

Table II. Structures and relative quantities of neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate PA-oligosaccharides derived from human and porcine islets

Peak code number	GU ^a ODS (Amid)	Molecular ^b mass (Da)	Structure ^c	Relative quantity (%) ^d	
				Pig	Human
Mono-sialyl glycan					
pM2-1	9.0 (5.4)	1646	$\begin{array}{c} \text{Man } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.2	–
pM3-1	11.9 (5.9)	1792	$\begin{array}{c} \text{Man } \alpha \text{ 1-6} \diagdown \quad \text{Fuc } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.3	–
hM2-1	7.9 (6.0)	1646	$\begin{array}{c} \text{Man } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-6Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	–	0.15
pM1 = hM3	8.6 (7.1)	1970	$\begin{array}{c} \text{Man } \alpha \text{ 1-6} \diagdown \\ \text{Man } \alpha \text{ 1-3} \diagup \quad \text{Man } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.6	0.2
pM2-2	9.0 (6.2)	1808	$\begin{array}{c} \text{Man } \alpha \text{ 1-3} \diagup \quad \text{Man } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.3	–
pM3-2	11.9 (6.7)	1954	$\begin{array}{c} \text{Man } \alpha \text{ 1-3} \diagup \quad \text{Man } \alpha \text{ 1-6} \diagdown \quad \text{Fuc } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.3	–
hM1	7.6 (7.7)	1970	(Hexose) ₆ (HexNAc) ₃ (NEuAc) ₁ (PA) ₁ ^e	–	0.2
hM2-2	7.9 (6.8)	2255	(Hexose) ₄ (HexNAc) ₆ (NeuAc) ₁ (PA) ₁ ^e	–	0.15
hM4-1	11.2 (6.4)	1792	$\begin{array}{c} \text{Man } \alpha \text{ 1-6} \diagdown \quad \text{Fuc } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-6Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	–	0.1
hM4-2	11.2 (6.7)	2011	$\begin{array}{c} \text{Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	–	0.4
pM4 = hM5	13.5 (7.6)	2157	$\begin{array}{c} \text{Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-6} \diagdown \quad \text{Fuc } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-6Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.5	0.5
pM5	14.4 (6.2)	1995	$\begin{array}{c} \text{GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-6} \diagdown \quad \text{Fuc } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.6	–
M6	15.1 (7.1)	2157	$\begin{array}{c} \text{Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-6} \diagdown \quad \text{Fuc } \alpha \text{ 1-6} \diagdown \\ \text{Neu5Ac } \alpha \text{ 2-3Gal } \beta \text{ 1-4GlcNAc } \beta \text{ 1-2Man } \alpha \text{ 1-3} \diagup \\ \text{Man } \beta \text{ 1-4GlcNAc } \beta \text{ 1-4GlcNAc-PA} \end{array}$	0.6	2.1

^aUnits of GU were calculated from the elution times of the peaks obtained from the ODS column in Figure 2 and the Amide column in Figure 3.

^bAverage mass calculated from the m/z values of $[M + Na]^+$ or $[M + H]^+$ ion for neutral, $[M - H]^-$ ion for mono-sialyl and mono-sulfated and $[M + Na - 2H]^-$ ions for mono-sialyl-mono-sulfated and di-sulfated PA-oligosaccharides (Supplementary data, Figure S1).

^cStructures of PA-oligosaccharides are represented.

^dMolecular percentage of was calculated from the peak area in Figure 2 by comparison with total N-glycan content in each islet tissue.

^eN-glycans did not coincide with those of known references in the GALAXY database.

Table III. Structures and relative quantities of neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate PA-oligosaccharides derived from human and porcine islets

Peak code number	GU ^a ODS (Amid)	Molecular ^b mass (Da)	Structure ^c	Relative quantity (%) ^d		
				Pig	Human	
Di-sialyl glycan						
D1	10.6 (7.5)	2302	Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-6	Man β 1-4GlcNAc β 1-4GlcNAc-PA	0.2	0.4
			Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-3			
hD2	12.1 (6.5)	2302	Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-2Man α 1-6	Man β 1-4GlcNAc β 1-4GlcNAc-PA	-	0.3
			Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-2Man α 1-3			
pD2 = hD3	13.5 (7.9)	2448	Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-6	Man β 1-4GlcNAc β 1-4GlcNAc-PA	0.8	0.2
			Neu5Ac α 2-6Gal β 1-4GlcNAc β 1-2Man α 1-3			
pD3 = hD4	15.8 (6.9)	2448	Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-2Man α 1-6	Man β 1-4GlcNAc β 1-4GlcNAc-PA	0.5	0.9
			Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-2Man α 1-3			

^aUnits of GU were calculated from the elution times of the peaks obtained from the ODS column in Figure 2 and the Amide column in Figure 3.

^bAverage mass calculated from the m/z values of $[M + Na]^+$ or $[M + H]^+$ ion for neutral, $[M - H]^-$ ion for mono-sialyl and mono-sulfated and $[M + Na - 2H]^-$ ions for mono-sialyl-mono-sulfated and di-sulfated PA-oligosaccharides (Supplementary data, Figure S1).

^cStructures of PA-oligosaccharides are represented.

^dMolecular percentage of was calculated from the peak area in Figure 2 by comparison with total *N*-glycan content in each islet tissue.

^e*N*-glycans did not coincide with those of known references in the GALAXY database.

Table IV. Structures and relative quantities of neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate PA-oligosaccharides derived from human and porcine islets

Peak code number	GU ^a ODS (Amid)	Molecular ^b mass (Da)	Structure ^c	Relative quantity (%) ^d	
				Pig	Human
Mono-sulfated glycan					
S1-1	7.3 (3.8)	1478	(Hexose) ₃ (HexNAc) ₄ (HSO ₃) ₁ (PA) ₁ ^e	0.2	-
S1-2	7.3 (4.5)	1641	SHO ₃ \backslash Man α 1-3 \backslash Man α 1-6 \backslash Man β 1-4GlcNAc β 1-4GlcNAc-PA	0.6	-

^aUnits of GU were calculated from the elution times of the peaks obtained from the ODS column in Figure 2 and the Amide column in Figure 3.

^bAverage mass calculated from the m/z values of $[M + Na]^+$ or $[M + H]^+$ ion for neutral, $[M - H]^-$ ion for mono-sialyl and mono-sulfated and $[M + Na - 2H]^-$ ions for mono-sialyl-mono-sulfated and di-sulfated PA-oligosaccharides (Supplementary data, Figure S1).

^cStructures of PA-oligosaccharides are represented.

^dMolecular percentage of was calculated from the peak area in Figure 2 by comparison with total *N*-glycan content in each islet tissue.

^e*N*-glycans did not coincide with those of known references in the GALAXY database.

and collected by centrifugation at $400 \times g$ for 1 min. The islets were then suspended in 4 mL of 1000 PU/mL Dispase-II (Godo-Shusei Co. Tokyo, Japan) and treated at 37°C for 15 min. Cell aggregates were allowed to settle and the supernatant was transferred to a conical tube. The pooled harvests were centrifuged at $400 \times g$ for 3 min. The cell pellet was washed twice with phosphate buffer saline (PBS) and re-suspended in PBS.

Flowcytometry

The islets were incubated with a 10% solution of normal human pooled serum (NHS) at 4°C for 1 h, washed and then incubated with 1.25 μ g of fluorescein isothiocyanate-conjugated anti-human

IgG and IgM (Cappel, West Chester, PA) as a second antibody for 1 h at 4°C. The stained cells were analyzed with a FACS Calibur flow cytometer (Nippon Becton Dickinson, Tokyo, Japan).

Sulfate-depleted cells

Islets were starved for 24 h in sulfate-free RPMI1640 medium containing 1% of fetal cow serum supplemented with fresh 10 mM sodium chlorate (Nakarai Tesque, Kyoto, Japan).

Supplementary data

Supplementary data for this article are available online at <http://glycob.oxfordjournals.org/>.

Table V. Structures and relative quantities of neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate PA-oligosaccharides derived from human and porcine islets

Peak code number	GU ^a ODS (Amid)	Molecular ^b mass (Da)	Structure ^c	Relative quantity (%) ^d	
				Pig	Human
Mono-sialyl-mono-sulfated glycan					
MS1	9.8 (5.0)	2133	(Hexose) ₄ (HexNAc) ₅ (NeuAc) ₁ (HSO ₃) ₁ (PA) ₁ ^e	0.3	–
MS2	12.7 (5.3)	2279	$\begin{array}{c} \text{Neu5Ac } \alpha 2-6\text{Gal } \beta 1-4\text{GlcNAc } \beta 1-2\text{Man } \alpha 1-6 \\ \text{SHO3} \swarrow \qquad \qquad \qquad \searrow \\ \text{GalNAc } \beta 1-4\text{GlcNAc } \beta 1-2\text{Man } \alpha 1-3 \end{array} \begin{array}{c} \text{Man } \beta 1-4\text{GlcNAc } \beta 1-4\text{GlcNAc}-\text{PA} \\ \text{Fuc } \alpha 1-6 \end{array}$	1.3	–
MS3	15.9 (5.4)	2279	(Hexose) ₄ (HexNAc) ₅ (Deoxyhexose) ₁ (NeuAc) ₁ (HSO ₃) ₁ (PA) ₁ ^e	0.4	–

^aUnits of GU were calculated from the elution times of the peaks obtained from the ODS column in Figure 2 and the Amide column in Figure 3.

^bAverage mass calculated from the m/z values of $[M + \text{Na}]^+$ or $[M + \text{H}]^+$ ion for neutral, $[M - \text{H}]^-$ ion for mono-sialyl and mono-sulfated and $[M + \text{Na} - 2\text{H}]^-$ ions for mono-sialyl-mono-sulfated and di-sulfated PA-oligosaccharides (Supplementary data, Figure S1).

^cStructures of PA-oligosaccharides are represented.

^dMolecular percentage of was calculated from the peak area in Figure 2 by comparison with total N-glycan content in each islet tissue.

^eN-glycans did not coincide with those of known references in the GALAXY database.

Table VI. Structures and relative quantities of neutral, mono-sialyl, di-sialyl or mono-sulfate, mono-sialyl-mono-sulfate and di-sulfate PA-oligosaccharides derived from human and porcine islets

Peak code number	GU ^a ODS (Amid)	Molecular ^b mass (Da)	Structure ^c	Relative quantity (%) ^d	
				Pig	Human
Di-sulfated glycan					
S2	12.7 (3.9)	2110	$\begin{array}{c} \text{SHO3} \swarrow \\ \text{GalNAc } \beta 1-4\text{GlcNAc } \beta 1-2\text{Man } \alpha 1-6 \\ \text{SHO3} \swarrow \qquad \qquad \qquad \searrow \\ \text{GalNAc } \beta 1-4\text{GlcNAc } \beta 1-2\text{Man } \alpha 1-3 \end{array} \begin{array}{c} \text{Man } \beta 1-4\text{GlcNAc } \beta 1-4\text{GlcNAc}-\text{PA} \\ \text{Fuc } \alpha 1-6 \end{array}$	7.0	–

^aUnits of GU were calculated from the elution times of the peaks obtained from the ODS column in Figure 2 and the Amide column in Figure 3.

^bAverage mass calculated from the m/z values of $[M + \text{Na}]^+$ or $[M + \text{H}]^+$ ion for neutral, $[M - \text{H}]^-$ ion for mono-sialyl and mono-sulfated and $[M + \text{Na} - 2\text{H}]^-$ ions for mono-sialyl-mono-sulfated and di-sulfated PA-oligosaccharides (Supplementary data, Figure S1).

^cStructures of PA-oligosaccharides are represented.

^dMolecular percentage of was calculated from the peak area in Figure 2 by comparison with total N-glycan content in each islet tissue.

^eN-glycans did not coincide with those of known references in the GALAXY database.

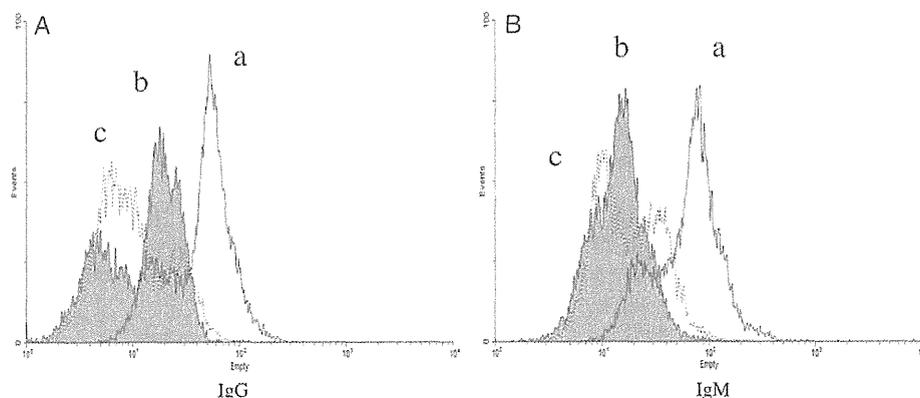


Fig. 8. FACS analysis for the antigenicity of sulfate structures. Islets from adult pigs were treated with 10% NHS as the first antibody and anti-human immunoglobulins as the second antibodies. Typical FACS profiles of human IgG (A) and IgM (B) deposition on islets are shown. The effect of removal of sulfate structures by sodium chlorate and sulfate-free medium on the antigenicity of pig islet cells was next investigated. The presence of sodium chlorate led to a reduction in the reactivity of islets to a natural antibody, suggesting that the sulfate structures of islets contain a considerable amount of natural antibody epitopes; a, Normal line: API in usual medium; b, painted out: Sulfate depleted API and c, dotted line: Second antibody control.

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Conflict of interest

None declared.

Abbreviations

2D, two dimension; API, adult pig islets; ATP, adenosine triphosphate; DEAE, diethylaminoethyl; FCS, fetal cow serum; FITC, fluorescein isothiocyanate; GALAXY, glycoanalysis by the three axes of MS and chromatography; GalNAc, *N*-acetylgalactosamine; GKO, α 1-3-galactosyltransferase knockout; GlcNAc, *N*-acetylglucosamine; GU, glucose unit; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; Lew^x, Lewis x; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometric; Man, mannose; MS2, mono-sialyl-mono-sulfate; NeuAc, neuraminic acid; NeuGc, *N*-glycolylneuraminic acid; NHS, normal human pooled serum; ODS, octa decyl silyl; PA, pyridylamino; PBS, phosphate buffer saline; S2, di-sulfate; α -Gal, α -galactosidase.

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The Influence of Collagen III Expression on the Efficiency of Cell Isolation With the Use of Collagenase H

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ABSTRACT

Objective. We previously demonstrated that collagenase H (ColH) plays a crucial role in rat islet isolation, whereas collagenase G (ColG) plays only a supporting role. We also showed that collagen III appears to be one of the key targets of ColH based on a mass spectrometry analysis. In the present study, we investigated whether our novel findings in an islet isolation model are universally applicable for other types of cell isolation, such as a hepatocyte isolation, with the use of enzyme blends of recombinant collagenases.

Methods. As the first step, the expression of one of the main matrix components, collagen III, on rat pancreatic and hepatic tissues was assessed with the use of immunohistochemical staining. ColG and ColH were expressed in recombinant *E. coli* carrying expression plasmids for each collagenase. Then the efficiency of the collagenase subtype on rat hepatocyte isolation was evaluated in terms of cell yield with the use of thermolysin combined with either ColG or ColH ($n = 3$, respectively).

Results. The expression of collagen III on rat hepatic tissues was dramatically lower than that of rat pancreatic tissues. In the rat hepatocyte isolation, a substantial amount of hepatocytes ($0.81 \pm 0.11 \times 10^6$) were obtained in the ColG group, whereas almost no hepatocytes were retrieved in the ColH group, indicating that the influence of the collagenase subtypes in rat hepatocyte isolation are completely opposite to that observed in rat islet isolation.

Conclusions. Considering that the expression of collagen III on hepatic tissues was relatively low and that almost no hepatocytes were retrieved when ColH and thermolysin were used, the present study supports our novel finding that collagen III appears to be one of the key targets of ColH in hepatocyte isolation. Therefore, the semiquantification of collagen III on the target tissues not only may positively contribute to efficient islet isolation, but also may affect other types of cell isolation by optimizing the ColH amount.

PANCREATIC islet transplantation is an attractive and promising therapy for type 1 diabetes. However, many issues are required to improve regarding this therapy. Pancreatic islet isolation method is one of these tissues. Indeed, even in the leading centers, a transplantable yield of isolated islets is obtained from <50% of processed pancreases [1]. Besides, the pancreatic tissues have to be dissociated by enzymatic degradation of the extracellular matrix (ECM) without damaging the structural and functional integrity of the islets [2]. To improve the pancreatic islet

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isolation method, we have so far focused on the enzymes for islet isolation as well as on the ECM of target tissues. Our group previously reported that collagenase H (ColH) plays a crucial role in rat islet isolation, whereas collagenase G (ColG) plays only a supporting role [3]. We also showed that collagen III appears to be one of the key targets of ColH based on a mass spectrometry analysis [3]. We think that these novel findings could lead to tailor-made islet isolation according to the ECM of target pancreatic tissues. Furthermore, we hypothesize that these findings may be the case in the other types of cell isolation. Therefore, in the present study we examined whether our novel findings in rat islet isolation model are universally applicable to other types of cell isolation, such as rat hepatocyte isolation with the use of tryptic-like activity (TLA)-free enzyme [4] preparations of highly purified recombinant collagenase of each subtype.

MATERIALS AND METHODS

Enzymes

Recombinant ColG and ColH were prepared with the use of *Escherichia coli* transformants based on the method described previously [5]. Thermolysin (TL) was purchased from Peptide Institution (Osaka, Japan).

Animals

Rat tissues were obtained from 10–13-week-old male inbred Lewis rats (Japan SLC, Shizuoka, Japan). All animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and the guidelines for animal experiments and related activities at Tohoku University (approved protocol ID: 2013 NICHe-Animal-011). All surgeries were performed with the use of anesthesia, and maximal efforts were made to minimize suffering.

Enzyme Activity and Blending

In the present study, recombinant ColG, ColH, and TL were used to prepare highly pure TLA-free enzyme blends. The enzyme blend activity was adjusted to equal that of the crude collagenase from *Clostridium histolyticum* (Sigma collagenase type IV; Sigma Chemicals, St Louis, Missouri) with the use of Azocoll, 4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-PLGPR), and N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA) as substrates. The TLA activity was measured by the cleavage of Bz-Arg-pNA (Wako Pure Chemical Industries, Osaka, Japan) at 37°C and pH 7.5. Notably, no TLA was detected in any components of the enzyme blends. In both experimental groups, 0.5 mg TL was added. The G and H groups consisted of ColG or ColH, respectively.

Immunohistochemical Analysis

Tissues were taken from the rat pancreata and livers, fixed with 10% formalin overnight, and then embedded in paraffin. The samples were sliced into 4- μ m sections and washed with phosphate-buffered saline solution (PBS) twice. Sections were pretreated with 10% rat serum for 30 minutes at room temperature to block endogenous peroxidase activity, followed by immunolabeling with primary antibodies against collagen III for 60 minutes at 37°C. Rabbit anti-rat collagen type III antibodies were used (Chemicon, Merck Millipore, Darmstadt, Germany). After washing the samples with PBS twice, secondary antirabbit antibodies conjugated with horseradish

peroxidase (Dako, Glostrup, Denmark) were used, antibody binding was localized with diaminobenzidine hydrochloride, and samples were counterstained with hematoxylin.

Hepatocyte Isolation

Rat hepatocytes were isolated by the 2-step collagenase perfusion technique as previously described [6–8]. The cells were then purified with the use of Percoll (GE Healthcare Biosciences, Pittsburgh, Pennsylvania) gradient centrifugation to obtain a highly purified cell population.

RESULTS

Immunohistochemical Analysis

As shown by representative examples (Fig 1), both rat pancreatic and hepatic sections showed positive staining for collagen III. However, the expression of collagen III on hepatic tissues was dramatically lower compared with pancreatic tissues.

Effects of the Collagenase Subtype on the Yield of Isolated Rat Hepatocytes

In rat hepatocytes isolation, a substantial amount of hepatocytes were obtained in the ColG group ($0.81 \pm 0.11 \times 10^6$), whereas almost no hepatocytes were retrieved in the ColH group. In rat pancreatic islet isolation, we previously showed that a substantial amount of well shaped functioning islets were obtained in the ColH group [3]. Taken together, we found that the influence of the collagenase subtypes on isolated hepatocyte yield is completely opposite to that observed in pancreatic islet isolation.

DISCUSSION

This study demonstrates that the expression of collagen III on rat hepatic tissue and the effects of the collagenase subtype on the yield of isolated rat hepatocytes support our novel findings that collagen III appears to be one of the key targets of ColH [3].

In the present study, we hypothesized that our novel findings could be applicable for not only pancreatic islet isolation but also for other types of cell separation. Although there are many types of cell isolations, we used a hepatocyte isolation model to examine our hypothesis because it is well known to require the degradation of collagen matrices and ~100 cases of clinical hepatocyte transplantation have already been performed as treatment for patients with liver-based metabolic diseases and acute and chronic liver failure [9–11]. Of particular note, in most cases of hepatocyte isolation, the enzymes targeted for islet isolations have been used [12,13]. However, it may be speculated that the enzymes suitable for islet isolations are not necessarily effective for hepatocyte separations, and vice versa. Most likely, the extracellular matrix components on pancreatic tissues and hepatic tissues are completely different. Indeed, the present study clearly showed that the expression pattern of one of the crucial matrix components, collagen III, was apparently distinct between the pancreatic and hepatic tissues.

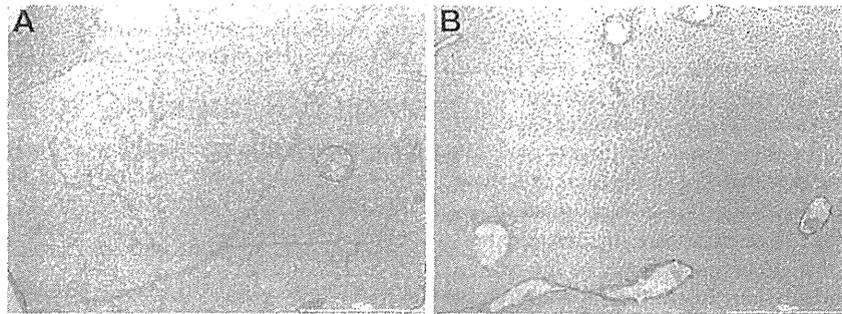


Fig 1. Collagen staining of rat pancreatic and hepatic tissues. (A) The lobular and acinar septa and the pancreatic ducts in the rat pancreatic tissues were positively stained for collagen III. (B) On the other hand, only a few sinusoidal and perivascular lesions were positively stained for collagen III in the rat hepatic tissues.

Therefore, optimizing the ratio of collagenase subtypes according to the ECM on target tissues may contribute to improving the success rate of several types of cell isolation and regenerative medicine that require degradation of collagens. We are currently attempting to identify the target ECMs of ColG, neutral protease, and the other crucial enzyme components.

In summary, in contrast to the pancreatic islet isolations, the role of ColG is relatively more crucial than ColH in hepatocyte isolation. These results support our novel findings that collagen III appears to be one of the key targets of ColH in hepatocyte isolation as well. Therefore, the semi-quantification of collagen III on the target tissues not only may positively contribute to efficient pancreatic islet isolation, but also may affect other types of cell isolation by optimizing the ColH amount.

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Three-Dimensional Computed Tomographic Volumetric Changes in Pancreas Before and After Living Donor Surgery for Pancreas Transplantation: Effect of Volume on Glucose Metabolism

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ABSTRACT

In the present study, we aimed to compare the pancreas volumetric changes before and after living donor surgery for pancreas transplantation, using three-dimensional (3D) computed tomography (CT) and glucose metabolism. Pancreatic volume (PV) measurement using 3D CT was performed in 13 consecutive donors who underwent distal pancreatectomy for simultaneous living donor pancreas and kidney transplantation. PV was measured using a workstation before and 3 months after living donor operation. As the parameters of glucose metabolism, hemoglobin A1c (HbA1c) level, fasting plasma glucose (FPG) level, body mass index (BMI), homeostasis model assessment of insulin resistance (HOMA-IR), and insulinogenic index (IGI) were examined simultaneously with the PV measurement. The preoperative and postoperative PVs of pancreas was 30 ± 5 mL and 42 ± 9 mL, respectively. The postoperative PV was significantly higher than the preoperative PV ($P < .01$) and increased by approximately 40% at 3 months after surgery. The postoperative FPG and HbA1c levels were significantly higher than the preoperative values ($P < .01$). BMI decreased significantly after surgery ($P < .01$). No differences in HOMA-IR and IGI were noted between before and after surgery. Diabetes mellitus was not observed any of the 13 living donors during this period. Distal pancreatectomy for living donors caused an increase in the PV and maintained insulin resistance, but it was not sufficient to maintain glucose metabolism at the preoperative state.

THE FIRST LIVING DONOR PANCREAS TRANSPLANTATION using a segmental pancreas (distal pancreas body and tail) was performed at the University of Minnesota on June 20, 1979 [1]. In 1994, the first simultaneous pancreas and kidney transplantation was also performed at the University of Minnesota [2]. Because of the severe shortage of deceased donors in Japan and the satisfactory outcomes of the living donor pancreas transplantations that have been performed at the University of Minnesota, living donor pancreas transplantation was introduced in Japan on January 7, 2004 [3]. A recently developed laparoscopic surgical procedure has been found to be minimally invasive and safe and has attracted more living donors, thus increasing the donor pool for pancreatic transplantation [4]. Although the satisfactory outcome of living donor operation has improved, the possible deterioration of glycemic controls in living donors, as a result of distal pancreatectomy, has been a long-standing concern.

Computed tomographic (CT) examination after pancreas transplantation has been shown to be a useful method for detecting postoperative complications of pancreas allograft transplantation [5]. Three-dimensional (3D) CT volumetry of the liver, kidney, and lung is considered to be a reliable and accurate method for volumetric assessment [6–8]. Pancreatic volumetry using 3D CT has also been shown to provide accurate measurements for pancreas transplantation [9]. In patients with neoplasms, pancreatic volumetric assessment is a useful predictor of new-onset diabetes mellitus following distal pancreatectomy [10].

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However, in the case of pancreas from living donors, no data are available preoperative and postoperative volumetric assessments.

This present study describes 3D CT volumetric changes in pancreas before and after living donor pancreas transplantation and the effect of the volume of the reserved pancreas on the glucose metabolism of living donors.

METHODS

Of the 13 donors examined, 3 were men and 10 were women. The median donor age at transplantation was 56 years (range, 28–66 years). All the donors were healthy and fulfilled the previously reported stringent criteria for living donors for pancreas transplantation [3]. Pancreatic volume (PV) measurement using 3D CT was performed in 13 consecutive living donors who underwent distal pancreatectomy for simultaneous transplantation of the pancreas and kidney between May 2007 and April 2011. CT examination was performed using a 4-channel CT scanner (Aquilion Super 4, Toshiba, Tokyo, Japan), with intravenous administration of iodine contrast media. Each CT scan was obtained using the following settings: tube voltage, 120 kV; tube current, 220 mA; section thickness, 1 mm; reconstruction interval, 0.5 mm; pitch factor, 5.5; field of view, 32 to 40 cm; and matrix, 512 × 512. To accurately estimate the volume of the pancreatic parenchyma, contrast enhancement with an intravenous contrast medium was applied during 3D CT. The pancreatic head volume was outlined by the left edge of a superior mesenteric vein, and PV was measured before and 3 months after the living donor operation (Fig 1), using the workstation Virtual Place Fujin (AZE Software Inc., Tokyo, Japan).

As parameters of glucose metabolism, hemoglobin A1c (HbA1c) level, fasting plasma glucose (FPG) level, and body mass index (BMI) were examined simultaneously during PV measurement. The insulin resistance of a pancreas was evaluated by the homeostasis model assessment of insulin resistance (HOMA-IR) [11], which was calculated as $[FPG \text{ (mg/dL)} \times \text{fasting insulin } (\mu\text{U/mL})/405]$, with lower values indicating a higher degree of insulin resistance. To evaluate the pancreatic β -cell function, we calculated the insulinogenic index (IGI) at 30 minutes during a 75-g oral glucose tolerance test (OGTT), as follows [12]:

$$\text{IGI} = \frac{\{\text{insulin at 30 minutes } (\mu\text{U/mL}) - \text{insulin at 0 minutes } (\mu\text{U/mL})\}}{\{\text{plasma glucose at 30 minutes } (\text{mg/dL}) - \text{plasma glucose at 0 minutes } (\text{mg/dL})\}}$$

This index can be used to evaluate the initial insulin secretion after glucose loading in healthy subjects and patients with impaired fasting glycemia/impaired glucose tolerance.

The statistical significance of the differences was analyzed by a paired *t* test, and *P* values of <.01 were considered to be statistically significant.

RESULTS

The mean PV of the entire pancreas was 66 mL. The mean PV of the preserved pancreatic head and allograft pancreatic body and tail were 30 mL (46%) and 36 mL (54%), respectively (Fig 2). No differences in preoperative 3D CT volumetric measurements were noted between the

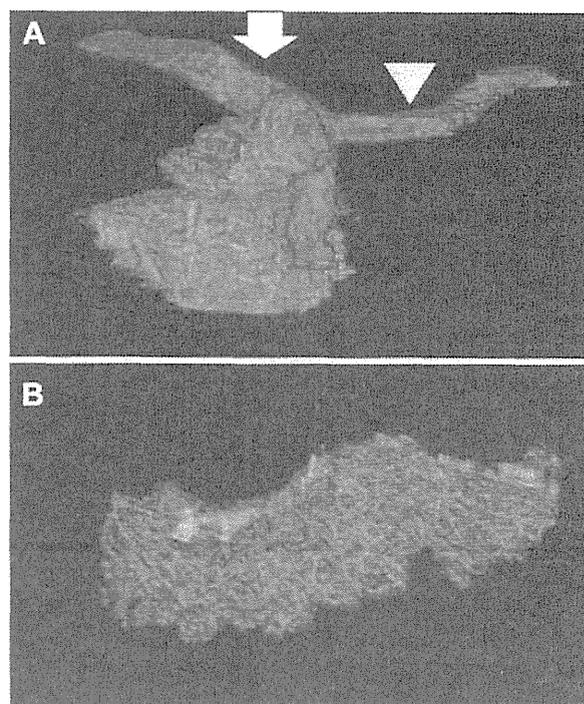


Fig 1. Preoperative images of three-dimensional computed tomography pancreatic volumetry. (A) The pancreatic head is outlined by the left edge of a superior mesenteric vein (arrow) conjoined with a splenic vein (arrowhead). (B) A pancreatic body-tail as an allograft.

pancreatic head and body and tail. Almost half of the pancreatic parenchyma was obtained by distal pancreatectomy during living donor operation.

FPG levels increased from 88 ± 1 before surgery to 104 ± 6 after surgery ($P < .01$). Likewise, the postoperative HbA1c level was significantly higher as compared to the preoperative HbA1c level (6.1 ± 0.4 vs 5.6 ± 0.2 ,

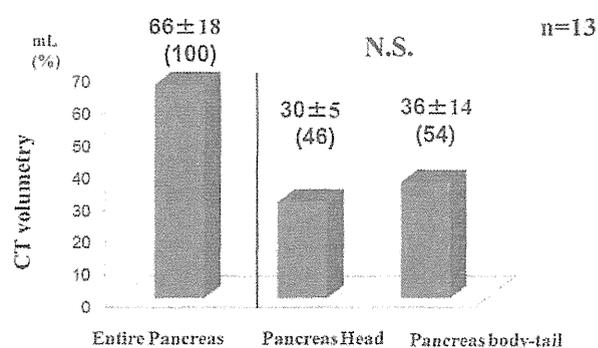


Fig 2. Preoperative three-dimensional (3D) computed tomography (CT) volumetry of an entire pancreas, pancreatic head, and body-tail from donors. No differences in preoperative 3D CT volumetric values were found between the pancreatic head and body-tail. N.S., not significant.

respectively; $P < .01$). No cases of diabetes mellitus was noted in among all the 13 living donors during this period. The BMI decreased from 23.0 ± 0.5 before surgery to 21.4 ± 2.2 after surgery ($P < .01$). No significant differences in HOMA-IR (1.3 ± 0.7 vs 1.0 ± 0.4 , respectively) and IGI (1.1 ± 1.1 vs 0.6 ± 0.5 , respectively) were noted between the values before and after surgery (Table 1).

The preoperative and postoperative PVs were 30 ± 5 mL and 42 ± 9 mL, respectively. The postoperative PV was significantly higher than the preoperative PV ($P < .01$) and increased by approximately 40% at 3 months after surgery (Fig 3).

CASE REPORTS

The preoperative PV on 3D CT was 42 mL (Fig 4A), and the postoperative PV was 52 mL (Fig 4B) at 3 months after surgery, thus indicating an obvious increase in the living donor PV after distal pancreatectomy.

DISCUSSION

The effects of distal pancreatectomy on glucose metabolism and β -cell function of living donors have been studied [13–15]. Insulin secretion was found to be lower after distal pancreatectomy [13]. Our study showed that the IGI, calculated using the 75-g OGTT and used to assess β -cell function, did not decrease significantly and tended to decrease after donor surgery. Although FPG and HbA1c levels were reported to be significantly higher postoperatively than the values preoperatively, normal glucose and HbA1c levels were maintained [13–15]. In addition, our study demonstrated that plasma glucose and HbA1c levels increased significantly at 3 months after distal pancreatectomy and did not exceed the reference range. The effects of distal pancreatectomy on increased secretion levels have been limited to our donor operations.

Obesity is now a contraindication to living pancreas donation [15]. A living donor who is to undergo distal pancreatectomy should be strongly advised to avoid becoming obese. Our study showed the BMI of all the donors was maintained within the reference range, but decreased significantly after the operation. The HOMA-IR allows for a quantitative assessment of the contributions of fasting plasma insulin and glucose concentrations to insulin

Table 1. Glycemic Metabolism of Donors ($n = 13$) Before and After Surgery

	Before Surgery	After Surgery
Fasting plasma glucose (mg/dL)	88 ± 1	$104 \pm 6^*$
HbA1c (NGSP%)	5.6 ± 0.2	$6.1 \pm 0.4^*$
Body mass index (kg/m^2)	23.0 ± 0.5	$21.4 \pm 2.2^*$
HOMA-IR	1.3 ± 0.7	1.0 ± 0.4
Insulinogenic index	1.1 ± 1.1	0.6 ± 0.5

Abbreviations: NGSP, National Glycohemoglobin Standardization supported by the National Institutes of Diabetes and Digestive and Kidney Diseases; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance.

*Statistically significant ($P < .01$).

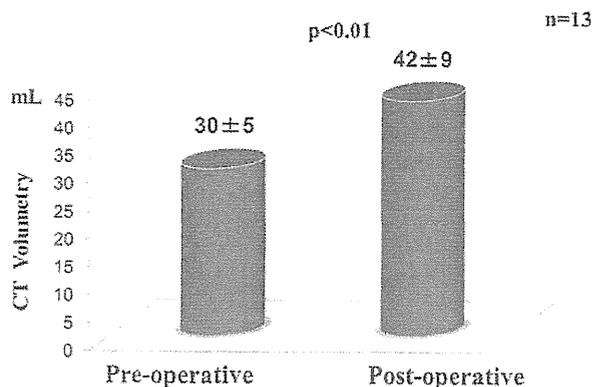


Fig 3. The postoperative computed tomography (CT) pancreatic volume (PV) was significantly higher than the preoperative PV and increased by approximately 40% at 3 months after surgery.

resistance. The HOMA-IR of our donors was maintained within normal levels. Thus the living donor operation did not diminish the ability of insulin resistance to maintain the postoperative body weight. These results on BMI and

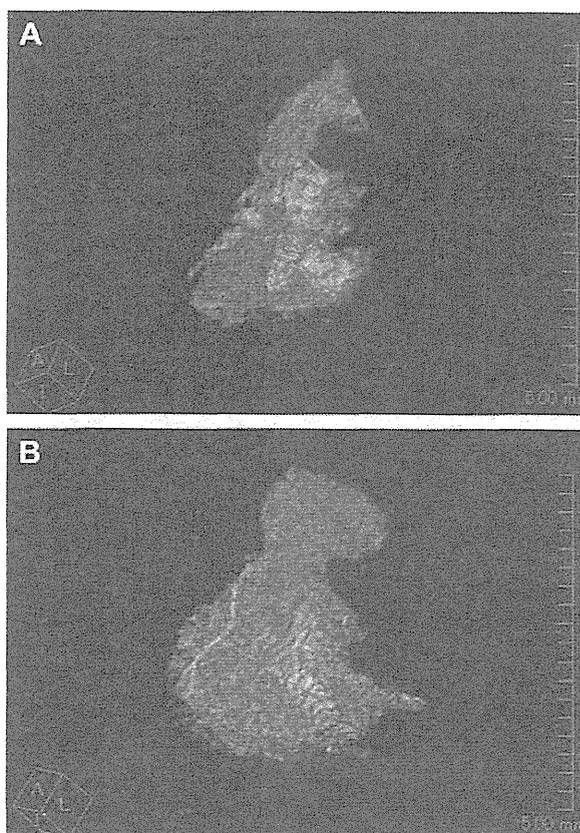


Fig 4. The three-dimensional computed tomography volumetric changes in a 42-year-old male donor before (A) and after distal pancreatectomy (B).

HOMA-IR may compensate for the decrease in insulin secretion after distal pancreatectomy.

Through 3D CT volumetry, we were able to estimate the volume of each allograft, such as that of the liver, kidney, and lung, and obtain additional information on preoperative allograft anatomical architecture [6–8]. The information is useful to determine the living allograft to be resected and postoperatively evaluate transplants. This technique could be used more widely to assess the preserved organ of living donors. Previous studies reported that the pancreatic weight in rats increased significantly with the increase in the volume of the preserved pancreas by distal pancreatectomy [16,17]. Bombesin, a tetradecapeptide hormone analogue of a mammalian gastrin-releasing peptide, plays a role in pancreatic growth and regeneration. When no bombesin was administered, the pancreatic weights in rats increased twice as much as the preoperative values after 90% of the PV was resected by distal pancreatectomy [17].

In humans, distal pancreatectomy did not induce an increase in PV, as evidenced on 3D CT [18]. However, this report included cases of chronic pancreas, pancreatic carcinoma, and pancreatic metastasis, but did not include normal pancreas. Our study demonstrated that the normal pancreas that fulfilled the stringent donor criteria had apparently exhibited increased PV on 3D CT after distal pancreatectomy. PV assessment was used as a predictor of new-onset diabetes mellitus after distal pancreatectomy for pancreatic neoplasm [10]. This study conclude that preoperative HbA1c levels higher than 5.7% and a pancreatic resection rate higher than 44% were independent risk factors for new-onset diabetes mellitus after distal pancreatectomy. Our study showed that in the cases with preoperative HbA1c levels lower than 5.7% and mean pancreatic resection rates of almost 50%, no new-onset diabetes mellitus was observed. Distal pancreatectomy for living donors resulted in an increase in the volume of the reserved pancreas and maintenance of insulin resistance; however, these effects were not sufficient to maintain the preoperative insulin secretion levels. Recently, local *in vivo* *GSK3 β* knockdown was found to promote β -cell and acinar-cell regeneration in 90% pancreatectomized rats [19]. Intrapancreatic *GSK3 β* knockdown leads to increased β -cell mass by promoting β -cell proliferation and differentiation. In the near future, this gene therapy may be helpful to promote human β -cell mass regeneration after donor distal pancreatectomy to maintain glucose metabolism.

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Clinical Outcome of Pancreas Transplantation From Marginal Donors in Japan

Y. Tomimaru, T. Ito, K. Kawamoto, N. Hama, H. Wada, S. Kobayashi, H. Eguchi, M. Tanemura, M. Mori, Y. Doki, and H. Nagano

ABSTRACT

In Japan, absolute shortage of donors still continues even after the law allowing organ transplantation from deceased donors came into force in 1997. With the passage of the waiting period after registration for pancreas transplantation (PTx), both deaths and serious cases of diabetic complications necessitating withdrawal of the registration have undoubtedly increased. Therefore, so-called “marginal donor” (MD) has been considered as a potential solution for shortage of donors in Japan. The aim of the present study is to evaluate feasibility of MD in terms of post-PTx outcomes using data from Japan Organ Transplantation Network. A total of 148 PTx were performed from deceased donors in Japan from 2000 to 2012. MD was defined as follows: (1) >45 years old; (2) hemodynamically unstable at harvest using a high-dose dopamine or more than 2 vasopressors; or (3) non-heart-beating status. Postoperative outcomes after PTx were compared between the MD group and the non-MD group. Among the 148 PTx donors, 108 donors (73.0%) satisfied the criteria of MD. Early graft loss of pancreas graft during 3 months post-transplant was observed in 15 patients (10.1%), and the marginality (MD vs non-MD) was not significantly correlated with the early loss of pancreas graft. The overall patient survival of the MD group (1, 3, 5 years: 94.7%, 94.7%, 94.7%) was not significantly different from that of the non-MD group (1, 3, 5 years: 95.0%, 95.0%, 95.0%). Pancreas graft survival in the MD group (1, 3, 5 years: 80.9%, 73.2%, 66.0%) seemed to be slightly lower than that in the non-MD group (1, 3, 5 years: 92.5%, 85.2%, 77.4%), but no statistically significant differences were found between the 2 groups. These results suggest the feasibility of the use of MD for PTx.

PANCREAS TRANSPLANTATION (PTx) is an established treatment for type 1 diabetes [1–3]. It is the only effective therapeutic option to restore normal glucose metabolism, to improve quality of life of the patients, and to even reduce chronic complications of the diabetes. Although its outcome was not satisfactory previously, graft survival has much improved during the last 30 years because of development in immunosuppressants, surgical techniques, and postoperative management.

In Japan, the Organ Transplant Law was enforced on October 1997, and it was revised on July 2010. Since the revision, the number of donation is increasing. However, absolute shortage of donors still continues even after the revision. With the passage of the waiting period after registration for PTx, both deaths and serious cases of diabetic complications necessitating withdrawal of the registration have undoubtedly increased. Accordingly, we have had to

depend on the so-called “marginal donor” (MD). To date, however, the feasibility of PTx from MD has not yet investigated well. In this regard, the present study was performed to evaluate its feasibility in terms of postoperative outcomes using data from Japan Organ Transplantation Network.

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PATIENTS AND METHODS

Patients

Between April 2000 and December 2012, a total of 148 PTx were performed for type 1 diabetes from deceased donors in Japan. Among the 148 cases of PTx, 146 cases were from brain-dead donors and the remaining 2 were non-heart-beating donors. In Japan, PTx is performed in 17 approved institutions. Characteristics of the 148 patients are shown in Table 1.

Criteria of Marginal Donor

The criteria of MD for PTx of Kapur et al were used in this study; donors of 45 years of age and more, hemodynamically unstable donors at the time of harvest (with dopamine dose > 10 µg/kg/min, or 2 or more vasopressors), or non-heart-beating donors [4]. Based on these criteria, the donors were divided into 2 groups: the MD group and the non-MD group.

Graft Failure

Pancreas graft failure was defined as return to insulin-dependence or serum C-peptide level < 0.3 ng/mL. Kidney graft failure was defined as return to dialysis. Death with a functioning graft was also considered be a graft failure. Early graft loss was defined as that within 3 months post-PTx in this study.

Statistical Analysis

Survival was calculated according to the Kaplan-Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0; SAS Institute Inc., Cary, NC, United States). A *P* value < .05 was considered statistically significant.

Table 1. Characteristics of 148 PTx Patients (n = 148)

Factors	
Donor-related factors	
Age (≤45 y/>45 y)	74/74
Gender (male/female)	80/68
Body mass index (kg/m ²) (<25/≥25)	115/33
Cause of death (CVA/trauma/others)	87/28/33
Type of death (brain-dead/non-heart-beating)	146/2
Hemodynamics (stable/unstable)	87/61
Cardiopulmonary resuscitation (-/+)	86/62
Marginality (MD/non-MD)	108/40
Recipient-related factors	
Age (≤50 y/>50 y)	123/25
Gender (male/female)	56/92
Duration of diabetes (<30 y/≥30 y)	90/58
Duration of dialysis (<10 y/≥10 y)	72/47
PTx-related factors	
TCIT (<12 h/≥12 h)	86/62
Type of PTx (SPK/PAK/PTA)	119/20/9
Duct management (bladder drainage/enteric drainage)	30/118
GDA reconstruction (-/+)	35/87
Immunosuppressive regimen	
CNI (TAC/CyA)	144/4
Antibody (-/+)	7/141

Abbreviations: PTx, pancreas transplantation; CVA, cerebrovascular accident; MD, marginal donor; TCIT, total cold ischemic time; SPK, simultaneous pancreas and kidney transplantation; PAK, pancreas transplantation after kidney transplantation; PTA, pancreas transplantation alone; GDA, gastroduodenal artery; CNI, calcineurin inhibitor; TAC, tacrolimus; CyA, cyclosporine.

RESULTS

Ratio of Marginal Donors

Among the 148 donors at the PTx, 74 were 45 or more years old. Sixty-one donors were hemodynamically unstable at the time of harvest. Two donors were non-heart-beating donors. In total, 108 donors (73.0%) of the 148 donors satisfied the criteria of MD and categorized into the MD group, and the remaining 40 donors (27.0%) were categorized into the non-MD group. Characteristics of the 148 patients are shown in Table 1.

Risk Factors for Early Loss of Pancreas Graft

Among the 148 PTx cases, early graft loss of pancreas graft was observed in 15 patients (10.1%). Thrombosis was the most frequent cause of the graft loss (8/15, 53%). The other causes were as follows: sepsis in 3, rejection in 2, duodenal perforation in 1, and cardiogenic shock in 1.

To investigate whether the marginality (MD vs non-MD) is a risk factor for the early loss of pancreas graft, as well as to identify factors that significantly correlate with the early graft loss, donor-related factors were compared between cases with the early graft loss and without the early graft loss (Table 2). The incidence of the early graft loss was significantly higher in donors with total cold ischemic time (TCIT) ≥12 hours (*P* = .05), and the marginality (MD vs non-MD) was not significantly correlated with the graft loss.

Long-Term Outcome After Pancreas Transplantation

We examined long-term outcomes of PTx in terms of overall patient survival, pancreas graft survival, and kidney graft survival (SPK cases). As shown in Table 3, in all the 148 cases, postoperative mortality was found in 5 patients in the MD group (4.6%) and in 3 patients in the non-MD group (7.5%). The incidence was not significantly different between the 2 groups (*P* = .45). Overall patient survival in the 148 cases was 94.8%, 94.8%, and 94.8% at 1, 3, and 5 years, respectively. The overall patient survival of the MD group (1, 3, 5 years: 94.7%, 94.7%, 94.7%) was not significantly different from those of the non-MD group (1, 3, 5 years: 95.0%, 95.0%, 95.0%; *P* = .42, Fig 1A). Twenty-four pancreas grafts were lost during the observation period

Table 2. Correlation of Donor-Related Factors With Early Loss of Pancreas Graft in the 148 PTx Cases

Factor	Early Graft Loss (-) (n = 133)	Early Graft Loss (+) (n = 15)	<i>P</i> Value
Age (≤45 y/>45 y)	66/67	8/7	.79
Gender (male/female)	70/63	10/5	.41
Body mass index (kg/m ²) (<25/≥25)	103/30	12/3	.56
Cause of death (CVA/others)	78/55	10/5	.59
Hemodynamics (stable/unstable)	80/53	7/8	.41
Cardiopulmonary resuscitation (-/+)	78/55	8/7	.78
TCIT (<12 h/≥12 h)	81/52	5/10	.05
Marginality (MD/non-MD)	96/37	12/3	.76

Abbreviations: PTx, pancreas transplantation; CVA, cerebrovascular accident; MD, marginal donor; TCIT, total cold ischemic time.

Table 3. Incidence of Mortality and Graft Failures in MD Group and Non-MD Group

	MD Group	Non-MD Group	P Value
Mortality	5/108 (4.6%)	3/40 (7.5%)	.45
Cardiogenic	1	2	
Cerebral bleeding	1	0	
Sepsis	2	1	
GVHD	1	0	
Pancreas graft failure	24/108 (22.2%)	4/40 (10.0%)	.08
Thrombosis	7	1	
Duodenal perforation/ bleeding	2	0	
Pancreatitis	1	0	
Recurrent diabetes	2	0	
Rejection	12	3	
Kidney graft failure	8/88 (9.1%)	1/31 (3.2%)	.44
Thrombosis	0	0	
Primary nonfunction	1	0	
Rejection	7	1	

Abbreviations: MD, marginal donor; GVHD, graft-versus-host disease.

among the 108 cases in the MD group, and 4 pancreas grafts were lost in the 40 cases in the non-MD group (Table 3). The incidence of the pancreas graft failure in the MD group tended to be higher than the non-MD group ($P = .08$, Table 3). Especially, thrombosis and rejection were frequently observed as a cause of the graft failure in the MD group. Pancreas graft survival in all the 148 cases was 84.8%, 76.4%, and 68.9% at 1, 3, and 5 years, respectively. Pancreas graft survival in the MD group and the non-MD group was 80.9% and 92.5%, 73.2% and 85.2%, and 66.0% and 77.4% at 1, 3, and 5 years post-PTx, respectively, and there was no significant difference between the 2 groups ($P = .35$, Fig 1B). Incidence of kidney graft failure in 119 SPK cases was also compared. The incidence was not significantly different between the 2 groups ($P = .44$,

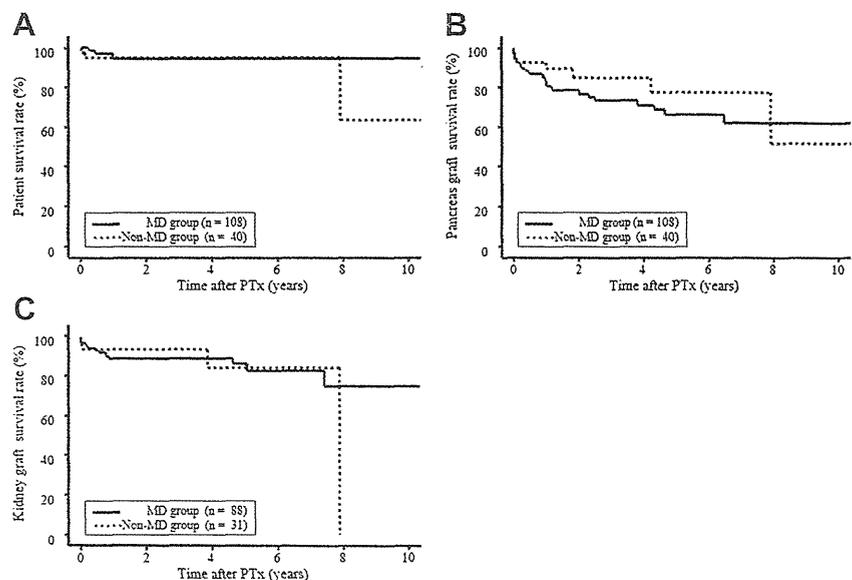
Table 3). Kidney graft survival in the SPK cases was 84.8%, 76.4%, and 68.9% at 1, 3, and 5 years, respectively. Kidney graft survival of the MD group (1, 3, 5 years: 89.1%, 89.1%, 86.0%) was not significantly different from that of the non-MD group (1, 3, 5 years: 93.5%, 93.5%, 84.2%; $P = .77$, Fig 1C).

DISCUSSION

The present study first showed that MD has been mostly utilized for PTx in Japan, compared with the condition of PTx donors in the United States [2,3]. However, the patient survival and graft survival were not significantly different from that in the United States. In case of simultaneous liver harvest in Japan, the reconstruction of gastroduodenal artery in pancreas graft has been done as much as possible (71.3%) to increase the blood flow in pancreas head region [5]. It remains unknown whether this procedure will have an effect on the early graft outcome.

The present study also demonstrated that there are no statistically significant differences in long-term outcomes after PTx between the MD group and the non-MD group. Furthermore, we investigated risk factors for the early loss of pancreatic graft and found that the marginality (MD vs non-MD) is not statistically significantly correlated with the early loss. These findings suggested the possibility that PTx from MDs is feasible in terms of postoperative outcomes. We also showed that the incidence of the early pancreatic graft loss within 3 months posttransplant is significantly increased when TCIT is over 12 hours. On the other hand, in the United States, it has been reported that preservation time of pancreatic graft >20 hours is significantly associated with post-PTx complications [6,7]. In this regard, a permissive range of the preservation time is likely to be narrow in Japan as compared to the United States where non-MDs are mostly available.

Fig 1. Long-term outcome after pancreas transplantation. Overall patient survival (A), pancreas graft survival (B), and kidney graft survival (C) were compared between the MD group (solid lines) and the non-MD group (dotted lines). Overall patient survival and pancreas graft survival were calculated in all the 148 PTx cases, and kidney graft survival was calculated in 119 simultaneous pancreas and kidney transplantation cases. Survival was not significantly different between the 2 groups. MD, marginal donor; PTx, pancreas transplantation.



In addition to the preservation time of the graft, to date, many donor-related risk factors have been considered as key determinants of outcomes after PTx such as donor age, obesity, donation after cardiac death, and cause of death. Especially, donor age is one of the most common risk factors. In general, aging affects nearly all the kinds of cells that play roles in outcomes of PTx including insulin-producing islet cells and endothelial cells of blood vessels, potentially affecting formation of thrombus. Salvalaggio et al reported from the United States data that old donors (>45 years) result in poorer long-term outcome in comparison to younger donors [8]. European data suggest equivalent outcomes [9]. Furthermore, donor age has been recognized as one of the factors composing scoring index for assessment of donor risk [10,11].

Indeed, the results of the present study may help expand the donor pool and resolve the donor shortage by using pancreas from MD. However, based on these previous reports, there seems to be another possibility that the current study enrolled too few cases to find statistically significant differences in post-PTx outcomes between the MD group and the non-MD group. Actually, the incidence of the pancreas graft failure in the MD group tended to be higher than the non-MD group, though the difference of the incidence was not statistically significant. To allow any conclusion on whether usage of grafts from MD is an acceptable option at PTx, studies with larger PTx numbers will be needed. If the outcome of PTx from MDs is judged to be worse than those from non-MDs, further investigations may be also necessary to clarify factors that contribute to better outcomes in MDs.

In summary, the current study suggested that PTx from MDs is feasible in terms of postoperative outcomes based on data obtained so far from a nationwide database in Japan. At the same time, considering the small number of PTx in Japan compared to other countries, the finding should be validated in studies with a larger number of PTx cases.

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脂肪由来間葉系幹細胞を用いた細胞療法の 膵島移植への応用

CD90high 脂肪由来間葉系幹細胞亜分画の有効性

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REVIEW ARTICLE

Possible application of adipose-derived mesenchymal stem cells to islet transplantation—potential of CD90 high-expression cells

[Abstract] Mesenchymal stem cells (MSCs), including adipose tissue-derived mesenchymal stem cells (ADSC), are multipotent and can differentiate into various cell types possessing unique immunomodulatory features. Several clinical trials have demonstrated the safety and possible efficacy of MSCs in organ transplantation. Thus, stem cell therapy is promising for tolerance induction. In this study, we assessed the reprogramming capacity of murine ADSCs and found that CD90 (Thy-1), originally discovered as a thymocyte antigen, could be a useful marker for cell therapy. Murine ADSCs were isolated from B6 mice, sorted by selection of CD90Hi or CD90Lo, and then transduced with four standard factors (Oct4, Sox2, Klf4, and c-Myc). Among unsorted, CD90Hi-, and CD90Lo- murine ADSCs, reprogrammed CD90Hi ADSCs showed increased numbers of alkaline phosphatase-positive colonies compared with reprogrammed CD90Lo ADSCs. The relative reprogramming efficiencies of unsorted, CD90Hi-sorted, and CD90Lo-sorted ADSCs were 100%, 116.5%, and 74.7%, respectively. CD90Hi cells were more responsive to reprogramming. CD90Hi ADSCs had greater reprogramming capacity than CD90Lo ADSCs, suggesting that ADSCs have heterogeneous subpopulations. Thus, CD90Hi selection presents an effective strategy to isolate a highly suppressive subpopulation for stem cell-based tolerance induction therapy.

key words: CD90, ADSC, reprogramming
(CD90, 脂肪由来間葉系幹細胞, リプログラミング)

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1 型糖尿病に対する β 細胞代替療法

1 型糖尿病の本体は、自己免疫を原因とするインスリン産生細胞 (β 細胞) の破壊であり、最終的

には自己反応性 T 細胞により β 細胞は全滅し、内服治療は無効である。したがって、1 型糖尿病に対する標準療法はインスリン強化療法となる。インスリン強化療法は、糖尿病性合併症の進行を抑制可能であるが、時として低血糖発作が頻発し、致命的になることもある。このことは、1 型糖尿病に対するインスリン治療の限界を示している。

インスリン強化療法に対して、24 時間連続的に血糖値をモニターしつつ、それに対して必要十分なインスリンを分泌させる機能を有する細胞を患者に移植することで、完璧な血糖コントロールを回復させることを目的とした治療法が β 細胞代

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替療法である。この β 細胞代替療法としては、膵臓移植および膵島移植といった移植医療がある。膵臓移植は、現時点において長期インスリン離脱率が最も高い治療法であり、生命予後改善効果も確認されているが、手術侵襲が高度である点が問題となる。

これに対し膵島移植は、膵臓移植と比較して、IVR手技を用いて局所麻酔下に完了する非常に低侵襲な治療法である。その一方で、膵臓移植と比較すると長期インスリン離脱率はいまだ不十分で、免疫抑制プロトコールのさらなる改善を要する。さらに、膵島移植のもう一つの問題点として、膵島分離法は確立したものの、分離時および移植直後の細胞喪失が多く、複数回のドナーを必要とするケースが多い。加えて、欧米では、膵島収量が多く見込める肥満ドナーを用いているのに対し、本邦では肥満ドナーはごくわずかであるため、欧米の成績を本邦で再現するときのハードルとなっている。これらは、特に脳死および心停止ドナー提供数の少ない本邦において解決すべき問題である。

さて、この問題点を解決するために注目される一つの可能性として、脂肪由来間葉系幹細胞(adipose-derived mesenchymal stem cell, ADSC)がある。ADSCは、皮下脂肪などの脂肪組織より簡単に調製可能な幹細胞で、臨床膵島移植の成績を改善する可能性を有していることが報告されている。筆者らのグループでも、ADSCと膵島を共移植することで、移植に必要な膵島数を減量可能であることをマウスモデルで報告した¹⁾。そこで、さらに膵島収量を増やすことを目的として、このADSCに対してリプログラミング手技を応用することを考えた。

リプログラミング効率と幹細胞療法

人工多能性幹細胞(induced pluripotent stem cell, iPS細胞)は、2006年に京都大学の山中らのグループが最初に報告した多能性幹細胞で、再生医療を実現するための重要なツールとして研究が進んでいる²⁾。体細胞に数種類の遺伝子等を導入し、特定の条件下で培養することで得られる。以降iPS細胞の基礎医学・創薬への応用が期待されている。

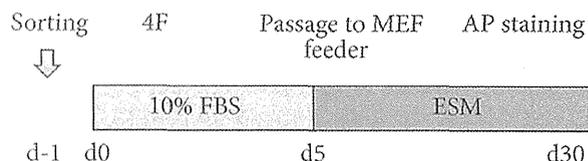


図1 プロトコール

(Kawamoto K et al. Disease Markers 35: 573-579, 2013⁶⁾より)

血液幹細胞における研究において、未分化な細胞であるほど、リプログラミング効率にすぐれることが報告された³⁾。これらの知見に加えて、筆者らが膵島移植効率を高めるために応用しようと考えているヒトADSCにおいても、CD90high分画が分化誘導効率に関与するとの報告もある⁴⁾。このCD90(Thy-1)は、GPIアンカーの膜蛋白であり、当初は胸腺細胞の抗原として同定された。つづいて、幹細胞でも発現していることが報告された。このことは、リプログラミング効率にすぐれる分画が、幹細胞療法に適した分画であることを示唆している⁵⁾。そこで筆者らは、CD90high分画がリプログラミング効率にすぐれるか解析した⁶⁾。

研究の概要

8~12週齢のC57BL/6マウスの皮下脂肪より、通常法を用いてADSCを分離した。分離当日にFACSアリアセルソーターを用いて、ADSCをCD90high分画およびCD90low分画にソートした⁷⁾。ソート後に6ウエルプレートに播種した。翌日、レンチウイルスベクターを用いて、山中4因子(Oct3/4, Sox2, Klf4, c-Myc)を導入した。導入5日目に、1,000個の細胞を、マイトマイシンCで処理したマウス胎生線維芽細胞(MEF)のフィーダーに移し、以降はESメEDIUMで合計30日間培養した。リプログラミング効率はアルカリフォスファターゼ染色(AP染色)を用いて解析した。また免疫細胞染色にて、未分化マーカーの発現を確認した。

本研究における実験プロトコールを図1に示す。通常の単層培養条件下においてマウスADSCでは、コロニー形成は認めず、またAP染色においても陰性であった。一方、ADSCに山中4因子

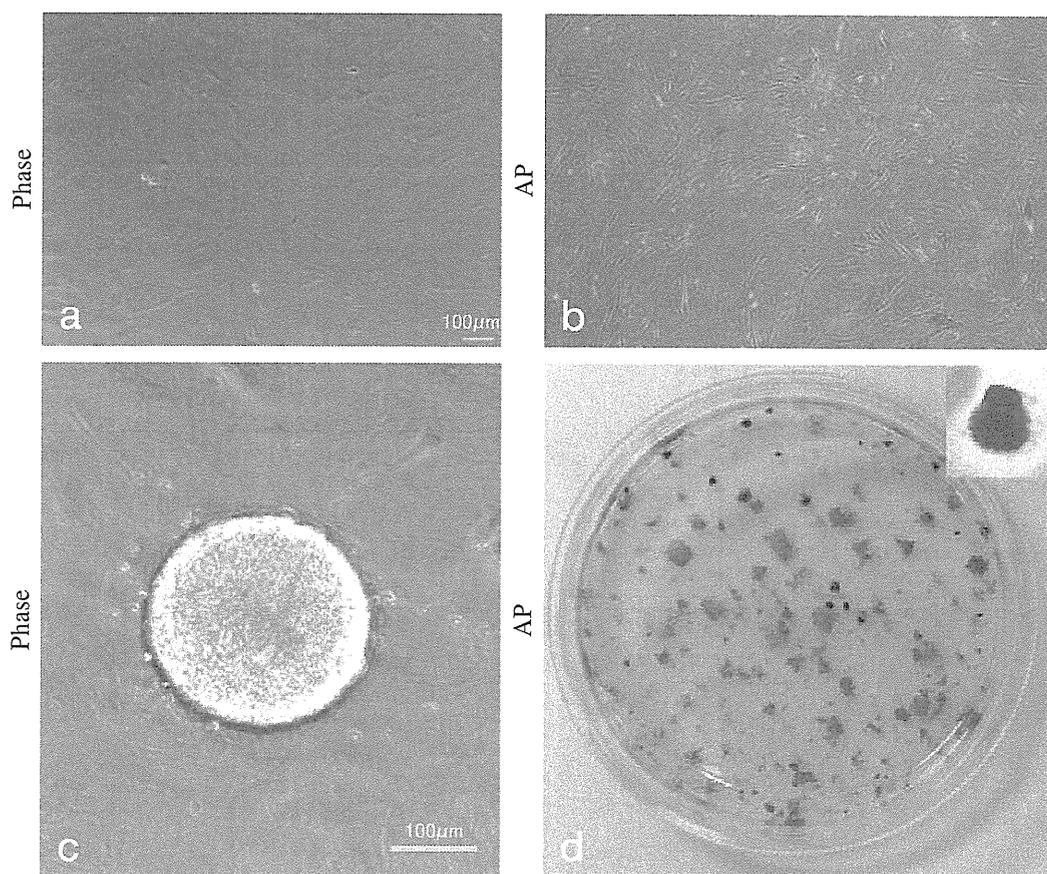


図2 マウス ADSC のリプログラミング前(a, b), リプログラミング後(c, d)
(Kawamoto K et al. Disease Markers 35 : 573-579, 2013⁶⁾より)

を導入することで、iPS コロニーを誘導することができた(図2)。このことはマウス ADSC でもレンチウイルスベクターを用いたリプログラミングが可能であることを示している。また、FACS アリアを用いて doublet を除去、7-アミノアクチノマイシン D (7-AAD) 染色にて死細胞を除去、CD31-CD45-CD34-分画のうち、CD90^{high} 分画と CD90^{low} 分画にソートし、プレートに播種した(図3a)。翌日に観察したところ、いずれも形態学的には同様の細胞であった(図3b)。ソート後であっても、1週間培養すると、特に、CD90^{low} 分画から、また CD90^{high} 細胞が誘導されていた(図4)。したがって、ウイルスベクターを導入するのは、なるべくソート後早期がよいことが示唆される。また、このコロニーは、免疫細胞染色にて OCT3/4 および SSEA1 陽性であった(図5)。CD90^{high} 分画および、CD90^{low} 分画でソートした ADSC のリプログラミング効率、それぞれ 1.48 ± 0.24 , 1.0 ± 0.15 と有意差を認めた ($p = 0.01$)(図6,7)。

細胞治療の今後

細胞療法は、ドナーのリンパ球輸注(donor lymphocyte or leukocyte infusion, DLI)を皮切りに、最近では、樹状細胞、制御性 T 細胞、間葉系幹細胞(mesenchymal stem cell, mSC)の効果が報告されている。なかでも mSC は、ある特定の細胞に分化誘導させることで、再生医療の細胞ソースとなる可能性に加えて、分化誘導していない mSC 自身にも、免疫修飾作用が知られている⁸⁾。このことは、分化誘導させた幹細胞の効果がいまだ確立していない現況を鑑みると、分化誘導させずとも、幹細胞自身を移植時に投与することで、成績改善が得られる可能性を示唆している。さらに最近、mSC が免疫抑制作用の可能性に基づき、免疫抑制剤の代替療法としての mSC を用いた臨床試験が、海外を中心に進行中である⁹⁻¹¹⁾。また、このメカニズムに関しては、さまざまなサイトカインのオートクラインおよびパラクライン効果などが推察されている。本研究では、この mSC の一種で

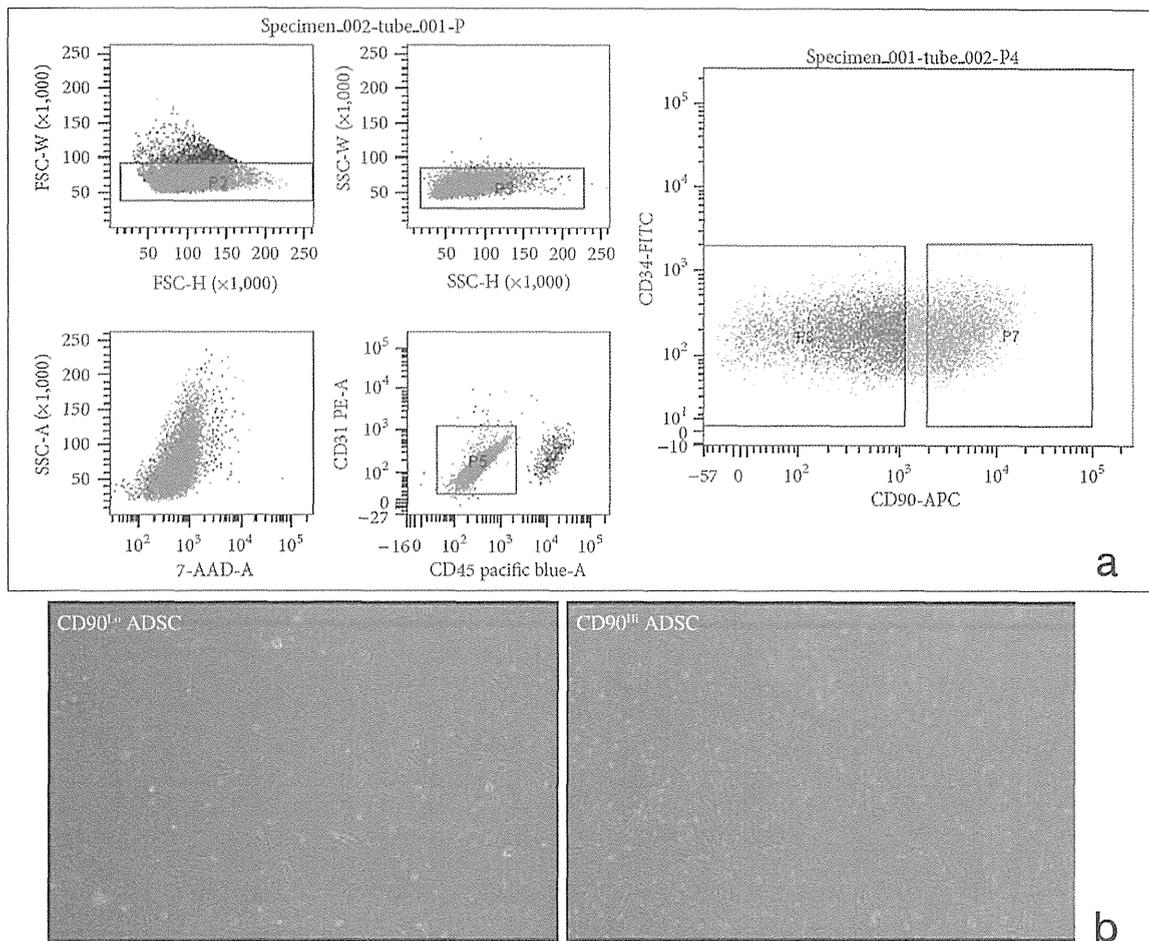


図3 ゲーティングによる分析
(Kawamoto K et al. Disease Markers 35 : 573-579, 2013⁶⁾より)

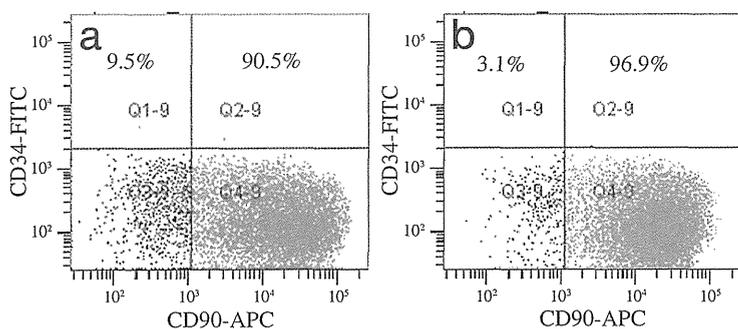


図4
長期培養の影響
a : CD90Lo ADSC
b : CD90Hi ADSC
(Kawamoto K et al. Disease Markers 35 : 573-579, 2013⁶⁾より)

ある ADSC を細胞ソースとして用いることで、1 型糖尿病に対する治療の可能性を検討した。

今回の研究で、レンチウイルスベクターを用いて山中 4 因子を導入することでマウス ADSC から iPS 細胞を誘導可能であることを、AP 染色および免疫細胞染色により証明した。細胞の初期化およびダイレクトリプログラミング(細胞の初期化を経ずに他の分化細胞に転換する現象)が、さまざまな転写因子により調節されていることは想

像にがたないが、いまだにどのように細胞の初期化を制御しているかの詳細は明らかになっておらず、今後の研究の進捗が期待される。

幹細胞との共移植は、臨床膵島移植を含む移植医療の成績を改善しうるテクノロジーとして期待されている。通常 ADSC そのものを使用するが、将来、移植療法により適した ADSC を峻別可能であれば、さらなる成績向上が期待できる。細胞療法施行時には、どのタイミングで、どれくらいの

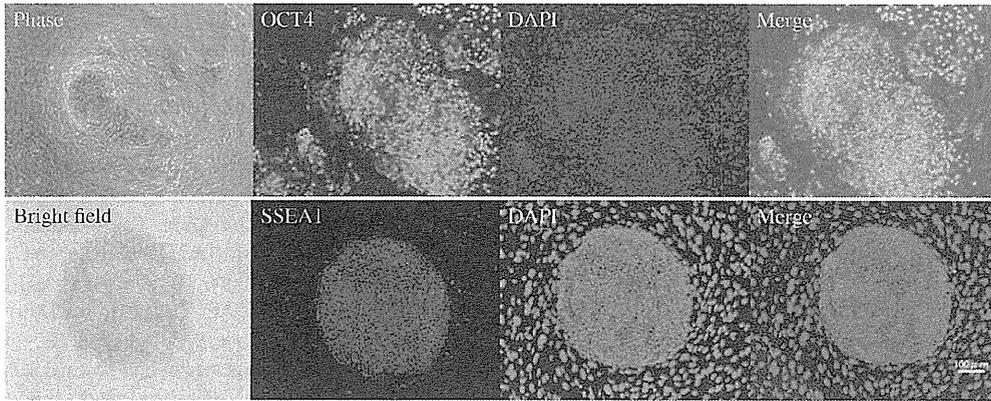


図5 細胞免疫染色

(Kawamoto K et al. Disease Markers 35 : 573-579, 2013⁶⁾より)

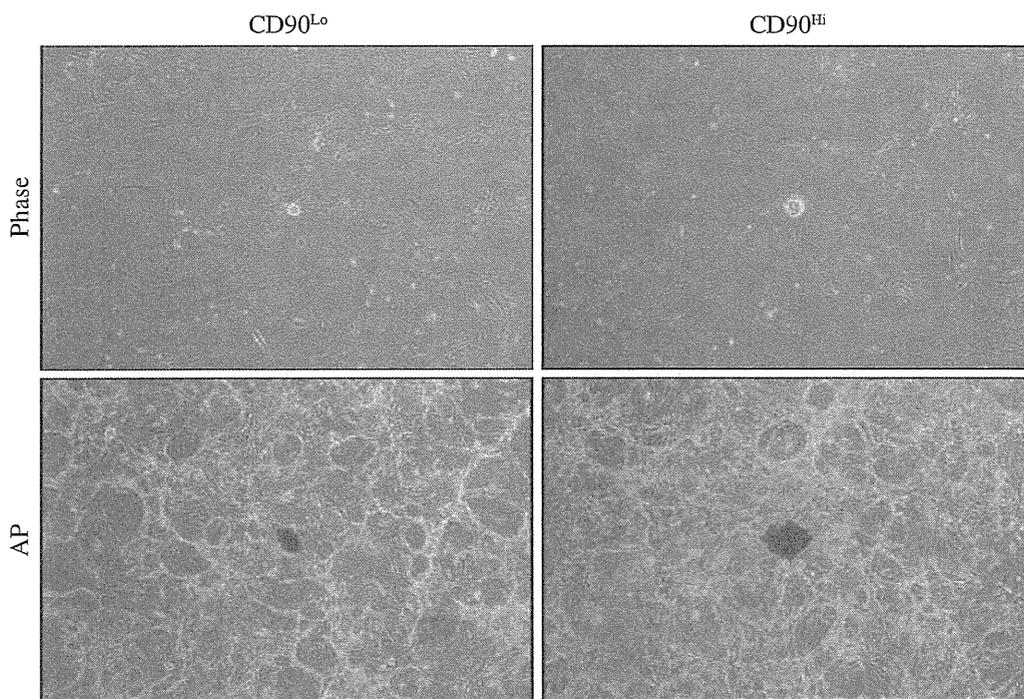


図6 ソート後のリプログラミング

(Kawamoto K et al. Disease Markers 35 : 573-579, 2013⁶⁾より)

質および量の細胞が必要とされるかは、マウスモデルでは同定不能であり、最終的にヒトでの臨床試験が必要となるが、コスト面から最適の条件を絞り切れていない点もハードルとなる。すなわち、ある細胞に移植成績を改善する効果があっても、使用方法が不適切であれば、ネガティブな結果しか得られない。筆者らの仮説どおりであれば、リプログラミング効率を、幹細胞を選択するときのマーカーとして利用することも可能である。今後は、マウス移植実験にて、実際の有効性を確認し、将来的には臨床検体を用いて同様の実験を計画している。また、臨床応用を考慮し、筆者らのグルー

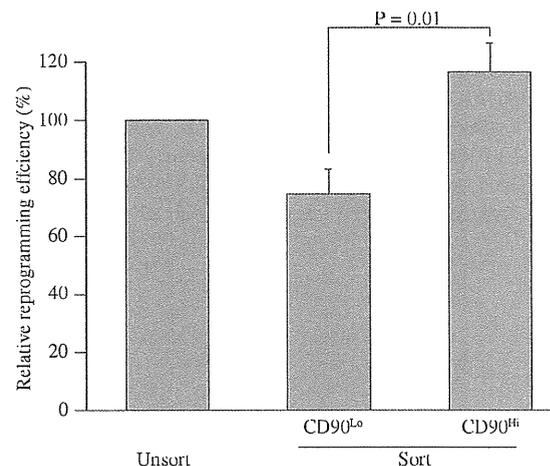


図7 リプログラミング効率