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Considerations in biodistribution evaluation of iPSC-derived cell therapy: A pancreatic islet cell case study

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Induced pluripotent stem cells (iPSCs) have substantial transformative potential in regenerative medicine, enabling tissue repair and restoration. However, their clinical application is limited by tumorigenic risks owing to their pluripotency. Biodistribution studies are crucial for elucidating the fate and tumorigenicity risk of iPSC-derived cell therapy products (CTPs). The lack of standardized biodistribution study protocols has led to inconsistent and unreliable study results, posing difficulties in the drug approval process. Therefore, we conducted a case study on the biodistribution of iPSC-derived pancreatic islet cells to develop a standardized biodistribution assessment protocol for iPSC-derived CTPs. We optimized a droplet digital polymerase chain reaction method targeting human-specific LINE1 sequences and validated its quantitativity for quantification across different tissues using a single calibration curve. We performed a long-term biodistribution study of iPSC-derived pancreatic islet cell in immunodeficient mice to assess biodistribution characteristics, which indicated that they remained localized at the transplantation site for one year, with no migration to other organs, suggesting long-term survival, minimal tumorigenicity risk, and advantages for clinical application. This study provides valuable insights into the standardization of biodistribution protocols for iPSC-derived CTPs.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) are cells that have been reprogrammed to regain pluripotency by introducing specific genes into somatic cells, enabling them to differentiate into various cell types.[1] iPSCs hold substantial promise for the creation of genetic disease models, elucidation of disease mechanisms, and development of novel therapeutics.[2,3] iPSCs have garnered considerable attention in regenerative medicine owing to their potential to provide innovative and fundamental treatment approaches to repair, reconstruct, and restore the function of diseased or damaged tissues or organs, offering curative solutions for diseases and injuries that are difficult to treat using conventional therapies.[4] Thus, regenerative therapy using iPSC-derived cell therapy products (iCTPs) promises significant benefits for patients if the transplanted iCTPs persist for extended durations at the appropriate site without exhibiting abnormal biodistribution. However, iCTPs are generally considered to have a high tumorigenicity risk because residual undifferentiated iPSCs are naturally tumorigenic, forming teratomas. Moreover, the accumulation of genomic abnormalities during the establishment of an iPSC line and subsequent prolonged cell passage may induce cell transformations.[5–8] Therefore, the effective evaluation and management of the tumorigenic risk of iCTPs is critical for their clinical application. A key method for assessing the tumorigenic risk of iCTPs is through biodistribution studies, which involve a detailed investigation of the distribution and behavior of transplanted iCTPs within the body.[9] Because biodistribution studies can determine whether iCTPs persist at the transplanted site, migrate to unintended sites, and/or proliferate in ways that could induce tumor formation, they play a crucial role in ensuring that the use of iCTPs in medical applications is safe and effective.

The biodistribution of iCTPs has been evaluated using various methods, including imaging, flow cytometry, and quantitative polymerase chain reaction (qPCR).[10–14] In particular, the *Arthrobacter luteus* element-targeting qPCR (Alu-qPCR) is frequently employed in preclinical biodistribution studies of human CTPs because of its enhanced sensitivity.[15–17] The number of human cells in a volume of biological samples can be quantified using a calibration curve prepared by spiking standard cell lysates into a blank matrix[18]; however, qPCR methods are susceptible to matrix effects and/or variable DNA recovery rates across different matrices.[19] Moreover, a further

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notable limitation of Alu-qPCR is nonspecific amplification in non-template control samples.[16,18]

We previously developed a droplet digital PCR method targeting human-specific LINE1 sequences (LINE1-ddPCR) as a quantification method for human CTPs in nonclinical biodistribution studies.[19] This method minimizes the matrix effect and variability in DNA recovery by leveraging the endpoint assay feature of ddPCR and normalization through the addition of an external control (EC) gene (dog genomic DNA [gDNA]) to each sample, resulting in the accurate quantification of the number of human cells in different tissue samples using a single surrogate matrix calibration curve. However, the applicability of this method to quantify iCTPs in various biological samples from long-term biodistribution studies remains unclear. Moreover, the appropriate assay criteria for quantifying CTPs in preclinical biodistribution studies remain undetermined.

Biodistribution studies for iCTPs are limited, and no consensus on aspects of study design such as study duration, number of animals to use, sex, and tissue selection for quantification has been established.[9] These considerations would affect the interpretation of the biodistribution characteristics of iCTPs. Generally, it is more comprehensive to evaluate cell distribution across all tissues, using a large number of animals over an extended period. However, from an animal welfare perspective, the number of animals used should be minimized. Furthermore, to expedite the development of innovative treatment options for patients, it is preferable to select relevant target tissues for bioanalysis.

Recently, the development of differentiation methods for human pluripotent stem cell-derived pancreatic islet cells (SC-islets) has advanced the treatment of type 1 diabetes.[20–26] Correspondingly, recent clinical studies have demonstrated the feasibility of achieving glucose control through the intrahepatic transplantation of SC-islets in patients with type 1 diabetes[27] and insulin-dependent type 2 diabetes,[28] indicating that SC-islet transplantation could benefit patients. Although subcutaneously transplanted cells might have difficulty surviving for extended durations owing to reduced vascularization and hypoxia, allogeneic human iPSC-derived pancreatic islet cells (iPICs), a type of SC-islet, have been shown to improve diabetes in diabetic mice and pig models following subcutaneous transplantation with biodegradable scaffolds.[29,30] This suggests that if cells that secrete insulin based on blood glucose levels survive at the transplant site, therapeutic efficacy can be achieved. Moreover, subcutaneously transplanted cells can be removed from the transplantation site in case of unexpected side effects, if the cells remain at the transplantation site without abnormal migration and tumorigenicity. Confirming the long-term persistence of iPICs at the transplantation site, without abnormal distribution and proliferation, is crucial for anticipating efficacy and safety.

In this study, we optimized the LINE1-ddPCR method, validated the quantitativity of the method as a bioanalytical assay for iCTPs in various matrices, and performed an *in vivo* biodistribution study in which iPICs were subcutaneously transplanted into immunodeficient mice. Additionally, we performed a long-term animal study of iPICs to standardize the fit-for-purpose biodistribution assessment for locally transplanted iCTPs.

RESULTS

Assay optimization

Figure 1 shows a two-dimensional scatterplot showing the fluorescence intensity from the amplification of LINE1 (FAM) and EC (VIC) in the duplex assay. A clear separation of negative and positive droplets was observed in both the LINE1 and EC sequences. Although the fluorescence intensity of VIC did not differ between LINE1-positive and negative droplets, and that of FAM was slightly increased in EC-positive droplets.

Assay validation

To confirm the quantitativity of this assay, quality control (QC) samples were prepared at three different concentrations of 3 (QC-L), 2×10^2 (QC-M), and 6×10^3 (QC-H) cells/20 mg tissue, 10 mg tissue, 0.2 mg tissue, or 50 μL blood on three separate days. The copy number of the LINE1 sequence was quantified using a single surrogate calibration curve with blank liver samples. The relative error (RE) and coefficient of variation (CV) values for the QC-H and QC-M samples ranged from −32.8% to 33.0% and 0.6% to 9.6%, respectively (Table 1). The RE and CV values for the QC-L samples ranged from −28.0% to 43.3% and 3.7% to 27.5%, respectively. These results met the required criteria: the RE and CV values were within 100.0% ± 35.0% for QC-H and QC-M, and within 100.0% ± 50.0% for QC-L. The regression analysis showed good linearity ($r^2 > 0.98$) and similar calibration curve slopes within the range of 3–10,000 cells (slope = 0.000423–0.000527, within 1.24 times) across the three validation batches. Dilutions of QC samples were prepared by diluting the sample with a concentration of 5×10^4 cells/0.2 mg skin 10-fold (QC-HD1) and 100-fold (QC-HD2) with the control homogenate to confirm the accuracy and precision of the dilution procedure. The RE and CV values for QC-HD1 and QC-HD2 were within ±35% and below 35%, respectively (Table 2), indicating that the dynamic range could be expanded by 100-fold by diluting the samples. These results indicate an acceptable quantitativity for this assay method.

In vivo biodistribution study

iPICs were subcutaneously transplanted to 18 male and 18 female NOD.Cg-PrkdcscidIl2rgtm1Sug/ShiJic mice (NOG mice; In-Vivo Science Inc., Tokyo, Japan). Of these, 10 male and 10 female NOG mice underwent the same surgical procedure as the sham group to confirm the specificity of iPIC detection. Blood and tissue samples were obtained from three male and three female mice at 5, 13, 26, and 52 weeks after transplantation of iPICs for the treated group, and at 5 and 26 weeks for the sham group.

During the study period, five and three animals in the treatment and sham groups, respectively, were found dead or were euthanized. In

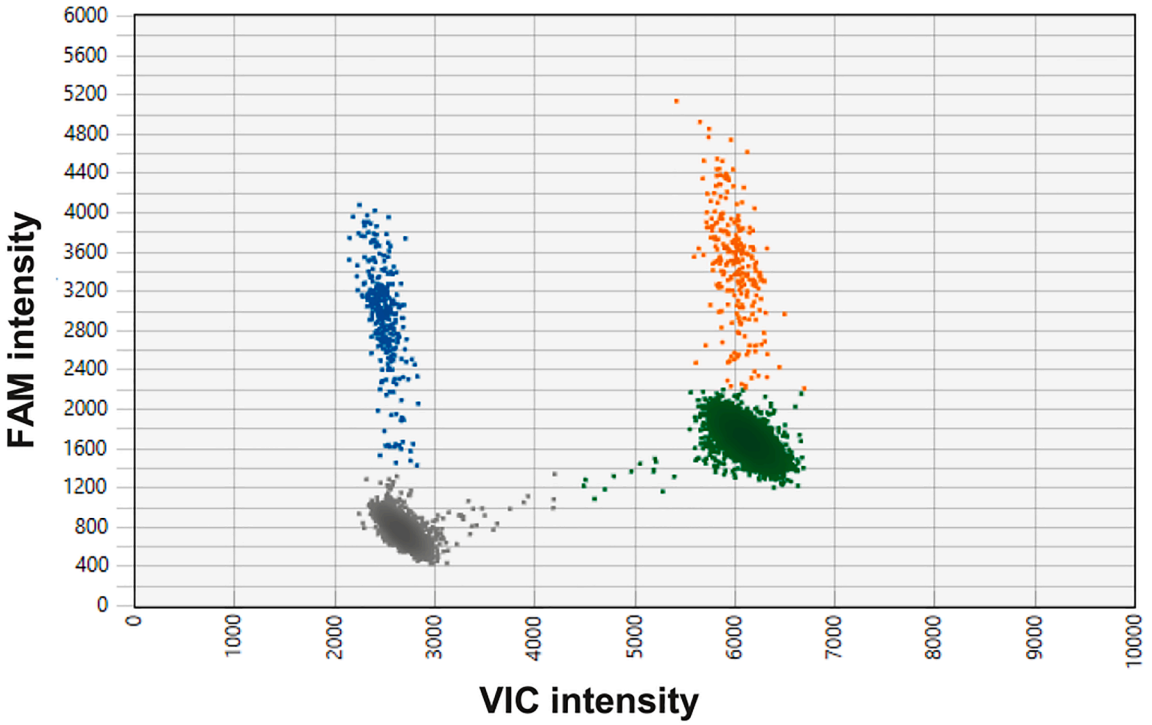

Figure 1. Simultaneous detection of LINE1 and EC sequences using a ddPCR assay
Blue, green, gray, and orange clusters represent LINE1-positive, EC-positive, double-negative, and double-positive droplets, respectively.

these animals, decreased spontaneous respiration and locomotion were observed, and necropsy revealed an enlarged spleen.

For quantification of the *in vivo* samples, QC samples were prepared to ensure assay quantitativity, and all QC samples met the criteria (Table S1). In the sham group, all tissue and blood samples were below the limit of quantification across all time points, indicating the selectivity of the LINE1-ddPCR method. The time profiles of cell concentrations and cell numbers at the transplantation site are shown in Figure 2. No clear differences were observed in the time profiles between cell concentration and cell number; however, the variability in the cell concentration was slightly greater than that in the cell number. The time-concentration profile fluctuated in males, whereas the cell number gradually increased severalfold over 26 weeks before reaching a steady state in both males and females. iPICs were not observed in the blood, liver, kidney, spleen, heart, lungs, brain, stomach, small intestine, large intestine, testis, uterus, or ovary at any given time point.

DISCUSSION

First, we optimized LINE1-ddPCR to quantify iPICs in the blood and various tissue samples using a single surrogate calibration curve. In a previous study, dog gDNA was used as an EC gene to normalize DNA recovery rates,[19] whereas a synthetic plasmid containing canine *MC1R* genes was utilized in this study. Because gDNA is a biologically derived material, its use in biodistribution studies of iCTPs, which require the measurement of large quantities of samples from various matrices, raises ethical concerns regarding animal welfare. Synthetic plasmids are preferred to gDNA in terms of cost and quality. Furthermore, another advantage of synthetic plasmid DNA is its flexibility to adjust critical elements in PCR, such as minimizing cross-reactivity with genetic sequences present in target cells and host animals, as well as controlling the amplicon length. As shown in Figure 1, the simultaneous detection of LINE1 and canine *MC1R* inserted into the plasmid DNA was successfully demonstrated, similar to the case where gDNA was used as an EC gene.[19] Because the positive and negative droplets could be clearly distinguished, this method could be used to quantify iPICs. However, there is scope for further improvement. FAM fluorescence intensity increased in the EC gene (VIC)-positive droplets, which was likely caused by fluorescence spillover from the VIC channel into the FAM channel. Accordingly, reducing the probe concentration for the EC gene or changing the fluorescent dye of the probe may help mitigate spillover.[31]

Second, we validated the quantitativity of the modified LINE1-ddPCR as a bioanalytical method to examine the large-scale biodistribution of iCTPs. As there are no established criteria for quantification in CTP biodistribution studies, in this study, we set the acceptance criteria for accuracy and precision based on our previous study[18,19]: ±35% RE and within 35% CV for QC-H and QC-M samples; ±50% RE and within 50% CV for QC-L samples. All QC samples met the criteria in three separate experiments, as shown in

Table 1. Validation of LINE1-ddPCR assay in NOG mouse samples

		First assay			Second assay			Third assay		
Sample source	Nominal concentration[a]	Mean observed concentration[a]	RE (%)	CV (%)	Mean observed concentration[a]	RE (%)	CV (%)	Mean observed concentration[a]	RE (%)	CV (%)
Liver	3	3.15	5.0	11.8	3.26	8.7	9.0	3.09	3.0	14.9
	200	198.00	−1.0	1.7	197.00	−1.5	3.0	204.00	2.0	3.7
	6,000	5,550.00	−7.5	4.8	5,440.00	−9.3	0.7	5,630.00	−6.2	1.9
Kidney	3	3.51	17.0	19.6	3.28	9.3	17.9	3.18	6.0	12.0
	200	192.00	−4.0	1.9	198.00	−1.0	6.7	201.00	0.5	3.2
	6,000	5,640.00	−6.0	3.2	5,610.00	−6.5	4.6	5,590.00	−6.8	1.4
Heart	3	3.33	11.0	11.2	3.46	15.3	17.5	3.37	12.3	9.6
	200	206.00	3.0	3.4	204.00	2.0	1.2	212.00	6.0	3.4
	6,000	5,890.00	−1.8	2.9	5,650.00	−5.8	0.8	5,880.00	−2.0	1.2
Lung	3	4.04	34.7	8.2	3.26	8.7	5.6	3.08	2.7	13.8
	200	221.00	10.5	1.1	206.00	3.0	2.6	218.00	9.0	2.5
	6,000	7,080.00	18.0	9.6	6,190.00	3.2	2.4	6,310.00	5.2	1.7
Brain	3	3.41	13.7	21.4	3.17	5.7	13.8	3.42	14.0	9.8
	200	203.00	1.5	3.3	199.00	−0.5	1.2	224.00	12.0	3.9
	6,000	6,090.00	1.5	4.9	5,700.00	−5.0	4.9	5,940.00	−1.0	2.7
Spleen	3	3.83	27.7	8.3	3.38	12.7	5.7	3.28	9.3	17.2
	200	216.00	8.0	1.8	197.00	−1.5	4.8	220.00	10.0	2.9
	6,000	6,160.00	2.7	3.0	5,620.00	−6.3	4.8	6,160.00	2.7	0.9
Testis	3	4.12	37.3	11.1	2.92	−2.7	10.0	4.14	38.0	5.7
	200	225.00	12.5	3.3	212.00	6.0	3.6	232.00	16.0	1.4
	6,000	6,540.00	9.0	3.1	6,090.00	1.5	2.6	6,410.00	6.8	3.5
Ovary	3	3.58	19.3	25.5	3.36	12.0	10.6	4.25	41.7	10.4
	200	238.00	19.0	4.0	219.00	9.5	4.1	243.00	21.5	3.0
	6,000	6,670.00	11.2	4.9	5,950.00	−0.8	5.2	6,710.00	11.8	2.0
Stomach	3	3.91	30.3	8.2	3.88	29.3	9.0	3.41	13.7	7.6
	200	234.00	17.0	1.6	220.00	10.0	9.0	244.00	22.0	0.6
	6,000	6,680.00	11.3	4.5	6,230.00	3.8	6.7	6,720.00	12.0	1.5
Small intestine	3	3.92	30.7	13.8	4.18	39.3	27.5	4.30	43.3	13.6
	200	236.00	18.0	4.7	234.00	17.0	3.8	266.00	33.0	3.6
	6,000	7,010.00	16.8	1.1	7,810.00	30.2	7.2	7,580.00	26.3	1.6
Large intestine	3	2.87	−4.3	20.6	3.88	29.3	12.2	3.06	2.0	12.3
	200	166.00	−17.0	6.0	220.00	10.0	6.0	186.00	−7.0	3.6
	6,000	4,940.00	−17.7	3.1	6,380.00	6.3	5.1	5,540.00	−7.7	2.9
Skin	3	2.54	−15.3	5.4	3.08	2.7	20.9	2.52	−16.0	9.1
	200	163.00	−18.5	4.1	180.00	−10.0	5.7	153.00	−23.5	4.3
	6,000	4,660.00	−22.3	2.7	5,250.00	−12.5	4.6	4,590.00	−23.5	2.7
Blood	3	3.42	14.0	3.7	2.84	−5.3	26.7	2.16	−28.0	8.0
	200	181.00	−9.5	2.7	191.00	−4.5	8.4	135.00	−32.5	2.3
	6,000	5,320.00	−11.3	2.1	5,480.00	−8.7	1.3	4,030.00	−32.8	2.8
Calibration curves										
Slope		0.00042–0.00044			0.00044–0.00045			0.00051–0.00053		
R^2		0.9831–0.9954			0.9905–0.996			0.995–0.9959		

Abbreviations are as follows: CV, coefficient of variation; RE, relative error.

[a]Liver, kidney, brain, heart, small intestine, large intestine, and testis: cells/20 mg tissue; spleen, lung, and ovary: cells/10 mg tissue; skin: cells/0.2 mg tissue; blood: cells/50 μL blood.

Table 2. Accuracy and precision in the diluted QC samples

Sample	Dilution factor	Nominal concentration[a]	Mean observed concentration[a]	RE (%)	CV (%)
QC-HD1	10	50,000	43,300.00	−13.4	5.1
QC-HD2	100	50,000	38,000.00	−24.0	22.6

Abbreviations are as follows: CV, coefficient of variation; RE, relative error.
[a]Cells/0.02 and 0.002 mg.

Table 1. Although these criteria are more stringent than recommendations from the Global CRO Council in Bioanalysis (GCC) and the proposal from the Multisite Evaluation Study on Analytical Methods for Non-clinical Safety Assessment of Human-Derived Regenerative Medical Products 2 (MEASURE2): ±50% RE and within 80% CV for the measured CAR-T in GCC recommendation, and ±50% RE and within 50% CV for QC samples in MEASURE2 proposal,[32,33] they are considered more suitable for the quantitative evaluation of the biodistribution of iCTPs. This was achieved as a result of DNA correction using the EC gene and attenuation of matrix effects by ddPCR, enhancing the accuracy and precision of the method.[32] Although further discussion is necessary to develop regulatory guidelines for assay validation, we believe that our results would provide valuable insights into establishing assay criteria for the quantification of CTPs.

Calibration curves were prepared for each plate during DNA extraction and ddPCR measurements. Because the difference in slope was within a maximum of 1.24-fold and the QC samples met the criteria regardless of which calibration curve was used for the calculation, a single calibration curve could be employed across the plates. Furthermore, the calibration curves were similar between runs, suggesting that the number of human cells in various biological samples can be calculated using a single calibration curve across runs. Thus, LINE1-ddPCR is advantageous in reducing the number of samples and costs and is applicable to long-term biodistribution studies of iCTPs, which require the quantification of a large number of samples with various matrices.

The bottleneck of LINE1-ddPCR is its narrow quantitative range of 3–10,000 cells (3,000-fold). This is critical because CTPs can exhibit significant proliferation *in vivo* beyond the dynamic range of ddPCR. Although qPCR—which offers a broader quantitative range despite the quantitative limitations caused by matrix effects—could be considered in the unlikely event that a sample exhibits significant proliferation of CTPs beyond the dynamic range of ddPCR, the LINE1-ddPCR assay demonstrated quantifiability even with a 100-fold dilution of the QC sample, effectively extending the quantitative range of the assay by 300,000-fold. This indicates that samples that exceeded the quantitative range of ddPCR could also be measured through dilution.

Third, we designed a study plan for assessing iPIC biodistribution. NOG mice were used because severely immunodeficient rodents, such as NOG mice, are recommended for nonclinical studies of CTPs to prevent immune rejection by the host due to xenogeneic transplantation.[34] Generally, biodistribution studies of iCTPs should be conducted for as long as possible to assess the tumorigenic risk and cell fate. In this study, the duration was 52 weeks, considering the survival rate of the strain.[35] Indeed, animal deaths were observed during the study period in both the treated and sham groups. Splenomegaly was observed in all these animals, suggesting that malignant lymphoma, which is frequently seen in this strain, was the cause of death,[35] although a histopathological examination, not conducted in the present study, might be necessary for tumorigenicity evaluation. Because diabetes occurs in both males and females, both sexes were evaluated to assess the impact of sex differences on biodistribution and potential migration to reproductive organs. The minimal number of animals necessary to evaluate distribution variability was used, with three animals per time point per sex. Additional reserve animals were prepared for both the treated and sham groups to ensure an adequate number of animals at the 52-week time point, accounting for the possibility of natural death. As shown in Figure 2, the time profiles of the cell numbers at the transplantation site were successfully obtained with low variability. To observe the time-cell number profile and predict the cell fate in each tissue, four time points, spaced approximately in a geometric progression with a ratio of two, were evaluated over the course of one year. These sampling time points effectively captured the time profiles of cell concentration and cell numbers. Selecting the appropriate time points, considering the expected time profile of the cell concentration or cell numbers, is vital to effectively evaluate cell proliferation, decrease, and persistence. Similarly, evaluation of all tissues in the body allows for a comprehensive assessment of tumorigenic risk, with more tissue samples providing more extensive coverage. However, these evaluations require considerable time and effort. To expedite product delivery to patients and achieve disease eradication, it is preferable to select the necessary organs based on the objectives of the study. In this study, we evaluated tissues with abundant blood flow, those critical for maintaining life, and transplantation sites to assess the risk of cell leakage from the transplantation site, subsequent tumor formation, or adverse effects. To predict the biodistribution profile in humans, it is important that the route of administration (ROA) reflects the intended clinical ROA. While it is conceivable to elucidate the systemic distribution following intravenous administration to estimate the risk in cases where cells leak from the transplantation site and migrate into the systemic circulation, this approach may overestimate the risk. Additionally, the systemic distribution could vary depending on the number of migrated cells, and it is possible that cells leaking from the transplantation site could enter the arteries or lymphatic vessels instead of the veins. Therefore, the primary objective of this study was to determine whether cells could leak from the administration site; accordingly, only subcutaneous transplantation, as intended for clinical studies, was explored.

Finally, we studied the long-term biodistribution of iPICs in NOG mice. LINE1-ddPCR showed that the cells were detected only at

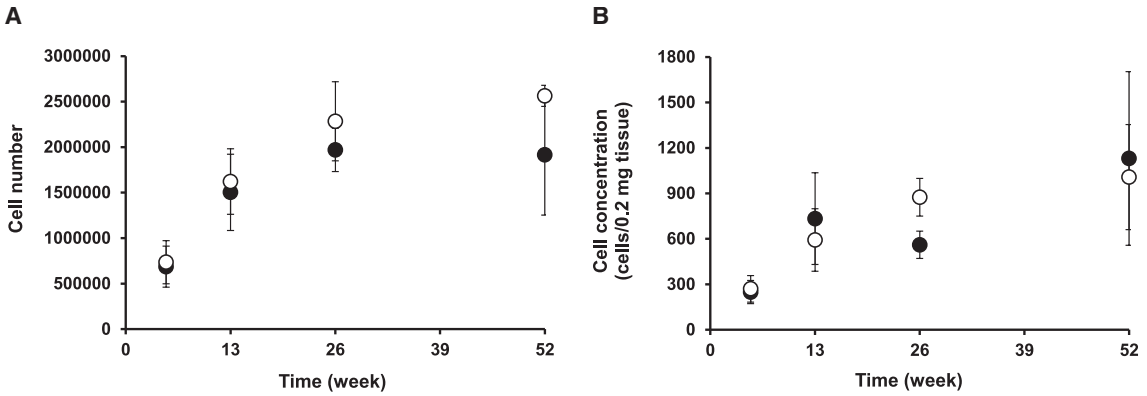

Figure 2. Time profiles of cell numbers and cell concentrations of iPSCs at the transplantation site

(A) Each data point represents mean ± standard deviation of cell numbers (n = 3). (B) Each data point represents mean ± standard deviation of cell concentrations (n = 3). The open and closed circles indicate cell numbers in female and male, respectively.

the transplant site and not in other organs, indicating that iPICs survived at the transplantation site for up to one year without migrating to other organs, thereby demonstrating a low risk of leakage from the transplantation site. If Alu-qPCR had been used in this study, it is likely that noise would have been detected in non-template control samples.[18] Moreover, the noise levels could vary between samples and fluctuate owing to matrix changes over the long-term study, making it difficult to conclusively determine that no migration occurred beyond the transplantation site, which is critical for evaluating the tumorigenicity of iCTPs and their applicability to clinical studies. The absence of noise when using LINE1-ddPCR provides a significant advantage over Alu-qPCR for application in long-term biodistribution studies.

At the transplant site, the number of cells per unit tissue weight (cell concentration) exhibited large variability compared with the number of cells per harvested sample. In addition, the time-concentration profile fluctuated slightly in males, whereas the cell number gradually increased severalfold over the course of 26 weeks before reaching a steady state in both males and females, with no evident sex differences. This suggests that the concentration of iPICs may vary depending on the ratio of iPICs to mouse-derived skin in the collected samples. In the present study, variation in the amount harvested at the transplantation site was minimal. Moreover, no clear differences in the time profiles of the cell numbers and concentration were observed, thus minimizing their impact on data interpretation. However, because cell concentration varies depending on the amount harvested, it could potentially lead to misinterpretations regarding cell number fluctuations, which could be critical in evaluating tumorigenic risk. Therefore, we recommend monitoring changes in the total number of cells per organ when evaluating the tumorigenicity risk of iCTPs with no clear proliferation, such as iPICs. However, when assessing products with expected proliferation, such as liver transplants, where the ratio of donor cells to host cells and organ weights can change significantly,[36,37] careful considerations should be given to the units used for reporting. Furthermore, for pharmacokinetic analysis, concentration data are commonly used[38–40]; therefore, it is important to adopt the appropriate units, based on the purpose of the study.

At the transplantation site, the number of cells initially decreased post-transplantation, recovered to the original transplanted number (dosage) after six months, and remained constant for the following six months. Based on the observed rate of increase in cell numbers from week 5 to week 13, it is probable that the cell number prior to week 5 was even lower than that at week 5. However, because iPICs were not detected outside the transplantation site at any given time point, it is unlikely that the temporary decrease in cell number was due to migration and engraftment of iPICs to other tissues. The temporary decrease was believed to have occurred until sufficient angiogenesis was established at the transplant site, after which the cell numbers gradually recovered. Previous studies have reported that when neonatal porcine islets were subcutaneously transplanted into mice along with a fibrin scaffold, blood vessels were observed around the transplant site at 7 days post-transplantation, vascularization increased at days 14 and 21, and insulin-positive islets engrafted even at 22 weeks post-transplantation. In contrast, without the fibrin scaffold, a reduction in insulin-positive islets was observed at 3 weeks post-transplantation, with complete disappearance by 7 weeks.[41] Thus, in subcutaneous transplantation, vascularization seems essential for the survival and therapeutic efficacy of transplanted cells.[42] Although further studies are required to analyze the factors influencing the time-dependent changes in the number of iPICs after subcutaneous transplantation, the absence of cells outside the transplant site and the lack of proliferation beyond the transplanted cell count suggest that the risk of migration from the transplant site and tumorigenicity are negligible for iPICs. Furthermore, iPICs survived at the transplantation site for 52 weeks, suggesting that their therapeutic effects in clinical settings could persist for over a year. These characteristics are advantageous for patients, given that insulin pens conventionally used by patients require repeated injections. Therefore, these findings support the clinical application of iPICs.

Table 3. Key considerations for the biodistribution study of induced pluripotent stem cell-derived cell therapy products

	Considerations
Bioanalytical assay method	unit notation: "cells/μL biofluid or mg tissue" is recommended
	instrument: ddPCR is recommended to mitigate matrix effects
	standard sample: cell lysate is recommended
	primer/probe design: sensitivity, selectivity, and specificity should be considered
	PCR conditions: master mix, spillover, annealing temperature/duration, number of cycles, primer/probe concentrations, PCR input, replicate assays
	DNA extraction: use of an external control gene is proposed to normalize DNA variability
	calibration curve: a single surrogate calibration curve can be employed to minimize the number of animals used; linear regression: $r^2 > 0.98$
	QC samples: at least four samples at each concentration with each matrix, concentration, and dilution
	accuracy: within ±35% (50% for QC sample at lower concentrations)
	precision: <35% (50% for QC sample at lower concentrations)
	sample preparation: homogenization of the entire tissue is recommended to quantify CTPs heterogeneously distributed in organs
In vivo biodistribution	duration: lifetime of the animal
	species: immunodeficient animals
	sex: both male and female (depending on the clinical application)
	number of animals: at least three animals to evaluate individual variability; consider the natural mortality during the study duration
	tissue: tissues with abundant blood flow, those vital for maintaining life, and the transplantation site
	time points: multiple time points to capture cellular kinetics
	units: "cells/μL biofluid," "cells/mg tissue," or "cells/tissue" can be reported

Abbreviations are as follows: CV, coefficient of variation; CTPs, cell therapy products; PCR, polymerase chain reaction; QC, quality control; RE, relative error.

This study had some limitations. Although this study demonstrated the applicability of LINE1-ddPCR in immunodeficient animals, it is necessary to evaluate their applicability to other animal species used to evaluate the efficacy of CTPs, such as dogs, pigs, and monkeys; we do believe that our method is applicable, based on the human specificity of LINE1 sequence.[43–46] When using LINE1-ddPCR in animal species other than mice, it is necessary to design ECs that do not cross-react with the host's gDNA. However, as synthetic plasmids are employed, this design is relatively straightforward. In biodistribution studies involving large animals, it will likely be a challenge to optimize homogenization methods due to the use of large tissue samples. Furthermore, the *in vivo* experiment was designed to show that the transplanted cells did not exhibit significant fluctuations in cell number and remained at the transplantation site without substantial proliferation; however, the same experimental design may not be applicable for products such as genetically engineered immune cells expressing chimeric antigen receptors that migrate to various tissues and demonstrate significant cellular dynamics. In the future, it is essential to accumulate further data on the *in vivo* distribution of various cellular products to advance the standardization of biodistribution studies and their analytical methods and to develop comprehensive guidelines.

Conclusion

In this study, we optimized LINE1-ddPCR, confirmed its quantitativity, developed a fit-for-purpose biodistribution study protocol, and conducted a long-term animal study of iPICs transplanted subcutaneously into immunodeficient mice to standardize the biodistribution assessment protocol for iCTPs. The present study successfully clarified the biodistribution profile of iPICs and justified their clinical use in terms of long-term persistence and low tumorigenicity risk, although the lack of histopathological examination should be noted. The key considerations for biodistribution studies derived from our study are summarized in Table 3. The insights gained from this study are crucial for standardizing biodistribution study protocols and advancing the research and development of iCTPs.

MATERIALS AND METHODS

Materials

iPICs were prepared by Orizuru Therapeutics, Inc. (Kanagawa, Japan) as previously reported, with some modifications. The plasmid, including a portion of the canine *MC1R* sequence, was synthesized by Thermo Fisher Scientific (Waltham, MA, USA) as the EC for normalization of the DNA recovery rate. Primers and probes for the human LINE1 sequence and canine *MC1R* were obtained from Thermo Fisher Scientific.

ddPCR analysis

A QX ONE Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA) was used for the ddPCR assay. The primer/probe sets used in our previous study were used to detect LINE1 and EC.[19] The ddPCR reaction was performed for 45 cycles (95°C for 0.5 min and 58°C for 1.5 min) after an initial denaturation at 95°C for 10 min. Following enzyme inactivation at 98°C for 10 min, the droplets were classified as positive or negative based on fluorescence intensity. Since no positive droplets were observed in our previous report when ddPCR Supermix for Probes was used for the ddPCR assay, ddPCR Multiplex Supermix (Bio-Rad Laboratories) was used as the master mix. The ddPCR reaction mixture contained 4× ddPCR Multiplex Supermix, primers (2,700 nmol/L for LINE1, 500 nmol/L for EC), probes (250 nmol/L), dithiothreitol (4 mmol/L), and restriction enzyme (ScaI, 10 U/μL; Takara Bio,

Shiga, Japan), making up a final volume of 20 μL. All reactions were performed in triplicate. The copy numbers of the LINE1 and EC sequences were determined using QX ONE Software (ver. 1.2, Bio-Rad Laboratories).

Assay validation

Assay validation was conducted on three separate days. The cells were lysed at a concentration of 7,140 cells/μL with DNA/RNA Shield (Zymo Research, Irvine, CA, USA) combined with proteinase K (ProK) in a ratio of 9:1 at a temperature of 65°C. Subsequently, working cell lysates were prepared by serially diluting cell lysate with the DNA/RNA Shield/ProK mixture, achieving final concentrations ranging from 4.29×10^{-2} to 1.43×10^{2} cells/μL. Mouse tissue and blood were obtained from male NOG mice. All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the facility, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Control tissue samples were homogenized in DNA/RNA Shield to generate blank homogenates. To prepare calibration curve samples, working cell lysates were added to blank homogenates or blood samples. The concentrations ranged from 3, 10, 30, 10^2, 3×10^2, 10^3, 3×10^3, 6×10^3, to 10^4 cells per 20 mg tissue (for liver, kidney, heart, brain, small intestine, large intestine, and testis), 10 mg tissue (for spleen, lung, ovary, and stomach), 0.2 mg tissue (skin), or 50 μL of blood. QC samples were prepared at concentrations of 3 (QC-L), 2×10^2 (QC-M), and 6×10^3 (QC-H) cells/20 mg tissue, 10 mg tissue, 0.2 mg tissue, or 50 μL blood. A QC sample with a concentration of 5×10^4 cells/0.2 mg skin (QC-UH) was prepared. The diluted QC samples were prepared by diluting the QC-UH sample 10-fold (5×10^3 cells/0.2 mg tissue; QC-HD1) and 100-fold (5×10^2 cells/0.2 mg tissue; QC-HD2) with the control homogenate to confirm the accuracy and precision of the dilution procedure. The plasmid was added to all samples as an EC to normalize DNA variability among samples. DNA was extracted following the protocol described in our previous study, with minor modifications,[19] using a MagMAX DNA Multi-Sample Ultra 2.0 Kit (Thermo Fisher Scientific). Briefly, DNA was extracted from 200 μL of tissue homogenate or 50 μL of blood using the KingFisher Apex system (MagMAX_Ultra2_200μL_Flex and MagMAX Ultra 2TissueV). The copy numbers of LINE1 and EC in the reaction mixture were determined as described in the "ddPCR analysis" section. The cell concentration in the QC samples was calculated using the equation Y = aX, where X is the nominal cell concentration, Y is the ratio of the LINE1/EC copy numbers in the reaction mixture, and a is the slope of the calibration curve. The assay criteria were based on our previous report,[32] recommendations from the GCC, and a proposal from MEASURE2.[33,34] The RE and CV value criteria were set within 100.0% ± 35.0% for QC-H, QC-M, QC-HD1, and QC-HD2 and within 100.0% ± 50.0% for QC-L.

Long-term biodistribution study

All procedures were approved by the IACUC of the contract research organization accredited by AAALAC. Seven-week-old male and female NOG mice were housed under a 12-h light-dark cycle with free access to water and a pelleted diet (CE-2; CLEA Japan, Inc., Tokyo, Japan). The room temperature was maintained at 23°C ± 3°C. The animals were divided into sham and treatment groups. A biodegradable implant containing 2.5×10^6 iPICs was transplanted into the subcutaneous space of treatment group animals. Blood and tissue samples were obtained from three male and three female animals at 5, 13, 26, and 52 weeks after transplantation. In the sham group, the same surgical procedure was performed, and blood and tissue samples were obtained at 5 and 26 weeks from three male and three female animals to confirm the specificity of iPIC detection. Samples were frozen immediately after weighing and stored at −70°C until DNA extraction. Because CTPs typically exhibit heterogeneous distribution within an organ, the entire organ was homogenized in DNA/RNA Shield to quantify the total CTPs in the organ. For the treatment group, the transplantation site (skin) was further diluted 10-fold with control skin homogenate. DNA extraction from samples and ddPCR assays were performed as described in the "assay validation" and "ddPCR analysis" sections. Sample analysis was conducted on two separate days: from 5, 13, and 26 weeks, and from 52 weeks.

DATA AVAILABILITY

All datasets generated in this study will be made available upon request.

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AUTHOR CONTRIBUTIONS

M.N., Y.M., H.U., T.W., H.H., and S.Y. contributed to the design of the experiments, interpretation of data, and writing of the manuscript; M.N. and S.Y. conducted the experiments.

DECLARATION OF INTERESTS

H.U. is an employee of is Orizuru Therapeutics.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2025.101538.

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