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RESEARCH ARTICLE

Improvement of hepatocyte engraftment by co-transplantation with pancreatic islets in hepatocyte transplantation

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Abstract

Because of the fragility of isolated hepatocytes, extremely poor engraftment of transplanted hepatocytes remains a severe issue in hepatocyte transplantation. Therefore, improving hepatocyte engraftment is necessary to establish hepatocyte transplantation as a standard therapy. Since the pancreatic islets are known to have favorable autocrine effects, we hypothesized that the transplanted islets might influence not only the islets but also the nearby hepatocytes, subsequently promoting engraftment. We evaluated the effects of islet co-transplantation using an analbuminemic rat model (in vivo model). Furthermore, we established a mimicking in vitro model to investigate the underlying mechanisms. In an in vivo model, the hepatocyte engraftment was significantly improved only when the islets were co-transplanted to the nearby hepatocytes ($p < 0.001$). Moreover, the transplanted hepatocytes appeared to penetrate the renal parenchyma together with the co-transplanted islets. In an in vitro model, the viability of cultured hepatocytes was also improved by coculture with pancreatic islets. Of particular interest, the coculture supernatant alone could also exert beneficial effects comparable to islet coculture. Although insulin, VEGF, and GLP-1 were selected as candidate crucial factors using the Bio-Plex system, beneficial effects were partially counteracted by anti-insulin receptor antibodies. In conclusion, this study demonstrated that islet co-transplantation improves hepatocyte engraftment, most likely due to continuously secreted crucial factors, such as insulin, in combination with providing favorable circumstances for hepatocyte engraftment. Further refinements of this approach, especially regarding substitutes for islets, could be a promising strategy for improving the outcomes of hepatocyte transplantation.

KEYWORDS
cell transplantation, coculture techniques, exosome, hepatocyte engraftment, hepatocyte transplantation, insulin, pancreatic islet transplantation, paracrine communication

1 | INTRODUCTION

Liver transplantation is currently the only curative therapy for end-stage liver diseases such as fulminant hepatitis, acute liver failure, and advanced cirrhosis (W. M. Lee, Squires, Nyberg, Doo, & Hoofnagle, 2008). Liver transplantation was first performed in 1963 and is now established as the standard therapy for end-stage liver diseases (Graziadei et al., 2016). However, there are long waiting lists of liver transplantation candidates worldwide due to a shortage of donors (Barahman et al., 2019). Hepatocyte transplantation (HTx), in which isolated hepatocytes are transplanted to the patients through minimally invasive techniques, has been investigated as a potential solution to such problems (Barahman et al., 2019). More practically, HTx is expected to serve as a bridging therapy in emergency situations such as fulminant hepatitis and as an alternative therapy to liver transplantation in cases involving metabolic liver diseases (Squires et al., 2017). Furthermore, in HTx, we may be able to effectively use fatty livers and cardiac arrest donor livers that have been excluded from the indications for liver transplantation, which consequently enlarges the donor pool (Squires et al., 2017).

In spite of these advantages, the clinical application of HTx still faces many obstacles, including hepatocyte isolation, graft preservation (Fukuoka et al., 2017), graft quality evaluation (Matsumura et al., 2019), and hepatocyte engraftment (Domen, 2018; Weber, Groyer-Picard, Franco, & Dagher, 2009). Among them, extremely poor engraftment of hepatocytes clearly remains the most severe issue. One of the main causes of poor engraftment of hepatocytes after HTx is considered to be the fragility of isolated hepatocytes. Isolated hepatocytes are easily damaged by warm ischemia and physical pressure (Smets, Chen, Wang, & Soriano, 2002; Sufiandi et al., 2015), and transplanted hepatocytes can also be destroyed by the host immune responses after HTx. Considering that approximately 5% of the whole liver hepatocytes may be the upper limit due to the risk of portal embolism (Baccarani et al., 2005), the improvement of hepatocyte engraftment is an issue that must be overcome.

Several reports have so far suggested that hepatocyte viability was improved by coculturing with pancreatic islets (Kaufmann et al., 1999; Kuo, Juang, & Peng, 2011). Moreover, Ricordi, Lacy, Callery, Park, and Flye (1989) previously reported that hepatocytes, that would otherwise be quickly and completely destroyed, remained morphologically intact when islets were simultaneously co-transplanted. Pancreatic islets are used in islet transplantation for patients with severe diabetes (Shapiro et al., 2000) or total pancreatectomy (Hata et al., 2013). Islet transplantation is a cell transplant therapy similar to HTx, but has greater therapeutic effects than HTx (Uematsu et al., 2018). It is therefore regarded as an established treatment worldwide. Unlike hepatocytes, pancreatic islets can be cultured for a long period (Goto, Eich, et al., 2004; Goto, Holgersson, Kumagai-Braesch, & Korsgren, 2006). In addition, the islets are more robust than exocrine tissues, which are derived from the same pancreas, and also have a unique characteristic of releasing a large amount of soluble factors (Johansson, Olsson, Gabrielsson, Nilsson, & Korsgren, 2003). It is speculated that these autocrine effects contribute at least partially to the improvement of islet graft survival (Liu et al., 2009). We therefore hypothesized that the transplanted islets might have positive effects not only for islets per se, but also for nearby hepatocytes and that they would promote hepatocyte engraftment.

In recent years, the role of exosomes in cell-to-cell communication has gained increasing interest as potential therapeutic, diagnostic, and biocompatible delivery tools. Exosomes are extracellular vesicles derived from the endocytic membrane that can contain lipids, proteins, and RNA (Gurunathan, Kang, Jeyaraj, Qasim, & Kim, 2019). They are distinguished from micro-vesicles by their smaller size (Pegtel & Gould, 2019). Exosomes derived from hepatocytes were shown to promote hepatocyte proliferation in an in vitro model and liver regeneration in a mouse model (Nojima et al., 2016). Two hundred fifty-one proteins were identified in exosomes from rat primary hepatocytes (Conde-Vancells et al., 2008). On the other hand, mRNA—including NGN3, MAFA, and PDX1, proteins from hormones including C-peptide and glucagon—were identified in exosomes from pancreatic islets (Ribeiro et al., 2017). Thus, examining the relevance of exosomes to the additional effects of islets is an interesting topic.

In the present study, we quantitatively evaluated the effects of islet co-transplantation on hepatocyte engraftment using an analbuminemic rat model. Furthermore, we established and used an islet coculture in vitro model to investigate the underlying mechanisms, including the involvement of exosomes.

2 | MATERIALS AND METHODS

2.1 | Animals

Rat livers were obtained from male inbred F344/NSLc rats (age: 9–13 weeks; Japan SLC., Shizuoka, Japan). Analbuminemic rats (age: 9–13 weeks) were provided by Professor Yuji Nishikawa (Asahikawa Medical College, Japan) and were bred at the animal facilities of Tohoku University. These analbuminemic rats had a syngeneic background to the donor rats.

All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bayne, 1996), and the guidelines for animal experiments at Tohoku University. The experimental protocol of the present study (protocol ID: 2016 Med Animal-106) was approved by the animal experimental committee in the Tohoku University. All surgeries were performed under anesthesia, and maximal efforts were made to minimize animal suffering.

2.2 | Hepatocyte isolation

Hepatocytes were isolated from F344/NSLc rats by the two-step collagenase perfusion as described previously (Fukuoka et al., 2017; Matsumura et al., 2019). First, Ca^{2+}-free Hanks' balanced salt solution (HBSS, Sigma-Aldrich) containing ethylene glycol tetra-acetic acid was perfused through the portal vein at a rate of 14 ml/min for 5 min. Second, Ca^{2+}-containing HBSS with 0.5 mg/ml of collagenase (type V; Sigma-Aldrich) was perfused via the same route at the same rate. The isolated cells were suspended in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES) (Gibco). The cells were then filtered through a #150 mesh (Ikemoto Scientific Technology) and purified by centrifugation ($50 \times g$, 2 min) at 4°C. Density gradient centrifugation ($50 \times g$, 20 min) at 4°C was performed using Percoll (GE Healthcare Biosciences) to obtain a highly purified cell population. The hepatocyte viability was evaluated by a trypan blue exclusion assay. For all experiments, we used hepatocytes with a viability exceeding 90%.

2.3 | Islet isolation

Rat islet isolation was performed as previously described (Jimbo et al., 2014). After cannulating the bile duct, 10 ml of cold HBSS containing 1 mg/ml collagenase (type IV, Sigma-Aldrich) was injected, then the pancreas was removed. After digestion at 37°C for 12 min and washing, purification by density-gradient centrifugation was performed using Histopaque-1119 (Sigma-Aldrich) and Lymphoprep (Axis-Shield PoC AS). The isolated islets were cultured for 1 day in Roswell Park Memorial Institute-1640 medium containing 5.5 mmol/L glucose (Thermo Fisher Scientific) and 10% FBS at 37°C under 5% CO_2 and humidified air.

2.4 | Effect of islet co-transplantation on the engraftment of transplanted hepatocytes in an in vivo model

In all experimental groups, donor hepatocytes and islets were isolated from F344/NSLc rats, and recipients were analbuminemic rats. Recipients received 2.0×10^6 hepatocytes in the renal subcapsular spaces on both sides (total 4.0×10^6 hepatocytes; ×HT group, $n = 5$), or 2.0×10^6 hepatocytes and 1600 islet equivalents (IEQs) islets into the renal subcapsular spaces on both sides (total 4.0×10^6 hepatocytes and 3200 IEQs islets; co-transplantation [CoTx] group, $n = 5$). The serum albumin levels were quantified using a LBIS Rat Albumin ELISA kit (AKRAL-220; FUJIFILM Wako Shibayagi, Gunma, Japan). Figure 1a, b and Table 1a show a summary of the designs of the experiments in the present study.

2.5 | Effects of islet co-transplantation site on the engraftment of transplanted hepatocytes in an in vivo model

To evaluate an effect of islet co-transplantation site on hepatocyte engraftment, recipients were transplanted with 4.0×10^6 hepatocytes into renal subcapsular space in one side (total 4.0×10^6 hepatocytes; Hemi-HTx group, $n = 6$), 4.0×10^6 hepatocytes together with 3200 IEQs islets into the renal subcapsular space on one side (total 4.0×10^6 hepatocytes with 3200 IEQs islets; Hemi-CoTx group, $n = 7$), or 4.0×10^6 hepatocytes into one renal subcapsular space with 3200 IEQs islets into the opposite side renal subcapsular space (total 4.0×10^6 hepatocytes, 3200 IEQs islets; Separate-CoTx group, $n = 7$). Figure 1c and Table 1b show a summary of the designs of the experiments in the present study.

2.6 | Immunohistochemical staining

Recipient kidneys were removed 4 weeks after transplantation and fixed with 4% paraformaldehyde, and embedded in paraffin for immunohistochemical staining. Albumin staining was performed using anti-albumin antibody (MP Biomedicals) combined with the VECTASTAIN ABC system (Vector Laboratories, Inc.). Insulin staining was performed using guinea pig anti-swine anti-insulin (DAKO Agilent Technologies) combined with the EnVision System Labelled Polymer anti-rabbit (DAKO Agilent Technologies).

2.7 | Effects of islet co-culture on hepatocytes in an in vitro model

Coculture of hepatocytes with islets was performed using type I collagen-coated six well-plate (Corning) and inserts with 1.0 μm pores (Corning). Hepatocytes were seeded on the bottom of the well at 1.0×10^6 cells/well in 1.68 ml of basal medium, and islets were seeded in insert at 0 (Hepatocyte group) or 400 IEQs (coculture group)/well in 0.82 ml of basal medium. Williams medium E (Thermo Fisher Scientific) containing 50 mg/L L-glutamine was used as a basal medium. Table 2a shows a summary of the designs of the experiments in the present study.

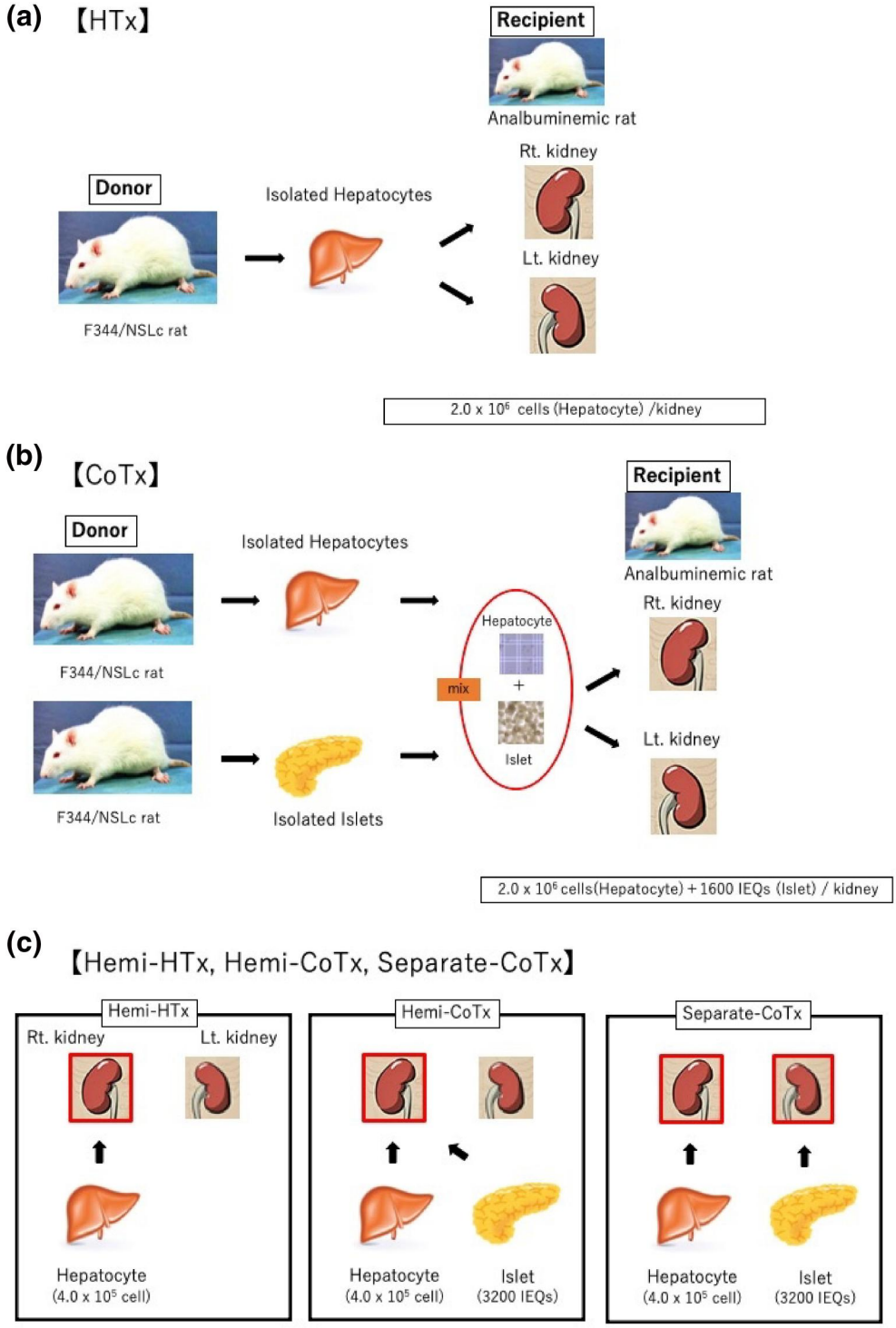

FIGURE 1 Summary of the designs of the experiments in an in vivo model in the present study. In all experimental groups, donor hepatocytes and islets were isolated from F344/NSLc rats, and recipients were analbuminemic rats. (a) Effect of islet co-transplantation on the engraftment of transplanted hepatocytes. Recipients received 2.0×10^6 hepatocytes in the renal subcapsular spaces on both sides (total 4.0×10^6 hepatocytes; Hepatocyte [HTx] group). (b) Recipients received 2.0×10^6 hepatocytes and 1600 islet equivalents (IEQs) islets into the renal subcapsular spaces on both sides (total 4.0×10^6 hepatocytes and 3200 IEQs islets; Co-transplantation [CoTx] group). (c) Effects of islet co-transplantation site on the engraftment of transplanted hepatocytes. Recipients were transplanted with 4.0×10^6 hepatocytes into renal subcapsular space in one side (total 4.0×10^6 hepatocytes; Hemi-Hepatocyte [Hemi-HTx] group), 4.0×10^6 hepatocytes together with 3200 IEQs islets into the renal subcapsular space on one side (total 4.0×10^6 hepatocytes with 3200 IEQs islets; Hemi-Cotransplantation [Hemi-CoTx] group), or 4.0×10^6 hepatocytes into one renal subcapsular space with 3200 IEQs islets into the opposite side renal subcapsular space (total 4.0×10^6 hepatocytes, 3200 IEQs islets; Separate-Cotransplantation [Separate-CoTx] group)

TABLE 1 A summary of the *in vivo* experimental design in the present study

(a)					
		HTx		CoTx	
Groups	Kidney	Right	Left	Right	Left
	Hepatocyte	2.0×10^6	2.0×10^6	2.0×10^6	2.0×10^6
	Islet (IEQs)	No	No	1600	1600
Total amount	Hepatocyte	4.0×10^6		4.0×10^6	
	Islet (IEQs)	No		3200	

(b)							
		Hemi-HTx		Hemi-CoTx		Separate-CoTx	
Groups	Kidney	Right	Left	Right	Left	Right	Left
	Hepatocyte	No	4.0×10^6	No	4.0×10^6	No	4.0×10^6
	Islet (IEQs)	No	No	No	3200	3200	No
Total amount	Hepatocyte	4.0×10^6		4.0×10^6		4.0×10^6	
	Islet (IEQs)	No		3200		3200	

TABLE 2 A summary of the *in vitro* experimental design in the present study

(a) Groups	Hepatocyte	Co-Culture
Hepatocyte per 9.5 cm^2 (six-well plate)	1.0×10^6	1.0×10^6
Islet per 9.5 cm^2 (IEQs)	No	400
Culture medium	Basal medium	Basal medium

(b) Groups	Basal medium	Islet supernatant	Coculture supernatant
Hepatocyte per 9.5 cm^2 (six-well plate)	1.0×10^6	1.0×10^6	1.0×10^6
Culture medium	Basal medium	Islet culture supernatant	Co-culture supernatant

(c) Groups	Basal medium	Coculture supernatant	Anti-insulin	Anti-VEGF	GLP-1 antagonist
Hepatocyte per 1.9 cm^2 (24-well plate)	2.0×10^5	2.0×10^5	2.0×10^5	2.0×10^5	2.0×10^5
Culture medium	Basal medium	Coculture supernatant	Coculture supernatant	Coculture supernatant	Coculture supernatant
Antibody and antagonist	No	No	Anti-insulin receptor	Anti-VEGF	GLP-1 antagonist

2.8 | Evaluation of the hepatocyte viability and function in vitro

After 3 days of culture, evaluation of the hepatocyte viability and function was conducted. DNA quantification was performed to determine the number of cultured hepatocytes. The DNA content was measured using a DNA quantification kit (Primary cell, Ishikari, Japan), as previously described (Fukuoka et al., 2017; Matsumura et al., 2019) ($n = 12$). Hepatocyte viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT (Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg/ml, and incubated for at 37°C with 5% CO_2 for 3 h. Then, DMSO was added to each well to dissolve the purple formazan product. The absorbance measured at 535 nm using 96-well plate reader (Thermo Fisher Scientific) was shown as the percentage in comparison to the Hepatocyte group ($n = 10$). An ammonia removal assay was performed to evaluate the function of the hepatocytes. The culture supernatant was replaced with 1.0 ml/1 $\times$ 10^6 cells of ammonia-load culture medium (Williams Medium E containing approximately 2.2 mmol/L ammonia, 10% FBS, 1 μmol/L insulin, and 1 μmol/L dexamethasone). Hepatocytes were cultured at 37°C in 5% CO_2, then the concentration of ammonia in the medium at 0 and 6 h was measured using an Amicheck Meter (Arkray). The ammonia concentration at 6 h was shown as the percentage in comparison to the concentration at 0 h ($n = 14$). To

evaluate the albumin secretion function of hepatocytes, the albumin concentration in the culture medium was quantified using a LBIS Rat Albumin ELISA kit (Shigayagi) ($n = 35$).

A cytochrome P450 1A (CYP1A) activity was measured using commercial cell-based assays (P450-Glo™ Assays; Promega Corp.) to evaluate drug-metabolism function of the hepatocytes. Hepatocytes were seeded in the six-well-plate at 1.0×10^6 cells/well in basal medium, and cultured for 2 h. Then the inserts were placed onto the provided wells, and islets were seeded in inserts at 0 (Hepatocyte group) or 400 IEQs (Co-culture group)/well. After 3 days of culture, medium was replaced with 1 ml of medium containing 100 μM luminescent probes (Luciferin-CEE (6-chloroethyl ether)). After incubation for 4 h, 50 μl of medium was aliquoted into 98 well white-walled microplate (Thermo Fisher Scientific) and a luciferase reagent was added at an equal volume to the wells and incubated for 20 min, then luminescence was measured using a plate reader (Luminoskan Ascent, Thermo Fisher Scientific). The CYP1A activity was normalized using a luciferin standard.

2.9 | Effect of culture supernatants on the hepatocyte function

Isolated hepatocytes were incubated with basal medium (Basal medium group, $n = 11$), islet culture supernatant (Islet supernatant group, $n = 12$) or coculture supernatant (Coculture supernatant group, $n = 12$). Coculture supernatants were prepared for this experiment using coculture system. Islet culture supernatant was obtained by culturing islets alone. Table 2b shows a summary of the designs of the experiments in the present study.

2.10 | Isolation and evaluation of exosomes derived from coculture supernatant

After coculture for 3 days, the supernatant was collected and centrifuged at 2200×*g* for 15 min at 4°C. To remove cellular debris, the supernatant was filtered with a 0.22 μm filter (Sartorius, Gottingen, Germany). The coculture supernatant was then ultracentrifuged at 110,000×*g* for 70 min at 4°C. The exosome fractions were washed with 12.5 ml phosphate-buffered saline (PBS), and after ultracentrifugation, they were resuspended in PBS.

The protein contents of exosome were determined using a micro BCA protein assay kit (Thermo Scientific). Exosomes were resuspended in PBS at a concentration of approximately 10 μg protein/ml for the analysis. Measurement of size distribution and particle number of exosomes was carried out using the NanoSight NS300 system (Malvern Panalytical) and Nano Tracking Analysis software program (Malvern Panalytical).

To assess whether exosomes obtained from coculture supernatant could affect viability of hepatocytes, we cultured hepatocytes in William's E medium supplemented with several concentrations of exosomes (0, 0.2, and 2.0 μg/ml) ($n = 3$). We also examined the influence of exosome depletion on hepatocyte viability. As the exosomes depleted coculture supernatant, the supernatant obtained after ultracentrifugation of the co-culture supernatant was used. Hepatocyte (2×10^5cells) were cultured in 500 μl of basal medium supplemented with exosomes (0, 0.2, and 2.0 μg/ml in a type I collagen-coated 24-well-plate (BD Falcon). Hepatocytes were also cultured in coculture supernatant with and without exosomes. After incubation for 3 days, the viability and function of each hepatocyte were examined according to an ammonia removal assay.

2.11 | Measurement of inflammatory and metabolic factors in the culture supernatant

The IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-18, IFN-G, EGF, MCP-1, IP-10, GRO/KC, VEGF, Fractalkine, LIX, MIP-1a, MIP-2, TNF-α, and RANTES levels in the culture supernatant were detected with the Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead Panel (Millipore) using a Bio-Ple×200 system (Bio-Rad, Hercules). Ghrelin, GIP, GLP-1, glucagon, leptin, PP, PYY, and insulin levels were determined using the Rat Metabolic Hormone Magnetic Bead Panel (Millipore).

2.12 | Effect of the inhibition of candidate factors in the co-culture supernatant on the hepatocyte function

Hepatocytes were seeded on type I collagen-coated 24-well (Corning) at 2.0×10^5 hepatocytes/well in 0.5 ml of culture supernatant. Hepatocytes were cultured with basal medium (hepatocyte group, $n = 13$), coculture supernatant (coculture supernatant group, $n = 13$), coculture supernatant together with anti-insulin receptor antibody (bs-0290R, Bioss Inc) (Anti-insulin group, $n = 8$), coculture supernatant together with 4 μg/ml anti-VEGF antibody (ab9570, Abcam) (Anti-VEGF group, $n = 5$), or coculture supernatant together with 50 ng/ml GLP-1 antagonist (ab141101, Abcam) (GLP-1 antagonist group, $n = 5$). The amounts of anti-VEGF antibody and GLP-1 antagonist to sufficiently exert inhibitory effects were determined based on a product data sheet or previous reports (Meurer, Colca, Burton, & Elhammer, 1999). Anti-insulin receptor antibody was purified using the concentration kit (ab102778, Abcam) to remove sodium azide, and at least 8.2 μg/ml antibody was included in the coculture supernatant. Hepatocytes were cultured at 37°C under 5% CO_2 for 3 days; then an ammonia removal assay was performed. Table 2c shows a summary of the designs of the experiments in the present study.

2.13 | Statistical analysis

All values were expressed as the means and standard deviation. All statistical analyses were performed using the JMP pro 14 software program (SAS institute Inc.). Student's *t*-test was used for comparisons of quantitative variables between the two groups. The serum albumin levels were analyzed by a two-way analysis of variance

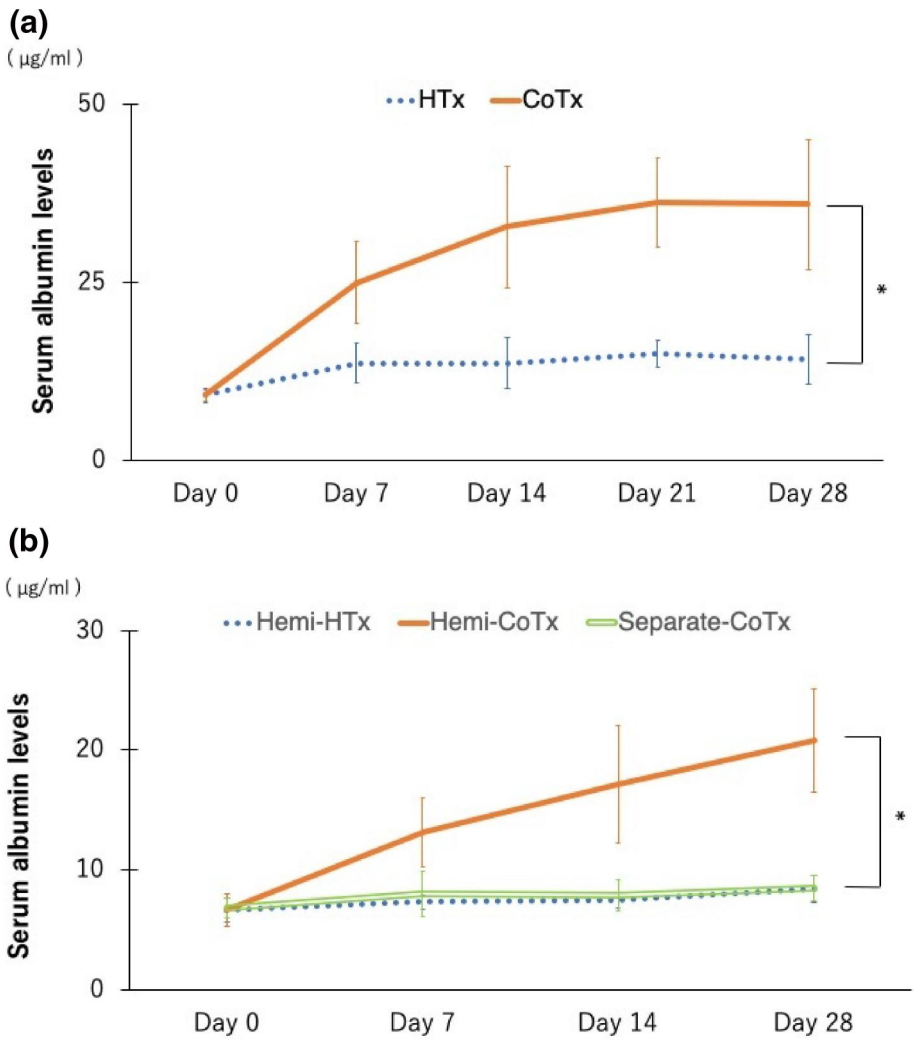

FIGURE 2 Evaluation of hepatocyte engraftment according to recipient's serum albumin level. (a) The effect of islet co-transplantation on the engraftment of transplanted hepatocytes. The serum albumin level of the CoTx group was significantly higher in comparison to the HTx group (*$p < 0.001$). (b) The effect of the islet co-transplantation site on the engraftment of transplanted hepatocytes. The serum albumin level of the Hemi-CoTx group was significantly higher in comparison to the Hemi-HTx and Separate-CoTx groups (*$p < 0.001$). No significant difference was observed between the Hemi-HTx and Separate-CoTx groups ($p = 0.947$) [Colour figure can be viewed at wileyonlinelibrary.com]

(ANOVA). A one-way ANOVA, followed by Tukey-Kramer test was used for the comparison of three or more groups. *p*-values of <0.05 were considered to indicate statistical significance.

3 | RESULTS

3.1 | Effect of islet co-transplantation on the engraftment of transplanted hepatocytes

Hepatocyte engraftment was evaluated according to the serum albumin levels in the recipients. In the islet CoTx group, the serum albumin levels gradually increased during the observation period. In contrast, they appeared to reach a plateau 7 days after hepatocyte transplantation and remained low in the HTx group (Figure 2a). The serum albumin levels of the CoTx group (Pre-transplantation: 9.11 ± 0.82 μg/ml, Day 7: 24.96 ± 5.81 μg/ml, Day 14: 32.73 ± 8.47 μg/ml, Day 21: 36.20 ± 6.27 μg/ml, Day 28: 35.92 ± 9.17 μg/ml) were significantly higher than those of the HTx group (Pre-transplantation: 9.16 ± 0.98 μg/ml, Day 7: 13.64 ± 2.85 μg/ml, Day 14: 13.58 ± 3.56 μg/ml, Day 21: 14.96 ± 1.86 μg/ml, Day 28: 14.10 ± 3.47 μg/ml) ($p < 0.001$) (Figure 2a).

3.2 | Effect of islet co-transplantation site on the engraftment of transplanted hepatocytes

The serum albumin levels of the Hemi-CoTx group (Pre-transplantation: 6.70 ± 1.37 μg/ml, Day 7: 13.14 ± 2.93 μg/ml, Day 14: 17.19 ± 4.86 μg/ml, Day 28: 20.81 ± 4.26 μg/ml) were significantly higher than those of the Hemi-HTx (Pre-transplantation: 6.68 ± 1.20 μg/ml, Day 7: 7.32 ± 0.63 μg/ml, Day 14: 7.47 ± 0.72 μg/ml, Day 28: 8.40 ± 1.21 μg/ml) or the Separate-CoTx group (Pre-transplantation: 6.83 ± 0.82 μg/ml, Day 7: 7.97 ± 1.88 μg/ml, Day 14: 7.94 ± 1.28 μg/ml, Day 28: 8.49 ± 1.07 μg/ml) ($p < 0.001$), suggesting that the beneficial effect of islet co-transplantation might be

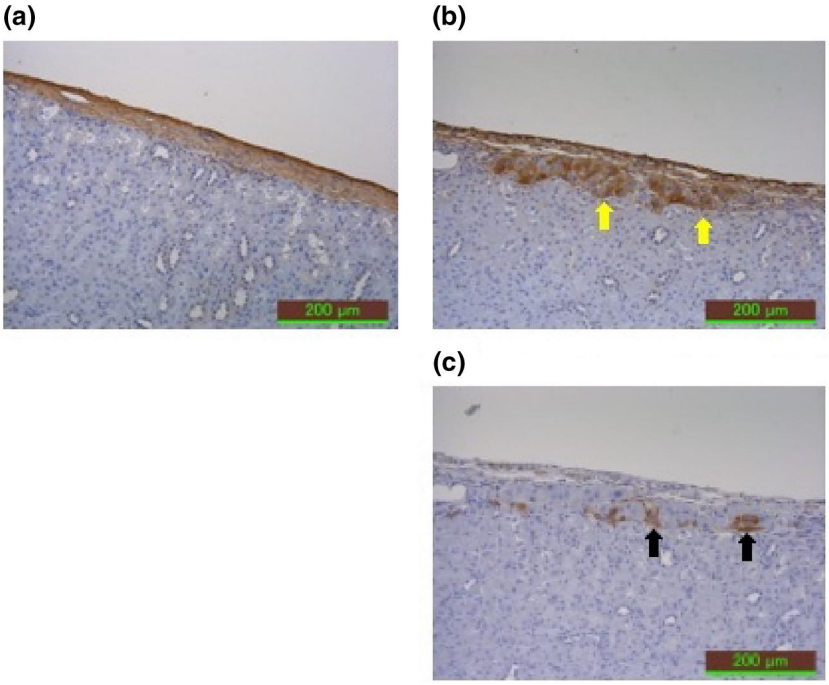

FIGURE 3 Immunohistochemical staining of transplanted hepatocytes and islets. Transplanted hepatocytes with (CoTx group) and without (HTx group) islet co-transplantation were evaluated by albumin (a and b) and insulin staining (c) (scale bar: 200 μm). (a) HTx group, albumin staining: albumin-positive hepatocytes formed a monolayer. (b) CoTx group, albumin staining: transplanted hepatocytes penetrated the renal parenchyma and formed multiple layers (yellow arrows). (c) CoTx group, insulin staining: insulin-positive islets located in the renal parenchyma are indicated by black arrows

dependent on the distance between hepatocytes and islets (Figure 2b). No significant difference was observed between the Hemi-HTx and Separate-CoTx groups ($p = 0.947$) (Figure 2b).

3.3 | Immunohistochemical staining of transplanted hepatocytes and islets

Transplanted hepatocytes with (CoTx group) and without (HTx group) islet co-transplantation were evaluated by albumin (Figure 3a,b) and insulin staining (Figure 3c). In the HTx group, albumin-positive hepatocytes that remained in the renal subcapsular space subsequently formed a monolayer (Figure 3a). In contrast, the majority of transplanted hepatocytes penetrated the renal parenchyma and formed multiple layers in the CoTx group (Figure 3b). Likewise, insulin-positive islet cells were detected in the kidney parenchyma in this group (Figure 3c). Taken together, the transplanted hepatocytes in the CoTx group appeared to be located in close proximity to the islets.

3.4 | Effect of islet co-culture on the hepatocyte viability and function in an in vitro model

3.4.1 | DNA quantitation

In the DNA quantitation, no significant difference was observed between the Hepatocyte group and coculture group (20.62 ± 5.74 vs. 22.27 ± 5.34 μg) ($p = 0.49$) (Figure 4a).

3.4.2 | MTT assay

The viability of hepatocytes in the coculture group was significantly higher than that in the hepatocyte group (229.94 ± 66.68 vs. 100.00 ± 0.00%, $p < 0.001$) (Figure 4b).

3.4.3 | Ammonia removal assay

The function of the hepatocytes was evaluated by an ammonia removal assay. The ammonia metabolic rate at 6 h in the co-culture group was significantly higher than that in the hepatocyte group (70.78 ± 4.30 vs. 36.49 ± 6.45%, $p < 0.001$) (Figure 4c).

3.4.4 | Albumin secretion function

In the coculture group, the albumin concentration in the medium was significantly higher than that in the hepatocyte group (3.90 ± 0.70 vs. 2.30 ± 0.57 μg/ml, $p < 0.001$) (Figure 4d).

3.4.5 | CYP1A activity

The CYP1A activity in the co-culture group was significantly higher than that of the hepatocyte group (6.86 ± 5.33 vs. 2.93 ± 1.78 pmol/well/h, $p < 0.01$) (Figure 4e).

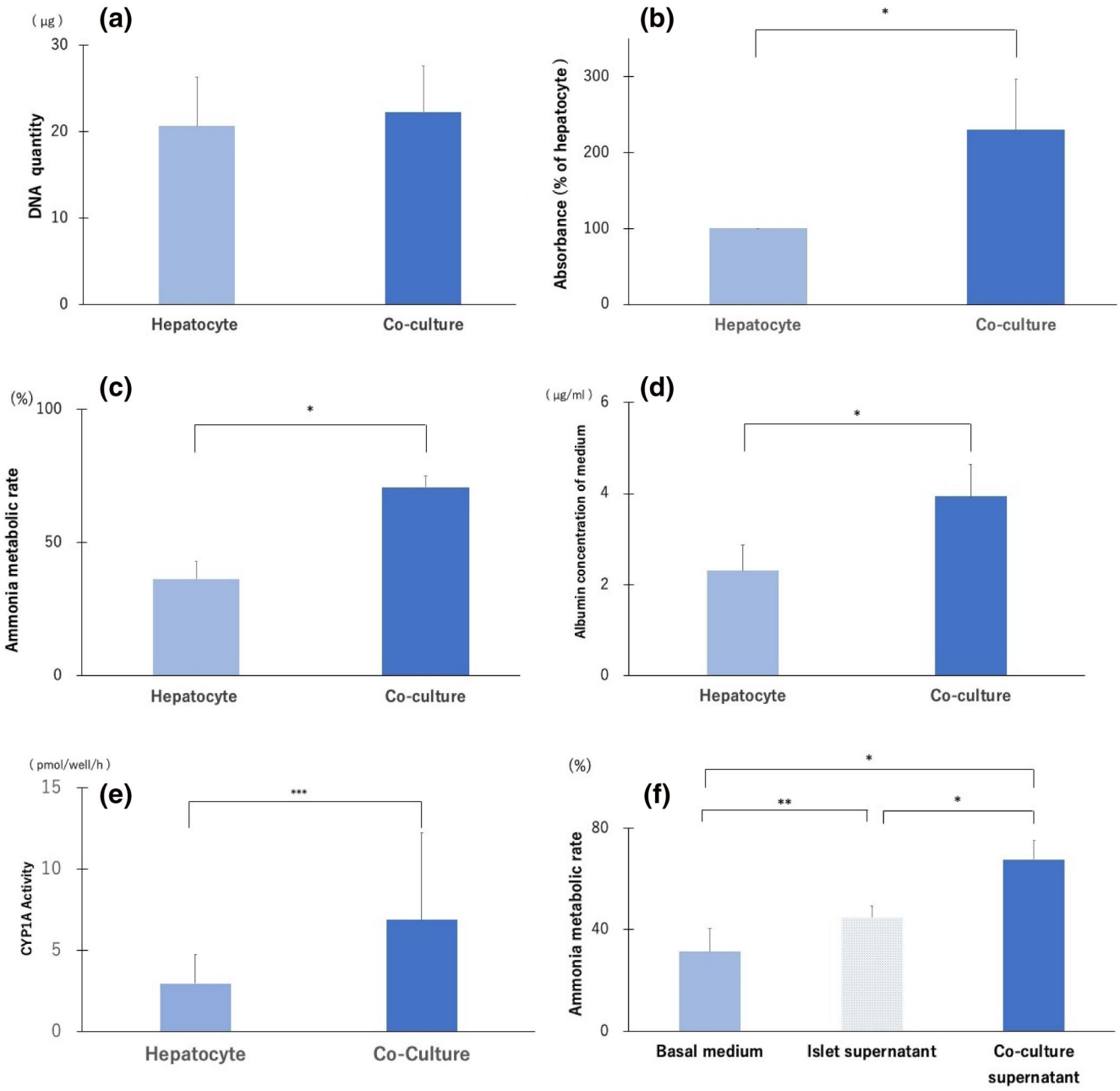

FIGURE 4 Effects of islet coculture or various culture supernatants on the viability and function of hepatocytes in an in vitro model. (a) DNA quantitation between the hepatocyte and co-culture groups. No significant difference was observed between the hepatocyte and coculture groups ($p = 0.49$). (b) MTT assay between the hepatocyte and coculture groups. The viability of hepatocytes in the co-culture group ($n = 10$) was significantly higher than that in the hepatocyte group ($n = 10$) (*$p < 0.001$). (c) Ammonia removal assay between the hepatocyte and coculture groups. The ammonia metabolic rate at 6 h in the co-culture group ($n = 14$) was significantly higher than that in the hepatocyte group ($n = 14$) (*$p < 0.001$). (d) The albumin secretion function between the hepatocyte and coculture groups. The albumin concentration in the medium of the co-culture group ($n = 35$) was significantly higher than that in the hepatocyte group ($n = 35$) (*$p < 0.001$). (e) The CYP1A activity between the hepatocyte and co-culture groups. The CYP1A activity was significantly higher in the co-culture group ($n = 10$) compared to the hepatocyte group ($n = 10$) (***$p < 0.01$). (f) Ammonia removal assay among the Basal medium, Islet supernatant, and coculture supernatant groups. The ammonia metabolic rate at 6 h in the Islet supernatant group ($n = 10$) was significantly higher than that in the Basal medium group ($n = 10$) (**$p = 0.001$). Of particular interest, the ammonia metabolic rate in the coculture supernatant group ($n = 10$) was significantly higher than not only that in the Basal medium group ($n = 10$) (*$p < 0.001$) but also that in the Islet supernatant group ($n = 10$) (*$p < 0.001$) [Colour figure can be viewed at wileyonlinelibrary.com]

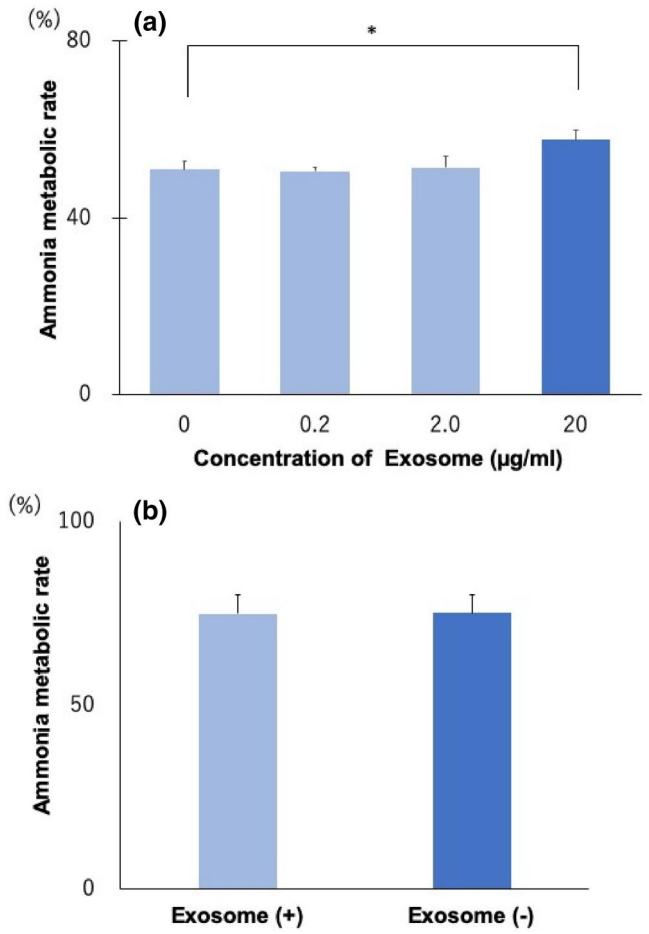

FIGURE 5 Effect of exosomes derived from co-culture supernatant on the hepatocyte function in an in vitro model. (a) Hepatocytes were cultured in medium containing different concentrations of exosomes ($n = 3$), or (b) coculture supernatant with (Exosome+, $n = 3$) and without (Exosome−, $n = 3$) exosomes. The hepatocyte function was evaluated according to the ammonia metabolic rate during a 3 h ammonia removal assay (a and b). The ammonia metabolic rate at 3 h in the 20 μg/ml exosome group was significantly higher than that in the 0 μg/ml group. However, no significant difference was observed between the Exosome (+) and Exosome (−) groups [Colour figure can be viewed at wileyonlinelibrary.com]

3.5 | Effect of various culture supernatants on the hepatocyte function in an in vitro model

The hepatocyte function was evaluated by an ammonia removal assay. The ammonia metabolic rate at 6 h in the Islet supernatant group was significantly higher than that in the Basal medium group (44.77 ± 4.71% vs. 31.42 ± 9.17%, $p = 0.001$) (Figure 4f). Of particular interest, the ammonia metabolic rate in the coculture supernatant group was significantly higher than not only that in the Basal medium group (67.67 ± 7.48% vs. 31.42 ± 9.17%, $p < 0.001$) but also that in the Islet supernatant group (67.67 ± 7.48% vs. 44.77 ± 4.71%, $p < 0.001$) (Figure 4f).

3.6 | Effect of exosomes derived from coculture supernatant on the hepatocyte function in an in vitro model

The amount of exosomes in the coculture supernatant was 0.37 μg/ml. The size distribution of exosomes in the coculture supernatant was determined using a nanoparticle tracking system, then revealed the peak of vesicle size was 55 nm, and mean size was 129 nm (Figure S1). An ammonia removal assay was performed to evaluate the contribution of exosomes in the coculture supernatant to the hepatocyte function. The ammonia metabolic rate at 3 h in the 20 μg/ml exosome group was significantly higher than that in the 0 μg/ml group (Figure 5a). However, no significant difference was observed between the Exosome (+) and Exosome (−) groups (Figure 5b).

3.7 | Identification of factors crucial for the hepatocyte function in the coculture supernatant

The supernatant derived from hepatocytes only cultured (hepatocyte supernatant group), islet only cultured (Islet supernatant group) and coculture (coculture group) were analyzed using a Milliplex MAP Rat Cytokine/Chemokine and Rat Metabolic Hormone Magnetic Bead Panel. The factors that were significantly enriched in the coculture group, in comparison to the hepatocyte supernatant group and Islet supernatant group were identified as the candidates for crucial factors on hepatocyte function. Insulin, VEGF, GLP-1, LIX, and IL-18 were selected as candidate factors (Table 3, Figure S2).

3.8 | Evaluation of the hepatocyte function by inhibition of candidate factors in the co-culture supernatant

Among the five candidate factors identified by a Milliplex analysis, it was hypothesized—based on previous reports—that both LIX and IL-18 in the coculture supernatant are most likely upregulated due to enhanced inflammation based on high density of cells in the well. We therefore focused on insulin, VEGF, and GLP-1, and then performed an inhibition assay using neutralizing antibodies or antagonist peptide. The ammonia metabolic rate at 3 h in the coculture supernatant group was significantly higher than that in the Basal medium group (64.59 ± 10.09% vs. 30.99 ± 12.20%, $p < 0.001$). Although no inhibitory effects were seen in the Anti-VEGF (65.64 ± 10.03%) and GLP-1 antagonist groups (68.87 ± 6.82%), the ammonia metabolic rate in the Anti-insulin group was significantly lower than that in the coculture supernatant group (30.31 ± 7.00% vs. 64.59 ± 10.09%, $p < 0.001$). No significant difference was observed between the Basal medium and anti-insulin groups (30.99 ± 12.20% vs. 30.31 ± 7.00%, $p = 0.99$) (Figure 6).

TABLE 3 A summary of factors crucial for the hepatocyte function in the co-culture supernatant

	Mean ± SD			*p*-Value		
	Coculture supernatant (pg/ml) (n = 11)	Hepatocyte supernatant (pg/ml) (n = 12)	Islet supernatant (pg/ml) (n = 12)	Co-culture supernatant versus Hepatocyte supernatant	Co-culture supernatant versus Islet supernatant	Hepatocyte supernatant versus Islet supernatant
Insulin	1842012.50 ± 957508.74	181.25 ± 279.44	916340.25 ± 537172.73	<0.001*	0.003*	0.003*
VEGF	6875.14 ± 1086.33	1218.99 ± 187.81	1667.31 ± 552.46	<0.001*	<0.001*	0.271
GLP-1	1053.34 ± 399.19	4.96 ± 12.93	61.32 ± 67.81	<0.001*	<0.001*	0.816
LIX	41.93 ± 21.74	7.23 ± 18.01	0.00 ± 0.00	<0.001*	<0.001*	0.521
IL-18	42.51 ± 6.34	32.81 ± 6.82	0.28 ± 0.91	<0.001*	<0.001*	<0.001*

*$p < 0.05$.

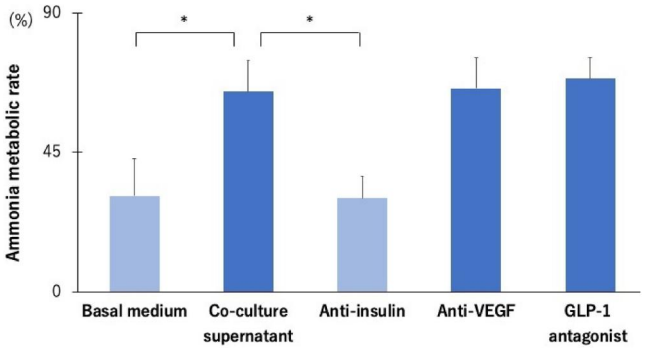

FIGURE 6 Evaluation of the hepatocyte function by inhibition of candidate factors in coculture supernatant. The ammonia metabolic rate at 3 h in the coculture supernatant group (n = 13) was significantly higher than that in the Basal medium group (n = 13) (*$p < 0.001$). Although no inhibitory effects were seen in the Anti-VEGF and GLP-1 antagonist groups (n = 5), the ammonia metabolic rate in the anti-insulin group was significantly lower than that in the coculture supernatant group (*$p < 0.001$, n = 8). No significant difference was observed between the Basal medium and anti-insulin groups [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

In the present study, we—for the first time—quantitatively demonstrated that islet co-transplantation could promote engraftment in hepatocyte transplantation. Ricordi et al. (1989) previously reported that islet co-transplantation may provide morphological benefits for transplanted hepatocytes. However, no groups have thus far shown functional advantages on hepatocyte grafts. Unlike the previous reports (Hayashi et al., 2007), in the present study, we did not use commercially available Nagase analbuminemic rats (not syngeneic combination), instead we used mutant F344 rats with defective albumin production (kindly provided by Professor Nishikawa) (Fukuoka et al., 2017; Matsumura et al., 2019). Thus, we could focus on physiological engraftment without paying any attention to the immunological influences, since these mutant rats are a syngeneic combination. In addition, unlike Nagase analbuminemic rats, we could clearly detect a significant difference in the hepatocyte engraftment of the groups using this combination, since the basal albumin levels of these rats are almost zero. We believe that this useful animal model greatly contributed to our findings.

Of particular interest, the beneficial effects of islet co-transplantation on hepatocyte engraftment were not observed when exactly the same amount of islets was separately transplanted into the renal subcapsular space of the opposite side. This novel finding suggests that transplanted hepatocytes need to be present near the islets in order for the recipient to receive benefits from them. This may be explained by the importance of cell-to-cell contact and/or exposure to high concentrations of crucial factors derived from the islets. Considering the outcome of the in vitro coculture experiments in the present study (Figures 4 and 5), the latter appears to play a more important role than the former. Another possible explanation for the abovementioned novel finding was obtained from immunohistochemical evaluation (Figure 3). Originally, islet grafts transplanted into the kidney subcapsular space have the property of not staying within the subcapsular space but penetrating into the kidney parenchyma, which is well vascularized in comparison to the renal surface space, most likely due to the autocrine effects of islets per se (Ricordi et al., 1991; Wennberg et al., 2005); thus, it is well known that the kidney subcapsular space is the preferred transplantation site for islet engraftment (Goto et al., 2006) (Wennberg et al., 2005) (Goto, Groth, Nilsson, & Korsgren, 2004). Considering these characteristics, the outcomes of Figure 3b may suggest that hepatocyte grafts attached to the islet surface by co-transplantation also penetrate into the renal parenchyma together with the islets, then hepatocyte engraftment is improved by better vascularization from the recipient. In sharp contrast, the Figure 3a demonstrates that this is not the case in hepatocyte transplantation alone. Since this effect cannot be expected when hepatocytes and islets are transplanted separately, it appears to be quite logical.

In order to narrow down and identify crucial factors originated from islets, it is necessary to establish a useful in vitro model that mimics the effects of islet co-transplantation. We established a hepatocyte and islet coculture system, and evaluated this model by assessing the viability and function with several assays (Fukuoka

et al., 2017; Matsumura et al., 2019) (Enosawa, 2017). In this model, the viability of hepatocytes in the coculture group was more than twice that in the hepatocyte group. Furthermore, the ammonia metabolic rate, CYP1A activity, and albumin secretion ability of hepatocytes in the coculture group were also significantly higher than those in the hepatocyte group, suggesting that this model could be a useful screening system for identifying crucial factors of islet co-transplantation effects. Considering that the rate of hepatocyte improvement in the coculture group was almost identical among all of the evaluation assays, it was hypothesized that the main beneficial effect of islet coculture was the improvement of the viability of hepatocytes (as opposed to their function). Notably, the beneficial effects of coculture were also confirmed in an in vitro model using a cell insert system, strongly suggesting that hepatocytes may receive benefits of coculturing through crucial factors without the need for cell-to-cell contact between hepatocytes and islets. This finding was consistent with the previous report by Kaufmann et al. (1999).

The present study clearly showed that coculture supernatant alone could also provide beneficial effects on hepatocytes that were comparable to coculture with islets (Figure 4c,f). This finding suggests the possibility that hepatocyte engraftment can be improved through the use of a frozen coculture supernatant, without preparing islets at the time of transplantation. This is extremely significant from a clinical viewpoint. Therefore, a development of useful device which can continuously release the coculture supernatant in the limited space near the hepatocyte grafts would be warranted. Interestingly, only a marginal effect was observed by adding the culture supernatant of islets alone; the coculture supernatant was clearly more effective (Figure 4f). This implies that the effect of co-transplantation is not solely caused by the addition of islets, and that interaction between the hepatocytes and islets may effectively activate the production of key factors, and consequently enhance the beneficial effects (J. G. Lee et al., 2018). The contribution of exosome involvement was estimated to be low in the present in vitro model, but it was also suggested that exosomes might have dose-dependent effects (Figure 5a). Thus, in the in vivo model, the partial contribution of exosomes cannot be ruled out.

Based on the novel finding that the supernatant of the coculture was clearly more effective than the supernatant of islets alone, among the various inflammatory and metabolic factors, we extracted several factors that were significantly upregulated in the coculture group in comparison to both the islet-alone and hepatocyte-alone groups. As a result, insulin, VEGF, GLP-1, LIX, and IL-18 were selected as candidate crucial factors (Table 3, Figure S2). However, LIX and IL-18 are chemokines and cytokines caused by ischemia and/or inflammation (Erikson et al., 2017), and both are also known to cause strong inflammation and damage to cells (Wilson et al., 2015). Thus, in this study, we focused on insulin, VEGF, and GLP-1, and subsequently performed inhibition assays for these factors. Unlike the Anti-VEGF and GLP-1 antagonist group, the ammonia metabolic rate in the anti-insulin group was significantly lower than that in the coculture group (Figure 6). Kaufmann et al. suggested that not only insulin but also glucagon was involved in the coculture of hepatocytes and islets (Kaufmann et al., 1999). In contrast, in the present study, no upregulation of glucagon was observed in the coculture supernatant group. This discrepancy is most likely due to the differences in culture conditions, such as the type of culture medium and the mixing ratio of hepatocytes and islets. Anti-glucagon antibodies are a topic of interest for our future study. Although the present study clearly suggests that insulin is a key factor for the improvement of hepatocyte engraftment, it cannot be denied that multiple secreted proteins, such as VEGF and GLP-1, which on their own could not provide sufficient inhibition, cooperated and partially contributed to the beneficial effects of coculture, since the in vitro model applied in the present study was a mimic of the in vivo model, not exactly the same system. Taken together, crucial secreted proteins derived from islets, mainly insulin, and microRNA derived from extracellular vesicles (e.g., exosomes) may synergistically contribute to the improvement of hepatocyte engraftment. Given that the liver is the current standard transplant site for both hepatocyte and islet grafts (Goto, Johansson, et al., 2004) (Tokodai et al., 2010), the application of the present finding to intraportal HTx is another important issue to be investigated in the near future.

In summary, the present study proved that islet co-transplantation could improve the engraftment of transplanted hepatocytes and this was most likely due to continuously secreted crucial factors (e.g., insulin) in combination with providing favorable circumstances for hepatocyte engraftment (kidney parenchyma). Further refinements of this approach—especially with regard to the substitutes for the islets—could be a promising strategy for ameliorating the outcomes of hepatocyte transplantation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in association with the present study.

AUTHOR CONTRIBUTIONS

Yoshikatsu Saitoh and Masafumi Goto participated in the research design, the performance of the research and the writing of the paper. Akiko Inagaki participated in the performance of the research and the writing of the paper. Takehiro Imura, Hiroyasu Nishimaki, Hiroyuki Ogasawara, Muneyuki Matsumura, and Shigehito Miyagi participated in the performance of the research. Ibrahim Fathi, Yohichi Yasunami, Michiaki Unno, and Takashi Kamei participated in the writing of the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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