Genetic engineering and quality control of clinical grade iPS cells as a source of HLCN061

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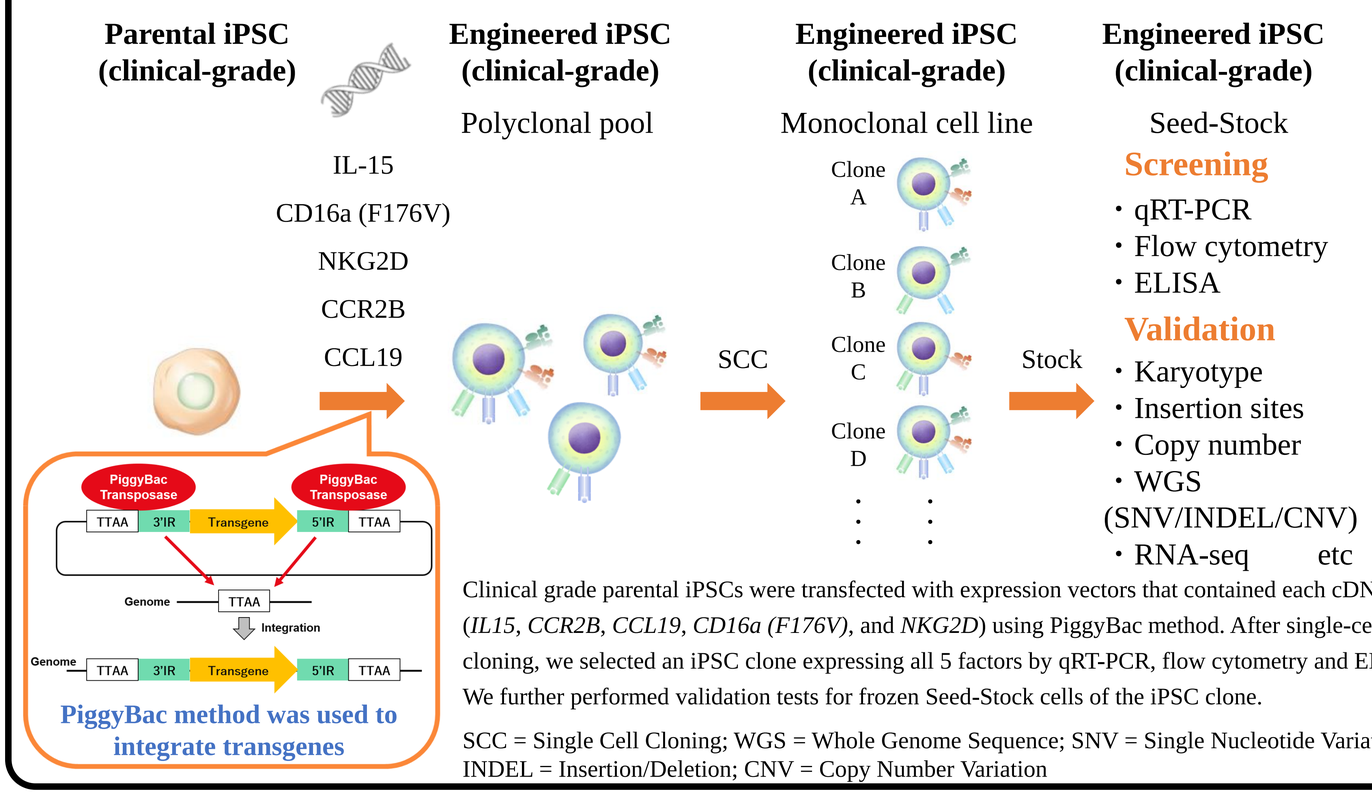
Abstract

HLCN061 is a human iPSC derived NK cells differentiated from clinical grade iPSC, in which *IL15, CCR2B, CCL19, CD16a, and NKG2D* have been introduced using PiggyBac system. After single-cell cloning, an iPSC clone expressing all 5 factors was selected for further application. Frozen Seed-Stock cells of the iPSC clone showed negative in mycoplasma, bacterial and endotoxin tests.

G-banding karyological test with the iPSC Seed-Stock showed normal human karyotype. RAISING method, PCR amplification followed by high-throughput sequencing, unveiled that the PiggyBac donor DNAs have been integrated in the genome at total 13 locations. No deleterious mutation that could increase tumorigenicity risk was found in the iPSC Seed-Stock compared to parental iPSC through WGS analysis followed by SNV/INDEL/CNV calling coupled with RNA-seq expression data. We have concluded that this iPSC Seed-Stock is suitable as a source material of iPSC MCB, followed by iPSC WCB and HLCN061. Additional analyses will be conducted in these iPSC banks as well as HLCN061 to assess tumorigenicity risk of HLCN061.

Methods

Fig. 1 : Strategy of Generating Engineered iPSC expressing five factors



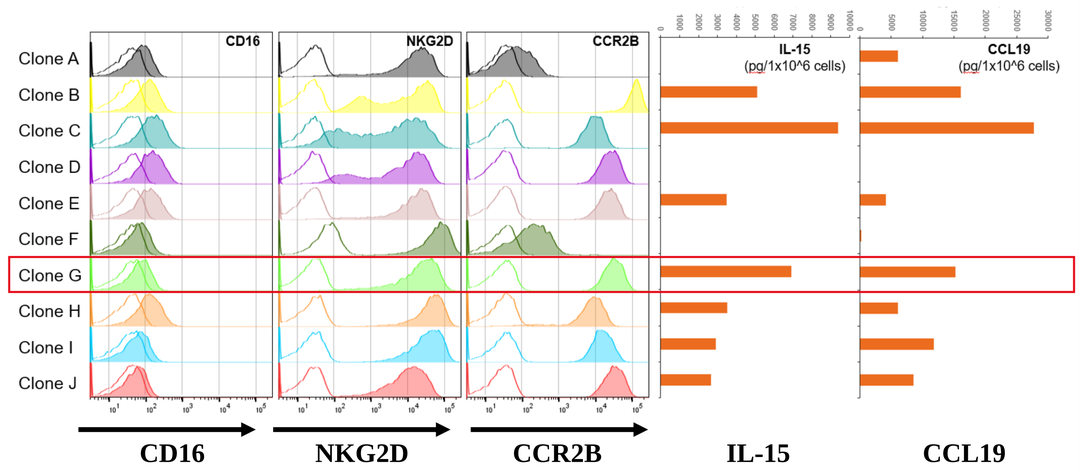
Clinical grade parental iPSCs were transfected with expression vectors that contained each cDNA (*IL15, CCR2B, CCL19, CD16a (F176V),* and *NKG2D*) using PiggyBac method. After single-cell cloning, we selected an iPSC clone expressing all 5 factors by qRT-PCR, flow cytometry and ELISA. We further performed validation tests for frozen Seed-Stock cells of the iPSC clone.

SCC = Single Cell Cloning; WGS = Whole Genome Sequence; SNV = Single Nucleotide Variation; INDEL = Insertion/Deletion; CNV = Copy Number Variation

Results

(1) Selection of candidate as a source of HLCN061

Fig. 2 : Expression of each transgene in selected iPSC clones



Expression level of transgenes in the clonal lines as measured by flow cytometry and ELISA. Transgenes (CD16a, NKG2D, and CCR2B) are highly expressed on cell surface of five clones (Clone E, Clone G, Clone H, Clone I, and Clone J). The level of IL-15 and CCL19 in the culture supernatant was measured by ELISA. Clone G secretes more IL-15 than the other four clones (Clone E, Clone H, Clone I, and Clone J).

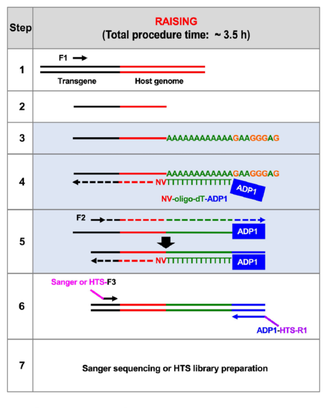
(2) Identification of integration sites and copy number

Table 1: Integration sites and copy number of transgenes in the Clone G

Chromosome : Position	Direction	Transgene
Chr1 : XXX,XXX,XXX	forward	XXX
Chr1 : XXX,XXX,XXX	reverse	XXX
Chr1 : XXX,XXX,XXX	forward	XXX
Chr7 : XXX,XXX,XXX	forward	XXX
Chr9 : XXX,XXX,XXX	reverse	XXX
Chr11 : XXX,XXX,XXX	reverse	XXX
Chr12 : XXX,XXX,XXX	reverse	XXX
Chr14 : XXX,XXX,XXX	reverse	XXX
Chr18 : XXX,XXX,XXX	forward	XXX
Chr18 : XXX,XXX,XXX	reverse	XXX
Chr18 : XXX,XXX,XXX	forward	XXX
Chr20 : XXX,XXX,XXX	forward	XXX
ChrX : XXX,XXX,XXX	reverse	XXX

Transgene	Number of sites
IL15	2
CCL19	3
CCR2	3
CD16	1
NKG2D	2
DAP10	2

RAISING method



In the Clone G, total 13 integration sites were identified by RAISING method (*Wada Y, et, al. 2022, Commun Biol*). 12 integration sites except for chr X were heterozygous insertion confirmed by WGS. The copy number of each transgene was analyzed using the Droplet Digital PCR (ddPCR) method. Genomic DNA PCR followed by Sanger Sequencing identified which cDNA is inserted at which integration sites (data undisclosed).

(3) Quality Control of the iPSC Seed-Stock (Clone G)

Table 2 : Summary of validation tests

Test	Specification	Results
Mycoplasma	Negative	Negative
Bacteriology	Negative	Negative
Endotoxin	≤ 5.00 EU/mL	< 0.50 EU/mL
Karyotype	46 XY, Normal	46 XY, Normal
Residual vector test	≤ 1 copy/100 cells	≤ 1 copy/100 cells (Undetectable)
Undifferentiation stem cell marker	SSEA-4 >70% TRA-1-60>70% TRA-1-81 >70% OCT-4A >70%	SSEA-4 > 99% TRA-1-60 > 99% TRA-1-81 > 99% OCT-4A > 99%
Insertion sites	For information	Done (as described in (2))
Copy number	For information	Done (as described in (2))
Tumorigenicity	For information	Done (as follows)

The safety and quality tests for clinical-grade engineered iPSC Seed-Stock are listed in the table above:

(4) Tumorigenicity risk assessment of the iPSC Seed-Stock (Clone G)

Table 3 : Risk assessment scheme by NGS data analysis

WGS analysis (CNV) → **No CNV was found in the iPSC Seed-Stock!!**

WGS analysis (SNV/INDEL)

Chromosome : Position	Annotation
Chr1 : XXX,XXX,XXX	Gene X intron
Chr7 : XXX,XXX,XXX	Gene Y intron
Chr17: XXX,XXX,XXX	Gene Z intron

RNA-seq

Gene	TPM (Seed-Stock)	TPM (Parent)
Gene X	3.55	2.78
Gene Y	0	0
Gene Z	2.62	3.89

We analyzed aligned sequences obtained from the iPSC Seed-Stock (Clone G) compared to parental iPSC through WGS. CNV analysis highlighted 0 variant. SNV/INDEL analysis highlighted 0 variant for impact score HIGH and 3 SNV/INDEL for MODERATE by using SnpEff (version 5.1) in cancer-related genes (COSMIC Tier1 v97 or Shibata's List). All the SNV/INDEL are located at intron region. The SNVs found in intron did not result in more than 2-fold expression change of mRNA analyzed by RNA-seq. These results indicate that No deleterious mutation that could increase tumorigenicity risk was found in the iPSC Seed-Stock compared to parental iPSC.

Conclusion

We have concluded that the iPSC Seed-Stock (Clone G) is suitable as a source material of iPSC MCB, followed by iPSC WCB and HLCN061. Additional analyses will be conducted in these iPSC banks as well as HLCN061 to assess tumorigenicity risk of HLCN061.

演題名：**Genetic engineering and quality control of clinical grade iPS cells as a source of HLCN061**
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