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Original Article

# Therapeutic potential of amniotic-fluid-derived stem cells on liver fibrosis model in mice

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ABSTRACT

*Objective:* Liver fibrosis results from the wound healing response to chronic liver damage. Advanced liver fibrosis results in cirrhosis and liver failure, and liver transplantation is often the only option for effective therapy; however, the shortage of available donor livers limits this treatment. Thus, new therapies for advanced liver fibrosis are essential.

*Materials and methods:* Amniotic fluid contains an abundance of stem cells, which are derived from all three germ layers of the developing fetus. These cells do not induce teratomas *in vivo* and do not pose any ethical concerns. To generate liver fibrosis models, male ICR mice were treated with $CCl_4$ via oral gavage for 4 weeks, and the serum levels of glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, and albumin were higher than in the control group following chemical induction. To assess the potential of amniotic-fluid-derived stem cells (mAFSCs) to ameliorate liver fibrosis *in vivo*, mAFSCs were isolated from amniotic fluid of 13.5-day-old transgenic mice, which globally express the fluorescent protein, enhanced green fluorescent protein (EGFP), for tracing purposes (EGFP-mAFSCs). Single cells were injected via the mesentery ($1 \times 10^6$ cells/mouse) of transplanted mice with liver fibrosis.

*Results:* Four weeks after EGFP-mAFSC transplantation, the serum glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, and albumin levels of recipient mice in the EGFP-mAFSC-injected group were significantly decreased when compared with mice in the saline-injected group. Additionally, fibrotic tissues were evaluated using Masson's trichrome staining 4 weeks after cell transplantation. Shrinkage of the fibrotic area was observed in the EGFP-mAFSC-injected group. The tissue-repair effects were also confirmed by hydroxyproline content analysis.

*Conclusion:* The possible repair mechanism from our data revealed that EGFP-mAFSCs may fuse with the recipient liver cells. Overall, EGFP-mAFSCs can ameliorate liver fibrosis in mice, thus providing insight into the future development of regenerative medicine.



## Introduction

Five percent of the global population (300 million) has chronic liver disease and 2 million patients have died due to liver disease. In Taiwan, carriers of hepatitis have reached 15–20% of the population and hepatitis ranks seventh among the top 10 causes of death. Liver fibrosis is a consequence of chronic liver disease resulting from viral infections, alcohol abuse, drug overdose, and dietary habits.

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Continuous liver damage causes an accumulation of extracellular matrix (ECM) to compensate for the gaps resulting from damaged hepatocytes for maintaining liver structure. Accumulation of ECM is referred to as liver fibrosis [1]. As a result, curing liver fibrosis is an essential health issue.

Due to a lack of suitable medications, liver fibrosis easily converts into liver cirrhosis. Liver transplantation is an option; however, there is a lack of donors, which leads to a high mortality rate. Hence, an alternative approach to cure liver fibrosis needs to be investigated. There are many sources of cells that have been reported to alleviate liver fibrosis/cirrhosis successfully, including umbilical-cord-derived cells [2,3], bone-marrow-derived cells [4–6], and hepatic stem/progenitor cells mature hepatocytes [7]. Such cell sources can be retained in the liver for a long time and reduce collagen scarring and improve liver function, as shown by blood biochemistry values [8–10]. It has been demonstrated that undifferentiated stem/progenitor cells have better therapeutic efficacy in repairing liver fibrosis and are retained in a higher quantity in the liver than differentiated cells [11,12]. Donor undifferentiated cells may elevete the content of matrix metalloproteinase (MMP)-2, MMP-9 and MMP-14 [13,14] to degrade the collagen in fibrotic scarring [13,15]. Donor cells hold the properties of antioxidants to alleviate liver fibrosis [16].

It is difficult to retrieve sufficient stem/progenitor cells from patients, hence, a source with a large quantity of cells is needed for transplantation. Amniotic fluid can be collected through amniocentesis, which is thought to supply abundant progenitor cell source of cells shed from the fetus during pregnancy and holds great potential for therapeutic use [17].

Intra-liver injection of stem cells results in an uneven distribution to the liver and causes bleeding during injection [18]. Splenic injection is another option to infuse stems cells; however, the therapeutic effect and survival rate is low compared to other routes of transplantation [12]. Stem cells are easily trapped in lung capillaries during tail vein injection [19]. Peritoneal injection of stem cells results in peritonitis and adhesions. Portal vein injection is superior to vena caudalis injection or other routes [20]. Thus, portal vein injection of stem cells is a universal method of transplantation. Cell infusion via the portal vein results in an even distribution in the liver and does not lead in liver injury. Of note, we found that portal vein injection can cause serious bleeding and result in injury. Thus, we have transplanted the cells through the mesentery (upstream of the portal vein) to avoid an abundant loss of blood. There are no reports demonstrating amniotic-fluid-derived stem cell amelioration of liver fibrosis/cirrhosis. We transplanted enhanced green fluorescent protein (EGFP)-mouse amniotic-fluid-derived stem cells (mAFSCs) to assess the efficacy of recovery from $CCl_4$-induced liver fibrosis in a Ds-red (*Discosoma* sp.)-harboring mouse model and determined how the donor cells improved liver fibrosis syndrome.

## Materials and methods

### *Animals*

Ubiquitously-expressed Ds-red-harboring ICR mice and EGFP-harboring ICR mice were bred and maintained in the Animal Reproductive Technology Laboratory of the Department of Animal Science and Technology, at the National Taiwan University. The mice were housed under standard conditions, and all experimental procedures were approved by the Institutional Animal Care and Use Committee. Eight-week-old Ds-red-harboring ICR male mice were utilized to establish a liver fibrosis mouse model. EGFP-harboring ICR pregnant mice were used to retrieve mAFSCs for transplantation.

### *Production of EGFP- and Ds-red-expressing transgenic mice*

To track how the transplanted cells interact with liver fibrosis hepatocytes, EGFP- and Ds-red-expressing transgenic mice were generated by means of pronuclear microinjection of ICR strain zygotes with *Sca*I–*Pst*I DNA fragments, which are composed of β-actin promoter-driven EGFP or red fluorescent protein from pCX-EGFP or pCX-Ds-red plasmids. Ubiquitously expressed EGFP or Ds-red was successfully produced.

### *Collection of EGFP-harboring mAFSCs*

EGFP-harboring mice (13.5 days pregnant) were anesthetized with $CO_2$ and sacrificed via cervical dislocation. Then, 70% alcohol was used to disinfect the extirpated uterus, which was immersed in 2% fetal bovine serum (FBS) containing phosphate-buffered saline. The uteri were sectioned and segregated, and each fetus from the amniotic sacs was examined for EGFP signals. We then peeled the amniotic membranes from the fetuses and amniotic fluid was collected. Amniotic fluid was subsequently centrifuged at 12,000 rpm for 10 minutes. We resuspended the pellet in α-minimal essential medium (MEM; Sigma, St. Louis, MO, USA) with 10% FBS. A limiting dilution method was used and cells were seeded in 32-well plates. Fibroblast-shaped colonies were selected to culture until confluence. The cells were passaged in a dilution of 1:2 for subsequent transplantation.

### *Examination of fluorescence-activated cell sorting*

Trypsin/EDTA (Gibco 25200; Los Angeles, CA, USA) was utilized to detach the cells, which were then stained with CD44, stem cell antigen 1 (Sca-1), and CD105 (eBioscience, San Diego, CA, USA), fixed, and examined using a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA, USA). To avoid false-positive staining, isotypes were utilized as negative controls (eBioscience).

### *Tri-lineage differentiation of mAFPCs*

To verify the tri-lineage differentiation potential of mAFSCs, osteogenic, adipogenic, and chondrogenic differentiation was conducted. For adipogenic differentiation, mAFSCs were cultured for 3 weeks in α-MEM with 10% FBS, 0.5 mM isobutylmethylxanthine (Sigma), 1 μM dexamethasone (Sigma), 10 μg/mL insulin (Sigma), and 100 μM indomethacin (Sigma). Lipid droplets were observed by staining with oil red O (Sigma). For osteogenic differentiation, mAFSCs were cultured for 3 weeks in α-MEM with 10% FBS, 10 mM glycerolphosphate (Sigma), 50 μM ascorbic acid (Sigma), and 0.1 μM dexamethasone. Mineralized bone matrix was observed by staining with alizarin red S (Sigma). For chondrogenic differentiation, mAFSCs were cultured for 3 weeks in α-MEM with 1% FBS, 50 μM ascorbic acid, 10 ng/mL transforming growth factor-1, and 6.25 μg/mL insulin (R&D Systems, Minneapolis, MN, USA). Proteoglycans were visualized with toluidine blue (Sigma) staining.

### *Establishment of liver fibrosis mice*

A liver fibrosis model was produced using $CCl_4$ [21]. The mice were randomized and separated into three groups: wild-type; $CCl_4$; and oil control ($n = 6$ per group). Ds-red-harboring ICR male mice (8 weeks old) were treated with $CCl_4$ (2 mL/kg) in sunflower oil solution twice weekly. ICR mice (8 weeks old) were treated with sunflower oil solution without $CCl_4$ every week, and designated as the pseudo-fed shame group to confirm the feeding procedure did not influence the results.