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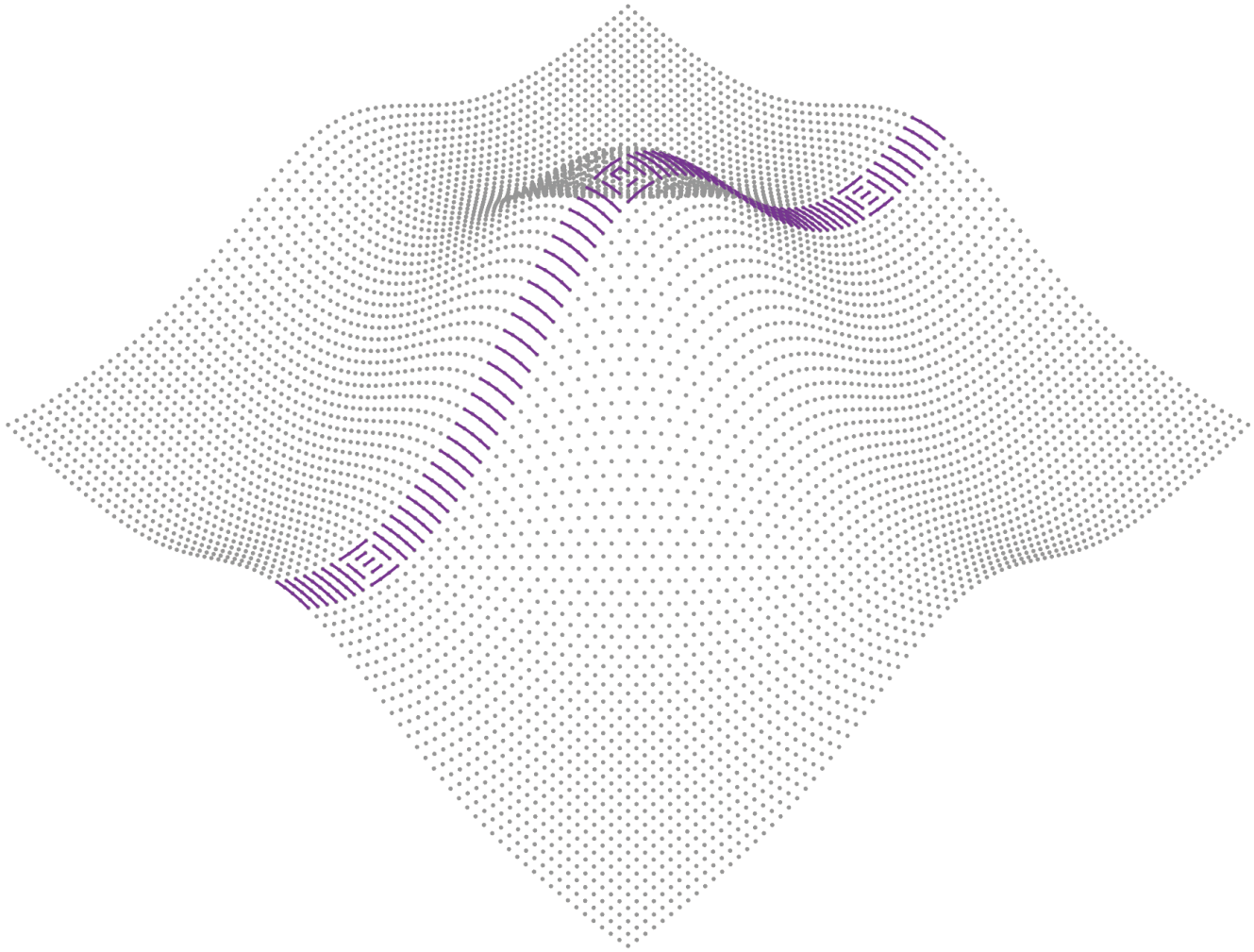

Future Pluripotency Research for iPS Cell Therapy and Disease Modeling: CiRA and Gladstone

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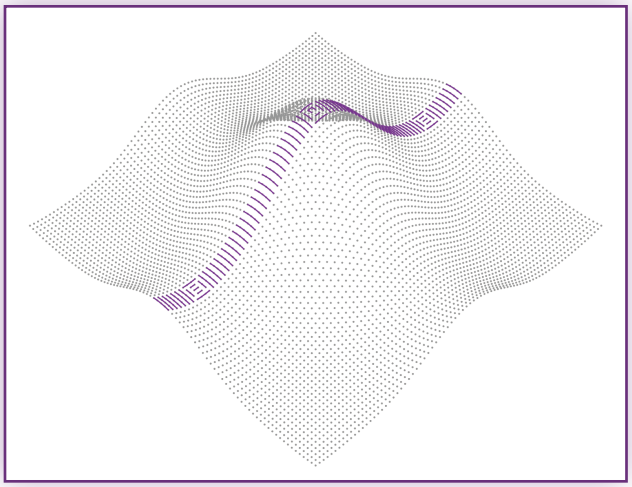
About the Cover:

A visual illustration of the Waddington Landscape, an idealization used to describe cell differentiation from a pluripotent state (i.e., at the maximum of the landscape), as well as reprogramming from a primary cell back to pluripotency (i.e., induced pluripotency).



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1 Pluripotency Research: Translational and Basic

Induced pluripotent stem cells (iPS cells) are utilized by those in disease-modeling and cell therapy in pursuit of delivering treatments to patients. The induction of pluripotency itself from already-differentiated cells via reprogramming, on the other hand, is itself a basic-scientific marvel whose elucidation, although in many cases sufficient for translational purposes, is not fully determined; basic research continues. The purpose of this investigation is to ascertain to what extent there remain basic research questions concerning pluripotency and reprogramming of consequence to translational imperatives.

We'll center on two institutions that have been at the forefront of the iPS cell field throughout its (pre-)history, whose work is intertwined: 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); and Gladstone Institutes in San Francisco. Since 2019, these two have shared the iPS Cell Research Center at Gladstone Institutes (「グラッドストーン研究所iPS細胞研究拠点」), an on-site laboratory that is part of Kyoto University (京都大学). Since the formation of CiRA, the key pivot in its dovetailing with Gladstone has been Professor YAMANAKA Shinya (山中伸弥). (SciSci follows the convention of writing Japanese surnames first in Romaji and capitalizing them.)

Yamanaka-sensei was awarded the 2012 Nobel Prize in Physiology or Medicine (shared with Sir John Bertrand GURDON) for his discovery of iPS cells (made with colleagues, listed in our Technical Refresher section). Presently, Yamanaka-sensei is the President of the CiRA Foundation (京都大学iPS細胞研究財団); Director Emeritus and Professor at CiRA; Senior Investigator at Gladstone Institutes; and Professor of Anatomy at UC San Francisco. (Yamanaka also performed his postdoctoral research at Gladstone.)

CiRA's mandate, as pronounced in its CiRA Vision 2030 (CiRA 2030 年までの目標), is to "develop new medical applications using iPS cells" (「iPS細胞の医療応用」). Gladstone's mandate is "empower[ing] its world-class scientists to find new pathways to cures" and pioneering "biomedical research to overcome disease". Gladstone, although pursuing a research scope that is wider than the iPS field, is also a leader in iPS research, though of a different character, as summarized by Dr. TOMODA Kiichiro (友田紀一郎), who is both an Associate Professor at CiRA and a Research Investigator at the Gladstone Institute of Cardiovascular Disease.

Tomoda-sensei:

> At CiRA, one of the main focuses is cell therapy using iPS cells. Also, at CiRA, many research groups are doing disease modeling. That's the same at Gladstone. But at Gladstone, not as many groups are doing cell therapy. That's really the difference.

Here, cell therapy encompasses the treatment of diseases (e.g., macular degeneration) and damage (e.g., spinal cord injury) via transplantation of healthy, iPS-derived cells. Disease-modeling involves the study of the progression of diseases (e.g., Parkinson's), for the purpose of developing treatments, by using iPSCs to culture otherwise difficult-to-obtain cells (e.g., dopaminergic neurons) implicated in their etiology and pathogenesis.

Invoking the dichotomy (or spectrum) of basic and translational research, one can situate CiRA more so in the translational research domain; Gladstone, the basic research domain. For instance, Yamanaka-sensei conducts his basic research through Gladstone.

Yamanaka-sensei:

> I used to focus on basic research, but during my tenure as the Director of CiRA, most of my time and energy were dedicated to translational research—specifically, how we could bring iPS cell technology to patients. After stepping down as Director, I felt a strong desire to return to basic science. Gladstone was the ideal place for this transition, as it is where I first delved into basic science using molecular biology. It was also there that I identified a novel gene, NAT1, which has remained a central theme of my research throughout my career. These factors are the primary reasons I chose to conduct my basic research at Gladstone.

Such research investigates post-transcriptional phenomena – especially pertaining to the eukaryotic translation initiation factors gamma (eIF4G) family – and its contribution to pluripotency and differentiation precision. Within respect to iPS cells, it is of an even more basic character than the disease-modeling research performed at Gladstone, the latter being more applied insofar as it utilizes iPS cells for studying diseases rather than examining the underlying mechanisms of reprogramming and pluripotency themselves. For instance, as Professor Steven FINKBEINER – Director of the Gladstone Center for Systems and Therapeutics; Director of the Taube/Koret Center for Neurodegenerative Disease Research; and Director of the Hellman Family Foundation Alzheimer's Disease Research Program – put it –

Professor Finkbeiner:

> [Yamanaka] is super interested in [reprogramming] [...] I use it as a tool.

In this respect, both iPSC-based therapy and disease modeling aim to utilize basic research findings from the study of reprogramming and pluripotency to deliver benefit to patients, tending towards pursuit of translational deliverables. On the other hand, disease-modeling is somewhat more basic in character insofar as it investigates the mechanics of diseases, seeking both explanations and eventual cures.

Since 2007, basic research on reprogramming and pluripotency has grown into a sizable field pursued by investigators worldwide. One of the first topics to arise within this category was that of reprogramming enhancement (e.g., with alternative or complementary transcription factors), which is still an impetus for a great many papers even at present. Indeed, this basic research category has by now grown to a scope that exceeds that of Yamanaka-sensei's own research. Moreover, it appears to exceed the research scopes of all of the cell therapy and disease-modeling researchers with whom I spoke at CiRA and Gladstone.

Of course, the remit of any given community or scientist will encompass only a subset of the total research field to which the community or scientist belongs. Nevertheless, what I observed when interviewing CiRA and Gladstone researchers was not an arbitrary parochialism shaped by individual idiosyncrasy or specialization. Rather, what was expressed to me consistently, across researchers from varying backgrounds and areas of specialization, was a common list of basic research topics that are viewed as progress-critical in cell therapy and disease-modeling (e.g., pluripotency for reproducible differentiation), and, likewise, a list of topics that are not (e.g., reprogramming enhancement).

We might refer to the decision whether or not to uptake basic research within translational settings as the "translational research filter" in the iPSC field. Thus, the motivating question for this SciFrontiers piece is as follows: which trends in basic research on re-

programming and pluripotency are passing the translational filter to disease-modeling and cell therapy research? That is to say – which developments in basic research are regarded as most useful to disease-modeling and cell therapy researchers as they pursue their patient-serving goals? The answer to the above question will involve a survey of the trends that indeed often clear the translational filter, those that typically do not, and those developments which disease-modeling and cell therapy researchers hope to see realized so that, once passed through the translational filter, they can be utilized. It is important to understand both what is and is not prioritized; after all, the ethos of scientific communities is often conveyed not only by the scientific investigations its scientists place within their research portfolios, but also those which they do not. Both, together, indicate scientists' estimation of the status of the field's research frontier.

2 Directions in Pluripotency Research

Here, I will attempt to synopsize the views expressed to me with (some) concision.

Biomanufacturing somatic cells using iPSC technology involves two segments: reprogramming to pluripotency and differentiation from pluripotency along the cell fate trajectories of interest. Translational researchers are largely satisfied with the first step, and focused on achieving consistency and reproducibility for the second. That is to say, they know how to reprogram cells (e.g., fibroblasts, peripheral mononuclear blood cells), but still grapple with attaining consistent differentiation control in order to produce the cells that they need (e.g., motor neurons, cardiomyocytes). The following next steps appear to be of some importance:

- Identifying pluripotent states from which differentiation can proceed reproducibly
- The epigenetics of reprogramming that underpin consistent differentiation
- Post-transcriptional influence on precise differentiation

More broadly, basic research findings on reprogramming efficiency improvements continue to be reported within the greater community, but such work infrequently finds application. Translational iPSC researchers face Herculean biomedical undertakings, such as establishing iPS cell therapy coverage across demographic HLA haplotype variations, and finding cures for prevalent neurodegenerative diseases such as Alzheimer's. In many cases, they are by no means disinterested in open questions in reprogramming, including the nature of pluripotency itself. However, because reprogramming indeed largely 'works in practice', translational researchers have shifted their focus further afield.

Here, I will try to give a summary of the strategic factors that have led translational researchers to their position on reprogramming efficiency research:

1. As a practical matter, given the capacity of iPSCs to self-renew, even low reprogramming yields are tolerable. For current clinical trials and disease-modeling consortia, bulk iPSC outputs are not a determinant of success, as clone availability is not the bottleneck.
2. Because translational researchers procure their reprogramming supplies from biotech vendors, they themselves cannot even implement new reprogramming innovations, such as new cocktails with reprogramming enhancers; they reprogram using the kits they buy.
3. On the vendor side, companies don't change their product line each time a new paper on reprogramming is published; only a small fraction precipitate industrial iteration.
4. As for new reprogramming publications: with a heavy flow of papers being published on reprogramming each year – covering a motley of mechanisms and regulators – translational researchers are simply not able to follow findings in fine detail.
5. Some of the most surprising developments in reprogramming have in fact been motivated by translational imperatives rather than basic research queries – one such example being transdifferentiation, which decouples reprogramming from pluripotency altogether and is valued among disease modelers.

Thus, at present, the portion of the basic research literature on reprogramming efficiency passing the "translation filter" is modest. Perhaps the greatest indicator of this is the maintained standard status of OSK(M/L), with OSKM being the transcription factor cocktail used by Yamanaka-sensei and collaborators in the original 2007 paper.

Such is not to suggest, however, that translational research has somehow transcended the inquiries of basic research and graduated to a strictly bioengineering field. The issue of reproducible differentiation does wade into certain theoretical depths. Thus, the era of asking basic questions has not drawn to a close.

Moreover, it is possible that reprogramming technology will continue to change. Beyond cocktail design, it has been subject to (largely translation-motivated) innovation, examples including integration- and transgene-free delivery methods (e.g., Sendai viruses, episomal plasmids), as well as feeder- and xeno-free media (e.g., StemFit, Essential 8). Thus, opportunities to influence technology have persisted, though less so on the grounds of improving reprogramming yields.

3 A Technical Refresher on iPSCs

Because reprogramming and pluripotency are centerpieces of this SciFrontiers piece, a cursory review of them is given here for those seeking a 'casual-technical' resource. (Of course, those seeking a comprehensive review are encouraged to read the academic literature.)

Induced pluripotent stem cells (iPSCs) are obtained by reprogramming already-specialized cells, such as fibroblasts or peripheral blood mononuclear cells (PBMCs), back to a pluripotent state. Pluripotency is the capacity of stem cells to differentiate (i.e., specialize) into the cell type of choice across all three germ layers (i.e., endoderm, mesoderm, ectoderm). *In vivo*, pluripotency is found, developmentally, in the inner cell mass (ICM) of the human blastocyst, which then yields the pluripotent epiblast. Embryonic cells, which eventually (alongside extraembryonic cells) comprise fetal organs and tissues, are differentiated from the pluripotent epiblast, beginning with gastrulation. In the iPSC literature, this epiblastic state is one of "naive" pluripotency.

Prior to the discovery of iPSCs, developmental pluripotency had been exploited via embryonic stem cells (ECSs). This practice has been mired in controversy due to the need to extract the ICM via immunosurgery, destroying the embryo via trophectodermal lysis. iPSC production is distinct in that it obviates the need for the embryo altogether; one obtains pluripotent cells by effectively reversing the differentiation process in somatic cells back to a pluripotent state. (This, coarsely speaking, is reprogramming.)

Another way to envision reprogramming is offered by the idealization of the Waddington landscape, originally proposed to describe differentiation: one reprograms from one cell type (a local minimum in the landscape) to a position along the global maximum (a pluripotent state), only to differentiate to another cell type, thereby proceeding downward again to another minimum. Thus, whereas in the ESC case, one exploits an embryo positioned at the top of the Waddington landscape in order to differentiate to cells of interest, in the iPSC case, one reprograms a specialized cell back to the top of the landscape before differentiating to another cell type, thereby rolling back down.

Reprogramming was first demonstrated, in the case of human skin cells, in 2007 by Yamanaka-sensei, TAKAHASHI Kazutoshi (高橋和利), TANABE Koji (田邊剛士), OHNUKI Mari (大貫茉里), NARITA Megumi (成田恵), ICHISAKA Tomoko (一阪朋子), and Tomoda-sensei. They found that ectopic expression of retrovirally transduced transcription factors (TFs) – Oct3/4, Sox2, Klf4, c-Myc (OSKM; eponymously dubbed "Yamanaka factors") – reprogrammed human dermal fibroblasts to pluripotent stem cells. The results, although showing iPSC yields with only modest efficiency, nonetheless heralded a new production prospect for the stem cell field.

The general prospect of iPSC technology is that of leveraging pluripotency to make cells. Transplantation of healthy cells can serve as a cell therapy. Difficult-to-sample cells (e.g., motor neurons) can be produced from iPSCs to study progressions in otherwise difficult-to-model diseases (e.g., neurodegenerative disorders). Translational iPSC research is being realized through initiatives such as clinical trials for treatments of diseases (e.g., macular degeneration) and disease-modeling research consortia (e.g., for Parkinson's).

One might say that the inception of the iPS cell field was particularly prosperous because it began with the demonstration of reprogramming. Indeed, the prospect of reprogramming doesn't appear to have ever been in doubt since; instead, open questions have included understanding how it works, and whether or not rarefied understanding can improve its efficiency. As a quantitative matter, the 2007 OSKM experiment returned only 10 iPSC colonies from 5000 hu-

man dermal fibroblasts, giving a yield efficiency of $\leq 1\%$. Thus, OSKM expression, although demonstrated as a feasible method for inducing pluripotency, only gave, from a basic science viewpoint, a partial glimpse into the implicitly broader architecture permitting reprogramming to occur.

Since 2007, the reprogramming basic research community has produced a rich corpus of literature on the mechanisms that bear on reprogramming and acquisition of pluripotency, such as other TFs (e.g., SALL4, Nr5a2, ERR-β); epigenetic processes (e.g., chromatin decompaction, demethylation, histone modification); signaling pathways (e.g., Wnt, Tgf-β, JAK-STAT); post-transcriptional phenomena (e.g., micro-RNAs, translation initiation factors); X chromosome inactivation genes (e.g., Xist, Tsix); metabolic pathways and conditions (e.g., OXPHOS, glycolysis, hypoxia); anti-oncogenic genes and loci involved in apoptosis and senescence (e.g., p53, INK4/ARF); and the mechanisms of the mesenchymal–epithelial transition (MET) (e.g., E-cadherin).

For a newcomer to the iPS cell field, this assortment of phenomena might instill some vertigo. Indeed, the community has implicated a plethora of up/downstream and co-expressive machinery in reprogramming and pluripotency; toggled a combinatorial abundance of up/downregulations; and surveyed several cell types. However, a key message from the translational researchers with whom I spoke is that disentangling this web of interdependencies is not on their agenda, given the satisfactory performance of reprogramming for their purposes.

Basic research findings, in certain cases, have been taken up by translational researchers and the biomedical industry. For instance, investigations into the influence of signaling pathways on pluripotency – revealing inhibition of glycogen synthase kinase-3 (GSK3) and MAP kinase/ERK kinase (MEK) to maintain naive pluripotency – led to the 2i medium, which is offered by companies such as Sigma-Aldrich, owned by Merck; and Cellartis, owned by Takara Bio (タカラバイオ). To take another example – the discovery of the excludability of c-Myc has influenced reprogramming; the development of episomal plasmid vectors at CiRA, now distributed by the Addgene nonprofit, used only OSK.

Since the inception of the iPSC field, OSK(M/L) – with L standing for l-Myc – has remained the standard reprogramming cocktail, with basic research findings only occasionally inspiring modification of reprogramming practices, which are not easily changed. Nevertheless, within the translational community, project-specific desiderata (e.g., blood cell reprogramming for iPSC banking) have dictated certain reprogramming innovations. These include the replacement of retro/lentiviral vector delivery methods with episomal plasmid and Sendai virus methods, as well as the development of feeder-free and xeno-free culture media. Because such innovations can only comfortably be discussed with a (casual-)technical tenor, they will be reviewed herein.

In the late aughts, a demand arose for reprogramming methods capable of inducing PSCs from somatic cells other than fibroblasts due to the lesions inflicted during skin biopsies. Peripheral blood was preferred, as liquid biopsy requires only venipuncture, which is less invasive. (Fibroblasts also pose risks from ultraviolet radiation exposure.) Moreover, it was foreseen that peripheral and cord blood collection could provide a donor basis for banking HLA-homozygous iPSC stocks, which is now indeed the case. Reprogramming of peripheral blood cells was originally performed via retroviral OSKM transduction. However, integration- and transgene-free methods were desired in order to prevent reprogramming artifacts from influencing disease modeling and cell therapy outcomes. (For instance, it was found in a 2007 study of murine germline chimeras produced from Nanog-iPS cells that retroviral transduction of c-Myc resulted in tumorigenic c-Myc reactivation with 20% frequency.) A plasmid vector approach for efficient PBMC repro-

gramming was reported by CiRA in 2012.

This series of delivery innovations has considerably defined the GMP practices of the present. CiRA's clinical-grade HLA homozygous iPS cells (HLAホモiPS細胞), such as QHJI 01s01 are developed using episomal plasmid reprogramming. The Addgene nonprofit distributes several plasmid reprogramming materials (e.g., pCXLE-hOct3/4-shp53-F for Oct3/4; pCE-hSK for Sox2 and Klf4) begifted by the Yamanaka lab at CiRA following the 2012 study.

FUJIFILM Cellular Dynamics – originally Cellular Dynamics, Inc., founded by Professor James Thomson; later acquired by Fujifilm (富士フイルム) – uses its own proprietary non-integrating episomal vector method for reprogramming. The iPSC collection of the California Institute of Regenerative Medicine (CIRM), under the management of FUJIFILM Cellular Dynamics, uses this method.

CiRA's iPS cell lines KTRH05 and KTRH26 are produced using the Sendai virus method. Improvements in Sendai rapid virus vector removal, post-reprogramming, have recently been reported via use of l-Myc in place of c-Myc (i.e., "OSKL"), alteration of temperature conditions, and modification of the SeV-KLF4 vector, whose backbone, SeV-TSΔF, was hypothesized to be implicated in vectorial retention due to temperature unresponsiveness. SeV-OSKL has allowed for feeder-free reprogramming of naive iPSCs, regarded as an auspicious development towards the prospect of personalized, autologous treatments.

On the media front, feeder cell media, such as fetal bovine serum (FBS), being animal-derived, was unsuitable for GMP-grade reprogramming, prompting the push for feeder-free products. Professor Thomson's lab introduced the essential 8 (E8) medium, which is serum-free, in 2011. Gibco (a subsidiary of Thermo Fisher) and Cellular Dynamics (now FUJIFILM Cellular Dynamics) partner in the commercialization of E8. In 2014, the Yamanaka lab and Ajinomoto (味の素) co-developed StemFit as a feeder-free and xeno-free medium.

The above expositions are intended to convey the amenability of reprogramming research to adaptation and iteration, underscoring, in the cases discussed, both the translational impetus for industrial innovation and the basic-translational alignments synchronized for clearing the translational filter.

4 Reprogramming Research: Current Views

4.1 Satisfaction with Reprogramming Among Translational Researchers

Translational iPSC researchers with whom I've spoken regard contemporary reprogramming performance as adequate for practical purposes, and view prevailing methods in reprogramming as having settled on a satisfactory standard. As Professor Benoît BRUNEAU, Director of the Gladstone Institute of Cardiovascular Disease, summarized –

Professor Bruneau:

> People just want to use what works. I think we've come to a *status quo* [...] about what it takes to make an iPS cell.

Professor Finkbeiner made a similar assessment.

Professor Finkbeiner:

> At the end of the day, [reprogramming] works pretty well at this point. I can't remember the last time, actually, that we had a line we couldn't reprogram. I'm not especially interested in that part of the process; it was a means to an end. Just getting it to work was the key thing for us.

Although reprogramming efficiency remains low, iPS cell yields from reprogramming are satisfactory. In fact, even with low yields, inefficient methods still provide investigators with clones in excess of their needs.

Tomoda-sensei:

> Reprogramming efficiency is still very low, but in most cases, it may be enough. After reprogramming, you document 100 colonies after starting with maybe 1 million cells. I think that's actually enough, or even too much. You cannot analyze all clones, so, maybe [alternative] reprogramming cocktail methods don't matter right now.

4.2 Industrial Constraints on Cocktail Choice

Reprogramming cocktail choices are determined not by working researchers, but by the product standards set by industry. As Yamanaka-sensei explained, the barrier to industrial adoption of new reprogramming research results is quite steep, given the costs and risks of GMP-grade standardization.

Yamanaka-sensei:

> From a translation point of view, once you start working on GMP production, it's very difficult to change factors, because they have to start everything from the beginning, from scratch. So, unless we find something completely different or completely transformative, we wouldn't change factors.

Tomoda-sensei offered a similar assessment.

Tomoda-sensei:

> If you choose one [method] – for instance, Sendai viruses – there is already a kit sold by Invitrogen. There is no choice in the reprogramming cocktail. So, we actually don't care about [new] reprogramming cocktails.

4.3 Bandwidth Constraints on Following the Literature

The volume of literature on reprogramming is prodigious in scope. For instance, to

use a crude metric, if one enters the keywords "pluripotent" and "reprogramming" into Google Scholar, one finds over 18,000 results published since 2020. As Yamanaka-sensei stated, given the continual stream of publications on reprogramming enhancement results, for working translational researchers, maintaining an up-to-date account is "next to impossible".

Nevertheless, there exists detectable sentiment within the translational community that the basic research literature is valuable, even if one does not have the time to follow it in depth. Localizing value in particular publications is less important than observing the general benefits that have sprung from basic research.

Professor Bruneau:

> We didn't understand how reprogramming worked, and we wanted to understand how we could use it in a meaningful way. I don't think it's worth looking back to see if all those efforts were worthwhile, because in general they were. The first 10 years of iPS research papers made contributions that have been worthwhile to science. The efficiency is much higher, and you have GMP production, which is really important.

However, the lack of industrial uptake of much of the basic research findings on reprogramming efficiency does reflect a certain assessment that such work has yet to exhibit revolutionary results. According to Yamanaka-sensei's evaluation, setting aside the question of translational applications, even on basic science grounds alone –

Yamanaka-sensei:

> We did not see such a huge improvement, even for scientific purposes.

4.4 Translation-Motivated Cocktail Choice

Nevertheless, certain reprogramming enhancement findings are adopted on occasion, especially when they serve translation-driven innovation pathways in reprogramming technology. For instance, it was found in 2009 that inactivation of the regulatory protein p53 (cellular tumor antigen p53) – which otherwise constrains reprogramming yields by inducing apoptosis in cells with signals of DNA damage (e.g., short telomeres) – increases reprogramming efficiency. This result demonstrated a drastic improvement in episomal vector reprogramming performance, as Tomoda-sensei recalled –

Tomoda-sensei:

> Knockdown of p53 really helped. After that finding, we incorporated shRNA against p53 in a cocktail with episomal reprogramming. I think that's really helpful for episomal reprogramming since originally, it had really low efficiency.

The context of the relevance of p53 knockdown – namely the pursuit of integration- and transgene-free delivery methods for blood cell reprogramming and iPS banking – is discussed in the Technical Refresher.

4.5 Reprogramming Without Pluripotency

Thus far, throughout our discussion of reprogramming and pluripotency, the two have been inextricably interlaced; after all, in the case of iPSCs, one cannot have one without the other. Nevertheless, as reprogramming has found utilization within disease-modeling, research-specific desiderata, such as the study of non-genetic risk factors, have motivated methodological adaptations that decouple it from pluripotent conditions. In particular, reprogramming techniques have been developed to achieve

circumvention, in certain cases, of pluripotency altogether, with researchers instead opting to directly reprogram one cell type to another via transdifferentiation. Thus, one is able to somehow curl around the Waddington landscape, eking out mobility without returning to the pluripotent summit. As Professor Bruneau recalled to me, developments in transdifferentiation, such as direct reprogramming from fibroblasts to cardiomyocytes or neurons, imparting the "the idea that you can reprogram any cell into another cell", have "exploded the field" of reprogramming-based disease modeling.

Such an approach has been viewed as especially advantageous in certain disease-modeling settings. To appreciate this advantage, it is well to recall that iPSC-based differentiation necessarily yields cells from an embryo-like naive state, whereas the cells in many disease-afflicted subjects have been subject to nontrivial aging. A record of aging is maintained in the subject's epigenetic marks, but erased when new cells are cultured from a pluripotent state *de novo*.

Professor Finkbeiner:

> While iPSCs preserve the genetics of the donor precisely, the epigenetic marks that presumably encode environmental factors, including aging, are lost; so, iPSC-derived neurons are quite young and immature. The most promising approach for overcoming this limitation is to transdifferentiate one type of primary cell (e.g., fibroblast) to another cell type (e.g., neuron), without reprogramming to pluripotency. Such an approach produces cells with an epigenetic signature of aging similar to the donor, and has been associated with much more dramatic disease-associated phenotypes when the starting cell is derived from a patient.

Epigenetic mark retention is potentially advantageous for explaining non-genetic risk factors associated with neurodegeneration.

Professor Finkbeiner:

> For diseases that we study, where aging is the single largest risk factor, [epigenetic mark erasure] is potentially a big loss. I always think of epigenetics as the way-station for all environmental influences on our genome, whether it's aging or toxins. If you took monozygotic twins and asked: if one got a disease, how often would the other – in Alzheimer's, it's 70-80%; in ALS; it's 40-60%; in Parkinson's, probably 30%. So clearly, non-genetic factors have an influence. I doubt, in most cases, that environmental factors, by themselves, cause these diseases, but if you overlay a factor on a certain susceptibility that the genome confers, it's the interaction [that matters]. We have some evidence for this from studying laborers in the Central Valley and their risk for Parkinson's from pesticide exposure. Being able to retain epigenetic marks is really valuable.

Nevertheless, transdifferentiation does not enjoy the same prospective scalability as pluripotency-based reprogramming, as specialized cells do not share the proliferative capability of iPSCs.

Professor Finkbeiner:

> Transdifferentiated cells are going to be really helpful, [but] they won't be able to scale, ever. The problem is: they start with a primary cell, like a fibroblast, and fibroblasts senesce after a certain number of divisions. You have to start with a fresh batch every single time. Because of that, you also can't CRISPR engineer them.

Tomoda-sensei offered a similar assessment –

Tomoda-sensei:

> Research groups focusing on disease modeling may not need to reprogram iPS cells; [they can] just directly reprogram the fibroblast to the target cell. The disadvantage is that you can't expand them. [Also,] currently, there are no high-efficiency direct reprogramming methods. So, I think we still need iPS cells.

Thus, the case of transdifferentiation illustrates both the manner in which translational desiderata can guide methodological directions in reprogramming research, as well as the tradeoffs such researchers must navigate between epigenetic information forfeiture and biomanufacturing scalability. Moreover, it illustrates that translational imperatives have allowed the community to discover new reprogramming capabilities.

5 Researcher Views: The Future of Pluripotency Research

5.1 Epigenetic Control for Reproducible Differentiation

In the case of both cell therapy and disease modeling, a complete production process involves two segments: reprogramming to pluripotency and differentiation from a pluripotent state along the desired cell fate trajectory. The degree of scientist satisfaction with the former is not matched with respect to the latter; impediments to reproducible and consistent differentiation remain. Tomoda-sensei recalled many instances in which "our cells couldn't differentiate; in many cases, they failed." Differentiation is important both for stem cell therapy and disease-modeling.

Tomoda-sensei:

> For disease modeling, you want to have your target cell: maybe cardiomyocytes, maybe neurons. So then, the question is: after reprogramming, can your iPS cells easily differentiate into those cell types? That we still don't know; we kind of have an idea. During reprogramming, some epigenetics don't change, or abnormally change. Then, your cells cannot respond to differentiation cues. If we can control the epigenetics surrounding differentiation master regulators, maybe we can more easily do disease modeling.

5.2 Pluripotency and Reproducible Differentiation

Differentiation reproducibility, although necessarily requiring control over pluripotency, begs rather specific research questions on pluripotency that are more pragmatic and functional than many of the questions featured in the more basic (e.g., embryogenetic) discourses on the topic.

For instance, as Professor Bruce CONKLIN – Senior Investigator at the Gladstone Institutes and Deputy Director of the Innovative Genomics Institute (IGI) – argued, understanding the manner in which cells are reprogrammed along the Waddington landscape, or even the relationship between pluripotent states in the landscape and the ICM, is less urgent than understanding the points of departure on the Waddington landscape from which differentiation into cell types can be made most reproducible.

Professor Conklin:

> In Waddington's landscape, at the very top, it's kind of a big area. When we take iPS cells, they act differently [from each other], even from their clones: they're in different parts of the Waddington landscape. From a practical point of view, I'm less concerned with defining every inch of that landscape and trying to figure out which states are closest to the inner cell mass. Instead, I want to use a drug to force a cell into a particular pluripotent state so that it's there every time, so that every time, we can turn the iPS cell into a cardiomyocyte [or another cell]. That's actually what companies – STEMCELL Technologies, all the big media companies – are trying to do: get reproducibility. It's all in the pluripotent state.

Thus, there is interest in pluripotency states (i.e., Waddington landscape positions), inasmuch as differentiation reproducibility is sensitive to precise induction of pluripotency.

Professor Conklin:

> What I really want to do is have some state from which iPS cells can make all the tissues that I want. I want to make neurons: I want to make sensory neurons; I want to make motor neurons. What I really want to do is see cells that are the same very time – so if I make them from your iPS cells, or my iPS cells, or the guy-down-the-road's iPS cells, they should be starting at the same pluripotency state and moving towards the same cell state. I care about the cardiomyocytes; I care about the motor neurons; I don't actually care about the starting point, as long as it produces the [intended] result.

Moreover, there is some concern that perfecting naive pluripotent stem cells is inimical to reproducibility due to the expectation that the closer the cell state is to the dynamic equilibrium of naive pluripotency, the greater its sensitivity, perhaps, to cell fate trajectories that differ from the desired differentiated cell.

Professor Conklin:

> I am less concerned about the naive state as long as I can differentiate to the cell type I want. If a pluripotent state allows the production of cardiomyocytes. I care more about making iPS cells better, not necessarily close to the naive state.

5.3 Post-Transcriptional Influence

As discussed, OSK(M/L) has remained the standard transcription factor cocktail. Post-transcriptional mechanisms, on the other hand, are now finding industrial application. For instance –

Tomoda-sensei:

> OSK with p53 shRNA is a really good combination, for now. Some companies have added some micro-RNAs.

The Yamanaka lab at Gladstone is investigating the role of post-transcriptional regulation on pluripotency and differentiation precision, with particular focus on the eukaryotic translation initiation factors gamma (elf4G) family and the prospect of new (e.g., cap-independent) translation mechanisms.

Yamanaka-sensei:

> It's more like basic research once again.

Of the members of the elf4G family, NAT1 (elf4G2), the protein with which Yamanaka-sensei began his career, is a particular focus.

Yamanaka-sensei:

> I did my postdoc training at Gladstone almost 30 years ago. There, I identified NAT1. Officially, NAT1 is called elf4G2, because it has significant homology to eukaryotic translation initiation factor 4G. Since then, I have been trying to understand what NAT1 is doing. After finishing my postdoc training, I went back to Japan in 1996 with this gene, NAT1, and I continued working on NAT1. In Japan, I found that NAT1 is essential for ES cells: embryonic stem cells. That's how I started working on ES cells. Eventually, this led to the iPS cell discovery. So, I owe NAT1 a lot.

This return to basic research has been facilitated, in part, by the establishment of the iPS Cell Research Center at Gladstone Institutes, an on-site lab in Kyoto.

Yamanaka-sensei:

> In Kyoto, I'm now directing the CiRA Foundation, where we're working very hard to bring iPS cells to patients. My lab at Gladstone is now considered part of Kyoto University; we have a contract between Gladstone and Kyoto University. Some of my activity at Gladstone is part of an on-site lab of Kyoto University. We have a very strong connection between San Francisco and Kyoto.

I asked Yamanaka-sensei if he suspected that translation-initiation factors could play as great a role in iPSC research as translation factors, to which he replied –

Yamanaka-sensei:

> It's still possible, but it was a surprise when we discovered that combination of 4 factors [OSKM]. Initially, we had 24 candidates, including RNA binding proteins and proteins with functions other than transcription, but we ended up having just those 4 transcription factors. I don't think it was just a coincidence. To change cell fates, transcription factors are probably the most powerful tool. But still, with that being said, other proteins can modify reprogramming. It's still possible.

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