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Dysfunctional pancreatic cells differentiated from induced pluripotent stem cells with mitochondrial DNA mutations

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Diabetes mellitus (DM) is a serious disease in which blood sugar levels rise abnormally because of failed insulin production or decreased insulin sensitivity. Although many studies are being conducted for the treatment or early diagnosis of DM, it is not fully understood how mitochondrial genome (mtDNA) abnormalities appear in patients with DM. Here, we induced iPSCs from fibroblasts, PBMCs, or pancreatic cells of three patients with type 2 DM (T2D) and three patients with non-diabetes counterpart. The mtDNA mutations were detected randomly without any tendency among tissues or patients. In T2D patients, 62% (21/34) of iPSC clones harbored multiple mtDNA mutations, of which 37% were homoplasmy at the 100% mutation level compared to only 8% in non-diabetes. We next selected iPSC clones that were a wild type or carried mutations and differentiated into pancreatic cells. Oxygen consumption rates were significantly lower in cells carrying mutant mtDNA. Additionally, the mutant cells exhibited decreased production of insulin and reduced secretion of insulin in response to glucose. Overall, the results suggest that screening mtDNA mutations in iPSCs from patients with T2D is an essential step before pancreatic cell differentiation for disease modeling or autologous cell therapy. [BMB Reports 2022; 55(9): 453-458]

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders characterized by high blood sugar levels over a prolonged period (1). Type 2 diabetes (T2D) is the most common form of diabetes, and especially in the elderly, is a serious disease with life-threatening complications (2). T2D is caused by insulin resistance, which prevents skeletal muscle, liver, and adipose tissue from responding to insulin, resulting in elevated blood glucose (3, 4). Additionally, excessive insulin production and hyperglycemia caused by T2D can damage pancreatic cells, leading to the progressive impairment of insulin secretion (5, 6). In the later stages, islet transplantation is the most effective treatment, but a lack of donors limits its large-scale clinical application. To overcome this issue, the use of induced pluripotent stem cells (iPSCs) may be considered (7, 8).

iPSCs can be generated from somatic cells by transducing transcription factors such, as *OCT4*, *SOX2*, *KLF4*, and c-*MYC* (9). The iPSCs have great potential for use in drug discovery, disease modeling, and cell therapy, because of their ability to proliferate indefinitely and differentiate into various cell types (10). The iPSCs can also be differentiated into pancreatic cells, which are useful for anti-diabetic drug development, diabetic disease modeling, and clinical treatment. However, abnormalities of the genome, such as aneuploidy, sub-chromosomal copy number variations, and single nucleotide variations in iPSCs limit their clinical use (11).

Mitochondria have an independent genome called mitochondrial DNA (mtDNA) (12). The mtDNA is small (16,569 base pairs), but encodes many genes essential for oxidative phosphorylation and cellular energy production (13). Mutations occur in the mtDNA at a rate about 10 to 20 times that of the nuclear genome and cause numerous inherited diseases in humans (14). Because mtDNA is a multi-copy genome, it often represents a heteroplasmic genotype in which both the healthy and the mutated allele are present in the same cell. Mitochondrial dysfunction may have a direct effect on DM because metabolites produced in mitochondrial metabolism act as additive signals regulating insulin secretion (15). Therefore, the presence of mtDNA mutations in iPSCs can be problematic when using iPSC as autologous cell therapy or disease modeling of DM (16).

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It has been reported that individual iPSC clones, each derived from a single cell, can carry mtDNA mutations that cause functional abnormalities, which are not generated during reprogramming but are already present in individual parental cells (17). Also, these somatic mutations tend to increase with age, which is associated with accumulated damage or replication errors in mtDNA (18).

In this study, we generated iPSC clones from fibroblasts, PBMCs, or pancreatic cells of patients with T2D and non-diabetes (ND). The iPSC clones were analyzed for the mtDNA mutations. We also selected 2 iPSC clones derived from one patient and differentiated them into pancreatic cells. Oxygen consumption rate, the production of insulin, and glucose-stimulated insulin secretion (GSIS) were measured to investigate the association between mtDNA mutations and the function of differentiated pancreatic cells.

RESULTS

mtDNA mutations in iPSCs derived from patients with type 2 diabetes

We isolated fibroblasts (Fib), PBMCs (Blo), or pancreatic cells (Pan) from three patients with T2D (T2D-1, 2, and 3). Due to limited access to tissue samples, Fib or Pan were not isolated from T2D-1 and T2D-3, respectively. The seven isolated cell lines were induced iPSCs and selected 5 clones from each cell line except for Blo of T2D-3, which were only 4 clones. A total of 34 clones and parental cells were sequenced whole mtDNA by the Illumina Miseq platform.

No mtDNA mutations were detected in parental Fib and Blo in T2D-1, while 4 and 5 mutations were identified in Fib-iPSCs and Blo-iPSCs, respectively (Table 1). Three mutations were in non-syn or rRNA regions, which could affect mitochondrial

Table 1. mtDNA mutations in iPSCs derived from patients with T2D

Subject	Cell origin	Position	Locus	Effect	Major haplotype	Parental cells (%)	Individual iPSC clone (%)				
							1	2	3	4	5
T2D-1	Fib	3315	ND1	Syn	G	ND			A (48)	ND	ND
		5226	ND2	Non-syn	G				A (100)		
		9018	ATPase6	Syn	T		C (39)				
		12650	ND5	Non-syn	T			C (56)			
	Blo	2613	16S	rRNA	T	ND	C (100)		ND	ND	C (100)
		10400	ND3	Syn	T						C (100)
		11002	ND4	Syn	A		G (100)				C (100)
		16005	ATT/P	MitoTIP 25.60%	T						C (7)
		16129	ATT/CR	Non-coding	G			A (78)			
T2D-2	Fib	5654	A	MitoTIP 29.80%	T		C (13)	C (10)		ND	
		5655	A	MitoTIP 26.70%	T	C (3)					
		6207	COI	Non-syn	T	C (6)					
		9928	COIII	Non-syn	A		G (11)				
		11976	ND4	Non-syn	A		G (64)	G (55)			
		13726	ND5	Non-syn	G						T (13)
		14260	ND6	Syn	A		G (100)	G (100)	G (100)		
	Blo	874	12S	rRNA	G	ND		A (5)			
		2220	16S	rRNA	A				G (8)		
		4703	ND2	Syn	T						C (5)
		6458	COI	Syn	C						T (100)
		6678	COI	Non-syn	A		G (100)				
		7970	COII	Non-syn	G		A (13)				
		13312	ND5	Non-syn	T					C (75)	
		14900	Cytb	Non-syn	G					A (13)	
	Pan					ND	ND	ND	ND	ND	ND
T2D-3	Blo	6023	COI	Syn	A	G (2)	ND	ND	ND	ND	
		10535	ND4L	Syn	T	C (50)					
	Pan	302	CR:HVS2/	Non-coding	A		ND	ND	ND	C (31)	
		9591	COIII	Non-syn	G						A (55)
		10535	ND4L	Syn	T	C (55)					

ND: not detected, Gray block: no iPSC clone, %: heteroplasmy.

function. Each 2 iPSC clones from Fib and Blo were not carried mutant mtDNA. Three clones out of 6 were carried 2 or 3 mutations in one iPSC clone. Two clones (clone 1 and clone 5 from Blo) were shared the same mtDNA mutations (mt2613 and mt11002), additionally, clone 5 were carried two more additional mutations (mt10400 and mt16005).

In the T2D-2, only Fib has detected 2 mutations with 3% and 6% heteroplasmy. There was no shared mutation between parental Fib and Fib-iPSCs. Four Fib-iPSC clones out of five were carried mtDNA mutations. Of which, three clones (clones 1, 2, and 3) contained the same mutation (mt14260, Syn) with homoplasmy. Additionally, two clones (clones 1 and 2) were carried additional 2 or 3 mutations with different heteroplasmy (Table 1). This observation was similar to Blo-iPSCs from T2D-1, which was shared the same mutation among iPSC clones and some clones carried additional mutations. All five Blo-iPSC clones from T2D-2 carried mtDNA mutations. Two of them carried high heteroplasmic mutations with no sharing among iPSC clones. Interestingly, no mutation was identified in parental Pan and Pan-iPSCs (Table 1).

No mutation was detected in Blo-iPSCs from T2D-3, even though two mutations were found in parental Blo. One was 50% heteroplasmy (mt10535, ND4L, Syn), which was shared with Pan as 55% heteroplasmy suggesting germline or *de novo* mtDNA mutation. Two Pan-iPSC clones carried mtDNA mutations, which were not carried in parental Pan. A similar observation was reported previously (17).

mtDNA mutations in iPSCs derived from patients with non-diabetes

We next isolated Fib, Blo, and Pan from 3 patients with ND (ND-1, 2, and 3). Expect Fib and Blo from ND-2, seven cell lines were induced iPSCs. Three to five clones were established in each line. Nine parental cell lines and 30 iPSC clones were analyzed for mtDNA mutations (Table 2).

Three mutations were identified in Fib of ND-1; mt1351, mt10158, and mt15077. Even 45% heteroplasmy in Fib, iPSC clones had no the same mutation indicating any shared mutation with parental cells. Mutation in mt15077 was shared with Blo

Table 2. mtDNA mutations in iPSCs derived from patients with ND

Subject	Cell origin	Position	Locus	Effect	Major haplotype	Parental cells (%)	Individual iPSC clones (%)				
							1	2	3	4	5
ND-1	Fib	1351	12S	rRNA	G	A (45)					
		10158	ND3	Non-syn	T	C (6)					
		14287	ND6	Syn	C		T (36)	T (44)	T (45)	T (47)	T (39)
		15077	Cytb	Non-syn	G	C (14)					
	Blo	7780	COII	Syn	A		G (78)				
		13726	ND5	Non-syn	G			T (7)			T (4)
		14287	ND6	Syn	C			T (35)	T (44)	T (14)	T (44)
		15077	Cytb	Non-syn	G	A (12)					
		15773	Cytb	Non-syn	G					A (24)	
	Pan	9478	COIII	Non-syn	T	ND			C (100)		
		13726	ND5	Non-syn	G		T (12)				
		14268	ND6	Syn	G		T (14)	T (100)	T (100)		
		14287	ND6	Syn	C		T (34)	T (50)	T (45)		
		15087	Cytb	Non-syn	A			G (4)			
		15096	Cytb	Non-syn	T			C (4)			
ND-2	Fib	13726	A	MitoTIP 29.80%	G	T (7)					
	Blo					ND					
	Pan	2976	16S	rRNA	G	A (48)				ND	
		4028	ND1	Non-syn	T		G (5)	G (12)			G (5)
		6026	COI	Syn	G		C (5)				
		11379	ND4	Non-syn	T						C (6)
		14343	ND6	Non-syn	C		A (16)		A (23)		
ND-3	Fib	3047	16S	rRNA	G	ND	A (8)				
	Blo	13107	ND5	Non-syn	C		A (40)	A (8)		A (39)	
	Pan	4298	I	MitoTIP pathogenic (83.50%)	G	ND	A (52)	ND		A (47)	
		4713	ND2	ND2:G82TERM	G				A (74)		

ND: not detected, Gray block: no iPSC clone, %: heteroplasmy.

of the same individual with similar heteroplasmy (14% and 12%), but any iPSC clones were not carried this shared mutation. However, mt14287C>T mutation was shared among iPSC clones from Fib, Blo, and Pan with high frequency, which 12 clones out of 13 clones carried it with 14%-50% heteroplasmy.

There was no shared mutation among parental cells in ND-2. Mt2976G>A with 48% heteroplasmy was identified in Pan, but any iPSC clones had detected the mutation. No mutation was identified in three different parental cells in ND-3. Seven iPSC clones out of 12 carried mtDNA mutations. Mt4298G>A encoded tRNA was shared with two iPSC clones from Pan, which was 83.5% pathogenicity in Mito TIP (https://www.mitomap.org/MITOMAP).

Comparison of mtDNA mutations in iPSCs between patients with T2D and ND

To investigate the difference in the mtDNA mutation pattern in iPSCs between patients with T2D and ND, we compared mtDNA mutations between the two groups. The average number of mtDNA mutations per iPSC clone was not significantly different in ND and T2D (1.2 vs. 0.9, Fig. 1A). Of all T2D-iPSC clones, 61% (21/34) was carried one or more mtDNA mutations, whereas 80% (24/30) in ND-iPSCs (Fig. 1B). Among the total mtDNA mutations identified in T2D-iPSCs, only 43% (13/30) contained less than 50% heteroplasmy and 37% (11/30) with homoplasmy (Fig. 1C). Interestingly, mtDNA mutations with homoplasmy in ND accounted for only 8% (3/29). Comparisons of iPSC clones with mutations revealed a significantly higher average heteroplasmy in T2D (52%) than in ND (34%) ($P < 0.05$, Fig. 1D). Additionally, the probability of obtaining an iPSC clone with homoplasmic mtDNA mutation in T2D was 24% and ND only 7% (Fig. 1E).

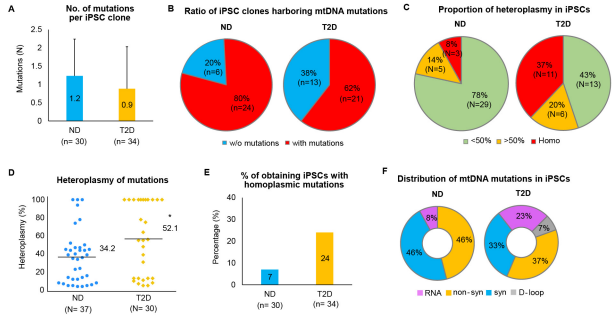

Fig. 1. mtDNA mutations in patients with ND- and T2D-iPSCs. (A) The number of mtDNA mutations in iPSC clones derived from patients with ND and T2D. There were no significant differences between ND and T2D. (B) The proportion of iPSCs with and without mtDNA mutations in ND and T2D. n = the number of iPSC clones. (C) Heteroplasmy proportion of whole mtDNA mutations in iPSCs. The proportion of homoplasmic mutations was only 8% in ND compared to 37% in T2D. N = the number of mtDNA mutations (D) Heteroplasmy distribution of mtDNA mutations found in ND and T2D. The heteroplasmy of mutations found in T2D was higher than that of ND (*$P < 0.05$). (E) Probability of obtaining iPSCs with homoplasmic mtDNA mutations. iPSC clones with homoplasmy were found with a probability of 7% in ND and 24% in T2D. (F) Distribution of mtDNA mutations in ND and T2D. The ratio of mutations in the RNA region was higher in T2D than in ND (23% vs. 8%). iPSC: induced pluripotent stem cell; mtDNA: mitochondrial DNA; ND: non-diabetes; T2D: type 2 diabetes.

The distribution of mtDNA mutations showed that non-synonymous (non-syn) mutations resulting in changes in amino acid coding were 46% (17/37) in ND and 37% (11/30) in T2D (Fig. 1F). The synonymous (syn) mutations were 46% (17/37) in ND and 33% (10/30) in T2D. For mutations in the RNA region, it was only 8% (3/37) in ND compared to 23% (7/30) in T2D. Mutations in the D-loop, a non-coding region, were found only in T2D (7%, 2/30).

Our results suggest that although mtDNA mutations in iPSCs occur randomly irrespective of the parental cells, more mtDNA mutations with homoplasmy are found in iPSC clones from T2D than in ND.

mtDNA mutations affecting the function of differentiated pancreatic cells

To determine the relationship between mtDNA mutations and pancreatic lineage differentiation, PBMC-derived iPSC (BiPSC) clones were considered because blood is technically and ethically more convenient to obtain compared to other tissues. Among BiPSCs from 3 T2D patients, T2D-2 BiPSC clone 1 was the only BiPSC clone with a non-syn mutation that changed the amino acid sequence, so we selected T2D-2 BiPSCs.

T2D-2 BiPSC clones were differentiated into pancreatic progenitor cells (PPCs) and PPC markers, including *PDX1*, *NGN3*, and *NKX6.1*, were analyzed by qRT-PCR (Fig. 2A). Clone 1 harboring 100% mt6678A>G and 13% mt7970G>A mutations in the non-syn protein-coding region showed low expression of all genes compared to the other clones ($P < 0.05$). Also, to determine the relationship between amino acid alterations by mtDNA mutation and the expression of PPC-related genes, we examined the expression of each gene according to the sum of the heteroplasmy in the regions of RNA non-syn protein-coding (Fig. 2B). The expression of PPC genes showed a tendency to decrease in the non-syn mutations. Clone 5 showed high gene expression, but it carried 5% mt4703T>C and 100% mt6458C>T mutations in the syn region (Table 1). Therefore, we decided to proceed with further research by selecting clone 1 as mutant and clone 2 carrying only 5% mt874G>A in the rRNA region as wild-type.

To investigates whether the mtDNA mutations affected the function of differentiated pancreatic cells, clones 1 (mutant) and 2 (wild) were differentiated into pancreatic spheroids. There was no difference in the number (381/384 vs. 382/384) and size (77 vs. 80 μm) of the pancreatic spheroids (Fig. 2B). INSULIN, a differentiation marker of pancreatic cells, was observed in both clones 1 and 2 by immunocytochemistry (ICC) (Fig. 2C).

We also investigated mitochondrial respiration function in iPSC clone 1 and clone 2 with a seahorse platform. Clone 1-derived pancreatic cells showed reduced oxygen consump-

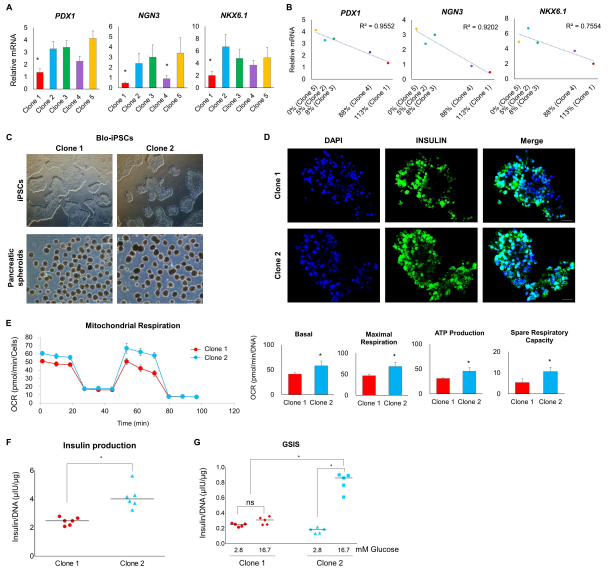

Fig. 2. mtDNA mutations affect the dysfunction of differentiated pancreatic cells. (A) The gene expression of PPC, *PDX1*, *NGN3*, and *NKX6.1*, relative to *GAPDH* in T2D-2 BiPSCs. All gene expressions in clone 1 were significantly lower compared to other clones (*P < 0.05, 2 biological replicates, 3 technical replicates). (B) PPC-gene expression was inversely proportional to the sum of the heteroplasmy of mtDNA mutations in the positions of change of the amino acid, and RNA. As heteroplasmy increases, the expression of PPC-related genes decreases. (C) Morphology of clones 1 and 2 and pancreatic spheroids derived from them. Clone 1 had 100% mt6678A>G and 13% mt7970G>A mutations in the non-syn protein-coding region and clone 2 had only 5% mt874G>A mutations in the rRNA region. Both clones showed similar spheroid formation. Scale bar = 200 μm. (D) Immunocytochemistry examining the INSULIN (green) expression of pancreatic spheroids. Nuclei were stained with DAPI (blue). Scale bar = 20 μm. (E) Analysis of OCR levels in differentiated pancreatic cells. In clone 1, all OCRs were significantly lower compared with clone 2 (*P < 0.05, 2 biological replicates, and 3 technical replicates). (F) Insulin production in pancreatic spheroids derived from iPSC clones. The insulin in clone 2 was significantly higher than that of clone 1 (*P < 0.05). (G) GSIS analysis of pancreatic spheroids. Clone 2-derived pancreatic spheroids increased the amount of insulin secretion when glucose concentration increased, whereas clone 1 did not (*P < 0.05, 2 biological replicates, 3 technical replicates). PPC: pancreatic progenitor cell; DAPI: 4′,6-diamidino-2-phenylindole; OCR: oxygen consumption rate; GSIS: glucose-stimulated insulin secretion.

tion rates (OCR) compared with clone 2 (Fig. 2D). Basal OCR, maximal respiration, ATP production, and spare respiratory capacity were significantly lower in clone 1 than in clone 2 (P < 0.05).

Next, we investigated the insulin content of pancreatic spheroids to determine the association of mtDNA mutations with insulin production (Fig. 2E). Intracellular insulin was significantly lower in clone 1 than in clone 2 (P < 0.05).

Finally, to examine the effect of mtDNA mutation on the insulin-secreting function of pancreatic cells, glucose-stimulated insulin secretion (GSIS) was induced by the low and high glucose treatment of iPSC-derived pancreatic spheroids (Fig. 2F) (19). Normal pancreatic cells secrete low insulin under low glucose conditions and increase insulin secretion when the glucose concentration increases (20). Clone 1-derived pancreatic spheroids exhibited low levels of insulin secretion at low glucose concentrations but did not increase insulin secretion even when the glucose concentration was increased, whereas clone 2 significantly increased insulin secretion at high glucose concentrations (P < 0.05).

These results indicate that mtDNA mutations could impair mitochondrial respiration capacity and further affect insulin production and insulin-secreting function in differentiated pancreatic cells.

DISCUSSION

It has been reported that iPSC clones derived from the same individual harbored various somatic mtDNA mutations, which were not found in parental cells (17, 21). As a similar result, we also identified several novel mtDNA mutations at high heteroplasmic or homoplasmic levels in iPSC clones. The mutations were not associated with tissue or disease with T2D. However, more homoplasmic mtDNA mutations were found in iPSCs from patients with T2D compared to ND.

Interestingly, some mutations, which were detected in parental cells with high heteroplasmy, were not detected in any iPSCs (ex, 50%-55% heteroplasmy at mt10535T>C in Blo and Pan from T2D-3, 45% at mt1351G>A in Fib from ND-1, and 48% at mt2976G>A in Pan from ND-2). However, other mutations have been identified in several clones with high heteroplasmy even not present in parental cells (mt2613T>C, mt4298G>A, mt11002A>G, 11976A>G, mt12387C>T, mt14260A>G, and mt14268G>T). These findings suggest that mtDNA mutations may be associated with positive or negative iPSC selection during induction or expansion (17).

mtDNA mutations may affect the differentiation of iPSCs into target cells such as neural cells, cardiomyocytes, and fibroblasts (17, 22, 23). Like the mt3243A>G mutation, there are well-known mtDNA mutations that are closely related to diabetes (24), however, to our knowledge, there are no reports of whether somatic mtDNA mutations in iPSCs cause pancreatic cell dysfunction.

Regulating insulin secretion in pancreatic cells is important for glucose homeostasis (25). In patients with diabetes, postprandial glucose-stimulated insulin secretion (GSIS) is insufficient in pancreatic cells, which is characteristic of diabetic pathology (26). Although the molecular mechanisms involved in GSIS are well known, the correlation between mtDNA mutations and GSIS is unclear and requires further exploration.

T2D requires islet transplantation in severe cases, and as shown in the transplant results of Choi *et al.* (27), it can be seen that 20% of all islet transplant patients were patients with T2D. However, our study revealed that the function of pancreatic cells derived from iPSC harboring mtDNA mutations was dysfunctional. The mitochondrial respiration capacity was decreased and insulin production and secretion were impaired. Given that ATP production is closely related to insulin secret-

ion (28, 29), these results suggest that mtDNA mutations may affect ATP production and impair ATP-dependent insulin secretion.

Taken together, our study suggests that screening the mtDNA is essential before further research for disease modeling and clinical application of iPSCs as autologous cell therapies for diabetes.

MATERIALS AND METHODS

Further detailed information is provided in the Supplementary Information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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