from a (c) and red from b (d). (Da, b) H&E staining for infiltrating mononuclear cells (a, indicated by circles) and quantification (b), n=6. (Ea, b) Immunofluorescence for hepatic satellite cells (HSCs) marked with α-SMA antibody (arrows, a), scale bar = 100 μm, n=5. Quantification of α-SMA (+) cells from a (b). Black lines indicate donor cells vs. PBS, and red lines indicate MLpvNG2+ cells vs. niBM-MSCs. (Fa, b) Immunofluorescence for macrophages marked with

F4/80 (arrows, a) and quantification (b), n=5, scale bar = 100 μ m. All results are representative of at least three to six independent experiments, and *P < 0.05 and **P< 0.001 vs. DEN + niBM-MSCs for 4 weeks.

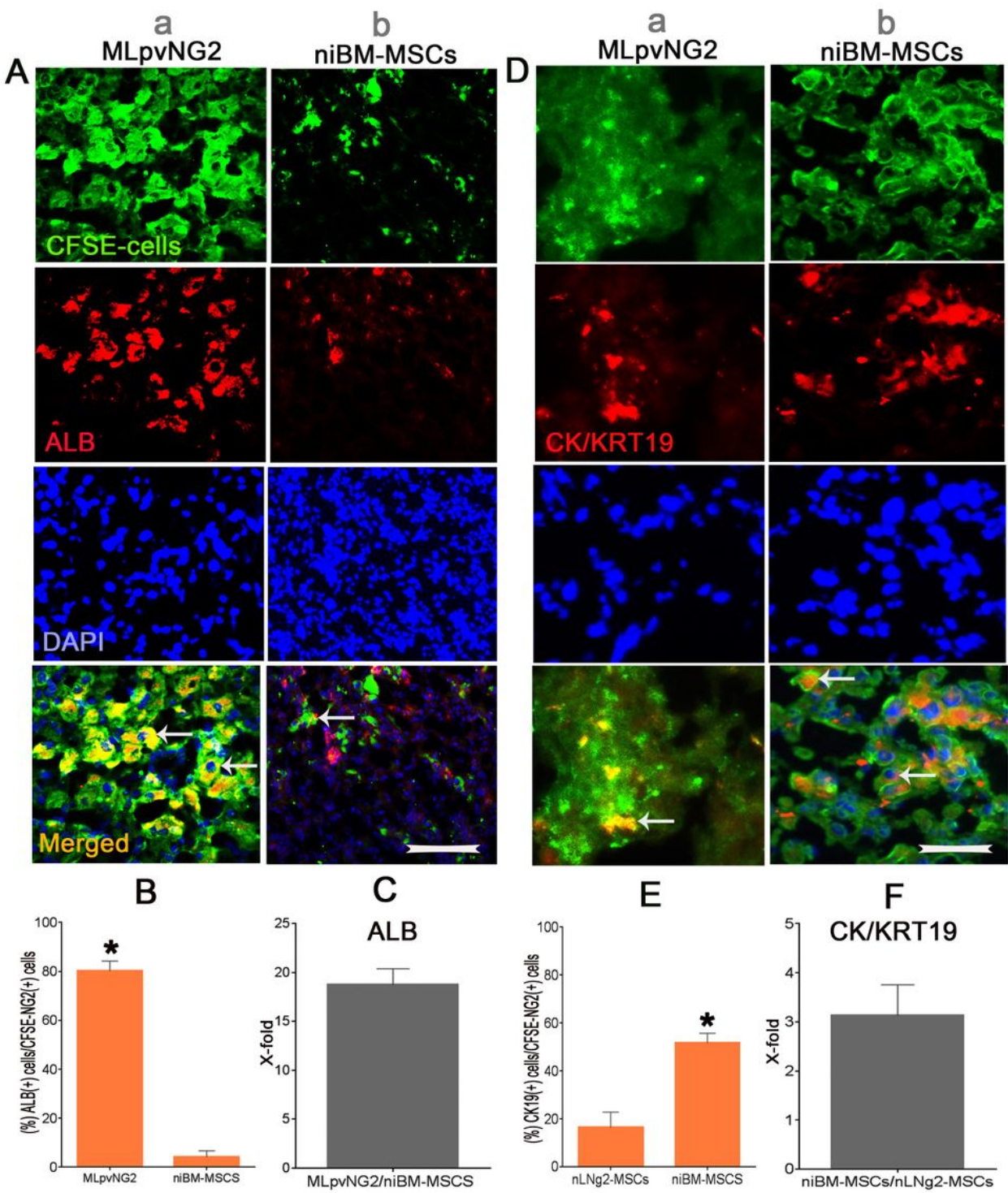


Figure 5

Direct functional differentiation of transplanted donors in DEN-induced fibrotic/cirrhotic livers. (Aa, b) ALB(+) cells differentiated from CFSE (green)-labeled donor MLpvNG2+ cells (a) and niBM-MSCs (b) (arrows), scale bar = 200 μ m. (B) Quantification of the number of differentiated ALB(+) cells, n=6. (C) Fold

increase in the number of differentiated ALB(+) cells vs. niBM-MSCs. (Da, b) CK/KRT19(+) cells differentiated from CFSE (green)-labeled donor MLpvNG2+ cells (a) and niBM-MSCs (b) (arrows), scale bar = 200 μ m. (E) Quantification of the number of differentiated CK/KRT19(+) cells, n=6. (F) Fold increase in the number of differentiated CK/KRT19(+) cells vs. MLpvNG2+ cells. At least three independent experiments were performed, *P < 0.05 vs. DEN + niBM-MSCs in cirrhotic livers for 4 weeks.

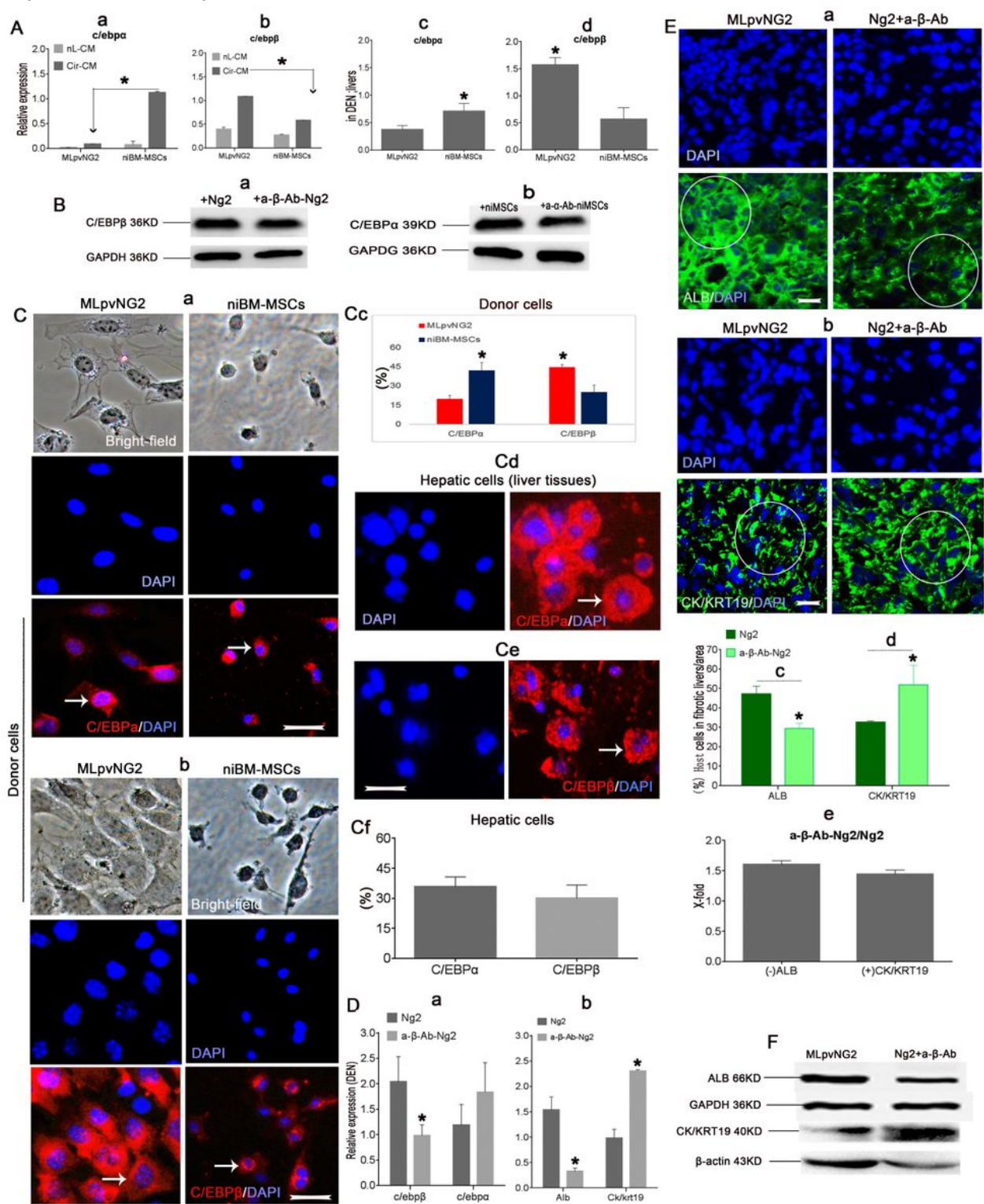


Figure 6

C/EBP β signaling mediates endogenous hepatocyte regeneration of transplanted donor MLpvNG2⁺ cells in DEN-induced fibrotic/cirrhotic livers. (Aa-d) mRNA levels of *c/ebp α* and *c/ebp β* of MLpvNG2 and niBM-MSCs in CMs (a, b, n=11) and in DEN-induced fibrotic/cirrhotic livers that received donor cells (c, d, n=6) assessed by RT-qPCR. (Ba, b) Protein levels of C/EBP α (b) and β (a) in DEN-induced cirrhotic livers treated with either the anti-C/EBP α or β neutralizing antibodies 4 weeks after cell transplantation (a- β -Ab-Ng2) compared to non-Ab-treated cirrhotic livers (+Ng2) assessed by western blotting. Antibodies were injected 24 h before donor cell injection. (Ca-f) Expression of C/EBP α and C/EBP β in donor cells (a, b) and naïve liver tissue hepatic cells (d, e), with quantifications from a, b (c, n=6) and d, e (f, n=3). (Da, b) RT-qPCR identified the expression of *c/ebp α / β* (a, n=6), *alb* and *ck/krt19* (b, n=6) in the mice that received either cells or antibody pretreatment plus cells. (Ea-e) Immunofluorescence for endogenous ALB(+) hepatocytes (a), CK/KRT19 (+) cholangiocytes (b) in an anti-C/EBP β antibody-pretreated group (Ng2+a- β -Ab) vs. non-treated ones (MLpvNG2, cycles), scale bar = 100 μ m; quantification (count in cycles from a, b (c, d, n=6) and fold changes from c, d (e). (F) Representative western blot analysis (n = 3) for ALB and CK/KRT19 expression (n=5) in the livers of DEN-induced cirrhotic mice treated with an anti-C/EBP β antibody (Ng2+a- β -Ab) 24 h before MLpvNG2⁺ cell transplantation vs. non-Ab-treated livers (MLpvNG2) using either GAPDH or β -actin as the loading control. All results are representative of at least 3 to 6 independent experiments. *P $\leq$ 0.05 vs. DEN + MLpvNG2 + anti-CEBP β -Ab in cirrhotic livers for 4 weeks.

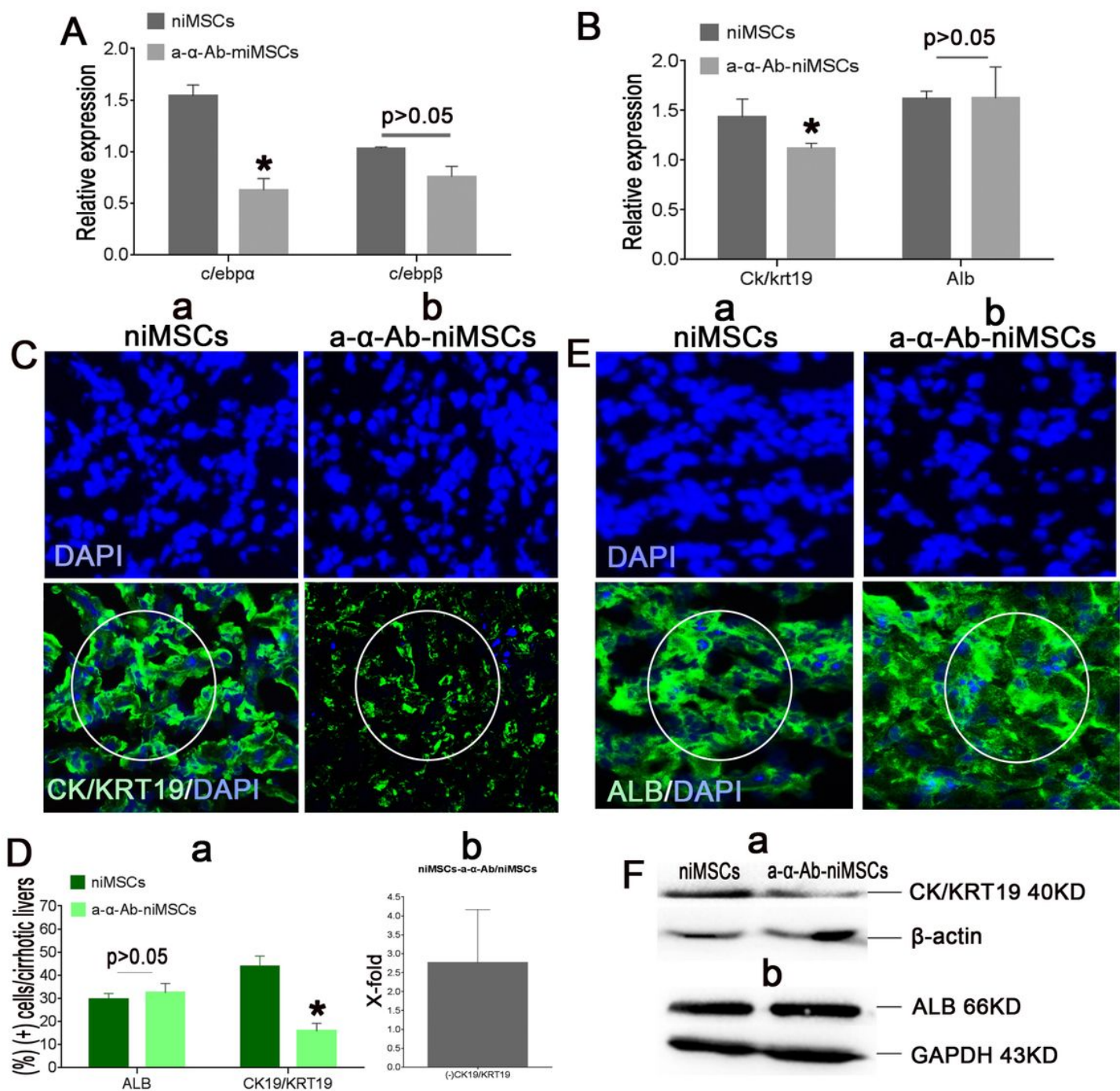


Figure 7

C/EBPα signaling mediates endogenous cholangiocyte regeneration of transplanted niBM-MSCs in DEN-induced fibrotic/cirrhotic livers. The expression of *c/ebpa/β* (A), *ck/krt19* and *alb* (B) in cirrhotic mice (liver) that received anti-C/EBPα neutralizing antibody (a-α-Ab-niMSCs) 24 h before niBM-MSC injection vs. non-Ab-treated mice (niMSCs) determined by RT-qPCR, n=6. (Ca, b) Immunofluorescence for CK/KRT19 in Ab- (b) and non-Ab (a)-treated groups, with quantifications from Ca, b (Da, counted in cycles, n=6) and X-fold changes from Da (Db). (Ea, b) Immunofluorescence for ALB in Ab- (b) and non-Ab (a)-

treated groups and with quantifications from Ea, b (Da, counted in cycles, n=6) scale bar = 100 μ m. (Fa, b) Representative western blot analysis of CK/KRT19 (a) and ALB (b) expressions in DEN-induced fibrotic/cirrhotic livers from non-Ab (niMSCs)- and Ab (α -Ab-niMSCs)-treated mice, n = 6. CK19 and ALB levels were normalized to those of either β -actin (a) or GAPDH (b). The data are presented as the means $\pm$ SEM of three independent experiments, *P<0.05 vs. DEN+ anti-C/EBP α neutralizing antibody +niBM-MSCs-treated mice for 4 weeks after cell transplantation.

Supplementary Files

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