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Basic Study

Long-term and non-invasive *in vivo* tracking of DiD-labeled human hepatic progenitors in chronic liver disease models

Long-term *in vivo* tracking of DiD labeled cells

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Abstract

BACKGROUND

Chronic liver diseases (CLD) are major public health burden due to continuous increasing rate of global morbidity and mortality. The inherent limitations of organ transplantation led to the development of stem cell-based therapy as a very supportive. However, identifying the fate of transplanted cells *in vivo* represents a crucial obstacle.

AIM

To evaluate the potential applicability of 1,1''-dioctadecyl-3,3,3'',3''-tetramethyl-indo-dicarbo-cyanine perchlorate (DiD) as cell labeling agent for long-term, and non-invasive *in vivo* tracking of transplanted cells in liver.

METHODS

Magnetically sorted EpCAM positive (1 X 10^6 cells/mL) fetal hepatic progenitor cells (fHPCs) were labeled with DiD dye and transplanted in severe combined immunodeficiency (SCID) CLD-mice liver. Near infrared (NIR) imaging was performed for *in vivo* tracking of DiD-labeled transplanted cells along with colocalization of hepatic markers up to 80 days. Existence of human cells within the mice liver was identified using Alu PCR and sequencing.

RESULTS

NIR fluorescence imaging of CLD-SCID mice showed a positive fluorescence signal of DiD at day 7, 15, 30, 45, 60, and 80 post-transplantation. Further, positive staining of CK, c-Met, and ALB colocalization with the DiD fluorescence clearly demonstrated that the fluorescent signal of hepatic markers emerged from the DiD-labeled transplanted cells. Recovery of liver function was also observed with serum levels of SGOT, SGPT and bilirubin. The detection of human-specific Alu sequence from the transplanted mouse livers provided evidence for the survival of transplanted cells at day 80.

CONCLUSION

DiD-labeling is promising for long-term and non-invasive *in vivo* cell tracking, and understanding the regenerative mechanisms incurred by the transplanted cells.

Key Words: Chronic liver diseases; Cell transplantation; Cell tracking and imaging; DiD; Hepatic progenitors

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Core Tip: Non-invasive tracking of transplanted cells is crucial to understand the homing, distribution, and differentiation into the desired cell types contributing to the organ regeneration. Lipophilic fluorescent dye DiD-labeled fetal hepatic progenitor cells (fHPCs) were transplanted into chronic liver disease (CLD) mice livers. DiD labeling of cells enabled long-term and non-invasive tracking of transplanted cells *in vivo* up to 80 days. Immunostaining and colocalization using liver-specific markers with DiD confirmed the persistence of transplanted cells in mice liver post-transplantation. Transplanted fHPCs supported liver function recovery, while identifying Alu gene sequence revealed survival and engraftment of human cells within the mice liver.

INTRODUCTION

Chronic liver diseases (CLD) represent one of the leading causes of morbidity and mortality worldwide, specifically in developing countries. It results from the progressive deterioration of liver functions which is caused by the continuous process of inflammation, destruction, and inadequate repair of the liver parenchyma leading to cirrhosis. Liver cirrhosis is characterized by the irreversible distortion of the liver architecture in form of fibrosis, scar formation, occurrence of several regenerative nodules, vascular reorganization, and immense liver failure. The major aim of the

current treatment approaches is to halt the progression of CLD into more severe forms and reduce the related complications which necessitate a multidisciplinary approach. The clinical signs and symptoms of CLD can be nonspecific; hence the major management perceptions are the elimination of underlying causes, management of the portal hypertension, and individualized therapy for each associated conditions. Although such strategies provide temporary support to the failing liver; they can't prevent the disease progression in long-term. Hence, more effective management approaches are required to overcome such hurdles and bridge gap for longer duration to improve the health related quality of life.

Cell therapy is an emerging technology to address the current demand for alternative options to liver transplantation for improving liver functions and as a supportive bridging therapy for CLD [1, 2]. However, it is necessary to establish appropriate cell types and sources, and a number of unsolved questions before utilizing such technologies in the clinical settings. Since last two decades, transplantation of different types of cells from various tissue sources such as autologous bone marrow-derived mononuclear cells and mesenchymal stem cells (MSCs), and allogenic fetal hepatic progenitor cells (fHPCs) have shown promising outcomes in clinical studies of CLD [3-7]. Besides, several preclinical studies using immune-compromised/deficient mice have also demonstrated potential applicability of such approaches to improve the current understanding of their safety, efficacy, and functionality of human stem/progenitor cells *in vivo* post-transplantation [8-11]. While several of these studies have proved the safety and involvement of the transplanted cells in liver recovery, the availability of methods for easy and long-term tracking of infused cells would be extremely beneficial in determining their viability, bio-distribution, homing and differentiation which represents a major roadblock for cell-based therapies in clinical settings.

The majority of the existing strategies employ radioisotopes, magnetic particles, fluorescent tags, or reporter genes to label the cells prior to transplantation in preclinical settings [12]. Further, non-invasive radionuclide imaging methods such as single-photon emission tomography (SPECT) and positron emission tomography (PET) using

radionuclides (Technetium (^{99m}Tc) and ^{111}In-oxine) are employed in the clinical settings [4, 6, 13]. However, the short life of radionuclide limits the application of such imaging methods to monitor the immediate cellular behavior for only a few hours. Magnetic resonance imaging (MRI) is another non-invasive imaging method which has been explored for cell tracing in preclinical CLD models for 1-2 wk [14, 15]. While MRI offers good spatial resolution and contrast, it is less sensitive and isn't effective for follow-up studies due to gradual loss of signal intensity [12]. Hence, several other tracking methods based on reporter gene expression, such as fluorescence imaging and bioluminescence imaging (BLI) are successfully employed for monitoring the fate of transplanted cells in animal models of liver injury [16, 17]. Although this method enables long-term cell tracking, the safety concerns owing to the genetic manipulation represent a major hurdle for clinical translation. Hence, direct labelling of the cells without involving genetic manipulation represents crucial need for sensitive, relatively safer, and less cumbersome process for tracking transplanted cells in both preclinical and clinical settings.

In the present study, long-term and non-invasive tracking of the transplanted fHPCs was evaluated in an experimental severe combined Immunodeficiency (SCID) mice model of CLD using DiD (1,1''-dioctadecyl-3,3,3'',3''-tetramethyl-indo-dicarbo-cyanine perchlorate). DiD is a carbocyanine dye having good photochemical properties of strong fluorescence, and stability [18-20]. It is a cationic dye and belongs to the family of lipid intercalating long alkyl side-chain carbocyanine derivatives that have a long-range (540-780nm) emission. Due to the long-range emission of DiD (670nm), tissue autofluorescence is minimum, permitting the use of other fluorochromes such as fluorescein isothiocyanate (FITC), for co-localization studies to evaluate the expression of other essential markers specific to the transplanted cells in the recipient tissue. Moreover, the process for labeling the cells using DiD is easy due to its excellent efficiency for its integration and diffusion into the cell membranes [18, 19]. Although DiD is insoluble in water, but its fluorescence is readily detected when incorporated into the cell membranes. Therefore, it has been classified as one of the most appropriate

carbocyanine families of dyes in labeling and tracking. After incorporating into cell membranes, it diffuses laterally within the cellular plasma membranes, resulting in staining of the entire cell. Structural similarity with the cell membrane phospholipids, and prolonged dye retention withn the cells are among the advantages of Did for live organisms. Hence, DiD has been used for labeling of differet types of cells without interference with the cellular differentiation; however, the effects of DiD labeling on human liver cells and its effect on the *in* vivo retention of labaled human liver cells remained to be investigated.

More specifically, we have utilized magnetically sorted fHPCs using epithelial cell adhesion molecule (EpCAM) as a surface marker due to its associated crucial functions relating to cell to cell adhesion, proliferation, maintenance of pluripotent state, regulation of differentiation and migration [23]. It has also been demonstrated that EpCAM positive HPCs are highly proliferative and have diminished class II MHC representation, and are classified as immature cells suitable for regenerative applications [24]. In our earlier study, EpCAM positive fHPCs have revealed a significant improvement in liver functions and increased disease-free life span in patients with end-stage CLD [6]. Thus, using fHPCs could highlight how well DiD serves for long-term, non-invasive, and real-time monitoring of cell survival, and structural and functional improvements in preclinical models of CLD post-transplantation. Accordingly, the present study aimed to shed light on possibility for determining the fate of DiD-labled human liver cells in CLD-SCID mice using live imaging up to 80 days post-transplantation.

MATERIALS AND METHODS

Animals

Experimental animals were obtained from inbred colonies of SCID mice (strain: NOD.CB17-*Prkdc*^{scid}/J) and maintained at the animal facility of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. The study was approved by the Institutional Animal Ethics Committee (Animal trial registration number

20/1999/CPCSEA dated 10/03/1999) of CCMB. All the animal experimental procedures were performed in accordance with the approved institutional ethical guidelines of CCMB for the care and use of animals. All the animals were maintained in standard ventilated cages with a 12 h light-dark cycle, and were fed ad libitum.

Development of CLD mice model

CLD mice models were generated using 25% Carbon tetra chloride (CCl_4, Rankem, India) diluted with mineral oil (Sigma, USA). Sub-lethal dose of diluted CCl_4 was administered according to 125 µL/Kg body weight in each animal. A total of 26 mice (either sex) of eight week were randomly assigned to vehicle control group (n = 4) and CCl_4 group (n = 22). CCl_4 group received intraperitoneal injections of diluted CCl_4; twice a week for four weeks and the vehicle control mice received only mineral oil. After four weeks, liver damage was confirmed by changes in the liver enzymes and liver tissue histology. For the biochemical evaluation of liver damage, 100-150 µL of blood was collected by orbital sinus puncture. Serum was used to measure total bilirubin, glutamate-pyruvate transaminase (SGPT), and glutamic oxaloacetic transaminase (SGOT) levels by the Jendrassik-Grof and Reitman-Frankel's methods respectively using kits (Coral Clinical Systems, India). The mice after four weeks of CCl_4 (referred to as SCID-CLD mice) were ready for cell transplantation (Tx).

Isolation of human fetal hepatic progenitors

Total fetal liver cells (tFLCs) isolation protocol was approved by Institutional Ethics committee (IEC) of Deccan college of Medical Sciences (DCMS), Hyderabad. Informed consent was obtained prior to the sample collection, and cell processing was performed according to the ethical guidelines for the use of human cells. Whole liver was dissected from spontaneously aborted fetuses (n = 3, 10-12 wk gestation), and perfused twice with ice cold phosphate buffer saline (PBS) for 5 min to eliminate circulating peripheral blood cells, followed by digestion with 0.025% collagenase in 1X PBS for 5 min at room temperature. Then the liver tissue was minced with scalpel blade, and disintegrated into a single cell suspension by passing through 40 µm cell strainers (BD Biosciences,

USA). Cell viability and counting was performed using the trypan blue dye exclusion test.

Flow cytometry

For flow cytometry analysis, single cell suspension of 2 X 10^6 tFLCs was fixed in 4% paraformaldehyde for 15 min at room temperature. Following to fixation, cells were washed twice with 1X PBS, and stained with anti-human CD326 or EpCAM antibody conjugated with FITC (Miltenyi Biotech, Germany) for 30 min. Cells were washed once with 1X PBS before analysing on FACS Calibur™ (BD Biosciences, USA) using 488 nm argon laser emission at 530/30 BP filter. The data was analysed and plotted using the Kaluza software 1.5a (Beckman coulter Inc, USA).

Enrichment of EpCAM positive fHPCs from tFLCs

To isolate EpCAM positive cells, 5 X 10^7 tFLC suspension in 500 µL buffer containing FCR blocking reagent was incubated with anti-human EpCAM antibody (Miltenyi Biotech, Germany) conjugated with magnetic beads at 4°C for 30 min and sorted using the magnetic cell sorter, AutoMACS according to the manufacturer's instructions (Miltenyi Biotech, Germany). Magnetically activated cell sorting (MACS) enriched EpCAM positive cells were collected and resuspended in RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA). Cell isolation and MACS sorting procedures were carried out under sterile condition in a Class100 bio safety cabinet.

Immunocytochemistry

MACS sorted EpCAM positive cells were fixed in 4% paraformaldehyde, and cytospin preparations were done on "Probe-on-Plus" slides (Fisher scientific, USA). Cells were blocked with 10% goat serum, and stained with mouse monoclonal anti-human EpCAM antibodies directly conjugated with FITC (Miltenyi Biotech, Germany), and co-stained with either anti-Cytokeratin (CK) 8+18+19 or anti-c-Met (Abcam Inc., MA, USA) primary antibodies, and Alexa 594 (Molecular Probes, USA) was used as secondary antibody. Images were captured using confocal laser scanning microscope (Leica, Germany, SP2 AOBS).

DiD labeling and intra-hepatic cell transplantation (TX) in SCID-CLD mice

MACS sorted EpCAM positive cells (1 X 10^6 cells/mL) in Hank's buffered salt solution (HBSS) were labeled with DiD dye by adding 5 µL of DiD cell labeling solution (Life Technologies, Eugene, USA) to the cell suspension and incubating for 20 min at 37°C. DiD-labeled cells were washed thrice with HBSS, and resuspended in the same buffer. 1 X 10^5 cells (100 µL) were injected directly into the liver lobes of the CLD mice (n = 14) at a single site using a 26 gauge needle. CLD mice receiving plain HBSS buffer served as non-transplanted (non-Tx) controls (n = 5). Post-transplantation, mice were maintained in different cages, imaged and sacrificed at different time points.

Long-term *in vivo* and *ex vivo* imaging

DiD-specific fluorescence from the transplanted animals was detected on the Multispectral FXPRO Fluorescence Imager (Carestream-KODAK, USA) using 630 nm excitation and 670/30 BP emission filters. High sensitive fluorescence images were combined with the high resolution X-ray images to precisely locate the DiD-labeled cells. Animals were imaged prior to TX (0 day) and after 7, 15, 30, 45, 60, and up to 80 days, post-TX. For each imaging experiment, the animals were anaesthetized with xylazine (5 mg/kg body weight) and ketamine (25 mg/kg body weight), the abdominal hair was shaved, placed within the chamber, and imaged. At 15, 30, and 80 days post-transplantation, after *in vivo* imaging, mice were sacrificed, and liver tissues were excised, and imaged (*ex vivo* imaging) to locate the fluorescing lobe for further processing. The fluorescing part of the liver lobe at day 15 and day 80 was immersed in OCT mounting solution, and stored at -80°C. Liver lobes of the non-transplanted mice was picked randomly, and processed similarly. The liver lobes at day 30 post-Tx, were processed for paraffin embedding and histology.

Histology Analysis

The excised liver lobes were fixed in 10% buffered formalin, and processed for paraffin embedding. Paraffin embedded liver tissues were sectioned at 4.0 µm thickness using a rotatory microtome (Leica RM2135, Germany), and stained with Hematoxylin and Eosin (H&E) and Sirius Red (SR, Sigma-Aldrich, USA) using standard protocols. Bright

field images were acquired from both control and CLD mice (n = 3) using 10X and 40X objectives of Olympus inverted microscope (IX3-SSU, Tokyo, Japan). A total of 20-25 random fields captured with the 10X objective were used for calculating the total collagen area using the Image J (1.5 2q) software, and expressed as total collagen percent area (% CPA).

Immunostaining

Serial cryosections (7.0 µm thickness) of transplanted and non-transplanted mice liver lobes of 15 and 80 days were stained for the expression of hepatic markers using anti-CK, anti-c-Met, and anti-human albumin (MP Biomedicals) primary antibodies, and detected with Alexa 488 (Molecular Probes, USA) secondary antibody. Images were captured using either confocal laser scanning microscope (Leica, Germany, SP2 AOBS), or the Axioimager Z2 Fluorescence microscope (Zeiss, Germany).

Alu sequence analysis

DNA was isolated from the fHPCs prior to transplantation and from the liver tissues of transplanted and non-transplanted mice at day 80. Isolated DNA was analyzed using human specific primers for Alu sequence (Forward primer5′-GGCGCGGTGGCTCACG-3′, Reverse primer-5′-TTTTTTGAGACGGAGTCTCGCTC3′). PCR was performed in 25 µL reaction mixture containing 1 µL DNA, 2.5 µL 10X complete PCR Buffer with $MgCl_2$, 1 µL dNTPs, 0.5 µL forward and 0.5 µL reverse primers, and 0.2 µL Taq DNA Polymerase (5 U/mL) with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of a three step program of 94°C for 30 sec, 54°C for 30 sec and 72°C for 45 sec, followed by a final extension step at 72°C for 5 min. The PCR products were electrophoresed on a 2% agarose gel, and observed under UV with ethidium bromide staining. The images were captured using Gel-Documentation system (BIO-RAD, USA), sequenced and analyzed using ClustalW2 online tool (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

Statistical analysis

All the statistical analysis was performed using graphpad Prism software (ver. 5.0). Data was represented as mean±standard error of mean (SEM). Paired Student t-test

with 95% confidence interval (CI) was considered significant. The *p* value of <0.05 was considered statistically significant. Values indicated by asterisk (***) differ significantly at $p<0.001$ and (*) differ significantly at $p<0.05$.

RESULTS

The schematic representation shows different steps involved in measuring different outcomes throughout the study process (**Figure 1**). Isolated tFLCs were enriched for EpCAM positive cells by MACS in step 1 (**Figure 1a**), labeled with DiD dye in step 2 (**Figure 1b**), and then transplanted into CLD mice livers (**Figure 1c**). Further, live non-invasive near infrared (NIR) imaging was performed at regular intervals using a small animal imaging system to detect the fluorescence signal (**Figure 1d**). However, *ex vivo* imaging of the excised liver was performed to confirm the localization of the fluorescence in the mice liver post-transplantation (**Figure 1e**).

Immunophenotyping of tFLCs to identify the proportion of fHPCs

Human tFLCs stained with FITC tagged anti-CD326 (EpCAM) antibody were analyzed using the flow cytometry. The gating strategy and the analysis of the cell fluorescence *vs* cell size, and the overlaid histogram of the unstained and EpCAM stained cells was acquired (**Figure 2a & 2b**). Of the tFLCs, 49±23% cells were found to be EpCAM positive.

NIR imaging of DiD-labeled EpCAM positive cells *in vitro*

Magnetically sorted EpCAM positive fHPC's were characterized for the expression of specific hepatic cell markers such as CK and c-Met together with EpCAM (**Figure 2c**). Magnetically sorted EpCAM positive cells which were labeled with DiD dye showed a separate peak corresponding to the DiD fluorescence (**Figure 2d**). Further, before transplantation, the DiD-labeled EpCAM positive cells in the tube were visualized for their fluorescence in the multispectral imaging system. The overlay image of the NIR fluorescence, and X-ray showed a positive signal only in the tube with the DiD- labeled cells, while the tube with the non-labeled cells was devoid of any such fluorescence (**Figure 2e**).

Long-term tracking of DiD-labeled cells post-transplantation in CLD-SCID mice

Assessment of the biochemical and histological changes in CLD-SCID mice models showed increase in serum parameters (SGOT, SGPT, and bilirubin), and collagen accumulation compared to the control animals, confirming the liver injury after 4 wk of CCl_4 injection (**Supplementary Figure S1**). These CLD mice were utilized for cell transplantation (at day 0), and tracking through *in vivo* imaging on different days from day 0 to day 80 (**Figure 3a**). NIR fluorescence imaging of CLD-SCID mice before intra-hepatic transplantation of DiD-labeled EpCAM positive fHPCs cells at day 0 didn't show fluorescence. While, mice imaged at day 7, 15, 30, 45, 60, and 80 post-transplantation showed a positive fluorescence signal (**Figure 3b**). The over lay images showed DiD fluorescence in the upper part of the abdominal cavity near the rib cage, suggesting that the cells continue to localize in the liver lobes until day 80 after cell transplantation. The non-transplanted mice abdomen lacked such fluorescence signal (**Figure 3c**). To further confirm the localization, liver was excised at days 15 and day 80 post-transplantation, and *ex vivo* imaging was performed which confirmed the localization of DiD-labeled cells within the liver (**Figure 3d**). These results indicate that the DiD-labeled cells can be efficiently visualized, and can be used for long-term tracking both *in vivo* and *ex vivo*.

Co-expression of hepatic markers with DiD in the transplanted mice livers

To evaluate the expression of hepatic markers in transplanted cells, frozen mouse liver sections were obtained from the portion of the liver that displayed a DiD positive fluorescence signal at day 15 and day 80 post-transplantation. Serial sections of the liver transplanted with DiD-labeled EpCAM positive cells and non-transplanted control mice were obtained, and stained for CK and C-Met (**Figure 4 & 5**). The presence of DiD fluorescence only in the transplanted mice confirmed the existence of transplanted cells, while non-transplanted mice didn't show DiD fluorescence in their liver tissue sections. Further, positive staining for both CK (**Figure 4a, 5a & 5c**) and c-Met (**Figure 4b, 5b & 5d**) colocalization with the DiD fluorescence clearly demonstrated that the fluorescent

signal of the hepatic markers, CK and c-Met emerged from the DiD-labeled transplanted cells.

Tracing the effect of transplanted DiD-labeled cells through improved liver function parameters

After 15 days of transplantation, recovery of liver function was analyzed by assaying for the liver enzymes SGOT, SGPT and bilirubin. The serum SGOT and SGPT levels reduced significantly in the transplanted mice compared to the non-transplanted mice (**Figure 6a**); while, serum bilirubin was reduced to normal levels in both the groups. Thus, the serum enzyme parameters suggest an improved liver function in the transplanted mice compared to the non-transplanted mice. Further, the lower collagen percentage area in transplanted mice compared to the non-transplanted mice suggests that this recovery may be attributed to the transplanted fHPCs (**Figure 6b & 6c**).

The above assumption was further confirmed through the positive expression of hu-ALB in CLD-SCID mice liver cells at day 80 post-transplantation (**Figure 7**). ALB positive cells were also tested for their colocalization with DiD to confirm the effect emerging due to labeled cells only. The non-transplanted mice livers were tested negative for the ALB staining. The presence of hu-ALB only in the livers of the transplanted mice, but not in the non-transplanted mice further confirmed the differentiation of fHPCs into functional hepatocytes (**Figure 7a & 7b**). In addition, the amplification and detection of human-specific Alu-sequence from the transplanted mouse livers provided molecular evidence for the continued presence of human fetal liver cells in the mice liver at day 80. Sequencing analysis of the amplified Alu gene confirmed the presence of human Alu gene sequence in mice liver tissues (**Supplementary Figure 2**). These results suggest that the transplanted human EpCAM positive DiD-labeled cells continue to survive for long-term without eliciting serious unfavorable effects in CLD-SCID mice.

DISCUSSION

This study reports a new application of the lipophilic fluorescent dye DiD for non-invasive *in vivo* imaging to monitor transplanted EpCAM positive fHPCs in SCID mice with CLD. In our earlier clinical study, transplanted EpCAM positive fHPCs in end-stage CLD patients could be tracked for only 24 h using radio-labeled ^{99m}Tc-HM-PAO post-transplantation [6]. In our current study, labeling of EpCAM positive fHPCs with DiD enabled long-term *in vivo* monitoring of the transplanted cells, which was further confirmed by *ex vivo* imaging and immuno-histological analysis. Using DiD as a cell label, the transplanted cells could be tracked for 80 days non-invasively in a CLD-SCID mouse liver. Our findings revealed that the DiD labeling is relatively simple, safer, and effective approach for long-term and non-invasive tracking of EpCAM positive fHPCs post-transplantation in the mice liver. In addition, our results also showed the efficacy of transplanted human EpCAM positive fHPCs as a cell source for improving the liver functions similar to the earlier studies [11, 25].

Long-term *in vivo* tracking of transplanted cells has been current need of an hour to answer several concerns and also reduce the existing controversies in cell-based therapies. Now, it has been identified as an essential component of cell-based therapeutic strategies for optimizing the cell number, route of delivery, biodistribution, cell viability, and evaluating the regenerative capabilities, which would aid in accelerating their clinical applications [26]. The depth of penetration, sensitivity, spatial and temporal resolution, ease of availability of the molecular probe and cost of imaging are some of the important factors to be considered for a suitable imaging approach [26]. Direct labeling of the transplanted cells with fluorescent dyes has been demonstrated in several animal liver disease models to understand the homing and differentiation. For instance, the fluorescent dye PKH26 was used in a CCl_4-induced liver injury model in rats to show stem cells migration, proliferation and expression of liver-specific markers [27, 28]. However, these studies didn't involve any live cell tracking, and only postmortem analysis of the liver tissue sections was performed to identify the presence of labeled cells. Also, due to the potential cross transfer of the dye to the host cells, PKH26 was not

considered ideal as a cell tracer, thus limiting its applications for transplantation studies [29].

The carbocyanine dyes, CM-DiI and DiR have been used to label transplanted cells in animal models of liver injury. Although both CM-DiI and DiR proved to be safe for cell tracking applications, the monitoring has been demonstrated only in *ex vivo* settings [30] or if *in vivo*, the signal was reported to have faded within 5 days of infusion [22]. Hence, the above carbocyanine dyes have not provided enough evidence for their long-term applicability for cell tracking *in vivo*. DiD has been demonstrated effective for long-term monitoring of labeled neuronal and cancer cells *in vitro* for up to 4 wk [31, 32]. Moreover, DiD at higher concentrations of up to 2 μM also don't affect the cell growth, proliferation, migration, and apoptosis, and also DiD doesn't cross transfer to neighbouring cells [33]. In line, DiD-labeled MSCs have demonstrated absence of cytotoxicity and sign of altered functional performance in terms of cytokine production or trilineage differentiation [34], thus assuring the safety of DiD for potential applicability in stem cells tracking. DiD-labeled neural stem cells have also showed promising results upon direct injection into the cerebrospinal fluid *in vivo* [35]. Also, among the Vybrant® dye series (DiR, DiI, CM-DiI, DiD), DiD has been demonstrated comparatively high intense fluorescence [32]. These observations thus support our choice of DiD for labeling and *in vivo* imaging.

In our study, DiD didn't show any interference with the tissue autofluorescence and FITC due to the fluorescence emission close to the near infra-red region. The quick and easy methodology of staining, non-toxicity, no interference with the functionality of the labeled cells, non-diffusion to the adjacent cells, and lack of photo-bleaching were identified as the major advantages of DiD labeling. Further, co-localization of CK and c-Met hepatic markers with the DiD-labeled transplanted cells in the recipient mice livers showed the long-term persistence of the transplanted cells. These observations support involvement of these markers in supporting the proliferation, survival and differentiation of transplanted cells in recipient's liver regeneration and functional improvements [36-38]. In addition, detection of hu-ALB specific expression within the

transplanted mice livers further confirmed that the transplanted fHPCs not only persisted for long duration, but also differentiated into functional hepatocytes contributing to the liver recovery. Our data also demonstrated that transplanted EpCAM positive DiD-labeled fHPCs into CLD-SCID mice livers provide functional support for the repair and regeneration of the recipient liver. In addition, fHPCs successfully attenuated the liver fibrosis in mice liver. However, future studies could aid in deciphering the detailed molecular mechanism by which fHPCs contribute to liver repair and regeneration.

Overall, the results from our study were supportive of the use of DiD for long-term, non-invasive, *in vivo* tracking of fHPCs in recipient's liver, but there are certain limitations to our study. While the results indicate the suitability of DiD for monitoring transplanted fHPCs in the liver, quantification of the signal to correlate the cell number or survival were not reported. Future studies using a larger cohort of animals, varying cell numbers, and quantification of the signal intensity in relation to the cell doses would be very useful to understand the efficacy and survival. Moreover, optimizing the route of cell delivery, and assessing the dynamic changes in the expression of EpCAM over a period of time will help further in addressing the questions on engraftment and differentiation of fHPCs. Lastly, while DiD is proven to be safe and non-toxic with no effect on the metabolic functioning of the cells *in vitro*. For regular *in vivo* imaging applications of DiD, it is essential to evaluate the metabolic cycle of the dye for long-term uses. Addressing these issues would make DiD labeling more valuable for wider *in vivo* cell tracking applications.

CONCLUSION

Monitoring the fate of transplanted cells by *in vivo* tracking or imaging can help in understanding the homing, engraftment, long-term survival and function of the transplanted cells. In this preclinical study, DiD-labeled EpCAM positive fHPCs showed efficient cell tracking for long-term up to 80 days. The ease of handling, non-toxicity, long-term signal retainment were proved to be major advantages of using DiD

as a cell labeling agent for non-invasive, long-term tracking of cells both *in vivo* and *ex vivo*. These findings could pave the way forward to unravel the underlying regenerative mechanisms and contribution of exogenously transplanted cells in restoring the structural and functional deficits of liver in CLD condition.

ARTICLE HIGHLIGHTS

Research perspectives

The findings of current study may pave the way forward to unravel the underlying regenerative mechanisms and contribution of exogenously transplanted cells in restoring the structural and functional deficits of liver in CLD condition.

Research conclusions

Monitoring the fate of transplanted cells through DiD-based *in vivo* live cell imaging can help in understanding the homing, engraftment, long-term survival and function of the transplanted cells.

Research results

This study showed that DiD-labeling of human liver cells is easy and efficient enough for long-term and non-invasive tracking *in vivo* post-transplantation up to 80 days.

Using DiD the fate of transplanted cells could be determined.

The transplanted human fetal liver cells are able to provide structural and functional improvement in CLD-SCID mice liver.

Research methods

A chronic liver disease SCID mice model was developed which received DiD-labaled human hepatic progenitor cells (EpCAM+ve) through intra-hepatic infusion.

The long-term survival and functional response of transplanted DiD-labaled cells has been investigated up to 80 days.

Research objectives

The present study aimed to shed light on possibility for determining the fate of DiD-labled human liver cells in CLD-SCID mice using live imaging up to 80 days post-transplantation.

Research motivation

DiD is a carbocyanine dye having good photochemical properties of strong fluorescence, and stability.

Due to the long-range emission of DiD (670nm), tissue autofluorescence is minimum, permitting the use of other fluorochromes such as fluorescein isothiocyanate (FITC), for co-localization studies to evaluate the expression of other essential markers specific to the transplanted cells in the recipient tissue.

Moreover, the process for labeling the cells using DiD is easy due to its excellent efficiency for its integration and diffusion into the cell membranes.

The effects of DiD labeling on *in vivo* retention of labaled human liver cells remained to be investigated.

Research background

To determine the fate of transplanted cells *in vivo* long-term cell tracking remains crucial filed of investigation.

Long-term live cell tracking *in vivo* has always been challenging due to absence of a safer cell-labeling agent.

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