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Cotransplantation With Adipose Tissue–derived Stem Cells Improves Engraftment of Transplanted Hepatocytes

Hiroki Yamana, MD,[1] Akiko Inagaki, PhD,[2] Takehiro Imura,[2] Yasuhiro Nakamura, MD, PhD,[3] Hiroyasu Nishimaki, MD, PhD,[1] Takumi Katano, MD,[2] Kazuo Ohashi, MD, PhD,[4] Shigehito Miyagi, MD, PhD,[1] Takashi Kamei, MD, PhD,[1] Michiaki Unno, MD, PhD,[1] and Masafumi Goto, MD, PhD[1,2]

Background. Hepatocyte transplantation is expected to be an alternative therapy to liver transplantation; however, poor engraftment is a severe obstacle to be overcome. The adipose tissue–derived stem cells (ADSCs) are known to improve engraftment of transplanted pancreatic islets, which have many similarities to the hepatocytes. Therefore, we examined the effects and underlying mechanisms of ADSC cotransplantation on hepatocyte engraftment. **Methods.** Hepatocytes and ADSCs were cotransplanted into the renal subcapsular space and livers of syngeneic analbuminemic rats, and the serum albumin level was quantified to evaluate engraftment. Immunohistochemical staining and fluorescent staining to trace transplanted cells in the liver were also performed. To investigate the mechanisms, cocultured supernatants were analyzed by a multiplex assay and inhibition test using neutralizing antibodies for target factors. **Results.** Hepatocyte engraftment at both transplant sites was significantly improved by ADSC cotransplantation ($P < 0.001$, $P < 0.001$). In the renal subcapsular model, close proximity between hepatocytes and ADSCs was necessary to exert this effect. Unexpectedly, ≈50% of transplanted hepatocytes were attached by ADSCs in the liver. In an in vitro study, the hepatocyte function was significantly improved by ADSC coculture supernatant ($P < 0.001$). The multiplex assay and inhibition test demonstrated that hepatocyte growth factor, vascular endothelial growth factor, and interleukin-6 may be key factors for the abovementioned effects of ADSCs. **Conclusions.** The present study revealed that ADSC cotransplantation can improve the engraftment of transplanted hepatocytes. This effect may be based on crucial factors, such as hepatocyte growth factor, vascular endothelial growth factor, and interleukin-6, which are secreted by ADSCs.

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INTRODUCTION

Liver transplantation is a well-established treatment for acute liver failure, metabolic diseases, and cirrhosis[1,2]; however, a chronic donor shortage is a serious problem for liver transplantation, as it is for almost all types of transplant medicine.[3,4] In hepatocyte transplantation (HTx), the donor liver is isolated by collagenase, and isolated hepatocytes are typically transplanted into the recipient's portal vein or spleen via a catheter. This attractive new approach is expected to be an alternative and/or bridging

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[1] Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan.

[2] Division of Transplantation and Regenerative Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan.

[3] Division of Pathology, Faculty of Medicine, Tohoku Medical and Pharmaceutical University, Sendai, Japan.

[4] Graduate School and School of Pharmaceutical Sciences, Osaka University, 565-0871, Osaka, Japan.

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Correspondence: Masafumi Goto, MD, PhD, Division of Transplantation and Regenerative Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-0872, Japan. (masafumi.goto.c6@tohoku.ac.jp).

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therapy to liver transplantation.[5,6] HTx is apparently less invasive than liver transplantation, as it does not require laparotomy and general anesthesia subsequently can be completed in a short time.[7] This simple procedure also has the advantage that donor livers, which are unsuitable for liver transplantation (eg, neonatal livers, fatty livers, and livers from circulatory death donors with prolonged warm ischemia), can be effectively used for HTx.[7,8]

However, HTx is associated with several problems that must be overcome, including hepatocyte isolation,[9] hepatocyte storage,[10] evaluation of hepatocyte quality before transplantation,[11] transplant site,[12] and transplant efficiency.[13] Among these, poor engraftment of transplanted hepatocytes is definitely one of the most severe problems to be solved for the current HTx. According to our previous report, the estimated transplant efficiency in the current HTx method is at most 0.5%,[12] although a transplant efficiency of nearly 5% against whole liver cells is considered necessary to cure metabolic liver diseases.[14] It is, therefore, necessary to establish a novel strategy for improving the transplant efficiency of HTx. Considering that hepatocytes are fragile and vulnerable to various stresses, including inflammation and hypoxia,[15-17] these factors may be intimately associated with the current insufficient engraftment in HTx. Of particular note, in pancreatic islet transplantation, which has many similarities to HTx, insufficient engraftment is known to be strongly related to an instant blood-mediated inflammatory reaction (IBMIR), which is characterized by the activation of both coagulation and complement cascades.[18-20] Given that IBMIR has also been reported to occur in HTx,[18,21] effective inhibition of IBMIR may be crucial for improving hepatocyte engraftment. We previously reported that low molecular weight dextran sulfate, gabexate mesylate, and C5a inhibitory peptide can be useful for inhibiting IBMIR.[22-26] Therefore, a novel approach for protecting transplanted hepatocytes from severe inflammation and/or hypoxia in combination with the abovementioned anti-IBMIR treatment would be an attractive strategy for improving the engraftment of transplanted hepatocytes.

Adipose tissue–derived stem cells (ADSCs), which are classified as a type of mesenchymal stem cell (MSC), can be isolated from adipose tissues and have strong self-renewal ability[27] and a unique characteristic to differentiate into adipocytes,[28] osteoblasts,[28] neurocytes,[29] myocytes,[28] and hepatocytes.[30] ADSCs secrete various growth factors, anti-inflammatory cytokines, extracellular matrices, and antiapoptotic factors to suppress inflammatory reactions, subsequently providing a growing environment for several types of cells.[27,31] In fact, ADSCs have been studied for the treatment of various diseases related to the skin,[32] cartilage,[33] nerves,[34] and heart.[35] Furthermore, it was reported that an infusion of MSCs into the injured liver with acute liver failure suppressed liver damage and promoted liver regeneration.[36,37] In addition, Fitzpatrick et al[38] previously reported that in vitro coculture with MSCs increased albumin production and prolonged survival of hepatocytes; however, it remains unknown whether ADSC (or MSC) cotransplantation could improve hepatocyte engraftment in HTx. Investigating the underlying mechanisms of this approach would also be interesting. Of particular interest, several studies have reported that coculture of pancreatic islets with MSCs maintained the islet function,[39,40] and cotransplantation with ADSCs was reported to increase islet engraftment.[41,42] Considering the close similarities between hepatocyte and islet transplantation, we hypothesized that cotransplantation of ADSC with hepatocytes would promote transplanted hepatocyte engraftment by regulating severe inflammation and/or hypoxia.

In this study, we attempted to evaluate the effects of ADSC cotransplantation on hepatocyte engraftment using an analbuminemic rat model. To clarify the underlying mechanisms of ADSC cotransplantation, we also examined several inflammatory mediators and growth factors that may influence the hepatocyte function using an in vitro coculture model.

MATERIALS AND METHODS

Animals

Rat hepatocytes were obtained from male inbred F344/NSLc rats (age, 9–14 weeks; weight, 186–304 g; Japan SLC Inc, Shizuoka, Japan). Syngeneic analbuminemic rats (age, 12–18 weeks; weight, 174–302 g) were bred at Tohoku University. All rats were maintained under a 12-h light/dark cycle with ad libitum access to food and water. All animals were handled according to the Guide for the Care and Use of Laboratory Animals and the guidelines for animal experiments at Tohoku University (protocol ID: 2020 MdA-143). All surgical procedures were performed under anesthesia, and every effort was made to relieve suffering.

Hepatocyte Isolation

Hepatocyte isolation was performed using a 2-step collagenase perfusion method, as previously described.[10] The isolated cells were suspended in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Equitech-Bio, Inc, Kerrville, TX) and HEPES (Gibco, Thermo Fisher Scientific Inc, Waltham, MA). Density gradient centrifugation (50G, 20 min, 4 °C) was performed using Percoll density gradient centrifugation media (GE Healthcare Biosciences, Pittsburgh, PA) to obtain a highly purified cell population. Hepatocyte viability was measured by the trypan blue exclusion assay, and hepatocytes with ≥90% viability were used in the following experiments.

ADSC Isolation

ADSCs were isolated from subcutaneous fat of 7-week-old male F344/NSLc rats as previously reported.[43] Subcutaneous fat from the rat lower abdomen was collected and washed with Hanks' balanced salt solution (Sigma-Aldrich) containing 1% penicillin-streptomycin (Thermo Fisher Scientific Inc) and 0.5% gentamicin (Thermo Fisher Scientific, Inc). Adipose tissue was digested in Hanks' balanced salt solution containing 1 mg/mL collagenase type II (Sigma-Aldrich) at 37 °C for 30 minutes using a shaker at 100 rpm. Then, ammonium-chloride-potassium lysing buffer (Lonza Group Ltd, Muenchensteinerstrasse, Switzerland) was added to remove blood cells and washed. The pellet was suspended in the medium for ADSCs (ADSC-1; Kohjin Bio Ltd, Saitama, Japan), and the resulting cells were placed in 100-mm gelatin-coated culture dishes (AGC TECHNO GLASS Co Ltd, Shizuoka, Japan, and Corning, Kennebunk, ME) and cultured at 37 °C

with 5% CO_2. Once they were 80% to 90% confluent, the cells were detached from the dishes by treatment with 0.05% trypsin-0.53 mmol/L ethylenediaminetetraacetic acid (Thermo Fisher Scientific Inc) at 37 °C for 3 min, then suspended in CELLBANKER 1plus (Takara-bio, Shiga, Japan) and cryopreserved in liquid nitrogen. In this experiment, ADSCs were used at passage 3. Viability was measured by the trypan blue exclusion assay and confirmed to be >90% at the time of passaging and transplantation. Flow cytometry was performed to characterize the phenotypes of ADSCs (**Supplemental Content S1, SDC,** http://links.lww.com/TP/C395).

Transplantation

In renal subcapsular transplantation, cells were injected into the renal subcapsular space through the catheter of a 18-G needle with a gastight syringe (Hamilton Company, Reno, NV), as previously described.[13] In the case of cotransplantation, both cells were mixed and placed for 5 min before transplantation. In portal vein transplantation, pellets were slowly injected into the portal vein through the catheter using a 25-G needle with a gastight syringe, as previously described.[11] Serum albumin levels were quantified during the observation period using an LBIS rat Albumin ELISA kit (AKRAL-220; Fujifilm Wako Shibayagi, Gunma, Japan).

Experimental Groups in the In Vivo Model

To evaluate the effect of ADSC cotransplantation in renal subcapsular transplantation, hepatocytes (5.0×10^5) were transplanted into the renal subcapsular space on both sides (total hepatocytes 1.0×10^6) in the HTx group (n = 15). In the cotransplantation group (CoTx group), hepatocytes (5.0×10^5) and ADSCs (5.0×10^6) were transplanted into the renal subcapsular space on both sides (total hepatocytes 1.0×10^6, ADSCs 1.0×10^7) (n = 14).

To evaluate the effect of ADSC cotransplantation site on hepatocyte engraftment, hepatocytes (5.0×10^5) were transplanted into the right renal subcapsular space in the Hemi-HTx group (n = 8). In the hemi hepatocyte transplantation group (n = 8), hepatocytes (5.0×10^5) and ADSCs (5.0×10^6) were mixed and transplanted into the right renal subcapsular space (n = 8). Furthermore, in the separate cotransplantation group (n = 8), hepatocytes (5.0×10^5) were transplanted into the right and ADSCs (5.0×10^6) were transplanted into the left renal subcapsular space.

To evaluate the effect of ADSC cotransplantation in intraportal transplantation, hepatocytes (1.0×10^6) were transplanted into the portal vein in the intraportal (IPO)-HTx group (n = 8). In the IPO-CoTx group, hepatocytes (1.0×10^6) and ADSCs (1.0×10^7) were transplanted into the portal vein (n = 8). In the IPO-ADSC transplantation (ATx) group, ADSCs (1.0×10^7) were transplanted into the portal vein (n = 4). Transplantation of higher doses of hepatocytes and ADSCs was also performed. In the intraportal higher doses (IPOHD)-HTx group, hepatocytes (5.0×10^6) were transplanted into the portal vein (n = 6). In the IPOHD-CoTx group, hepatocytes (5.0×10^6) and ADSCs (2.5×10^7) were transplanted into the portal vein (n = 5).

Immunohistochemistry

The recipient's kidneys and liver were retrieved 4 weeks after transplantation and fixed with 4% paraformaldehyde and embedded in paraffin for immunohistochemistry on cytokeratin 18, 5-bromo-2'-deoxyuridine (BrdU),[12] and albumin staining (**Supplemental Content S2, SDC,** http://links.lww.com/TP/C395).

Identification of Key Factors for the Hepatocyte Function and Inhibition of Candidate Factors in the ADSC Coculture Supernatant

ADSC coculture with hepatocytes was performed to investigate the effect on the hepatocyte function, and culture supernatants were analyzed to identify the key factors of the above effect using a Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel (Merck KGaA, Darmstadt, Germany) and Mouse/Rat Hepatocyte Growth Factor (HGF) Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota) (**Supplemental Content S3, SDC,** http://links.lww.com/TP/C395). ADSC coculture supernatants were divided into 2 groups mainly based on lot-to-lot variations: (1) effective for increasing the hepatocyte function (cocultured supernatant [Co-cul sup] [effective (E)] group) and (2) not effective for increasing the hepatocyte function (Co-cul sup [ineffective] group). In the inhibition assay, only the coculture supernatants in the Co-cul sup (E) group were used together with anti-HGF antibodies (anti-HGF group, n = 8) (1.0 μg/mL) (R&D Systems), anti–vascular endothelial growth factor (VEGF) antibodies (anti-VEGF group, n = 8) (1.0 μg/mL) (R&D Systems), and anti–interleukin (IL)-6 antibodies (anti–IL-6 group, n = 8) (3.0 μg/mL) (**Supplemental Content S4, SDC,** http://links.lww.com/TP/C395).

Statistical Analyses

All values were expressed as the mean ± SD. All statistical analyses were performed using the JMP Pro 16 software program (SAS Institute, Inc, Carry, NC). A 2-way ANOVA followed by the Turkey-Kramer test, as a post hoc comparison, was used to analyze the serum albumin levels. A 1-way ANOVA followed by the Turkey-Kramer test, as a post hoc comparison, was used to compare the rate of ammonia metabolism and serum cytokine levels of ≥3 groups. A paired *t* test was used to compare the rates of ammonia metabolism between 2 groups, the BrdU-positive rate, and the number of cytokeratin 18–positive cells. The Mann-Whitney *U* test was used to compare the rate of ADSC-adhered hepatocytes. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effect of ADSC Cotransplantation on Engraftment of Hepatocytes Transplanted Into the Renal Subcapsular Space

Engraftment of transplanted hepatocytes was evaluated by measuring the serum albumin levels of recipient rats. In the CoTx group, serum albumin levels gradually increased throughout the study period. In contrast, serum albumin levels remained low in the HTx group (Figure 1). The serum albumin levels of the CoTx group were significantly higher than those in the HTx group ($P < 0.0001$) (Figure 1).

Effect of ADSC Cotransplantation Site on Engraftment of Transplanted Hepatocytes

The serum albumin levels of the Hemi-CoTx group were significantly higher than the Separate-CoTx group or the

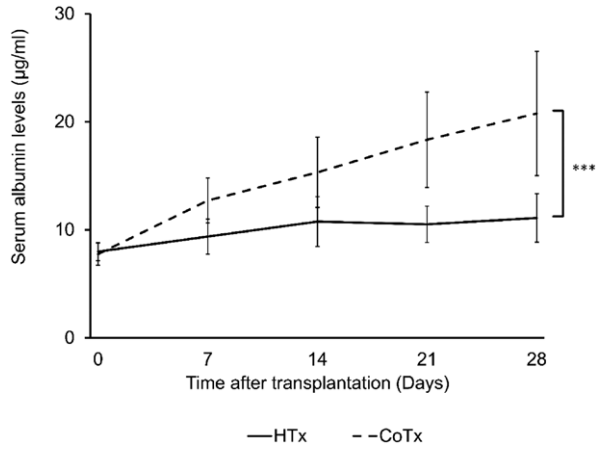

FIGURE 1. Effect of ADSC cotransplantation on engraftment of hepatocytes transplanted into the renal subcapsular space. The serum albumin levels of the CoTx group (n = 14) were significantly higher than those of the HTx group (n = 15) (***$P < 0.001$). ADSC, adipose tissue–derived stem cell; CoTx, cotransplantation; HTx, hepatocyte transplantation.

hemi hepatocyte transplantation group ($P < 0.0001$), suggesting that the beneficial effect of ADSC cotransplantation may be attributed to the distance between hepatocytes and ADSCs (Figure 2).

Immunohistochemical Staining of Hepatocytes and ADSCs Transplanted Into the Renal Subcapsular Space

In the CoTx group, most transplanted ADSCs differentiated into adipose cells and formed adipose tissues in the renal subcapsular space (Figure 3A and 3B). Cytokeratin 18–positive hepatocytes were engrafted on the surface of the kidney parenchyma (KP) (KP [+] group; Figure 3A, black arrow) or inside the adipose tissues (KP [–] group; Figure 3A, blue arrow). In the HTx group, transplanted hepatocytes were only detected on the surface of the KP (Figure 3C and 3D). In both groups, BrdU and cytokeratin 18 double–positive hepatocytes were observed (Figure 4A). Although the number of cytokeratin 18–positive cells in the CoTx group (1037.5 ± 518.4 cells/rat) tended to be higher than the HTx group (356.3 ± 146.5 cells/rat) ($P = 0.067$) (Figure 4B), the BrdU-positive rate in the CoTx group (3.7 ± 1.1%) was unexpectedly lower than that of the HTx group (11.6 ± 2.4%) ($P = 0.005$) (Figure 4C). Of particular interest, in the CoTx group, the BrdU-positive rate in the KP (+) group (4.8 ± 1.2%) was significantly higher than that in the KP (–) group (2.7 ± 0.9%) ($P = 0.038$) (Figure 4D–F). These data suggest that the renal subcapsular space may not be an ideal transplant site for ADSC cotransplantation with hepatocytes, most likely because of the limited space.

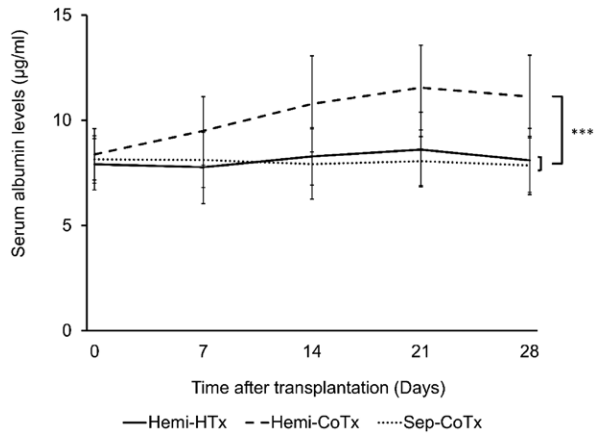

FIGURE 2. Effect of ADSC cotransplantation site on engraftment of transplanted hepatocytes. The serum albumin levels of the Hemi-CoTx group (n = 8) were significantly higher than those of the Sep-CoTx group (n = 8) or the Hemi-HTx group (n = 8) (***$P < 0.001$). ADSC, adipose tissue–derived stem cell; CoTx, cotransplantation; Hemi-HTx, hemi hepatocyte transplantation; HTx, hepatocyte transplantation; Sep-CoTx, separate cotransplantation.

Effect of ADSC Cotransplantation on Engraftment of Hepatocytes Transplanted Into the Portal Vein

According to the outcomes of the renal subcapsular transplantation, we further examined the effect of ADSC cotransplantation on engraftment of hepatocytes transplanted into the portal vein, which is regarded as the current gold standard transplant site. The serum albumin levels of the IPO-CoTx group were significantly higher than the IPO-HTx group ($P < 0.001$) and the IPO-ATx group ($P < 0.001$) (Figure 5A). In the IPO-ATx group, the serum albumin levels remained unchanged during the observation period (Figure 5A). Unlike the renal subcapsular space, a much higher dose of cells can be transplanted to the liver via the portal vein. In fact, the serum albumin levels of the IPOHD-CoTx group were significantly higher than the IPOHD-HTx group ($P < 0.001$) (Figure 5B) when 5.0×10^6 hepatocytes and $2.5 \times 10^7$of ADSCs were infused into the portal vein. As expected, the difference in serum albumin levels between the 2 groups was apparently more evident in this case.

Immunohistochemical Staining of Hepatocytes Transplanted Into the Portal Vein

In both groups, the albumin-positive hepatocytes (white arrows) were distributed widely in the recipient liver (Figure 6A and 6B). Unexpectedly, several small fat droplets were observed in the transplanted hepatocytes, irrespective of ADSC cotransplantation (Figure 6A and 6B), suggesting that steatosis may be induced by inflammation and/or oxidative stress according to the transplant procedure rather than by ADSCs. In the IPO-CoTx group, adipose tissue, most likely derived from ADSCs, was occasionally observed in the liver (Figure 6C, yellow arrows); however, the frequency was much lower than the renal subcapsular transplant group.

Identification of Key Factors for the Hepatocyte Function in ADSC Coculture Supernatant in an In Vitro Model

The rate of ammonia metabolism in the coculture group (47.5 ± 5.9%) was significantly higher than that in the indirect (Ind) coculture group (41.1 ± 9.6%) ($P = 0.02$), hepatocyte group (34.0 ± 4.4%) ($P < 0.0001$), or ADSC group (4.8 ± 6.1%) ($P < 0.0001$). Moreover, the rate of ammonia metabolism in the Ind coculture group (41.1 ± 9.6%) was significantly higher than that in the Hepatocyte group (34.0 ± 4.4%) ($P = 0.009$) (Figure 7A).

Supernatants derived from each group were analyzed using a multiplex assay and ELISA. The factors that were significantly higher in the coculture group and/or

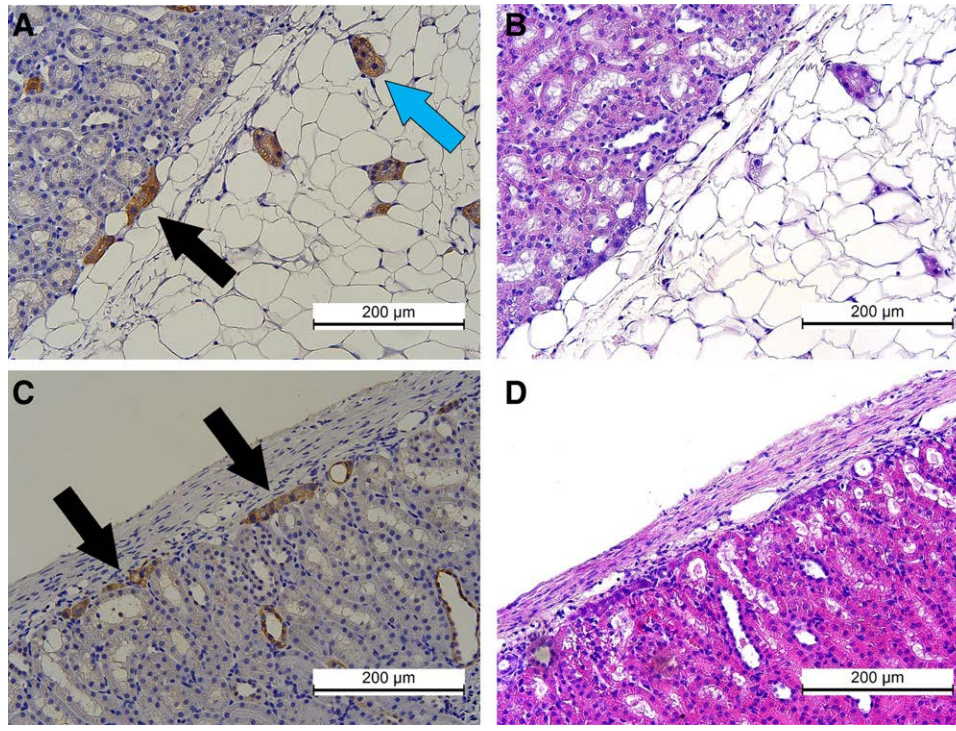

FIGURE 3. Immunohistochemical staining of hepatocytes and ADSCs transplanted into the renal subcapsular space. A, Cytokeratin 18 staining of the CoTx group. Cytokeratin 18–positive hepatocytes engrafted on the surface of the kidney parenchyma are shown with a black arrow. Hepatocytes engrafted inside adipose tissues are shown with a blue arrow. B, HE staining of the CoTx group. Most transplanted ADSCs differentiated into adipose cells and formed adipose tissues in the renal subcapsular space. C, Cytokeratin 18 staining of the HTx group. Cytokeratin 18–positive hepatocytes engrafted on the surface of the kidney parenchyma are shown with black arrows. D, HE staining of the HTx group. Magnification: ×200, Scale bar: 200 μm (all photomicrographs). ADSC, adipose tissue–derived stem cell; CoTx, cotransplantation; HE, hematoxylin and eosin; HTx, hepatocyte transplantation.

the Ind coculture group than the hepatocyte group and ADSC group were identified as candidate factors crucial for the hepatocyte function. Among these factors, IL-10, monocyte chemotactic protein-1, induced protein 10, growth-related oncogene/keratinocyte-derived cytokine, fractalkine, LPS-induced CXC chemokine, macrophage inflammatory protein-2, and regulated upon activation normal T-cell expressed and secreted were most likely upregulated because of enhanced inflammation caused by high-density cell contact between hepatocytes and ADSCs, according to previous reports. We, therefore, focused on HGF, VEGF, and IL-6 as potential candidate factors (Figure 7B–D).

Evaluation of the Hepatocyte Function by Inhibiting Candidate Factors in Coculture Supernatant

The rate of ammonia metabolism in the Co-cul sup (E) group (49.7 ± 6.5%) was significantly higher than that in the hepatocyte supernatant group (34.1 ± 8.5%) ($P < 0.001$) and Co-cul sup (ineffective) group (34.3 ± 8.2%) ($P < 0.01$) (Figure 8A). Accordingly, the supernatant in the Co-cul sup (E) group was considered to be appropriate for use in the subsequent inhibition assays using neutralizing antibodies. Although no significant inhibitory effects were observed by anti–IL-6, anti-HGF, or anti-VEGF neutralizing antibodies alone (Figure 8B), the rate of ammonia metabolism in the anti-Mix group (42.4 ± 4.2%), in which mixed neutralizing antibodies for IL-6, HGF, and VEGF were used, was significantly suppressed in comparison to the Co-cul sup (E) group (49.7 ± 6.5%) ($P < 0.01$) (Figure 8C).

DISCUSSION

We demonstrated that ADSC cotransplantation could effectively enhance engraftment of transplanted hepatocytes. Unlike previous reports, we first demonstrated the beneficial effects of ADSC cotransplantation on the outcomes of HTx using an in vivo model. This favorable effect was only seen when ADSCs and hepatocytes were located in close proximity. In particular, the benefits brought by this approach were more evident when hepatocytes were transplanted into the portal vein, most likely because of the increased graft volume and preferable circumstances for both cells. Furthermore, we developed an in vitro coculture model and proved that ADSCs have a direct enhancing effect on the hepatocyte function and that HGF, IL-6, and VEGF play important roles in this effect.

In the renal subcapsular transplantation model, the beneficial effects of ADSC cotransplantation were not observed when hepatocytes and ADSCs were separately transplanted into the renal subcapsular space of the opposite side, clearly suggesting that the proximity between hepatocytes and ADSCs is important for exerting the effects of ADSCs. This finding is consistent with previous reports in which the effects of ADSCs were only seen when they had a contact with target cells.[41,44] We previously reported that hepatocyte grafts attached to the islet surface penetrated into the renal parenchyma together with the islets and then hepatocyte engraftment was improved by better vascularization from the recipient in the case of cotransplantation between hepatocytes and pancreatic islets.[13] In sharp contrast, in the present study, such features (penetration into the renal parenchyma) of

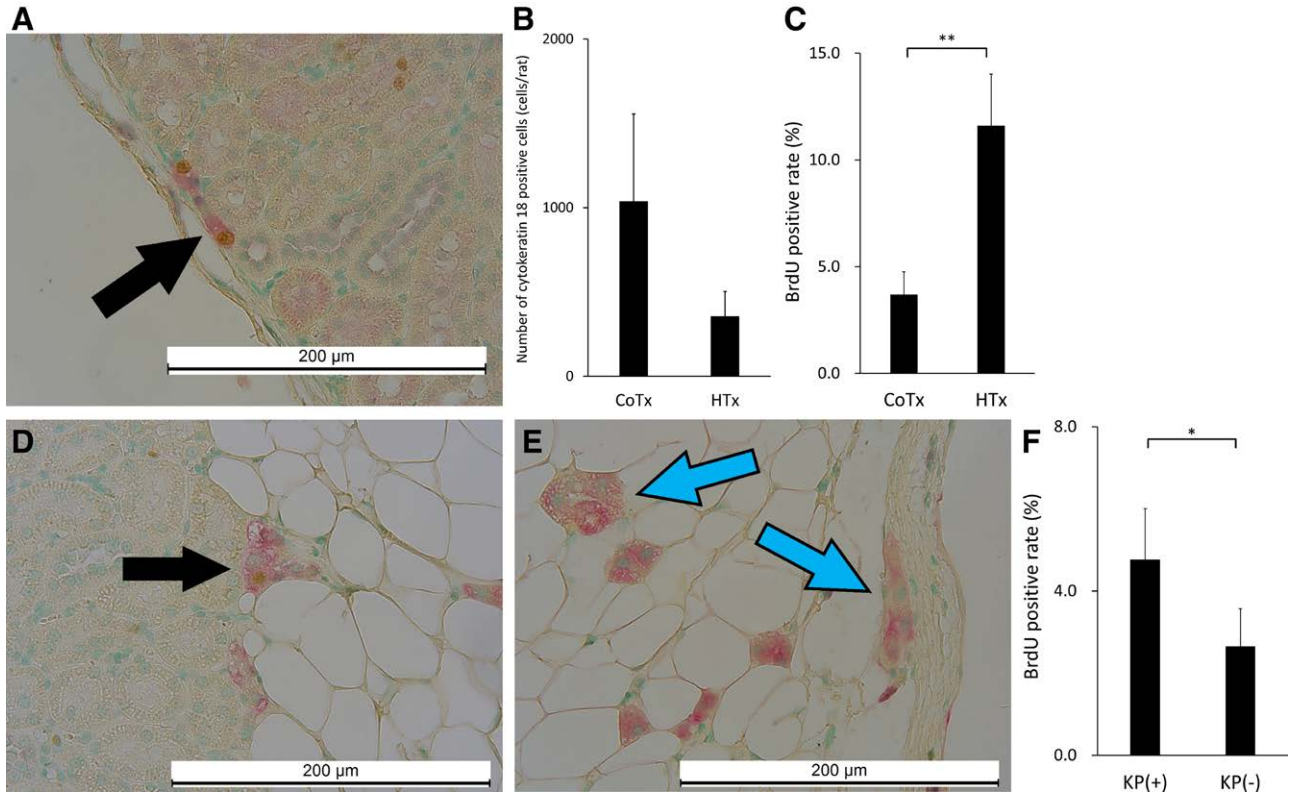

FIGURE 4. BrdU staining of hepatocytes transplanted into the renal subcapsular space. A, A representative photomicrograph of cytokeratin 18 and BrdU-double staining of the HTx group. Cytokeratin 18 and BrdU-positive hepatocytes are shown with a black arrow. Magnification: ×400. Scale bar: 200 μm. B, The number of cytokeratin 18–positive cells of the CoTx group tended to be higher than that of the HTx group ($P = 0.067$). C, The BrdU-positive rate of the CoTx group was significantly lower than that of the HTx group (**$P < 0.01$). D, A representative photomicrograph of cytokeratin 18 and BrdU-double positive hepatocytes engrafted on the surface of the KP (KP [+] group; black arrow) in the CoTx group. Magnification: ×400. Scale bar: 200 μm. E, A representative photomicrograph of cytokeratin 18 and BrdU-double positive hepatocytes inside of the adipose tissues (KP [–] group; blue arrow) in the CoTx group. Magnification: ×400. Scale bar: 200 μm. F, The BrdU-positive rate of the KP (+) group was significantly higher than that of the KP (–) group (*$P < 0.05$). BrdU, 5-bromo-2'-deoxyuridine; CoTx, cotransplantation; HTx, hepatocyte transplantation; KP, kidney parenchyma.

the transplanted hepatocytes were not seen at all; instead, some hepatocyte grafts remained in adipose tissues with a poor blood supply and even appeared to be compressed by them (Figure 3A and 3B). One possibility is that fat tissues differentiated from ADSCs that spread widely under the renal subcapsular space and physically inhibited contact between hepatocytes and the renal parenchyma, subsequently resulting in a low BrdU-positive rate in the CoTx group (Figure 4C). Supporting this hypothesis, in the CoTx group, the BrdU-positive rate of the KP (+) group was significantly higher than that of the KP (–) group (Figure 4F). Considering the finding that the total number of surviving hepatocytes tended to be higher in the CoTx group (Figure 4B), it is also assumed that the cytoprotective effect of ADSCs may have prevented the cell death of transplanted hepatocytes, which would otherwise have died soon after transplantation. Taken together, these data suggest that the renal subcapsular space may not be an ideal transplant site for ADSC cotransplantation with hepatocytes, most likely because of the limited space, although this site is useful as a tool for recovering histology samples and investigating the importance of the distance between both cells. Given that the liver is the current gold standard transplant site in clinical HTx,[12] we further examined the effect of ADSC cotransplantation on engraftment of hepatocytes transplanted into the portal vein.

We and others have reported that intraportal transplantation is a preferable site for HTx because the space is sufficient to transplant cells and due to physiological compatibility between hepatocytes and livers.[8,12] Before starting experiments, we were not sure if ADSC cotransplantation would work because transplanted hepatocytes and ADSCs would easily be separated in the liver after transplantation. Unexpectedly, the beneficial effect of ADSC cotransplantation was certainly reproduced when exactly the same amount of ADSCs and hepatocytes were transplanted into the portal vein (Figure 5A). Supporting this result, fluorescent staining showed that, in the liver, ≈50% of transplanted hepatocytes had attached ADSCs (Supplemental Content S5, SDC, http://links.lww.com/TP/C395), most likely because of the adhesive property of ADSCs.[44] Notably, the rate of ADSC-adhered hepatocytes at 7 days after transplantation significantly increased in comparison to that at 2 days after transplantation (**Supplemental Content S5, SDC,** http://links.lww.com/TP/C395), suggesting that hepatocyte grafts with insufficient ADSC-derived trophic factors might be destroyed soon after transplantation. This novel finding is consistent with the outcome of separate transplantation in the renal subcapsular model (Figure 2). Considering all these data, the proliferative capacity of transplanted hepatocytes is also expected to increase in the intraportal transplantation; thus, this is a topic of interest for our next study.

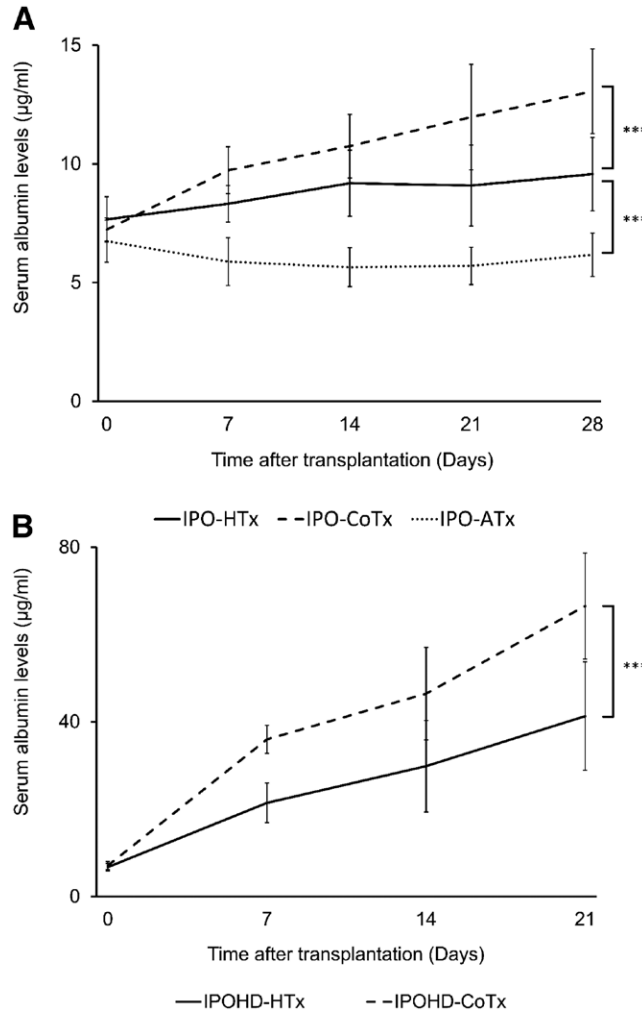

FIGURE 5. Effect of ADSC cotransplantation on engraftment of hepatocytes transplanted into the portal vein. A, The serum albumin levels of the IPO-CoTx group (n = 8) were significantly higher than those of the IPO-HTx (n = 8) and the IPO-ATx (n = 4) groups (***P < 0.001). The serum albumin levels of the IPO-ATx group (n = 4) remained unchanged during the observation period. B, The serum albumin levels of the IPOHD-CoTx group (n = 5) were significantly higher than those of the IPOHD-HTx group (n = 6) (***P < 0.001). ADSC, adipose tissue–derived stem cell; ATx, ADSC transplantation; CoTx, cotransplantation; HTx, hepatocyte transplantation; IPO, intraportal; IPOHD; intraportal higher doses.

Interestingly, immunohistochemical staining of liver tissues after intraportal transplantation showed numerous fat droplets in the transplanted hepatocytes. Because these fat droplets were observed in both groups, it is considered that they are not derived from ADSCs (Figure 6A and 6B). It is well known that steatosis is formed in the recipient hepatocytes after intraportal transplantation of pancreatic islets[45,46]; however, to our knowledge, there are no reports on steatosis after HTx. The mechanism underlying the formation of steatosis after islet transplantation is thought to involve the insulin secreted by transplanted islets,[45] which hepatocytes do not secrete. Given that both hepatocytes

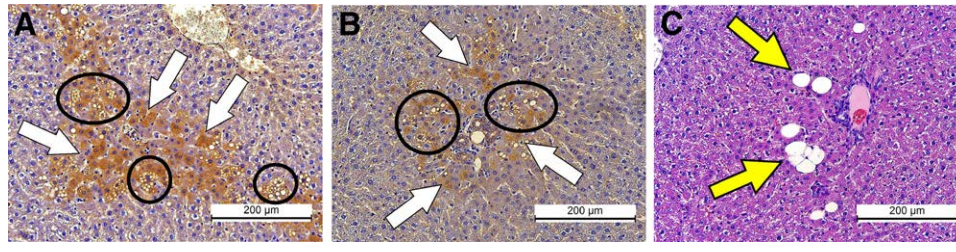

FIGURE 6. Immunohistochemical staining of hepatocytes and ADSCs transplanted into the portal vein. A and B, Albumin staining of the IPO-CoTx group (A) and the IPO-HTx group (B). In both groups, albumin-positive hepatocytes (showed with white arrows) were distributed widely in the recipient livers, and several small fat droplets (showed with circles) were also observed in the transplanted hepatocytes. C, HE staining of the IPO-CoTx group. In the IPO-CoTx group, adipose tissues (showed with yellow arrows) were occasionally observed in the livers, but the frequency was much lower than the renal subcapsular transplant group. Magnification: ×200. Scale bar: 200 μm (all photomicrographs). ADSC, adipose tissue–derived stem cell; CoTx, cotransplantation; HE, hematoxylin and eosin; HTx, hepatocyte transplantation; IPO, intraportal.

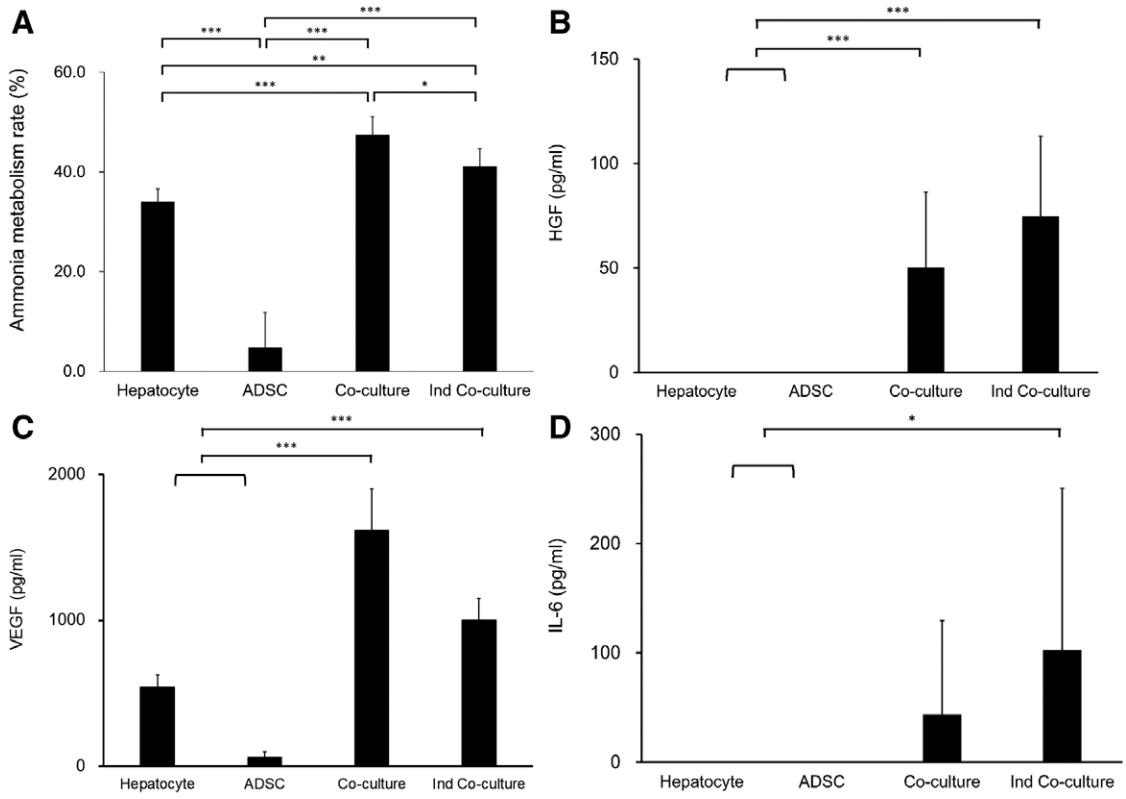

FIGURE 7. Identification of key factors for the hepatocyte function in ADSC coculture supernatant in the in vitro model. A, The rates of ammonia metabolism in several cell cultures. The rate of ammonia metabolism in the coculture group was significantly higher than that in the Ind coculture group, the hepatocyte group, or the ADSC group. B, The VEGF levels of the coculture supernatant and the Ind coculture supernatant were significantly higher than those of the hepatocyte supernatant and the ADSC supernatant. C, IL-6 levels of the Ind coculture supernatant were significantly higher than those of the hepatocyte supernatant and ADSC supernatant. D, HGF levels of the coculture supernatant and Ind coculture supernatant were significantly higher than those of the hepatocyte supernatant and ADSC supernatant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). ADSC, adipose tissue–derived stem cell; HGF, hepatocyte growth factor; IPO, intraportal; IL, interleukin; Ind, indirect; VEGF, vascular endothelial growth factor.

and islets secrete several inflammatory mediators during isolation, culture, and transplantation procedures,[13,47-50] these mediators might at least in part contribute to the formation of steatosis in the liver. This hypothesis together with the influence of the steatosis on the transplanted hepatocytes should be investigated in the future study.

Unlike the renal subcapsular space, a much higher dose of cells can be transplanted to the liver via the portal vein. As expected, the beneficial effect of ADSC cotransplantation was more evident when higher doses of ADSCs and hepatocytes were transplanted into the portal vein (Figure 5B); however, in intraportal transplantation, the amount of hepatocyte graft is strictly limited according to the portal vein pressure.[7] In particular, because ADSCs are well known to induce thrombi and/or embolisms,[51] attention should be paid when ADSC cotransplantation is performed in intraportal transplantation. In fact, in our preliminary rat experiments of intraportal ADSC

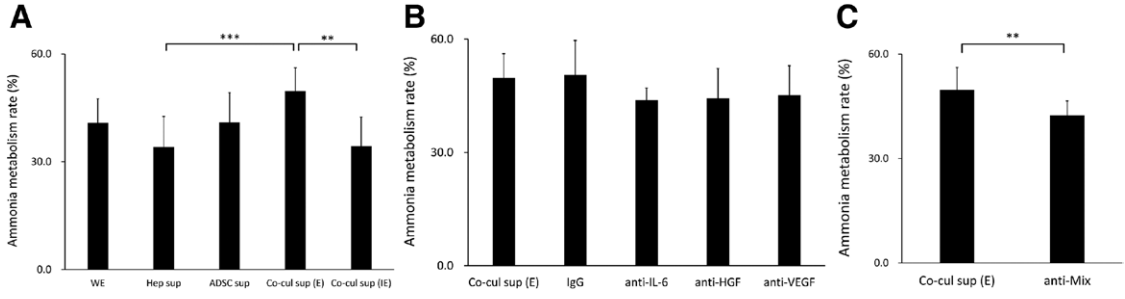

FIGURE 8. Evaluation of the hepatocyte function by inhibiting candidate factors in the coculture supernatant. A, Effect of adding various supernatants on the hepatocyte function. The rate of ammonia metabolism in the Co-cul sup (E) group was significantly higher than that in the Hep sup group or the Co-cul sup (IE) group. B, Effect of adding each neutralizing antibody alone on the hepatocyte function. No significant inhibitory effects were observed by anti–IL-6, anti-HGF, or anti-VEGF neutralizing antibodies alone ($P = 0.27$). C, Effect of adding mixed neutralizing antibodies for IL-6, HGF, and VEGF on the hepatocyte function. The rate of ammonia metabolism in the anti-Mix group was significantly suppressed in comparison to the Co-cul sup (E) group. **$P < 0.01$, ***$P < 0.001$. ADSC, adipose tissue–derived stem cell; Co-cul sup, cocultured supernatant; E, effective; Hep sup, hepatocyte supernatant; HGF, hepatocyte growth factor; IE, ineffective; IL, interleukin; VEGF, vascular endothelial growth factor; WE, Williams' medium E.

cotransplantation with hepatocytes, transplantation of 5.0×10^7 ADSCs was associated with high mortality, likely due to portal vein embolism (data not shown). One possible strategy to overcome this problem is transplanting hepatocytes together with ADSCs into a broad space, such as subcutaneous space[52,53] or intramuscular space.[54] This approach has actually been applied in clinical islet transplantation,[55] which is similar to HTx, and should therefore be verified in future HTx. Another possible strategy is direct injection of hepatocytes and ADSCs into the liver parenchyma.[56] The advantages of this approach are the sufficient space, rich blood flow, and low risk of portal vein embolism. In addition, the adhesion rate between hepatocytes and ADSCs would be expected to increase; as a result, hepatocyte engraftment would improve. Further studies are needed to determine the optimal transplant procedure for safe cotransplantation of high doses of hepatocytes and ADSCs.

In an in vitro coculture model, the multiplex assay showed that various cytokines and growth factors were significantly highly secreted in coculture supernatant in comparison to hepatocyte or ADSC culture supernatant. Among these substances, IL-10, monocyte chemotactic protein-1, induced protein 10, fractalkine, regulated upon activation normal T-cell expressed and secreted, LPS-induced CXC chemokine, macrophage inflammatory protein-2, and growth-related oncogene/keratinocyte-derived cytokine were most likely upregulated because of the enhanced inflammation caused by high-density cell contact between hepatocytes and ADSCs according to previous reports.[57-61] Although IL-6 is well known to be upregulated by inflammation, this cytokine is also known to promote hepatocyte proliferation[62-64] and is reported to be closely related to the islet-protective effects of ADSCs.[39,40] Likewise, HGF and VEGF are known to proliferate and protect hepatocytes.[65,66] We, therefore, focused on HGF, VEGF, and IL-6 as possible candidate factors for improving the hepatocyte function and demonstrated—by performing inhibition assays using neutralizing antibodies—that these factors may play important roles in ADSC cotransplantation with hepatocytes. This finding suggests that the addition of these 3 key factors together with hepatocytes may improve the outcomes of HTx. This approach should be investigated in future studies.

In this study, we used analbuminemic rats that had extremely low serum albumin levels compared with normal F344 rats or commercially available Nagase analbuminemic rats.[10,67] As we could detect the minute changes in albumin levels, these analbuminemic rats were useful for evaluating the engraftment of transplanted hepatocytes. In addition, this model made it possible to evaluate hepatocyte engraftment without considering the immunological influences because these analbuminemic rats are syngeneic to F344 rats. These characteristics greatly contributed to focusing on the engraftment-promoting effects of ADSCs in the present study; however, in the clinical setting, it is necessary to consider the immunological influences that may affect the engraftment of hepatocytes and the effects of ADSCs. Furthermore, the suitable dose of hepatocytes and ADSCs needs to be investigated in future clinical application.

In conclusion, this study revealed that ADSC cotransplantation can improve the engraftment of transplanted hepatocytes. This effect may be due to crucial factors such as HGF, IL-6, and VEGF, which are secreted by ADSCs. Thus, cotransplantation of ADSC with hepatocytes in combination with anti-IBMIR treatment may improve the outcomes of HTx.

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