SciSci Interview, No. 3
sci-sci.org/ipsc-therapy-haplobanking-genome-editing
DOI: 10.5281/zenodo.17152465

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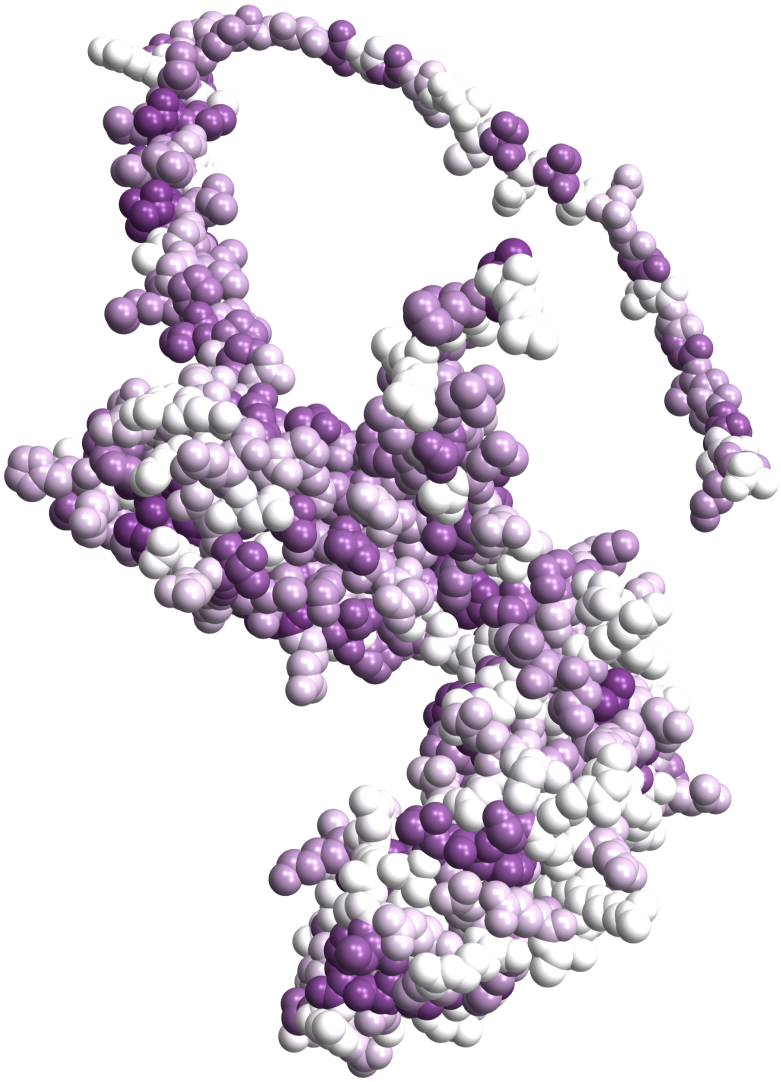

Trends in Haplobanking and Genome Editing

For iPS Cell Therapy

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SciSci Research, Inc.

About the Cover:

A backbone atom plot of the human leukocyte antigen C (HLA–C) protein (using UniProt data). HLA–C–retention is a defining feature of the CRISPR–Cas9 knockout technique discussed in this report for creating genome–edited iPSC lines.

Published by SciSci Research, Inc.

SciSci Research, Inc.
Boulder, Colorado, United States
www.sci–sci.org

Citation Format:

Boyd, J.D. (2025). Trends in Haplobanking and Genome–Editing for iPS Cell Therapy. SciSci Interview 1(3). DOI: 10.5281/zenodo.17152465

Contents

1 The iPS Cell Therapy Prospect

1.1 Clinical-Grade hiPSC Lines as a Biomedical Resource

iPS cell therapy refers to the translational use of induced pluripotent stem (iPS) cells for purposes of providing regenerative treatments for certain diseases or injuries. The iPSC therapy prospect (currently under many clinical trials for specific translational applications), if successful, may herald a capacity to repair the structure and functionality of tissues and organs by equipping them with healthy cells cultured from a pluripotent state.

It is hoped that hiPSC-based (i.e., human iPSC-based) therapeutics can offer functional and structural regeneration to patients, such as those suffering from degeneration (e.g., macular degeneration; neurodegenerative disorders such as Parkinson's), dystrophy (e.g., inherited retinal dystrophy), structural damage (e.g., arterial cartilage damage), or malignancy (e.g., ovarian carcinoma). It is also hoped that hiPSC-based treatments can provide regenerative therapies to counteract the effects of pathologies such as ischemia (e.g., ischemic cardiomyopathy); stem cell deficiency (e.g., corneal opacity from limbal stem cell deficiency); endothelial dysfunction (e.g., bullous keratopathy); or autoimmune destruction (e.g., type 1 diabetes). Clinical trials are ongoing, planned, or completed.

Upstream from any particular hiPSC-based therapeutic is the hiPSC line itself, from which therapy-specific cells can be differentiated for translational use. hiPSC lines are, in principle, a 'perpetual' resource, as hiPSCs can proliferate indefinitely and differentiate to cells across all three germ layers (i.e., endoderm, mesoderm, ectoderm); they have a trilineage differentiation potential. It is hoped that a clinical-grade hiPSC line collection can serve as a versatile long-term biomedical resource for regenerative medicine and drug discovery, applicable to a broad translational and therapeutic frontier.

1.2 Comparing iPSCs to Other Cell Types

Comparisons can be helpful for distinguishing the prospective advantage of clinical-grade hiPSC collections afforded by pluripotency.

Consider neural progenitor cells, for instance. They but do not share the indefinite proliferation capability of hiPSCs; they eventually enter senescence. Furthermore, they are uni/bi/multipotent (but not pluripotent); their differentiation potential is restricted to neuronal and glial cells, whereas hiPSCs, by merit of pluripotency, enjoy in principle a full trilineage differentiation potential. This may afford translational versatility on the therapeutic side, as the capacity to differentiate into many cell types widens the gamut of candidate injuries or diseases for which regenerative treatments could perhaps be developed. Cells subject to greater constraints, such as mesenchymal stromal cells (MSCs), whose differentiation potential is limited to certain cell types (e.g., adipocytes, osteoblasts, chondrocytes) are expected to remain restricted to a more modest array of therapeutic functions (e.g., anti-inflammatory, immunomodulatory).

To take another example: whereas immortalized cell lines can be indefinitely expanded (i.e., like hiPSC lines) they do not enjoy the differentiation versatility afforded by pluripotency. For instance, in 2014, immortalized megakaryocyte progenitor cell lines (imMKCLs) were created, with the aspiration to augment blood transfusion capabilities in Japan. Whereas imMKCL lines can in principle be subject to indefinite expansion, they are already morphologically and functionally predestined; they form platelets. hiPSC lines, on the other hand, can in principle differentiate along cell fate trajectories to any cell type. Thus, immortalized cell lines are expandable, but, unlike hiPSC lines, are not pluripotent.

From a translational view, the trilineage differentiation potential, pluripotency, and proliferation capacity of hiPSCs endow them with the capacity to serve as an upstream resource for a broad menu of cellular therapies. (In industrial terms, one might see proliferation capacity as endowing imperishability of value, and differentiation potential as affording fungibility with respect to cellular therapeutics.) Thus, the promise of the clinical-grade hiPSC line is one of a wide-application biomedical capability.

1.3 Allografts and the Hypoimmunity Challenge

Irrespective of the source of the iPSC-derived cells – whether it be the patient (in the autologous case) or a donor (in the allograft cases) – iPS cell therapy is to be administered as a transplantation medicine treatment, with cells cultured *in vitro* and engrafted *in vivo*. Although autologous methods are being pursued, it has been anticipated that the development of allograft therapy capabilities will be necessary to cultivate an industry ready to treat populations at scale. In the allograft case, successful engraftment requires that the cells introduced evade immune rejection. Thus, establishing requisite hypoimmunity for mitigation of allogeneic immunogenicity is one of the most basic requirements for hiPSC-based allograft therapies, and thus will serve as the focus of this SciSci article.

Hypoimmunity, for our purposes, refers to risk-reduction in immune reactions to hiPSC allografts. Hazards borne of ineffective hypoimmunity strategies include allogeneic immunogenicity in the host, transplant rejection, requirement of severe immunosuppressants, and graft-vs-host disease (GvHD). Such hazards pose harm to patients or thwart the prospect of therapeutic engraftment.

Immunogenicity challenges arise in transplantation medicine due to the polymorphism of human leukocyte antigen (HLA) haplotypes across and within populations. The HLA loci, forming the human version of the major histocompatibility complex (MHC), have been known for decades to be the most polymorphic of all in the human genome. HLA molecules process antigen peptides and present them to to immune cells such as leukocytes. HLA molecule binding is governed by HLA polymorphism, thus diversifying immune reactions among individuals, and thereby posing a challenge in transplantation medicine. In the case of hiPSC allografts, the hypoimmunity challenge is one of minimizing complications due to donor-patient HLA polymorphism.

HLA alleles function as a part of what we might think of as a critical human 'biosecurity apparatus' guarding the endogenous systems of the organism against potentially threatening exogenous elements. Moreover, they help enforce – at least at an immunorejective register – the boundary between self and non-self. With this heuristic in mind, the hypoimmunity challenge can be summarized, at least roughly and conceptually, as follows. With iPS cells manufactured *in vitro*, regenerative therapies for patients require therapeutic treatments – new cells – are manufactured extracorporeally. Thus, particularly in the allograft case, hypoimmunity strategies have everything to do with setting the conditions such that the patients receive transplanted cells as though they were, more or less, their own. The hypoimmunity challenge is one of promoting, to the greatest extent possible, acceptance on the part of the host of a technology (i.e., an hiPSC-derived cell) as part of the host's own physiological structure and functionality.

Herein, two hypoimmunity strategies for iPSC therapy shall be covered: haplobanking and HLA cloaking. Haplobanking refers to the collection of iPS cell lines from HLA homozygous donors in order to facilitate HLA matching with patients requiring treatments involving cells differentiated from iPSCs. Thus, the polymorphism barrier to controlling allogeneic immunogenicity is overcome by accumulating enough iPSC lines to accommodate common HLA haplotypes. Thus, one

aims for one's bank or stock to offer representation across the most frequent haplotypes in order to achieve a donor potential for the bulk of the demographic distribution.

On the other hand, HLA cloaking, which requires greater intervention, involves the use of gene editing to knock out certain HLA alleles in iPSC lines in order to mitigate immunorejection. That is to say, rather than overcome mismatch-induced allogeneic immunogenicity by matching HLA alleles, one suppresses allograft alleles via knockout in order to minimize the immunogenic consequences of a tolerated mismatch. The resulting iPSC lines are not HLA-homozygous; they are "pseudo-HLA-homozygous" iPS lines.

1.4 Haplobanking and HLA Cloaking in Kyoto

The focus of this piece is coverage of advances in haplobanking and HLA cloaking being pursued at the Kyoto University Center for iPS Cell Research and Application (京都大学iPS細胞研究所), or CiRA (サイラ), founded in 2010 as a part of Kyoto University (京都大学); as well as the CiRA Foundation (iPS細胞研究財団), an affiliated public interest incorporated foundation (公益財団法人) founded by Kyoto University and certified by the Cabinet Office (内閣府) in 2020. CiRA is distinguished from many institutions engaged in iPSC research by its commitment to delivering medical applications of iPS cells (「iPS細胞の医療応用」).

Professor YAMANAKA Shinya (山中伸弥) – now Director Emeritus and Professor (名誉所長・教授) at CiRA and President (理事長) of the CiRA Foundation – served as CiRA's first Director. Yamanaka-sensei and colleagues first discovered iPSCs – beginning with the murine case in 2006 and the hiPSC case in 2007 – for which Yamanaka-sensei was awarded the Nobel Prize in Physiology or Medicine in 2012. CiRA was recognized as an independent institute by Kyoto University, with its own building, in 2010, after having originally been a part of the Institute for Integrated Cell-Material Sciences (物質細胞統合システム拠点), or iCeMS (アイセムス).

The full gamut of research conducted at CiRA, and the full range of activities pursued under the mandate of the CiRA Foundation, exceed the scope of this piece. Rather, SciSci will focus on haplobanking and HLA cloaking strategies. The former began as a CiRA initiative and is now facilitated by the CiRA Foundation as the iPS Cell Stock Project (iPS細胞ストックプロジェクト). Clinical- and research-grade lines are manufactured at the CiRA Foundation's Facility for iPS Therapy (FiT; facility number: FA5150001), along with a second facility (FiT2; facility number: FA5170003), which were together reocngized as a Cell Processing Center (FA5200001) and obtained a manufacturing license (26FZ110001) in January 2021 in accordance with the Act on Pharmaceuticals and Medical Devices (PMD Act). These lines are then distributed from the CiRA Foundation to non-profit and for-profit organizations (though with differing policies). Distribution decisions are reviewed and made by the members of the iPS Cell Stock Review Committee (iPS 細胞ストック審査委員会).

The iPS Stock Project distributes the fruits of HLA cloaking research done at CiRA insofar as it includes gene-edited lines. Thus, the following article will focus on the iPS Stock Project and the CiRA research on immunogenicity challenges in order to understand how, jointly, distribution initiatives and translational research are navigating the immunogenicity challenge that attends the prospect of broadening the accessibility of prospective iPS therapies.

1.5 Objectives and Methods

1.5.1 Objectives

Being aware of the often exhilarating anticipation with which iPSC therapy is followed, SciSci wishes to inquire into trends in haplobanking and HLA cloaking in somewhat rarefied scientific detail, avoiding both sanguine romanticization of the iPSC therapy prospect and fatalist dismissal on account of setbacks or challenges. Instead, SciSci opts to give as square and sober an account of progress and challenges as possible. Reading over papers published by CiRA and CiRA Foundation researchers on haplobanking and HLA cloaking, many questions – regarding methodology, results, and consequences – arise, which are elicited by, but not answered within, the text of the papers themselves. Thus, this report is structured around interviews with CiRA and CiRA Foundation personnel concerning the myriad questions that SciSci had after multiple readthroughs of the papers.

In SciSci's view, the content given here presents – as one might expect in biomedicine – a very-much variegated picture of advances and aspirations on the one hand and complications and outstanding questions on the other. The weighted average of advances and challenges seems to SciSci less relevant than a rarefied portrait of the scientific details, and the positions of CiRA and CiRA Foundation on such matters. Moreover, in pursuit of its motto, "Science for scientists", the purpose of this report is to try to articulate both advances and challenges for a scientific readership, such as students, who might draw inspiration from advances and take an interest in attending to current challenges, as each scientific generation does.

1.5.2 Method: Interviews

The content presented herein is a product of multi-format interviews conducted with CiRA and CiRA Foundation participants, held in person in Kyoto, conducted via Zoom, and facilitated through email. Interview questions referred to the following papers.

Overview of strategies for evading immunogenicity (haplobanking and HLA cloaking):

- Yamanaka, Shinya. "Pluripotent stem cell-based cell therapy—promise and challenges." *Cell Stem Cell* 27.4 (2020): 523-531.

Haplobanking:

- Yoshida, Shinsuke, *et al.* "A clinical-grade HLA haplobank of human induced pluripotent stem cells matching approximately 40% of the Japanese population." *Med* 4.1 (2023): 51-66.

HLA cloaking:

- Xu, Huaigeng, *et al.* "Targeted disruption of HLA genes via CRISPR-Cas9 generates iPSCs with enhanced immune compatibility." *Cell Stem Cell* 24.4 (2019): 566-578.
- Hotta, Akitsu, Sonja Schrepfer, and Andras Nagy. "Genetically engineered hypoimmunogenic cell therapy." *Nature Reviews Bioengineering* (2024): 1-20.

I had the fortunate opportunity to interview Yamanaka-sensei and Associate Professor HOTTA Akitsu (堀田秋津) whilst in Kyoto. Following my visit, thanks to the assistance of the CiRA Foundation, I was able to conduct an email interview with three members of the CiRA Foundation team:

- HANATANI Tadaaki (花谷忠昭) – Executive Director, CiRA Foundation
- YOSHIDA Shinsuke (吉田信介) – Assistant Head of Research and Development Center, CiRA Foundation
- DOHI Hiromi (土肥浩美) – Manager of Planning Promotion office, CiRA Foundation

Text from the CiRA Foundation team's collective email interview responses will be quoted as received from "CiRA Foundation" in aggregate.

1.5.3 Summary Resources

The report includes summary tables summarizing the challenges in haplobanking and HLA cloaking discussed with participants during interviews. These summaries are presented as strategic resources for scientists interested in the field.

1.5.4 Data Visualization

SciSci has prepared some figures as visual references to accompany the content of the interviews and the above-mentioned tables. The data for these figures was obtained from the Supplemental Information sections of the 2019 paper (Xu *et al.*) and 2023 paper (Yoshida *et al.*). SciSci endeavored to visualize data that had not been so done in the original papers, or, in the case of Figure 7 of this report, visualize data in a different way relevant to the report content. SciSci offers the figures to relate the discussions back to the findings of the original papers as well as to draw read attention to these papers and the data provided in their Supplemental Information sections. Each figure caption credits the original paper, specifies the dataset used, and links to the Supplemental Information section of the paper.

2 Trends in Haplobanking

2.1 What is Haplobanking?

Haplobanking refers to projects which pursue the accumulation of clinical- and research-grade iPSC lines that match common HLA haplotypes. (The "haplo-" prefix refers to HLA haplotypes, the polymorphism of which, among and within populations, necessitates HLA matching and motivates the pursuit of stock/banking strategies.) On the therapeutic front, collection of clinical-grade lines in an HLA-homozygous iPS Cell Stock (HLAホモiPS細胞ストック) – consisting of HLA-homozygous-donor-derived lines – has been pursued to support allograft-based hiPSC therapies for donor-matched patients.

A cursory review of the history of haplobanking at CiRA is as follows. In 2013, the Japanese Ministry of Education, Culture, Sports, Science and Technology (文部科学省; MEXT) committed ¥110B to the Research Center Network for Realization of Regenerative Medicine (再生医療実現拠点ネットワークプログラム). Such was the beginning of the haplobanking initiative. Donor recruitment commenced in 2013 in cooperation with the Japanese Red Cross Society (日本赤十字社; JRCS), with blood donors recruited from Osaka and Kyoto. The iPS Stock was launched in 2015 and transferred to the CiRA Foundation in 2020. As discussed in the 2023 paper, by 2021, thanks to collaboration with the JRCS, Japan Marrow Donor Program (日本骨髄バンク; JMDP) and public blood cord banks, 67 donors (representing 28 haplotypes, or 60% of the domestic Japanese population) had been recruited, from which 7 donors (representing 28 haplotypes, or 40% of the domestic Jaanese population) were selected. As a result, 27 clinical-grade HLA-homozygous lines were created for the CiRA Foundation's iPS Cell Stock Project.

The process by which attainment of 40% haplobanking coverage was achieved is discussed in considerable detail in the aforementioned 2023 paper, "A clinical-grade HLA haplobank of human induced pluripotent stem cells matching approximately 40% of the Japanese population", which served as the textual basis for SciSci's questions for CiRA and the CiRA Foundation.

2.2 The iPS Stock project

The iPS Cell Stock Project has accumulated a collection of research-grade cell (研究用細胞) and clinical-grade cell (臨床用細胞) for commercial and nonprofit use. The Stock contains three clinical-grade collections:

- An HLA-homozygous iPS cell stock, created from HLA-homozygous donors
- A genome-edited stock (ゲノム編集株) of pseudo-HLA-homozygous cells that have been subject to certain HLA knockouts
- A Sendai virus (SeV) stock (センダイ株), reprogrammed with SeVs rather than episomal plasmids

The Project also includes the CFiS stock (CFiS株), consisting of 6 research-grade lines reprogrammed with SeVs from peripheral blood mononuclear cells (PBMCs). Cells are made available to companies and nonprofits (free of charge) under a Collaborative Research Agreement, with Kyoto University's patents, in this case, licensed by iPS Academia Japan (iPSアカデミアジャパン株式会社).

Distribution of materials from the CiRA Foundation iPS Stock is intended to lower barriers to hiPSC line access and facilitate optimal choice among lines for patient needs, as Yamanaka-sensei described.

Yamanaka-sensei:

> We established the CiRA Foundation five years ago in order to bridge the gap between

> academia and industry. The main vision of our CiRA Foundation is to provide the best iPS cells at an affordable price. [...] We are hoping doctors and companies can choose the best iPS cell line, not due to the cost, but [due to] the requirements of each patient.

The CiRA Foundation iPS Stock has 14 clinical-grade HLA-homozygous hiPSC lines, manufactured using plasmids pCE-hSK, pCE-hUL, pCE-hOCT3/4, pCE-mp53DD, and pCXB-EBNA1, with the StemFit AK03N medium, from the peripheral blood cells of 4 donors:

- QHJI (Japanese male; HLA-A: *24:02, HLA-B: *52:01, HLA-DRB1: *15:02). Lines available: QHJI 01s01; QHJI 01s04; QHJI 14s03; QHJI 14s04 – manufactured in 2015.
- RWMH (Japanese male; HLA-A: *33:03, HLA-B: *44:03, HLA-DRB1: *13:02). Lines available: RWMH 09s01; RWMH 15s01; RWMH 15s02; RWMH 23s01 – manufactured in 2016.
- DRXT (Japanese male; HLA-A: *24:02, HLA-B: *07:02, HLA-DRB1: *01:01). Lines available: DRXT 18s02; DRXT 18s03; DRXT 28s04; DRXT 28s05; DRXT 28s17 – manufactured in 2017.
- RJWI (Japanese female; HLA-A: *24:02, HLA-B: *52:01, HLA-DRB1: *04:05). Line available: RJWI s03 – manufactured in 2018.

The CiRA Foundation iPS Stock also has 13 hiPSC lines manufactured, using the same plasmids and medium, from the cord blood of 3 donors:

- YZWJ (Japanese male; HLA-A: *24:02, HLA-B: *52:01, HLA-DRB1: *15:02). Lines available: YZWJ s513; YZWJ s516; YZWJ s524; YZWJ s527; YZWJ s531 – manufactured in 2017.
- ILCL (Japanese female; HLA-A: *24:02, HLA-B: *52:01, HLA-DRB1: *15:02). Lines available: ILCL s14; ILCL s21; ILCL s23; ILCL s31 – manufactured in 2017
- GLKV (Japanese male; HLA-A: *33:03, HLA-B: *44:03, HLA-DRB1: *13:02). Lines available: GLKV s09; GLKV s13; GLKV s16; GLKV s31 – manufactured in 2017.

HLA-homozygous donors with prevalent HLA haplotypes were recruited. QHJI, YZWJ, and ILCL share their HLA haplotype with 8.437% of the Japanese population; RWMH and GLKV with 4.448% ; DRXT with 3.843% ; and RJWI with 2.722% . (See Table 1 of the 2023 Yoshida *et al.* paper.)

As of 2022, the clinical-grade haplobanked lines collected by the iPS Cell Stock Project have been used by 30 institutions, the research-grade lines having been used by 62 institutions. The most popular clinical-grade line, circa 2022, was QHJI 01s04, used by 19 institutions for 28 projects. Its research grade correspondent, Ff-I 01s04 p11, was also the most popular among research-grade hiPSC lines, used by 25 institutions for 44 projects. (See Table S9 in the Supplemental Information section of the 2023 Yoshida *et al.* paper.)

Additionally, 25 hiPSCs are made available from the RIKEN BioResource Research Center (理化学研究所バイオリソース研究センター), or BRC. HPS0063 : 201B7, from the 2007 iPS publication and deposited in 2011, has – at the time of writing this article – been used in 330 published studies. HPS0002 : 253G1, which was induced without MYC, and deposited in 2008, has been used in 154 published studies.

HLA–Homozygous iPS Stock Donors and Clinical–Grade hiPSC Lines				
	Demographic	Haplotypes	Lines	Manufactured
Peripheral Blood				
QHJI	Japanese Male	HLA–A: *24:02 HLA–B: *52:01 HLA–DRB1: *15:02	QHJI 01s01 QHJI 01s04 QHJI 14s03 QHJI 14s04	2015
RWMH	Japanese Male	HLA–A: *33:03, HLA–B: *44:03, HLA–DRB1: *13:02	RWMH 09s01 RWMH 15s01 RWMH 15s02 RWMH 23s01	2016
DRXT	Japanese Male	HLA–A: *24:02, HLA–B: *07:02, HLA–DRB1: *01:01	DRXT 18s02 DRXT 18s03 DRXT 28s04 DRXT 28s05 DRXT 28s17	2017
RJWI	Japanese Female	HLA–A: *24:02, HLA–B: *52:01, HLA–DRB1: *04:05	RJWI s03	2018
Cord Blood				
YZWJ	Japanese Male	HLA–A: *24:02, HLA–B: *52:01, HLA–DRB1: *15:02	YZWJ s513 YZWJ s516 YZWJ s524 YZWJ s527 YZWJ s531	2017
ILCL	Japanese Female	HLA–A: *24:02, HLA–B: *52:01, HLA–DRB1: *15:02	ILCL s14 ILCL s21 ILCL s23 ILCL s31	2017
GLKV	Japanese Male	HLA–A: *33:03, HLA–B: *44:03, HLA–DRB1: *13:02	GLKV s09 GLKV s13 GLKV s16 GLKV s31	2017

Figure 1: Table of clinical-grade iPS Stock information provided in 2.2 of this report, based on information provided on the CiRA Foundation website.

2.3 Haplobanking Advantages

As shall be discussed herein, haplobanking and HLA cloaking constitute complementary strategies for mitigating allogeneic immunogenicity, each with their own respective advantages. For instance, although playing a pioneering role along the gene-editing frontier, Hotta-sensei nonetheless spoke to the relative advantages of the haplobanking approach. It seemed to be Hotta-sensei's assessment that the challenges of haplobanking – namely, adequate donor screening and clinical-grade HLA-homozygous hiPSC line development – may be less cumbersome than the challenges of producing HLA-pseudo-homozygous lines via genome editing.

Hotta-sensei:

> Even though we mentioned this [pseudo-homozygous] approach in the paper, we think that approach may not be the priority. [...] This is because the HLA-homozygous stock, which does not require genetic engineering, is advancing already. Even though screening a large number of potential donors is a very laborious step, we could get cooperation from the Japanese Blood Bank, and the Cord Blood Bank as well. They already have haplotype information for ten thousand people. We could establish clinical-grade iPSCs from the most frequent HLA haplotypes.

Furthermore, unlike HLA-pseudo-homozygous lines, which are subject to knockout of certain HLA alleles (as discussed in the next section), HLA-homozygous lines retain full HLA expression, as the CiRA Foundation representatives described.

CiRA Foundation:

> Our HLA haplobank and genome-edited cells constitute different but related approaches. The haplobank provides HLA-A, -B, and -DR matches. On the other hand, the genome-edited cells are pseudo-HLA-C-homozygous cells edited to delete HLA-A, -B and CIITA. However, it is not necessary to edit HLA-C in our haplobank because the lines also tend to be homozygous for HLA-C.

Hotta-sensei gave a similar summary.

Hotta-sensei:

> If one wants to take advantage of antigen presentation from HLA-A, -B, and -C, then one might want to just use an HLA-homozygous line, without gene editing; that's the utility.

Thus, the logistical demands of donor screening and recruitment for haplobanking, although nontrivial, appear to be less cumbersome, perhaps, than those of de-risking gene-edited lines, despite the lower donation costs imposed by the latter.

Hotta-sensei:

> I think prioritizing HLA-homozygous lines, without genome editing, is probably easier.

On the other hand, the prospective donor potential and patient haplotype coverage range of pseudo-HLA-homozygous lines is expected to be broader. Until the consequences of HLA-knockouts are fully ascertained, the trade-off between HLA-pseudo-homozygous and HLA-homozygous stocks is essentially one between risk uncertainty and potential breadth of haplotype coverage.

CiRA Foundation:

> Whether to use the HLA-homozygous iPS cell stock or HLA-A-, -B-, and CIITA-edited lines will depend on the policy of the

company or research institute using these iPS cells. Some people use genome-edited strains because they have higher HLA coverage, while others prefer to use unedited HLA-homozygous iPS cells because the safety of genome-edited iPS cells is still unknown.

2.4 Reprogramming for Haplobanking

2.4.1 Reprogramming Factor Choice

From a certain point of view, the process of manufacturing clinical-grade hiPSC lines begins with reprogramming, where primary cells – in this case peripheral blood mononuclear cells (PBMCs) and Cord Blood Mononuclear Cells (CBMNCs) – serve as inputs. With that being said, obtainment of the PBMCs and CBMNCs is a necessary preliminary phase, involving – among other steps – recruitment, informed consent, interviews, collection, testing, isolation, and cryopreservation. Thereafter, establishing the hiPSCs is a multi-step process: cell reprogramming to pluripotency, selection of hiPCs for expansion, and cryopreservation of selected hiPCs as primary cell stocks (PCSs), which are then subject to screening and, following successful screening, subject to plating, expansion, and cryopreservation as secondary cell stocks (SCSs). Finally, SCSs that clear final release tests (e.g., mycoplasma, endotoxin, and sterility tests) are added to the Master Cell Bank.

Due to the constraint of passage number minimization and the facilitation of the workflow within the Cell Processing Center (CPC), a reliable, tested reprogramming method was desirable. Clinical-grade iPSCs were reprogrammed from roughly 5 million PBMCs and 1 million CB cells via transfection with five episomal plasmid vectors, which express Oct3/4, Sox2, Klf4, and MycL1 (OSKM), as well as Lin28A, the dominant-negative form of TP53, and EBNA1. Use of LIN28 and EBNA1, in addition to OSKM, has been common practice for more than a decade, ever since episomal vector methods were developed to replace lentiviral vector methods. Regarding the motivation for the dominant-negative form of TP53: in two previous CiRA papers[1], it was found that p53 inactivation provides a significant efficiency advantage for episomal plasmid reprogramming. In this case, rather than use an shRNA, the plasmid pCE-mp53DD – deposited to the Addgene nonprofit by the Yamanaka Lab following the 2012 study – was used.

CiRA Foundation:

> Since our clinical cell stocks were manufactured using isolators in the CPC, we had to select the simplest and most efficient procedure. The vector containing Lin28 and mp53DD (instead of TP53 shRNA, which was problematic due to patent issues) in addition to OSKM, was the most suitable method, and we decided to use this method for manufacturing the clinical iPS cell stock.

Thus, one can see in the iPS Stock Project as a certain thread of continuity from reprogramming methodological developments made by CiRA in the early 2010s.

2.4.2 Residual Plasmids and Integration-Free Vectors

One reprogramming challenge encountered in the paper was that of residual plasmids. Episomal plasmids are valued as non-integrating alternatives to other vectors, such as lentiviruses. However, as noted in the

[1]Namely, the 2013 paper, "An Efficient Nonviral Method to Generate Integration-Free Human-Induced Pluripotent Stem Cells from Cord Blood and Peripheral Blood Cells", and the 2012 paper, "A more efficient method to generate integration-free human iPS cells".

study, exogenous genetic material from integrated plasmid DNA was indeed found in many iPSC lines.

CiRA Foundation:

> The frequency of the remaining residual plasmids was a little surprising, because we thought that the residual plasmids would be easily reduced by repeating dilution during passaging (about 1/100 per passage between same-size wells).

However, the study involved multiple methods. "Method 2", which was used for donors RWMH, DRXT, and YZWJ, whose screening process involves a single cell/well plating and a limiting dilution process, is believed to provide sufficient screening against residual plasmids.

CiRA Foundation:

> The data made us decide to change from method 1 to method 2, as described in the Yoshida *et al*. paper. As a result, we believe we can simply screen out the risks, at least for providing a raw material for allogeneic therapy.

Although genomic integration is stated as the expected source of residuality in the paper, non-integrative residuality was noted as another possible explanation.

CiRA Foundation:

> Although the paper states that the residual plasmids are due to genomic integration, this does not completely deny the possibility that there may be cases where the residual plasmids remain without integration due to a high level of episomal vector replication. However, both cases would be the same in that they have essentially the same risk.

With respect to vector methods, Sendai viruses display promise as a non-integrating, quickly removable, efficient vector. Although such characteristics may render them more suitable, relative to episomal plasmids, the paramount performance indicator is the quality of the lines themselves, and it is not yet known which (i.e., Sendai viruses or episomal plasmids) performs better in this respect.

CiRA Foundation:

> Sendai virus vectors have advantages over episomal plasmids in terms of residual risk; for example, attempts have been made to promote removal by temperature control through the introduction of temperature-sensitive mutations. The simple protocol and high efficiency of reprogramming are also benefits. However, we don't have a conclusion regarding which is better in terms of the quality of the iPSCs produced.

Recall that the CiRA Foundation also maintains a collection of two clinical-grade Sendai Virus hiPSC lines. Perhaps, with time, the comparative performance of these vectors will become more pronounced.

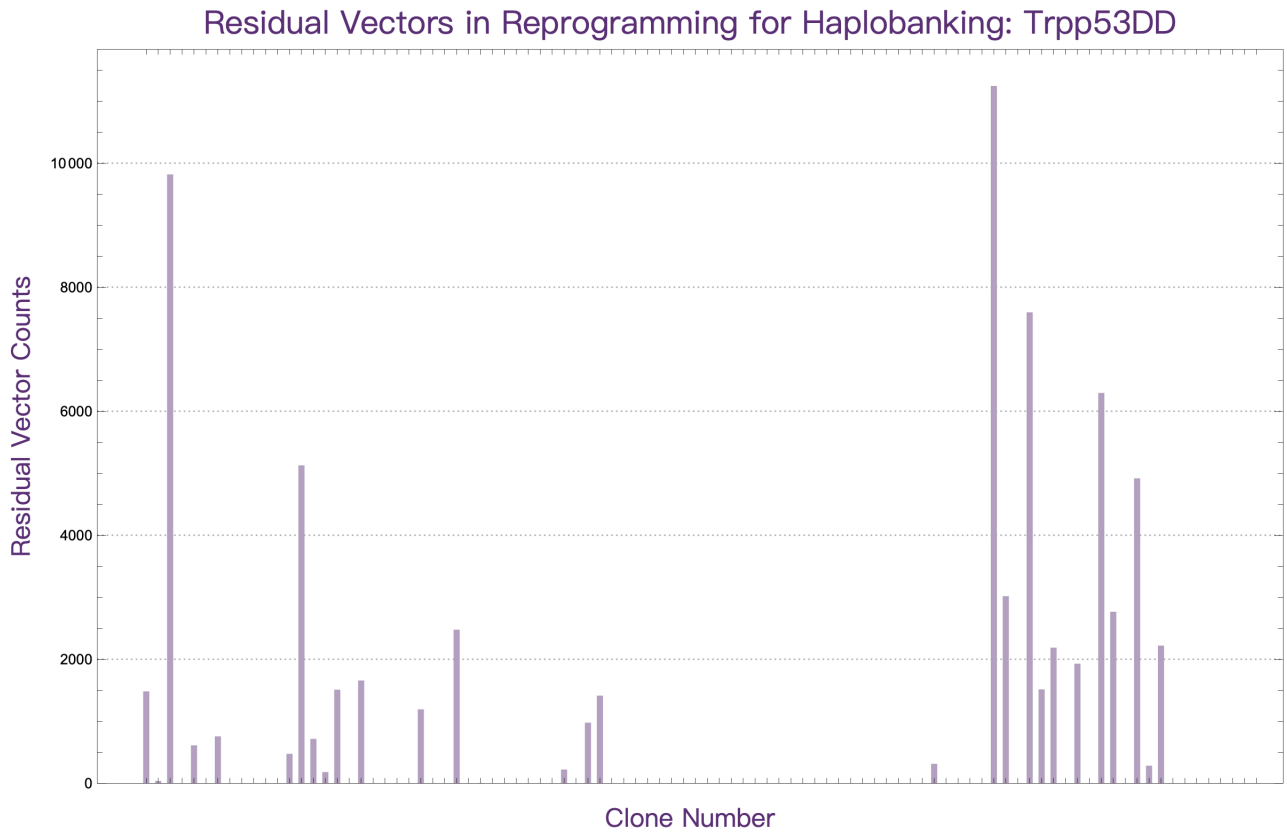


Figure 2: Residual vector counts for Trp53DD from column "P" of "Table S7" in the Supplemental Information section of the 2023 paper.

2.4.3 Chromosome 11 Uniparental Disomy

Another frequent observation during the screening process was the appearance of uniparental disomy (UPD), or the inheritance of two chromosomes from a single parent. This occurred with respect to chromosome 11, specifically Chr11q22.1–25. With the exception of QHJI-derived hiPSCs, all others exhibited chromosome 11 UPD.

In the report, it is written that chromosome 11 UPD might be attributable to the use of mp53DD in the reprogramming process. The report cites a study on myeloid leukemia in which p53 alterations were associated with UPD (p17), itself attributed to homologous recombination. (The myeloid leukemia report, in turn, cites another paper from the 1990's in which p53 inactivation is associated with an uptick in homologous recombination.)

It has been known for over a decade that p53 suppression enhances reprogramming efficiency. p53 functions as a cell cycle regulator and is implicated in cellular senescence, apoptosis, and cell cycle arrest. Attenuating such functionality permits more cells to attain pluripotency, improving reprogramming yields. Thus, it is desirable to suppress p53.

A direct relationship between p53 inactivation and Chr11q UPD has yet to be ascertained. What is more, UPD appeared predominantly in lines obtained via Method 3 (which was used for cells derived from donors RJWI, YZWJ, ILCL, and GLKV) whereas QHJI cells (which did not suffer significant Chr11q UPD) were obtained via Method 1.

CiRA Foundation:

> From the standpoint of reprogramming efficiency, it is preferable to keep p53 disruption. Chromosome 11 UPD was seen in a small number of lines from almost every donor, but what is noteworthy is that while it was seen in most of the cell lines derived from peripheral blood using method 3, it was not highly seen in other lines. There remains uncertainty regarding the relationship between the UPD and p53 disruption.

2.5 Differentiation Results

2.5.1 Differentiation Propensity Assays

hiPSCs are valued for their trilineage differentiation potential, or capacity to differentiate across all three germ layers (i.e., endoderm, mesoderm, ectoderm). The HLA-homozygous hiPSC lines in the CiRA Foundation iPS Cell Stock Project were indeed subject to a trilineage differentiation assay (using the StemDiff Trilineage Differentiation Kit), which confirmed a trilineage potential for lines created from donors QHJI, YZWJ, RWMH, DRXT, and GLKV. With one exception (i.e., the SFEBq test for neuron differentiation potential) assays were not performed to test differentiation potential for specific types. However, as a general matter, rather than have CiRA Foundation perform cell-type-specific differentiation assays in-house, it is preferred for organizations who receive materials distributed from the iPS Cell Stock to perform assays on their own in accordance with their own needs.

CiRA Foundation:

> We think that it is important to provide our users with the iPSC stock as quickly as possible so that they themselves can confirm the differentiation propensity for particular cell types because the differentiation protocols are highly individualized.

On the other hand, in certain cases, the CiRA Foundation has begun to perform cell-type-specific differentiation potential assays in response to common user demands.

CiRA Foundation:

> At the same time, in response to user needs, cardiac differentiation potential is tested in recently manufactured genome-edited cell lines and the information is shared with the users. We will consider performing further analysis of differentiation potential as needed.

In the paper, it is stated that the CiRA Foundation allows users to pre-select hiPSCs, and perform the requisite differentiation potential assays for the desired cell type, by distributing research-grade versions of clinical-grade lines, which share the same manufacturing process.

CiRA Foundation:

> There seems to be no significant difference in the manufacturing process between our research-grade and clinical-grade iPSC stock.

2.5.2 Undifferentiated Marker Expression

Quality control (QC) tests and characterization assessments for hiPSC lines included – in addition to whole-exome sequencing (WES), whole-genome sequencing (WGS), and karyotyping – detection of undifferentiated state markers via flow cytometry. hiPSCs were excluded due to residual plasmids, single nucleotide variants (SNVs), and insertions-deletions (indels). Another reason for exclusion during screening was under-expression of undifferentiated markers, such as TRA-1-60, TRA-2-49, and stage-specific

embryonic antigen-4 (SSEA-4). Intuitively, because undifferentiated markers are indicative of reprogramming to pluripotency, their under-expression may suggest the failure of a given cell to become an hiPSC. However, on a methodological note, the appearance of undifferentiated marker under-expression may be sensitive to sampling effects; their appearance may vary according to the time at which QC sampling is performed. Moreover, there are indications that their appearance may lessen under later-stage sampling.

CiRA Foundation:

> The mechanism is not fully understood. We have mainly screened out cells due to insufficient expression of TRA-1-60, which is known to have less stable expression than the other two markers. Also, there are some cell lines whose TRA-1-60 expression appears to be decreasing when close to confluence, so it may be improved when the timing of cell sample collection is more strictly regulated.

As far as matters of QC are concerned, one wishes to adopt a sampling method that enjoys the precision that accompanies standardization whilst allowing some screen variability in order to avoid false positive results in undifferentiated marker detection.

CiRA Foundation:

> There may be something to be discussed regarding the trade-off between flexibility in timing of QC sample collection and standardization of testing.

Figure 3: Undifferentiated factor expression per clone for SSEA-4 and TRA-1-60, using data from "Table S7" in the Supplemental Information section of the 2023 paper.

2.6 "Reverse Translational Research"

The conclusion of the 2022 paper introduces, briefly, the intriguing concept of "reverse translational research". The motivation is as follows. How does one know that one has developed a pluripotent cell line ready for industrial-scale cell therapy? One would like to perform a kind of assay for Critical Quality Attributes (CQAs) of a haplobanked line, which indicate that its satisfies the requisite scalability desiderata. However, CQAs as such are difficult to ascertain *in vitro*.

CiRA Foundation:

> In general, it is important to understand what the CQAs are, but they could not be identified in many cases due to insufficient *in vivo* parameters which indicate true endpoints for regenerative medicine products.

This issue is expected to be redressed, in part, with the availability of clinical trial data. Thus, one expects to surmise from clinical trial data not only translational insights regarding the therapeutic effectiveness of haplobanked, HLA-homozygous hiPSCs, but also a deeper basic understanding of the nature of pluripotency and differentiation itself as witnessed within an *in vivo* setting. Such is the intriguing nature of "reverse translational research".

CiRA Foundation:

> We believe that reverse translational studies are essential to verify the relationship between cell quality *in vitro* and cell behavior *in vivo*.

Certain *in vivo* data may only appear in treatment-seeking patients themselves, rather than control groups. Thus, it will be helpful, in reverse translational research, to gather insights from "an environment with a pathological condition[;] not [just a] normal [one]." It is hoped that such *in vivo* data can be analyzed in concert with biomanufacturing data in order to help realize induced pluripotency for therapeutic applications at an industrial scale.

CiRA Foundation:

> By comparing cell manufacturing process data with clinical trial data, it may be possible to identify the necessary cell quality characteristics of final cell products, as well as intermediate cell products, and establish an appropriate – neither excessive nor insufficient – QbD (Quality by Design) manufacturing process.

The commentary on "reverse translational research" given at the conclusion of the paper may be suggestive of new epistemological directionality – namely from translational to basic research – in the iPSC field, wherein basic aspects of pluripotency itself attain refined definition through the pursuit of translational imperatives and uptake of insights therefrom. Put differently, the prospect of reverse translational research reminds us of the fascinating epistemological character of biomedicine, with our basic understanding of biomedical phenomena being in many cases understood through the development of treatments. By observing the performance of hiPSC therapies in patients, we may be able to better understand the fundamental regenerative character of hiPSCs – from reprogramming to engraftment – *vis-à-vis* a dual biomanufacturing/clinical lens.

Haplobanking Challenges			
	Feature/Problem	**Possible Explanation**	**Possible Resolution**
Plasmid Residuality	Episomal plasmids are supposed to be non–integrating vectors for reprogramming (unlike lentiviruses)	Genomic integration Or Non–integrating residuality due to rapid episomal replication	Screening with single cell/well plating and a limited dilution process (contemporary view)
Chromosome 11 Uniparental Disomy (Chr11q UPD)	Chromosomal abnormality; encountered in a small number of cell lines for all donor–derived iPSCs but QHJI	May be attributed to p53 inactivation during reprogramming	Screening with single cell/well plating and a limited dilution process after primary screening by colony picking
Underexpression of Undifferentiated Markers	Underexpression of TRA–1–60 may indicate incomplete reprogramming	May be an artifact of sample timing	Stricter regulation of the timing of quality control sample collection considering the cell condition

Figure 4: Summary of challenges discussed in 2.4-2.5

3 Trends in HLA Cloaking

3.1 The HLA Cloaking Approach

As discussed previously, HLA cloaking is another hypoimmunity strategy being pursued parallel to haplobanking. In order to motivate this approach, one need only consider the combinatorics of HLA-homozygous matching and the logistical challenges at play in screening donors. With HLA cloaking, one may be able to eschew the task of HLA matching by knocking out certain HLA alleles implicated in allogeneic immunogenicity. In doing so, one can avoid, perhaps, the requirement of HLA matching by mitigating immunogenicity through gene-editing.

Knockout methods are employed to disrupt specific HLA alleles in order to equip otherwise HLA-mismatched allograft iPS cells with the capability to evade immune responses once transplanted *in vivo*. One wishes to suppress cell-surface presentation of foreign antigens to cytotoxic $CD8^+$ T cells by HLA class I molecules (e.g., HLA-A, HLA-B, HLA-C); antigen presentation to $CD4^+$ helper T cells by HLA class II molecules (e.g., HLA-DP, HLA-DQ, HLA-DR); and natural killer (NK) cell cytotoxicity in response to "missing-self recognition". One does so by performing gene knockouts (KOs) that suppress particular HLA alleles via clustered regularly interspaced short palindromic repeat (CRISPR) associated protein 9 (Cas9) methods.

One encounters what we might call 'knockout/hypoimmunity optimization' challenges when implementing HLA KO. If one knocks out all HLA class I molecules, one achieves evasion of $CD8^+$ T cell cytotoxicity at the expense of NK cell cytotoxicity: suppression of HLA-C foments missing-self recognition, to which NK cells react. Thus, the HLA cloaking strategy at CiRA and the CiRA Foundation retains HLA-C in order to achieve hypoimmunity with respect to $CD8^+$ T cells whilst also avoiding NK cell responses. One obtains "pseudo-HLA-homozygous", HLA-$A^-/B^-/C^+$, haploid cells.

Yamanaka-sensei:

> First of all, I believe you have heard about the "missing-self [recognition]" immune reaction. So, if you knock out all HLA, the recipient would recognize [cells from] those HLA-knocked-out cell lines as invaders, because they do not have any HLA. [...] Also, by deleting all HLA we have to worry about viral infection and tumorigenicity.

Hotta-sensei made a similar assessment.

Hotta-sensei:

> HLA has a very important function of presenting foreign antigens to immune cells. Obviously, completely deleting the whole HLA will completely abolish antigen presentation; I think that's too risky. HLA-C-retention – only expressing HLA-C, [without] HLA-A or -B – [...] would probably be better and safer for balancing antigen presentability and immune compatibility.

Notwitstanding considerable HLA polymorphism, due to relatively low HLA-C variance worldwide, Yamanaka-sensei believes that an HLA-C-retained approach could, if successful, achieve global coverage with a modest collection of lines.

Yamanaka-sensei:

> Our strategy is the so-called "HLA-C-[retained]" approach. We would knock out HLA-A and -B, leaving one intact -C allele. HLA-C is less diverse. We expect that as few as 10 lines with different HLA-C haplotypes will be sufficient to cover almost the entire world population.

On the biomanufacturing side, the strategy is attractive in its capacity to increase the ratio of lines yielded from a single donor.

Hotta-sensei:

> As you mentioned, our HLA-pseudo-homozygous approach basically relies on CRISPR-Cas9 knockout. If there is an HLA-homozygous donor who has 2 minor HLA haplotypes, we could create 2 or more HLA-pseudo-homozygous lines. That's probably the biggest advantage.

The CiRA Foundation currently offers 3 clinical-grade genome-edited cells, all from donor QHJI, each established using CRISPR-Cas9-KO and feeder-free media.

- QHJI14s04/AB II-KO-03
- QHJI14s04/AB II-KO-11
- QHJI14s04/AB II-KO-12

An additional 7 research-grade gene-edited cells, from two donors (QHJI and DRXT) are also made available:

- DRXT: Ff-XT28s05-ABo_To
- DRXT: Ff-I01s04-AB II-KO-16
- QHJI: Ff-I01s04-AB II-KO-50
- QHJI: Ff-I01s04-AB II-KO-54
- QHJI: Ff-I14s04-AB II-KO-7
- QHJI: Ff-I14s04-AB II-KO-13
- QHJI: Ff-I14s04-AB II-KO-24

With gene-edited lines made available in the iPS Stock, one can see a relationship between HLA cloaking research at CiRA and the iPS Cell Stock Project at the CiRA Foundation.

3.2 Hypoimmunity Testing

3.2.1 HLA-C-Retained hiPSC Hypoimmunity

The sought advantage of the HLA-pseudo-homozygous strategy is one of securing donor potential across a global HLA haplotype distribution with (at least) an order of magnitude fewer lines than a haplobanking strategy might require. Disruption of HLA alleles, if chosen carefully, might mitigate allogeneic immunogenicity in a manner that would otherwise require extensive donor matching. However, KO's do not in their own right guarantee hypoimmunity. For instance β2-microglobulin knockout (β2M-KO) is regarded as risk-prone due to KO-induced innate cytotoxic responses from NK cells in the complete absence of HLA class I, as Yamanaka-sensei described in an earlier quote. The chosen HLA-C-retained strategy is favored due to the expectation of hypoimmunity optimality, mitigating allogeneic immunogenicity without 'knocking out too much' and provoking an NK cell response. Thus, a key demonstration of the prospect of the HLA-C-retained gene-editing approach, distinguishing it from β2M-KO, is the demonstrated evasion of NK cell cytotoxicity.

In the 2019 paper, the clinical-grade hiPSC line, Ff-XT28s05, was used to produce a gene-edited line via knockout of HLA-A*24:02 and HLA-B*07:02 (as well as HLA-C*12:02), with HLA-C*07:02 retained. Genome editing was performed through CRISPR-Cas9 protein transfection via 4D-Nucleofector. Guide RNAs (gRNAs) – specifically HLA-A-ex3g1 and HLA-B-ex2-g1 – were selected for the knockouts.

Another gene-edited, HLA-C7$^+$ cell, was produced, from iPSC 585A1 (also subject to knockout using HLA-A-ex3g1 and HLA-B-ex2-g1) for immunogenicity testing. CD8$^+$ T-cell, CD4$^+$ T-cell, and NK cell immune responses were tested per cell type, rather than in concert, as is discussed with Hotta-sensei below.

A Chromium-51 release assay was performed to test for CD8$^+$ T-cell immunogenic-

ity, which showed HLA-C7$^+$-cell-derived blood cells to perform better than β2M-KO-cell-derived blood cells in evasion of CD8$^+$ T-cell cytolysis. (The HLA-C7$^+$-cell-derived blood cells also demonstrated better graft survival; comparing intraperitoneal transplants in mice of CD43$^+$ blood cells differentiated from a HLA-C7 retained line and a β2M-KO line, the former exhibited better performance.)

Next, NK cell cytotoxicity was tested. HLA-C7$^+$-cell-derived blood cells were tested for expression of the CD107a (LAMP-1) antigen, which is indicative of exocytotic degranulation by CD8$^+$ T-cells and NK cells. A Chromium-51 release assay was also performed. NK cell cytotoxicity was found to be significantly lower in the HLA-C7$^+$ case than in the β2M-KO control group cells. The HLA-C7$^+$ cells also exhibited cytotoxic evasion when exposed to a mixture of both NK cells and CD8$^+$ T cells.

Furthermore, HLA-C$^+$/CIITA$^-$ cells were tested for CD4$^+$ T cell immune responses via a carboxyfluorescein succinimidyl ester (CFSE) assay. It was found that, whereas CD4$^+$ T cell reactions did arise in the HLA-C$^+$/CIITA$^+$ case, it did not in the HLA-C$^+$/CIITA$^-$ case.

3.2.2 Global Coverage Differentiated Cell Testing

If such favorable hypoimmunity properties hold for further HLA-C$^+$ clinical-grade hiPSC lines, it is foreseen that a global donor potential can be reached with a small HLA-C$^+$ hiPSC collection. It is estimated that an HLA cloaking strategy can provide 90% haplotype coverage among Japanese, European-American, African American, Asian American/Pacific Islander, and Hispanic American populations with the addition of 11 clinical-grade HLA-C$^+$ hiPSC lines:

- HLA-C*01:02
- HLA-C*02:02
- HLA-C*03:03
- HLA-C*03:04
- HLA-C*04:01
- HLA-C*05:01
- HLA-C*06:02
- HLA-C*07:01
- HLA-C*08:01
- HLA-C*12:02
- HLA-C*16:01

Aspirations as such help one to appreciate the interest in HLA cloaking as a potential strategy for demographically broadening patient elligibility for hiPSC-based allograft therapies beyond those that can be accommodated via haplobank donor matches. Testing NK cell responses to the remaining 11 HLA-C7-retained lines is thus a natural course of action. As Hotta-sensei mentioned, data on other lines has been collected.

Hotta-sensei:

> For other HLA-C-retention strategies, of course, I think it would be ideal to [test] NK responses. Actually, we do have some unpublished data [on] additional HLA-C-retained lines.

Because the ultimate hypoimmunity outcomes concern not the hiPSCs themselves, but the specialized cells differentiated therefrom, it is better, in Hotta-sensei's estimation, to subject primary cells differentiated from HLA-C-retained hiPSCs to NK cell cytotoxicity testing. With the CiRA Foundation now a distributor of pseudo-HLA-homozygous cells, such testing can be decentralized across a greater network of institutions.

Hotta-sensei:

> [Regarding] the question of whether or not we have to do that test all the time when we create clinical-grade HLA-C-retained iPSC lines: it may not be the case. It could be tested after differentiation. That's something our

collaborators [do]. We already distributed HLA-C-retained lines through the CiRA Foundation last year. So now, our collaborators are trying to make them differentiate to the cell type of interest, and testing immune responses.

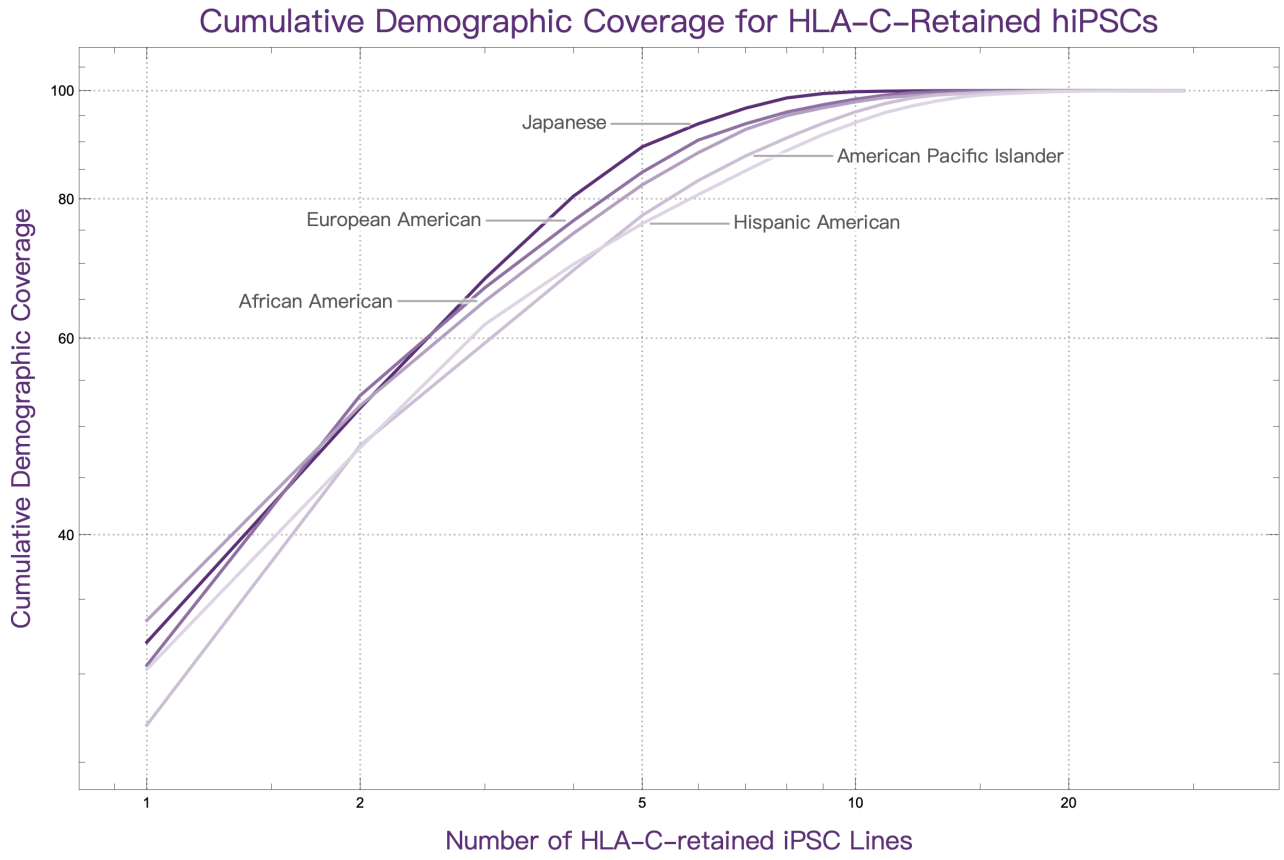


Figure 5: Plot of estimate of cumulative coverage provided to 5 demographic groups per cumulative availability of the HLA-C-retained lines listed in 3.2.2 of this report, visualizing data from Table S4 in the Supplemental Information section of the 2019 paper. The same data is visualized in a different manner in of Figure 6C in that paper.

3.2.3 Xenograft Limitations

The above-discussed immunogenicity tests were performed under xenograft, rather than allograft, circumstances; cytotoxicity was assayed in murine models. In the absence of clinical trials, NOD.Cg-*Rag1*^{tm1Mom}*Il2rg*^{tm1Wjl}/SzJ (NRG) mice (JAX:007799) – immunodeficient mice – have served as preliminary (albeit imperfect) subjects for *in vivo* testing, though with attendant limitations.

Hotta-sensei:

> It's challenging to do an *in vivo* assay. There are immunodeficient mice available. A human hematopoietic stem cell can be implanted to reconstitute the human immune system in mice: so-called humanized mice.

Nevertheless, even in humanized, immodeficient mice, one cannot fully recapitulate the human immune system.

Hotta-sensei:

> However, still, the thymus, lymphoid gland, and other [lymphatic structures] are derived from the mice, so it's not possible to completely reconstitute a fully mature human immune system in a mouse. I don't know if the [humanized mouse] system is enough to capture a human response; that's still under debate.

In the xenograft case, xenogeneic graft-versus-host disease (xeno-GvHD) is also an issue, though one that is a focus of recent publications. It was reported in a 2024 paper that hiPSC-derived CD4$^+$ T cells, induced with high expression levels of the regulatory T cell TF FOXP3, both mitigate CD8$^+$ T cell responses and xeno-GvHD in immunodeficient NOD-SCID IL2Rγcnull (NSG) mice that had received a xenograft of HLA-A2$^+$ human PBMCs. Nevertheless, murine models are not the only xenograft candidates; nevertheless, other models, such as those of NHPs, are still subject to similar risks.

Hotta-sensei:

> NHPs also [pose] xeno-GvHD risk[s], similar to [those of] mouse models.

3.2.4 *In Vitro* Assay Limitations

When performing *in vitro* assays, methodological choices span a considerable parametric range, and it is difficult to establish experimental conditions that simulate, within said range, the immunological conditions germane to the patient.

Hotta-sensei:

> The challenge of investigating immune responses [is]: there's a lack of precise measurement. We mainly use an *in-vitro*-based mixed [lymphocyte reaction] assay, which means the differentiated iPS cell product could be mixed with a human peripheral immune cell. We want to see the human immunoresponse, not the mouse response. The *in vitro* test is the gold standard method, but we find the sensitivity and degree of immune response depend on the experimental conditions, [such as] how many cells one mixes or how long one incubates, or how much one stimulates the cell with cytokines. It's kind of hard to make the right dose completely mimic the *in-vivo*-patient situation, because that can also vary patient-to-patient, disease-to-disease.

One can come closer to recapitulation, on a per-cell basis, by introducing human cytokines; though doing so for T, B, and NK cells in tandem remains a challenge.

Hotta-sensei:

> There's no humanized mouse model that can fully reconstitute mature T cells, B cells, and NK cells altogether. One has to supplement certain human cytokines to activate certain cell types. If people are interested in T cells, people can [...] stimulate human T cells, but in this case, NK and B cell contributions are much smaller. For NK cell reconstitution, there are NK-cell-specific cytokines available, but in that case, T cell and B cell numbers are much lower than the usual case. [...] I don't think there is a single sufficient animal model at this moment. The immune system is very complicated.

Thus, at present, the first step taken is that of demonstrating hypoimmunity on the basis of individual lymphocyte types.

Hotta-sensei:

> That's why, in the paper, we did the individual T cell assay and NK cell assay *in vitro*, with [...] highly stimulated cells so that they are primed to attack each allogeneic cell. We demonstrated that there was no immunoresponse against the HLA-C-retained line.

In light of such constraints, clinical trial results for HLA-pseudo-homozygous cells are greatly anticipated.

3.3 Knockouts and Genomic Integrity

3.3.1 Custom Guide RNAs

In the 2019 study, custom gRNAs were designed for performing HLA knockouts. Such design involved both HLA sequence alignment searches for identifying candidate sgRNA templates and *in vitro* transcription for synthesizing them. Candidate gRNAs were found using the Johns Hopkins Bowtie short read aligner on HLA haplotype sequences in the IPD-IMGT/HLA database. The sgRNAs were then synthesized using MEGAshortscript. sgRNAs were selected for targeting exons 2 and 3.

Hotta-sensei:

> In the first part of the paper, we performed an *in silico* analysis of potential guide RNA designs for all possible HLA haplotypes.

Prior to meeting Hotta-sensei, I had wondered if sgRNA template requirements will vary by patient HLA haplotype, such that custom sgRNAs must be designed for each pseudo-HLA-homozygous line. Indeed –

Hotta-sensei:

> That's one of the questions we had when we initiated that project.

In fact, multiple sgRNA design is necessitated when a consensus region for the multiple KOs involved is unavailable.

Hotta-sensei:

> What we found is [...] technically, it is possible to design a single guide RNA that targets HLA-A, -B, and -C all together, but the consensus regions are not [conveniently] located[.] [...] The HLA gene consists of 5 exons, and antigen-presenting extracellular domains are located mainly on exon 2 and 3, so it's important to knock out 2 and 3; but sometimes, the consensus region is located on exon 4 or 5...

(Multiple KO requires consensus among gRNA sequences. HLA class-I gene loci can be found on chromosome 6 (its short arm), it's heavy chain being encoded by 8 exons.) In light of the exon issue, sgRNAs are tailored to each pseudo-HLA-homozygous hiPSC line.

Hotta-sensei:

> What we're currently doing is custom design of guide RNAs for individual iPS lines; that way, we can identify the region that we want to target.

This might illustrate a possible disadvantage of the HLA cloaking approach relative to the haplobanking approach. In the former case, one may need to design sgRNAs and conduct off-target tests for each line, whereas, in the haplobanking case –

Hotta-sensei:

> We don't have to do extensive off-target genomic integrity analysis if no gene editing is performed. Still, minimal tests (karyotyping and WGS) are required for clinical-grade iPSC lines.

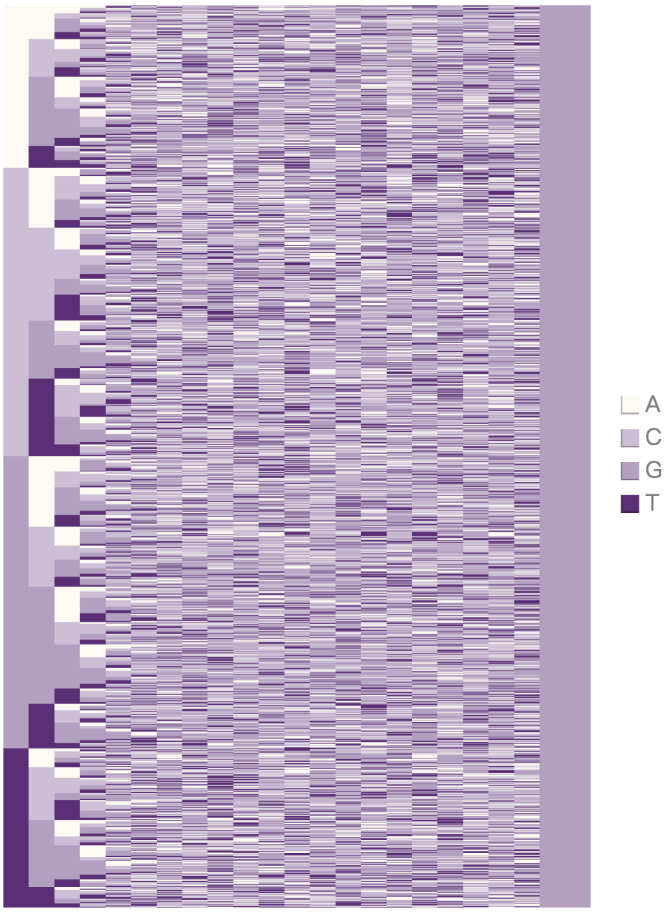


Figure 6: Array Plot visualizing the target sequences for the HLA-targeting CRISPR-Cas9 sgRNAs considered during *in silico* analysis, as shared in Table S1 in Supplemental Information section in the 2019 paper. Here, each nucleotide base is given a color, as shown in the legend.

3.3.2 Checking for Off-Target Effects

After disrupting the targeted class I HLA alleles, one seeks to confirm the absence of deleterious off-target edits. One wishes to ensure that the CRISPR-Cas9 knockouts only edit the sites pertinent to the targeted alleles; one wishes to confirm genomic integrity. During the sgRNA design phase, possible off-target behavior was identified using CRISPOR and GGGenome. In the study itself, integrity was assessed via karyotyping and whole-exome sequencing (WES) – the latter identifying single nucleotide variations (SNVs) and insertion-deletions (indels) – and Cas-OFFinder, which was used to determine the extent to which the SNVs and indels were off-target (up to 9 basepairs (bps)), given the great number of possible off-target sites within a 9bp range. Setting aside the on-target SNVs/indels, which were indeed located within 3 basepair proximity of the protospacer adjacent motif (PAM) site where the CRISPR-Cas9 cleavage occured, the distribution of the remaining SNVs/indels was found to be cleavage-independent. Thus, their appearance was attributed to mutagenic processes involved in the handling of hiPSCs themselves, such as culturing and subcloning.

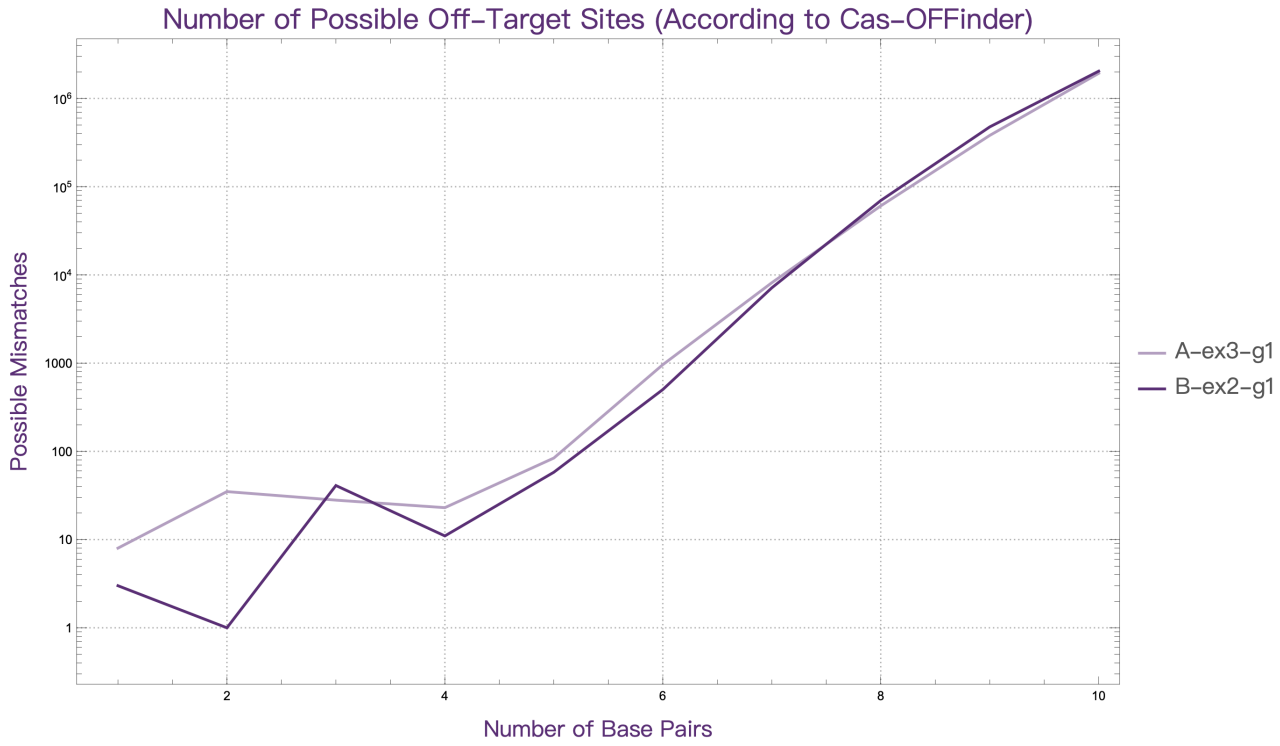


Figure 7: Number of possible off-target sites for sgRNAs A-ex3-g1 and A-ex2-g1, plotted on a logarithmic scale, and color-coded in the legend. Data is from Table S6B from "Document S2. Article plus Supplemental Information" in the Supplemental Information section of the 2019 paper.

3.3.3 Cultural Mutagenicity

In order to perform genomic integrity analysis, one must produce cells to test. Thus, one must expand one's hiPSCs *in vitro*. One does so at a cost, however, for the expansion process itself can be mutagenic, as Hotta-sensei described.

Hotta-sensei:

> Just isolation and cultivation of iPS cells accumulates genomic error. That has been known.

Moreover, one cannot hope to eliminate such mutagenicity; it is an ineluctable accident of in vitro expansion.

Hotta-sensei:

> Tissue culture is a quite artificial system: we culture cells as a monolayer in a plastic dish, which is totally different from *in vivo* [conditions]. Even cells dividing in a plastic dish is surprising; it's unbelievable to me. The situation of growing cells in a dish is very different from the *in vivo* situation. Similarly to other cell lines, iPS cells can be expanded unlimitedly; they have a very active proliferation profile. So, acquisition of genomic copy errors is expected. The human genome is 3 gigabytes; copying every two days. It would be surprising if there was no error at all.

Nevertheless, one wishes to reduce mutagenicity by minimizing the risk-prone parameters involved in expansion, such as passage number and culture time.

Hotta-sensei:

> The important thing is minimizing the culture period – what we call the passage number – with as few cell divisions as possible. That's critical. [...] Tissue culture time should be minimal.

Determining the risk severity of mutations is itself difficult, and confounded further by the unique genomic profiles of individuals.

Hotta-sensei:

> If you have a chance to look at individual genomic sequences, you'll be surprised by how diverse they are. There are 3 million SNPs [(single nucleotide polymorphisms)] between individuals. Each person has a unique genomic sequence. Mutations could even be found in oncogenes or tumor suppressor genes. [...] We establish iPS cells from healthy volunteers [but] [...] actually, in iPS genomic sequences, there's a large variation. No one knows the individual meaning of a mutation, even when there's a large copy number variation or a mutation in a well known gene. Deciding how benign or dangerous a mutation is: that's still a challenge in the field.

Nonetheless, such does not relieve practitioners of implementing due diligence protocols, such as oncogene assays.

Hotta-sensei:

> At least, after sequencing, we check for well known cancer-driver genes. If there was a loss-of-function mutation in a famous tumor suppressor gene, we would stop shipping that line for clinical applications. That's one of the standards of the CiRA Foundation.

3.4 Other Antigens

HLA class I proteins (e.g., HLA-A, -B, and -C) are not the only antigens of possible interest. For instance, the 2019 paper considers knockout of the class II major histocompatibility complex transactivator (CIITA) in or-

der to evade $CD4^+$ T cell responses. ($CD4^+$ T cells are activated via HLA class II antigen presentation.) Although $CD4^+$ T cells do not directly pose a cytolytic risk to allograft cells, they nonetheless pose an indirect threat as "helper cells" capable of marshaling other cells (e.g., B cells, cyototoxic T cells) via cytokine release. The sgRNA CIITA-ex3g5 was used to create HLA-C7-retained, CIITA-KO lines.

Beyond CIITA, other MHC class II proteins may influence engraftment in the case of certain therapies. HLA class II molecules differ from those from HLA class I in that they are expressed mostly by antigen-presenting cells (APCs). Thus, in the 2019 paper, it is suggested that further class II KO may only be needed for hiPSC-derived therapies involving the transplantation of APC allografts, such as T cells or macrophages.

For instance, class II KO may be needed in the case of certain cancer therapies.

Hotta-sensei:

> HLA class II is mainly expressed by antigen-presenting immune cells such as macrophages, dendritic cells, and NK cells. In the case of anti-cancer therapy, iPSC-derived NK cells have been investigated. So, I do think there are some cases where cell types expressing HLA class II can be utilized for cell therapy applications.

Attention is also paid to HLA-DR, a class II allele that is implicated in graft-vs.-host disease (GvHD) in transplantation medicine. Even in the haplobanking cases, donors are screened for HLA-DR homozygosity.

Hotta-sensei:

> HLA-DR is one of the important HLA haplotypes that is under investigation in a clinical setting: HLA-A, -B, -C, and -DR haplotypes match [in] basically any cord blood or organ transplantation [...] because, as has been shown, HLA-DR-matching is advantageous for minimizing immune rejections in tissue or organ transplantation.

Nonetheless, one might not expect certain kinds of immunogenicity or engraftment complications that arise in organ or tissue transplants to pose comprable risks in the case of hiPSC allografts.

Hotta-sensei:

> In my view, there's a difference between organ transplantation and iPS cell transplantation, because with iPS cell transplantation, you're making relatively pure cell populations.

Overall, the hypoimmunity advantage of class II disruption remains still unknown. However, because, unlike HLA-C-KO, class II-KO can in principle be executed without NK cell response risks; it can thus, perhaps, complement a HLA-C-retained cloaking strategy. However, the benefits remain undetermined.

Hotta-sensei:

> It's actually still a hypothetical discussion. No one knows how beneficial matching HLA class II will be in the case of iPS cell-derived products. But one could easily check whether HLA-DR is expressed in the final cell product or not. If there is HLA-DR expression, I still think there's a benefit in removing class II from the cell. Class II is not involved in NK cell suppression, so there could be a benefit.

However, such considerations might ultimately be taken on a cell-by-cell basis, depending on class II expression.

Hotta-sensei:

> At the same time, if the cell – like a motor neuron – does not express class II at all, one may not need to do additional genome editing.

Setting aside HLA class I and II, the MHC does feature further minor antigens, which are not accounted for in existing matching strategies. However, it may be the case that they may be left unmatched without significant immunogenic consequences.

Hotta-sensei:

> Even in the current HLA-homozygous iPSC trials, minor antigens cannot be fully matched, but they are not associated with severe immune rejection reactions. Therefore, I think the mismatches in minor antigens can be tolerable.

3.5 HLA Cloaking Advantages

Yamanaka-sensei expressed his confidence in the HLA cloaking approach. Given the still-partial coverage, domestic and global alike, estimated for the current state of haplobanking, and in light of the ongoing status of clinical trials, Yamanaka-sensei regards the parallel pursuit of haplobanking and HLA cloaking to be advantageous.

Yamanaka-sensei:

> At least in theory, the HLA cloaking approach should work, but we have to test it by clinical trial. As an alternative approach, we have been working on HLA-homozygous iPS cell lines, and are already providing [coverage for] 4 [common] HLA haplotypes. With those 4, we can cover up to 40% [of the Japanese population], but in order to cover the remaining 60%, and in order to cover the entire world population, we would need a lot more – maybe more than 100. [However], it's next to impossible to produce more than 100 GMP[-grade] iPS cell lines at the moment. [...] So, we are taking both approaches still, because we have to wait for the outcome of clinical trials.

Furthermore, even if future iPSC stocks extend coverage more broadly across patient haplotypes, Hotta-sensei expects that HLA-pseudo-homozygous hiPSC line production may nonetheless serve as a secondary resort for serving patients with rarer haplotypes.

Hotta-sensei:

> If we establish a huge number of HLA-homozygous lines, [but] need a very rare HLA haplotype, a pseudo-homozygous approach might become realistic. That's my own interpretation.

The prospect of pseudo-HLA-homozygous strategy is often compared with that of the pursuit by other organizations of a "universal" hiPSC line; that is, a single hiPSC line from which derived cell allograft treatments that can be received by any patient without immunogenic risk. A candidate "universal" gene-editing technique is KO of the β2-microglobulin (β2M) gene. The β2M polypeptide light chain and alpha heavy chain form the HLA class-I heterodimer. Thus, β2M disruption compromises the entire HLA class I architecture; β2M-KO is wholesale HLA-class-I-KO. As discussed previously, complete HLA class I disruption leaves one at the mercy of NK cells. Thus, weighing production efficiency and hypoimmunity tradeoffs, one should, in Yamanaka-sensei's view, produce multiple HLA-C-retained lines rather than vie to establish a universal line via β2M-KO.

Yamanaka-sensei:

> Other groups, and some companies, take a different approach in which they knock out all major HLA alleles, including HLA-C. With that approach, they argue that they would need one iPS cell line to cover the world population. They call that line the "universal cell line". At the moment, we don't know which [approach] is better. [...] [By] avoiding missing-self recognition, we believe HLA-C [retention] is better than other approaches. [...] [Also], we're hoping that by having one intact HLA-C, we can maintain some responses against viral infection and tumor formation. But we have a downside: we have to produce more than one – [perhaps] 10 lines.

Furthermore, if one's KO strategy leaves allografts vulnerable to NK cells, additional transgene techniques for NK suppression are needed. These, in turn, are undermined by the nature of iPSC-based cell biomanufacturing itself.

Hotta-sensei:

> Most other hypoimmune approaches require transgene overexpression to suppress NK cells. During iPS cell culture and differentiation, transgenes tend to be silenced. So, maintaining transgene expression in a constant manner is very challenging. Depending on how one transfers the transgene [...] one has to check the integration site for genomic integrity. Also, those types of vectors tend to have multi copy insertions, so one has to make sure that one is not disrupting important genes, such as tumor suppressor genes, or activating oncogenes.

As one can surmise, the prospective benefit, notwithstanding the NK cell risks, of the β2M-KO approach is its broad coverage. By comparison, the HLA-C-retained is favored due to the global coverage prospect it poses with rather parsimonious KO requirements, as Hotta-sensei reviewed.

Hotta-sensei:

> [The] hypoimmune approach [via] genomic engineering is a very hot field currently. Various groups [across] the world are trying different approaches. I do think there are pros and cons for each. Ours, the HLA-C-retention approach, has the advantage that it only relies on gene knockout: HLA-A and HLA-B, and, if necessary, CIITA.

Such parsimony, in turn, is expected to ease the manufacturing process.

Hotta-sensei:

> Manufacturing is also simple[r]. If one doesn't need class II knockout, we actually have a guide RNA that can knock out HLA-A and -B genes simultaneously. Just transfecting one Cas9 guide RNA into the iPS cell [...] in just one step, we can create a final cellular product. The ease of manufacturing is an advantage.

However, for a decisive verdict, one must await the results of clinical trials in order to compare the HLA-C-retained HLA cloaking strategy to other gene-editing approaches, as Yamanaka-sensei emphasized.

Yamanaka-sensei:

> I don't know which is better – in the beginning, we have to test it.

Hotta-sensei made a similar point – he awaits the outcome of the clinical trials with great anticipation.

Hotta-sensei:

> Ultimately, which methodology will be the most beneficial, in terms of avoiding host immunoresponses, is still an open question. We have to wait for the results of clinical trials, which will take several years. I'm really excited to see how the progress goes. [...] I think there are still many things that need to be investigated. Ultimately, the real answer could be provided by the clinical trial. I'm really looking forward to it.

3.6 Hypoimmunity and Immunosuppressants

From a pragmatic viewpoint, it is perhaps better to speak of fostering hypoimmunity than 'eliminating' immunogenicity *tout court*. As Hotta-sensei described, in practice, the sought outcome is one in which hypoimmunity is sufficiently accomplished by haplobanking or HLA cloaking such that conventional immuosuppressant regiments can be administered. Above all, of paramount importance is the patient experience, not immunogenicity *per se*.

Hotta-sensei:

> [By] now, HLA-homozygous line transplantation has been done safely in real human patients. In many cases, actually, immunosuppressants are also used. That's clinically practical in any cell transplant study. The combination of HLA editing with immunosuppressants [is] also an important strategy.

Put differently, one wishes to establish the requisite hypoimmune conditions for obviating a severe or prolonged immunosuppressant regiment.

Hotta-sensei:

> Immunosuppressants sometimes do cause side effects. Too much immunosuppression for too long could be a disadvantage. So, I'm hoping that HLA genome editing could reduce the requirements of immunosuppressants, reducing the amount or the period of administration. That's another advantage of the HLA editing approach.

Moreover, one should not lose sight of engraftment as the ultimate transplantation goal. One seeks a viable therapy whose allografts evade rejection by merit of a patient-tolerable hypoimmunity strategy.

Hotta-sensei:

> The problem is not necessarily avoiding immune rejection completely. To me, the important thing is having enough cells engrafted. Avoiding immune rejection is just a part of a long list.

Engraftment, in the absence of immunogenicity, has indeed been demonstrated already; for instance, in the case of a Parkinson's study performed on a *macaca fascicularis* model.

Hotta-sensei:

> Actually, Jun Takahashi-sensei's group previously demonstrated, by using a non-human primate (NHP) model, that even in the HLA-mismatch case, a neuron could be engrafted, though HLA matching was beneficial; but, there was no significant immune rejection observed in the NHP brain.

Thus, in view of the often vexing immunological quagmires confronted in transplantation

medicine, achieving satisfactory hypoimmunity may not require mastery over the intricacies of the human immune system; perhaps one need only produce results that reduce the immunogenicity risk to a familiar level that can be tractably redressed with immunosuppressants.

3.7 Immune-Privileged Tissues

Although the considerable investments in both haplobanking and HLA cloaking strategies indicate a great concern for immunogenicity, it should be noted that immunogenicity risks are anticipated to be significantly reduced for certain tissue types. In particular, immunogenicity risk may perhaps be weaker in certain cases by virtue of "immune privilege".

There exists an ongoing medical discourse on the immune privilege enjoyed by the vessels of certain organs – such as the brain or testis – due to "barriers" formed by junctions (e.g., tight, gap, adherens) whose selectivity facilitates protection against, for our purposes, the cytotoxic elements that participate in immune reactions.

Possible examples include the blood-brain barrier (BBB) and blood-testis barrier (BTB). Avascular tissues such as cartilage are regarded to enjoy similar immune privilege. As Yamanaka-sensei shared, it is hoped that immune-privileged organs and tissues implicated in diseases targeted by iPSC therapies may not pose hypoimmunity challenges requiring treatments beyond conventional immunosuppressants, thereby obviating the necessity of HLA matching.

Yamanaka-sensei:

> I have to tell you that, for many applications, we can just use HLA-unmatched iPS cell lines, because many [tissues and] organs, like cartilage and – it's controversial, but maybe – the brain have, we expect, [...] weaker immune reactions. So, by using some immunosuppressants like steroids, even local steroid injections, we may [only] have to use just one line [...] without considering HLA matching.

Hotta-sensei offered a similar outlook.

Hotta-sensei:

> The brain is protected by the so-called blood-brain barrier; so, a regular T cell, B cell, or NK cell cannot penetrate the brain region. [. . .] [In the case of] transplanting cells for neuro[degenerative] disorders, the concern of allogeneic immune rejection [due to] HLA mismatch is much less.

Thus, with HLA polymorphism potentially decoupled from immunogenicity risk in the case of therapies treating immune privileged tissues, one wonders if, perhaps, a universal-line-type approach may be feasible in such cases.

One might also take note of the diseases and injuries for which hiPSC therapies have been subject to clinical trials: *articular cartilage damage, Parkinson's, retinis pigmentosa, macular degeneration*... Many appear to pertain to tissues that, perhaps, might enjoy protection from the BBB, the blood-retinal barrier (BRB), or cartilaginous immune privilege.

Thus, although CiRA has been both developing therapies for clinical trial and developing hypoimmunity strategies for attending to immunogenicity induced by HLA polymorphism among patients, one might wonder to what extent the first wave of iPS cell therapies will require hypoimmunity strategies in order to advance forward.

However, it should be noted that such privilege may not be conferred to patients faced with a disease or injury that compromises barrier functionality. Thus, in certain cases, degenerative progressions in patients may

require that hiPSC treatments adhere to HLA matching (or editing).

Hotta-sensei:

> If one doesn't have to care about HLA mismatches, one does not need to use hypoimmune iPS cells. [...] [However,] in the case of severe neurodegeneration, [for instance] [. . .] there might be a disruption of the blood-brain barrier structure. In that case, there is a risk of immune infiltration. To avoid unnecessary immune responses, HLA-matched or genome-edited iPSC lines are beneficial.

Nevertheless, as stated previously, the ultimate goal of hypoimmunity allograft therapy is not mitigation of immunogenicity risk for its own sake; hypoimmunity is pursued to support engraftment. With this in mind, it may be the case that HLA matching will remain a desideratum, even for immune privileged tissues, in order to foster engraftment.

Hotta-sensei:

> Even with the NHP study, HLA matching was beneficial: graft sizes were significantly better than the HLA-mismatch case. Also, that study was based on a healthy animal, not a diseased animal model.

On the other hand, with the advent of organoids, it has been speculated in a recent review paper by Hotta-sensei, Dr. Sonja Schrepfer, and Professor Andras Nagy that barriers could be fashioned to engineer immune privilege. However, as Hotta-sensei noted.

Hotta-sensei:

> This is still a hypothetical statement with a future perspective.

HLA Cloaking Challenges and Open Questions			
	Question/Issue	**Context**	**Possible Approaches**
Immunogenicity Testing and Global Therapy Coverage	NK cell response tests for other HLA–C–retained lines	A HLA–C*07:02–retained line was tested in the paper. It is reported that 11 additional HLA–C–retained lines can provide >90% coverage for five international ethnic groups	Testing the other 11 HLA–C–retained lines Or Post–differentiation testing by recipients of hiPSCs from CiRA Foundation
Xenograft Limitations	How can limitations on *in vivo* assays be overcome in the xenograft case?	Difficult to recapitulate human immune system in murine models	Testing immune reactions per cell type (contemporary approach)
Cell Culturing and Mutagenicity	Reducing mutagenicity during the *in vitro* culturing process	Expanding iPSCs itself incurs genomic copy errors	Minimization of passage number and tissue culture time; screening iPSCs for oncogenes
Custom sgRNA Design	Are bespoke sgRNAs needed for each patient?	Exon location of consensus regions for an sgRNA for HLA–A, –B, and C– can vary by patient	Custom sgRNA design for CrisprCas9 knockouts
Class–II KO Hypoimmunity Benefits	Should gene–editing knock out class II?	The question of hypoimmunity benefits for class–II–KO remains open	Not yet known

Figure 8: Summary of challenges discussed in 2.3.2-2.3.3 of this SciSci report

4 Conclusion: Further Developments

4.1 The International Stage

4.1.1 Global iPSC Banking

Worldwide, haplobanking endeavors are underway or under consideration. A recent paper by Hotta-sensei and colleagues mentions several nations in which similar initiatives are underway. As of 2020, the Korea National Stem Cell Bank has 19 hiPSC lines, expected to provide 51% domestic coverage. As of 2020, the Banco Nacional de Líneas Celulares (BNLC) in Spain reports reports an inventory of 171 hiPSC lines. Not all of these initiatives are being pursued for therapeutic purposes. In certain cases, such as the European Bank for induced pluripotent Stem Cells (EBiSC), the objective is to provide a repository of research-grade lines (e.g., for disease modeling).

It is remarked by some that haplobanking, insofar as it is feasible in Japan, is infeasible in other nations due to greater domestic genetic diversity. 40% domestic haplobanking coverage in Japan may, for those of that view, be taken to buttress their position. On the other hand, the 90% projection (for US/Japan) attached to the gene-editing paper is *prima facie* intriguing, and indeed partly motivated the inquiries pursued in this piece. However, notwithstanding the haplobanking challenge in Japan, similar initiatives are under consideration even in nations whose HLA polymorphism hurdles are much more formidable. For instance, in Brazil – a nation with complex demography and broad HLA haplotype diversity – a preliminary study on haplobanking was conducted, in which it was estimated that 95% population coverage would require 559 (triple-)HLA-homozygous lines (and screening some ∼4 million citizens, or 1.9% of the population).

Given the significant line carrying requirements estimated for certain foreign haplobanking initiatives, one might wonder if it would be better to prioritize an HLA cloaking strategy for domestic coverage in nations with highly HLA-polymorphic populations. Hotta-sensei is of the view that it can still be valuable for national haplobanking projects to offer lines representative of the haplotype frequencies among their domestic populations, so long as the requisite support infrastructure is in place.

Hotta-sensei:

> [Different] nations have different HLA haplotype distributions, so I think it is beneficial [for each nation] to have their own iPSC stocks or banks. Running and operating such cell stocks requires nation- wide support, including an HLA- haplotyped blood/cord blood bank and financial support.

4.1.2 International Regulation

The development of national regenerative medicine capabilities necessarily depends on domestic regulatory environments; and the development of an international regenerative medicine depends on the international regulatory climate. Naturally, the first step is domestic; for instance, CiRA Foundations compliance with the PMD Act was discussed previously. Although national haplobanking initiatives may each differ in certain respects in accordance with domestic regulations, the global prospect of gene-editing strategies, which have the potential to redress lapses in haplobanking HLA coverage, might benefit from regulatory coordination among nations, such that they can more efficiently cooperate to provide hiPSC lines for individuals with HLA haplotypes not covered by available stocks.

Multilateral discussions between working scientists and regulatory bodies remain ongoing. One notable forum is the International Society for Stem Cell Research (ISSCR), which

offers an independent, nonprofit setting for such discussions.

Hotta-sensei:

> Regulatory decisions are made by regulatory agencies in each country (FDA in the USA and PMDA in Japan). Therefore, international collaboration between the regulatory agencies will be critical to achieve global harmonization. I am working as a member of ISSCR to meet with PMDA and FDA to discuss what kind of regulatory requirements and safety tests are commonly needed to assess genome-edited hypoimmune iPSCs.

In the case of Japan-US developments, one can detect signs favoring, albeit gradually, the permissibility of use of Japanese hiPSC lines – notably, haplobanked lines – for US clinical trials. For instance, in 2022, the US Food and Drug Administration (FDA) informed the CiRA Foundation that QHJI satisfied its requirements for QHJI-iPSC-derived dopaminergic neurons to be used in a product (CT1-DAP001) by Sumitomo Pharma Co., Ltd. (住友ファーマ株式会社), an Osaka-based multinational company, in a Parkinson's therapy clinical trial. Two QHJI lines, QHJI01s04 and QHJI14s04, were added by the FDA to its Drug Master File (DMF). In 2023, the Investigational New Drug (IND) application for a clinical trial at the UC San Diego Sanford Stem Cell Institute CIRM Alpha Clinic was approved.

4.2 Developments in Autologous hiPSC Biomanufacturing

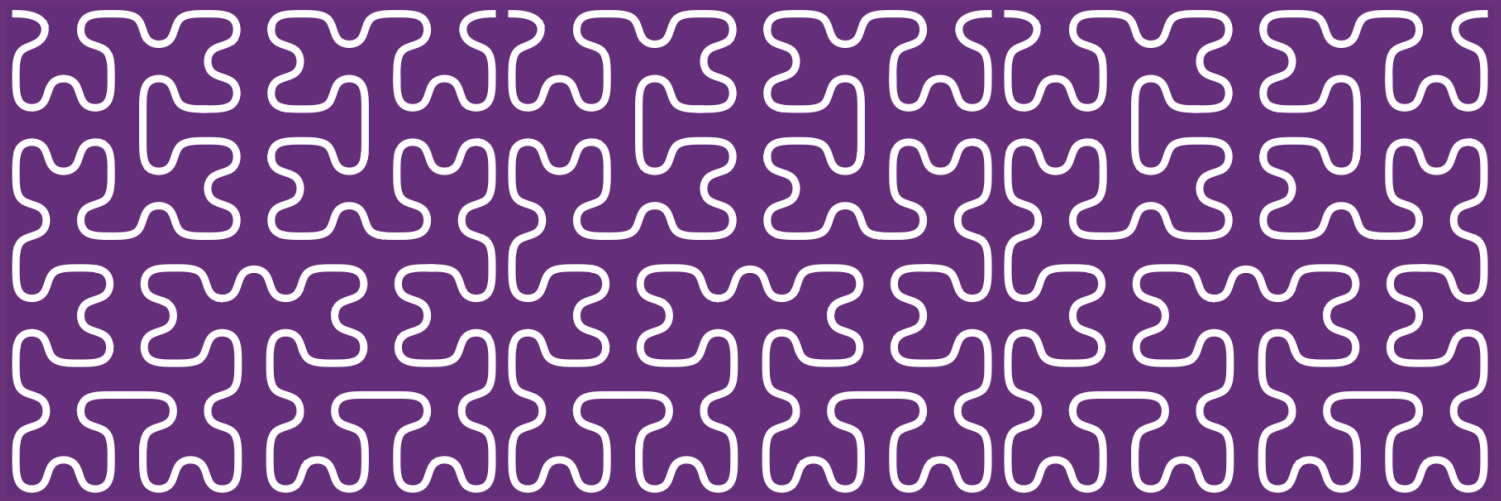
Recall that the very issue of immunogenicity is, to a large extent, a *fait accompli* of allograft transplantation methods, which are in turn motivated by the expectation of scalability limitations for autologous therapies. Nevertheless, such an expectation has not precluded investment in autologous capabilities. In fact, as Yamanaka-sensei described, new initiatives are forthcoming.

Yamanaka-sensei:

> We haven't given up on the autologous approach. We are working on the so-called "my iPS Project", in which we are trying to reduce the cost and time to produce autologous iPS cell lines[.] [...] [W]e are hoping that, by as early as [2025], we can provide that third approach as well.

The Yanai Facility for my iPS Cell Therapy (「Yanai my iPS製作所」), or "Y-FiT" (named after donor YANAI Tadashi (柳井正)) will operate in Osaka as a manufacturing facility for automated production.

Given that allograft transplantation, premised on the scalability challenges of autologous therapy, has been a key motivator for hypoimmune strategies, it will be highly informative to see how the hypoimmune strategies of haplobanking and HLA cloaking progress, over the coming years, relative to autologous hiPSC biomanufacturing undertaken by the my iPS project.



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Published by SciSci Press