Clinical grade genetically engineered hypoimmunogenic human induced pluripotent stem cell line

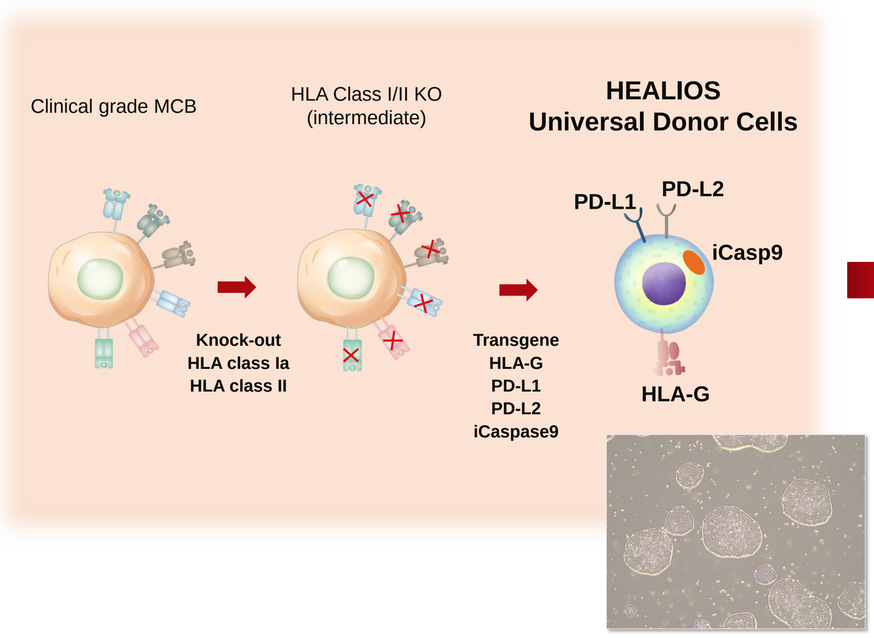
Norihiro Tsuneyoshi, Mitsuhiro Iwasaki, Hidetaka Murai, Kodai Saitoh, Sono Watanabe, Yudai Hasegawa, Yuriko Takeno, Kumiko Goto, Yuka Igarashi, Fusako Nishigaki, Tomonori Hosoya, Hironobu Kimura and Kouichi Tamura

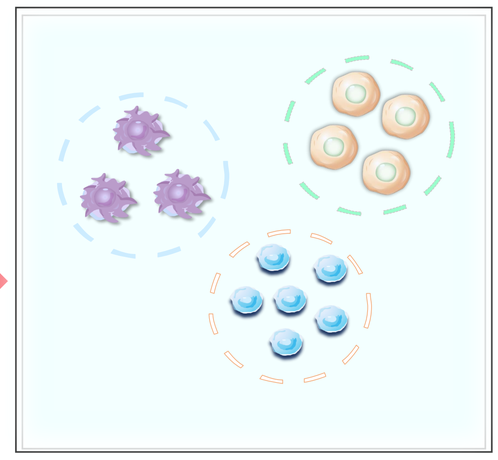
(Kobe Research Institute, HEALIOS K.K.)

Abstract:

We have generated universal donor cells (UDC), an iPSC clone in which *HLA-A*, *HLA-B*, *HLA-C* and *RFXANK* have been knocked-out, while HLA-G, PD-L1, PD-L2 and iCasp9 have been ectopically expressed. After single-cell cloning, an iPSC clone having biallelic frame shift mutation at the 4 gene loci as well as expressing all the 4 factors was selected for further application. RAIS (Rapid Amplification of Integration Sites) method unveiled that donor DNAs have been integrated in the genome at total 32 locations. T cell response against the UDC-derived cells was diminished. Furthermore, the UDC showed no vulnerability against NK cell cytotoxicity. Master Cell Bank (MCB) of the UDC iPSC clone showed negative in mycoplasma, bacterial and endotoxin tests. G-banding karyological test with the MCB showed normal human karyotype. No deleterious mutation that could increase tumorigenicity risk was found in the MCB compared to parental iPSC by Cancer Panel Amplicon-seq data. Our UDC could be used for a future starting material of regenerative therapy.

Gene Editing Procedure for Healios UDC

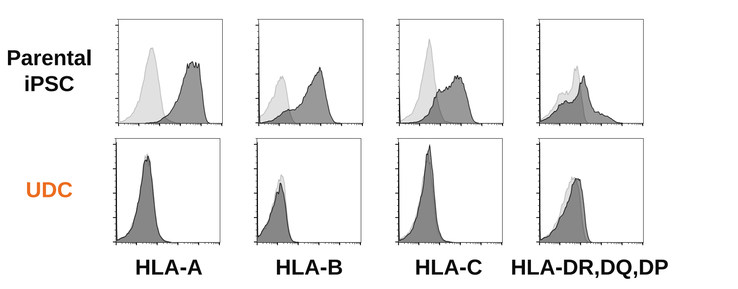




- Off-the-shelf, scalable and cost-efficient
- Address broadest population with single product
- Enhanced level and duration of efficacy

Established clinical grade universal donor cell line in 2020, and established MCB in 2021

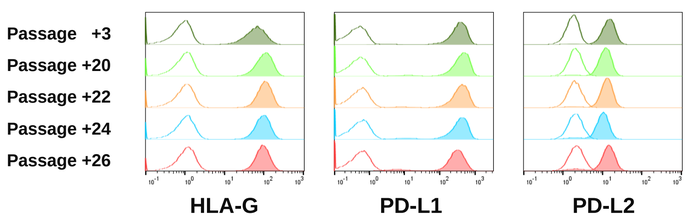
1 Knock-out HLA class Ia and class II



HLA expression was analyzed by FACS using iPSC-derived CD45+ cells. Cells were treated with 50ng/mL IFN-g for 2 days before FACS analysis.

Diminished expression of cell surface HLA molecules

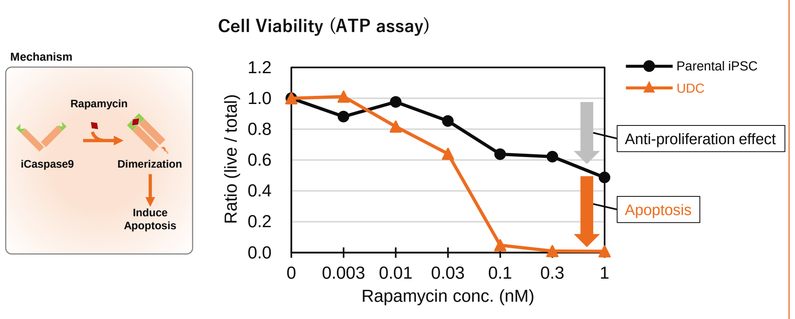
2 Transgenes are expressed on cell surface of UDC



Transgenes (HLA-G, PD-L1, and PD-L2) expression was analyzed by FACS using undifferentiated UDC. UDC were passaged once a week.

Expression of transgenes was observed for over half-year culture

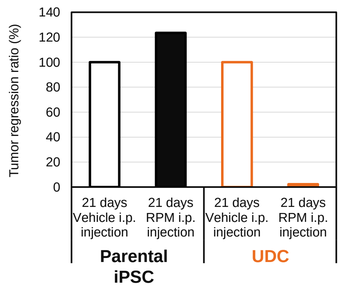
3 iCaspase9 Safety Switch (*in vitro* assay)



To verify the function of Rapamycin inducible Caspase 9, cells were treated with Rapamycin for 24 hours since the next day of cell seeding in 96-well plate, and then assayed using CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega).

Apoptosis was induced by Rapamycin treatment

4 iCaspase9 Safety Switch (*in vivo* assay)



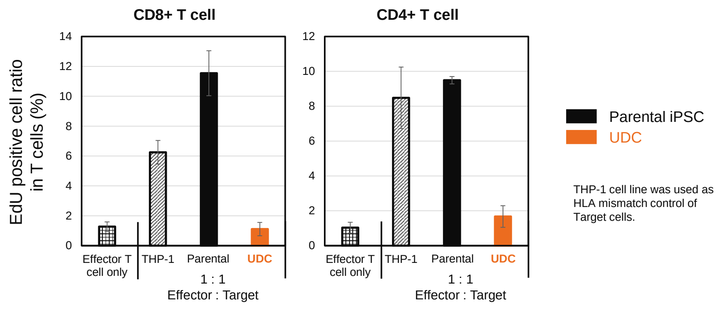
Cells were transplanted into NOG mouse to make teratoma formation (Day 1).
Rapamycin had been injected (8 mg/kg i.p.) every day for 21 days since 3 weeks later (Day 22-42).
At Day 71, the tumor mass was measured.

UDC-derived tumor was eliminated by Rapamycin treatment

5 Characteristics Test for UDC

Category	Test
Escape from immune cells	T cell response (*in vitro*) CD8+ T cell cytotoxicity (*in vitro*, *in vivo*) NK cell cytotoxicity (*in vitro*) Macrophage phagocytosis (*in vitro*)
Safety Switch	Response to Rapamycin (*in vitro*, *in vivo*)
Knockouts	Genomic DNA sequence Cell surface expression of HLA Off-target mutation
Transgenes	Cell surface expression of transgenes Integration sites
Trilineage differentiation potential	Differentiation potential (*in vitro*, *in vivo*) iPSC marker
Tumorigenicity risk	WGS short-reads SNV/indel and RNA-seq at iPSC Karyotype, G-band

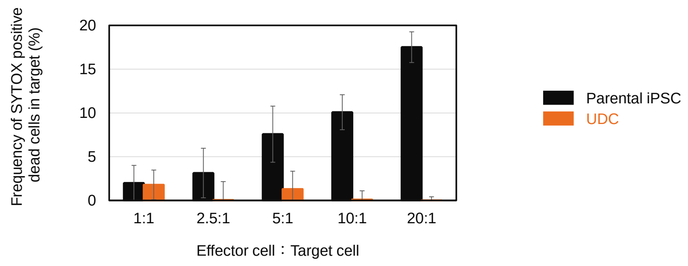
6 T cell response assay (*in vitro*)



HLA-mismatched CD3+ T cells were co-cultured with iPSC-derived CD45+ cells (Mitomycin C treated) for 7 days. Responded T cells (CD8+ T cell and CD4+ T cell respectively) were measured with Click-iT Plus EdU Flow Cytometry Assay Kit (Thermo Fisher Scientific).

Diminished T cell response to almost T cell only level

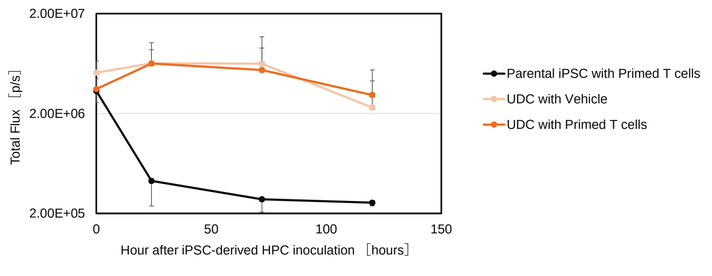
7 CD8+ T cell cytotoxicity assay (*in vitro*)



The iPSC-derived CD45+ cells were co-cultured with Primed CD8+ T cells (primed by Parental iPSC-derived CD45+ cells) for 3 hours. The dead cells of iPSC-derived CD45+ cells were measured by FACS analysis with SYTOX staining.

Not vulnerable against HLA mismatch CD8+ T cells

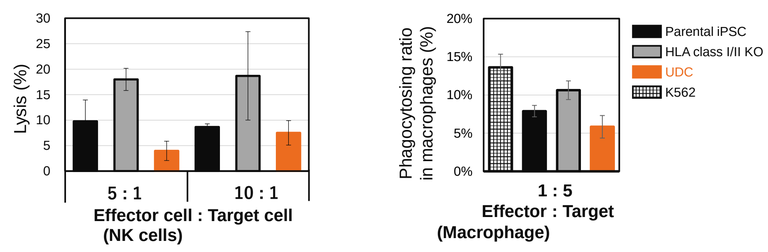
8 CD8+ T cell cytotoxicity assay (*in vivo*)



The iPSC-derived CD45+ cells (additionally Luciferase gene was introduced) were injected into NSG mouse, and Primed CD8+ T cells were administered at 1 hour later. Luminescence was confirmed by IVIS system for immune response evaluation.

UDC-derived cells could escape from T cell reaction

9 NK cell cytotoxicity assay, and Macrophage phagocytosis



The iPSCs (Calcein-labeled) were co-cultured with NK cells for 2 hours. The dead cells of iPSC were measured by FACS analysis with SYTOX staining.

The iPSC-derived CD45+ cells (CellTrace Violet-labeled) were co-cultured with Macrophages (CFSE-labeled) for 2 hours. The phagocytosing ratio were measured by FACS analysis.

Not vulnerable against NK cells and macrophage phagocytosis

10 Integration sites of transgenes

Chromosome	Position	Direction	Transgene
Chr1	xxx,xxx,xxx	forward	xxxx
Chr1	xxx,xxx,xxx	reverse	xxxx
Chr3	xxx,xxx,xxx	reverse	xxxx
Chr3	xxx,xxx,xxx	reverse	xxxx
Chr3	xxx,xxx,xxx	reverse	xxxx
Chr3	xxx,xxx,xxx	reverse	xxxx
Chr4	xxx,xxx,xxx	reverse	xxxx
Chr5	xxx,xxx,xxx	reverse	xxxx
Chr5	xxx,xxx,xxx	reverse	xxxx
Chr6	xxx,xxx,xxx	reverse	xxxx
Chr6	xxx,xxx,xxx	reverse	xxxx
Chr7	xxx,xxx,xxx	reverse	xxxx
Chr7	xxx,xxx,xxx	forward	xxxx
Chr7	xxx,xxx,xxx	forward	xxxx
Chr9	xxx,xxx,xxx	forward	xxxx
Chr10	xxx,xxx,xxx	reverse	xxxx

Chromosome	Position	Direction	Transgene
Chr10	xxx,xxx,xxx	forward	xxxx
Chr12	xxx,xxx,xxx	forward	xxxx
Chr13	xxx,xxx,xxx	forward	xxxx
Chr14	xxx,xxx,xxx	forward	xxxx
Chr15	xxx,xxx,xxx	reverse	xxxx
Chr15	xxx,xxx,xxx	forward	xxxx
Chr15	xxx,xxx,xxx	reverse	xxxx
Chr16	xxx,xxx,xxx	forward	xxxx
Chr16	xxx,xxx,xxx	reverse	xxxx
Chr17	xxx,xxx,xxx	reverse	xxxx
Chr18	xxx,xxx,xxx	forward	xxxx
Chr20	xxx,xxx,xxx	reverse	xxxx
Chr21	xxx,xxx,xxx	forward	xxxx
Chr22	xxx,xxx,xxx	forward	xxxx
ChrX	xxx,xxx,xxx	forward	xxxx
ChrX	xxx,xxx,xxx	forward	xxxx

Total 32 integration sites were identified by RAIS*) method

*) Ref: M. Saito et al. *Int J Hematol*. 112, 300-306 (2020)

11 Quality Control Test for MCB

Quality Control Test	Results
Karyotype, G-band	46,XY
Sterility	Sterile
Endotoxin	< 0.50 EU/mL
Mycoplasma	negative
Virus	negative
FCM of iPSC markers (SSEA-4, TRA-1-60, TRA-1-81, OCT-4)	Pass (99.8%, 99.2%, 99.6%, 98.3%, respectively)
Alkaline phosphatase staining	Pass
FCM of transgenes	Pass (HLA-G: 100.0%, PD-L1: 99.5%, PD-L2: 96.5%)
Function of iCasp9	Pass
Trilineage differentiation potential	Pass
Cancer Panel Amplicon-seq	Pass

12 UDC and iPSC Collaborations

Supplying UDC and iPSC to several companies and academic institutions (>10 facilities) and evaluating their potential for various diseases