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MEETING REVIEW

Ten years of induced pluripotency: from basic mechanisms to therapeutic applications

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ABSTRACT

Ten years ago, the discovery that mature somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs) redefined the stem cell field and brought about a wealth of opportunities for both basic research and clinical applications. To celebrate the tenth anniversary of the discovery, the International Society for Stem Cell Research (ISSCR) and Center for iPS Cell Research and Application (CiRA), Kyoto University, together held the symposium 'Pluripotency: From Basic Science to Therapeutic Applications' in Kyoto, Japan. The three days of lectures examined both the mechanisms and therapeutic applications of iPSC reprogramming. Here we summarize the main findings reported, which are testament to how far the field has come in only a decade, as well as the enormous potential that iPSCs hold for the future.

KEY WORDS: Cell reprogramming, iPS cells, Pluripotency

Introduction

The creation of induced pluripotent stem cells (iPSCs) represents a landmark development in the stem cell field, as it provided a straightforward, technically simple approach to creating PSCs from virtually any starting cell type (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This opened up countless possibilities for human disease modeling and personalized cell therapies, as well as for studying the fundamentals of pluripotency and the basis of cell fate specification (Fig. 1). Ten years later, it is difficult to exaggerate the impact that iPSCs have had on the developmental biology and stem cell fields. To recognize the significance of iPSCs, the International Society for Stem Cell Research (ISSCR) together with the Center for iPS Cell Research and Application (CiRA) held the symposium 'Pluripotency: From Basic Science to Therapeutic Applications' on the campus of Kyoto University, Japan, where iPSCs were discovered. The program was divided into four themes: (1) the therapeutic applications of iPSCs; (2) mechanisms of pluripotency; (3) cell differentiation and disease modeling; and (4) emerging technologies. Accordingly, talks varied from the very basics of pluripotency to the use of PSCs, especially iPSCs, for the study and treatment of disease. In this Meeting Review we summarize the key findings of the presentations and briefly consider their significance to both basic and applied research.

Towards the clinic: PSCs for cell therapy

Shinya Yamanaka (CiRA, Japan) began the scientific talks with the Special Lecture, in which he spoke in detail about CiRA's effort to prepare an iPSC stock for regenerative medicine. The stock will provide clinical-grade iPSCs to relevant organizations, such as hospitals and research centers, which can then differentiate the cells into the desired cell type. Yamanaka highlighted the clinical research of Masayo Takahashi (Riken, Japan) to demonstrate the importance of the iPSC stock. In a later talk, Takahashi elaborated on this theme as she discussed her group's efforts in using patient-specific iPSCs in a pilot study to treat age-related macular degeneration (AMD). The iPSCs were differentiated into retinal pigment epithelial (RPE) cells and transplanted back into the patients' eyes. Takahashi stressed that the cell therapy was not intended to recover lost photoreceptor cells, but to prevent the progression of loss. A year after the operation, observations indicate that this is indeed the case. As noted by Yamanaka, however, the transplantation of autologous iPSCs requires a lengthy period of preparation, as the patient's cells are first reprogrammed to iPSCs, whereupon multiple lines are screened for quality control and the chosen line is then redifferentiated. By contrast, allogeneic transplants generated via the iPSC stock would significantly shorten the waiting period for treatment. With this in mind, Takahashi concluded her lecture by stating her next goal, which is to conduct allogeneic transplants in cooperation with the iPSC stock.

An important matter regarding the iPSC stock is the preparation of safe cells. In his talk, Yamanaka posed an interesting question; one that he himself faced in deciding whether to proceed with the clinical trial of a second AMD patient. Yamanaka explained how the iPSC line chosen for the trial was found to harbor deletions in two genes. Although he did not find any evidence in the literature that these genes were associated with tumorigenicity, he advised not to proceed with the cells in question, and the clinical trials were suspended. Yamanaka then asked the audience whether they would support using these cells in the trial, and the response was mixed. Some researchers felt that Yamanaka's decision was appropriate, whereas others were more tolerant, arguing that the decision was too strict. Based on the opinions of the audience, much discussion is still needed for standardized criteria when deciding on the safety of a cell line by genomic analysis.

The talks by Yamanaka and Takahashi were an appropriate introduction to the sessions on the therapeutic applications of iPSCs. This topic had twice the number of invited speakers as any other session and covered a spectrum of organs, including the heart and brain – two organs that are especially in need of cell therapies owing to their poor regenerative ability. Charles Murry (University of Washington, USA) and Jun Takahashi (CiRA, Japan) discussed their progress with iPSC-based therapies for these organs, with clinical trials from both groups expected by 2020. Murry discussed his group's efforts in using human PSC-derived cardiomyocytes to remuscularize the heart following an infarction (Chong et al., 2014). Although the protocol to differentiate PSCs to cardiomyocytes is well established, the resulting cardiomyocytes are generally of the fetal type. At the symposium, Murry described a new differentiation protocol that produced more mature cardiomyocytes from human

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Development (2016) 143, 2039-2043 doi:10.1242/dev.138172

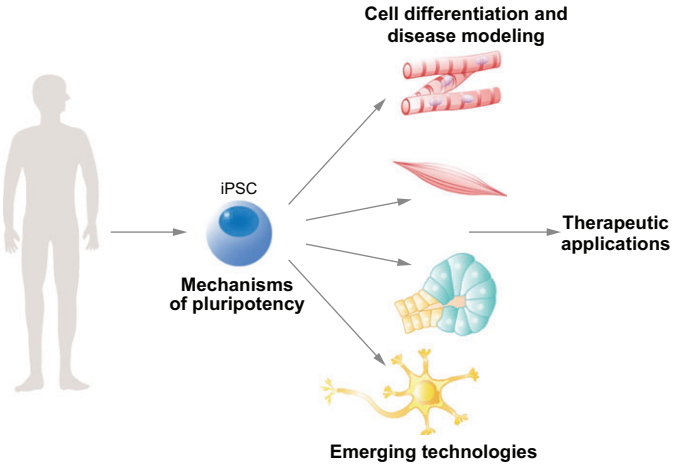

Fig. 1. The four key themes of the CiRA/ISSCR symposium. The meeting addressed fundamental questions in developmental biology through to the clinical application of stem cells.

PSCs. He showed that human PSC-derived cardiomyocytes coupled well when transplanted into primates (macaques). More importantly, he shared unpublished data demonstrating that these cell grafts significantly improved systolic function in the primate heart, providing evidence that they are contributing to bona fide cardiac regeneration. Regarding the brain, Jun Takahashi discussed his interest in Parkinson's disease, and explained the need for candidate compounds that promote the survival of dopaminergic cells and their synaptic formation, as a complement to cell therapy (Nishimura et al., 2015, 2016). He also stressed that this research has convinced him that regenerative medicine is not only science but art as well.

Stem cell therapies for diabetes are also showing great promise, as described by Allan Robins (ViaCyte, USA). He showed that the transplantation of human islet cells derived from embryonic stem cells (ESCs) into a murine model of diabetes led to blood glucose levels comparable to those found in healthy humans. Progenitor cells were encapsulated in a device that was then implanted into the body, which allowed differentiation into functional beta cells as well as good vascularization. The device has already been implanted in patients but the current goal is to confirm safety and tolerability, and the optimal dose and location of the implantation are yet to be confirmed.

Another setting in which iPSCs are showing promise is spinal cord injury. This type of injury can be divided into three phases, namely acute, subacute and chronic, with the greatest therapeutic potential for cell-based regeneration occurring in the subacute phase. Hideyuki Okano (Keio University, Japan) spoke about his work in using allogeneic iPSCs to repair spinal cord injury, and argued that an allogeneic approach is the only feasible way forward, since the subacute phase is short and occurs relatively soon after the injury (Okano and Yamanaka, 2014). While there has been much success in deriving neurons from iPSCs, the derivation of oligodendrocytes, which are necessary for myelination and therefore good recovery, has remained challenging (Kawabata et al., 2016). Okano showed that a new differentiation protocol for oligodendrocytes when paired with that for neurons leads to good functional recovery in murine and non-human primate spinal cord injury models. This approach also holds promise for spinal cord injury patients in the chronic phase, he added, if combined with drugs that promote axonal regeneration and rehabilitation.

Blood poses a unique 'industrial-scale' problem because the number of cells required – several hundred billion – is magnitudes greater than for other stem cell therapies. Luc Douay (Pierre and Marie Curie University, France) and Koji Eto (CiRA, Japan) each described their strategies for the large-scale production of erythrocytes and platelets, respectively, at clinical grade. Douay highlighted the difficulties in producing fully mature blood cells, as he showed how most PSC-derived erythrocytes have embryonic hemoglobin (Hb), with small traces of fetal Hb and no adult Hb. However, he showed that miR-30a can improve the nucleus expulsion capacity of mature erythroblasts, thus improving the final maturation step (Rouzbeh et al., 2015). He also reported the generation of engraftable, multilineage hematopoietic stem cells from human iPSCs using a dedicated, one-step, good manufacturing practice (GMP)-grade procedure. In contrast to making erythrocytes, the approach to induce platelet formation differs in that one can take advantage of the nucleated unipotent platelet progenitor, the megakaryocyte. Accordingly, Eto described an iPSC-derived immortalized megakaryocyte cell line from which platelets can be produced (Nakamura et al., 2014). Building on this, his team has been designing a bioreactor that creates the ideal physical and chemical microenvironment to maximize platelet release from the immortalized megakaryocytes. The use of PSCs provides a distinct advantage over other stem cell sources, such as cord blood, in that it is possible to choose a donor who possesses a desired phenotype. In the case of red blood cells, Douay noted that this privilege can produce a homogeneous cell population, which can extend the half-life of the transfused material by almost double, thus dramatically improving the transfusion capacity.

Back to basics: understanding pluripotency

The original experimental rationale that led to the discovery of iPSCs was based on fundamental developmental biology – specifically, on understanding which genes are important for specifying and maintaining the pluripotent state. Understanding what regulates pluripotency remains a key part of iPSC research and, accordingly, talks around this subject were an important component of the program. Austin Smith (University of Cambridge, UK) gave the first Keynote Lecture in which he discussed the difference between ESCs and epiblast stem cells (EpiSCs). He argued that the current model of a PSC transitioning from naïve pluripotency to primed pluripotency before segregating into the three germ layers is not reflective of actual development. He showed that changes in Rex1 (Zfp42) expression and DNA methylation levels are markers of a transitional state called 'formative pluripotency', which in mouse emerges at E5.5 and is the branchpoint from which the three germ layers and germ cells can be derived (Kalkan and Smith, 2014). Smith's talk highlighted the complex nature of pluripotency and the existence of sequential pluripotent states, each characterized by different molecular and functional attributes.

Kathrin Plath (UCLA, USA) continued with the theme of pluripotent states in her talk on X-chromosome inactivation in naïve versus primed human ESCs. The induction of differentiation in female ESCs is marked by X-chromosome inactivation, which is regulated by the long noncoding RNA Xist. By contrast, X-chromosome reactivation is a hallmark of reprogramming, as cells dedifferentiate and reacquire pluripotency. But what about the transitions between different states of pluripotency, namely between primed and naïve? Plath presented new, unpublished data on the expression of Xist and the erosion of X-chromosome inactivation in the primed state and then discussed how the

Development (2016) 143, 2039-2043 doi:10.1242/dev.138172

transition to naïve pluripotency affects the epigenetic status of the X chromosome.

Jose Polo (Monash University, Australia) discussed the series of events that occurs during reprogramming of somatic cells back to the pluripotent state. These events can be allocated into two waves that seem to have common and different transcriptional events. However, because different cell types start from different initial states, the reprogramming process must eventually converge to a common pathway. Polo also discussed his recent efforts in somatic lineage reprogramming, namely the use of Mogrify, a computational tool that combines human gene expression data with regulatory network information to predict the reprogramming factors capable of converting one somatic cell into another (Rackham et al., 2016).

Cell differentiation and disease modeling

One of the main applications of PSCs is directed differentiation into mature cell types, whether for eventual cell therapies or disease modeling. George Daley (Harvard Stem Cell Institute, USA) spoke about how differentiating PSCs to the hematopoietic lineage normally results in primitive, not definitive, hematopoiesis, which complicates disease modeling. However, he has found a transcription factor mixture that expands human PSC-derived myelo-erythroid cells that undergo globin switching and terminal red blood cell differentiation in engrafted mice. Using iPSCs derived from patients with Diamond-Blackfan anemia (DBA), he showed that gene editing as well as a drug discovered through high-throughput screening could rescue the DBA defects and enhance erythropoiesis. The study indicated that autophagy may be disrupted in this disease, suggesting that targeting autophagy-related mechanisms could be a strategy to recover erythropoiesis in anemia patients. Continuing on this theme, Andrew Elefanty (Murdoch Childrens Research Institute, Australia) elaborated on the challenges of controlling primitive and definitive hematopoiesis by providing a roadmap of the major transcription factors involved. A crucial component is the precise control of HOXA gene expression, which can be regulated by CHIR99021, a GSK3β inhibitor, and SB431542, a TGFβ inhibitor. Elefanty described how adding these compounds to the culture enhances HOXA gene expression and promotes aortic fate. He went on to show how these compounds also changed the dynamics of RUNX1 and SOX17 expression, which could be used as markers for hematopoietic precursors. The basis of this strategy comes from the lab of Gordon Keller (McEwen Centre for Regenerative Medicine, Canada), who spoke about his aim to produce all the different cell types present in the adult heart via directed differentiation. Keller stressed the importance of replicating *in vivo* developmental processes to achieve this aim, and showed how the derivation of atrial and ventricle cardiomyocytes, pacemaker cells and epicardial cells can be simplified to controlling the expression of BMP and Wnt at specific time points from the mesoderm stage (Witty et al., 2014).

Additional talks described important advances in the directed differentiation of other somatic cell types for therapeutic purposes, particularly the exciting new technique of 3D *in vitro* organogenesis to generate tissue-specific 'organoids'. Hans-Willem Snoeck (Columbia University Medical Center, USA) discussed a new protocol for producing lung bud organoids from human PSCs. Remarkably, the time-scale for the *in vitro* formation of lung organoids is not dissimilar to that of human lung development *in utero*. Snoeck also cautioned that the roles of important factors in lung branching, particularly those of the fibroblast growth factor (FGF) family, are not necessarily conserved between mouse and human. Takanori Takebe (Yokohama City University, Japan) also spoke about *in vitro* organogenesis – this time of liver – and discussed the importance of environmental cues for proper organoid formation. Specifically, he highlighted that it is crucial to control the mechanical properties of the culture system, such as matrix stiffness, and also that it is important to include multiple supporting cell types in the culture, similar to what is observed *in vivo*. He added that a similar strategy showed promise for producing islet buds that had good vascularization. Moving to the kidney, Ryuichi Nishinakamura (Kumamoto University, Japan) discussed his latest efforts in generating 3D kidney organoids, which he showed could form nephron-like structures complete with glomerular podocytes and proper vasculature when transplanted. He also showed how to expand nephron progenitors from mouse embryos and human iPSCs *in vitro*, generating a number that should be sufficient for future regenerative medicine (Tanigawa et al., 2016). Finally, Nori Tsumaki (CiRA, Japan) explained how iPSCs could be used to produce chondrocytes that generate hyaline cartilage rather than fibrous cartilage, which is unlike current cell therapies for cartilage (Yamashita et al., 2015). This accomplishment is due, in part, to the expansion of iPSCs and not of chondrocytes in the protocol.

In the last Keynote Lecture and also the last talk of the symposium, Richard Young (Whitehead Institute for Biomedical Research and MIT, USA) explained how the study of topologically associated domains (TADs), which create insulated neighborhoods in the chromatin structure, can be applied to disease modeling (Dixon et al., 2015). Super-enhancers can function bidirectionally along the DNA, but insulated neighborhoods constrain the enhancer effects via loops formed by CTCF and cohesin binding. Furthermore, the super-enhancer activity within insulated neighborhoods varies as the cell transitions between naïve and primed pluripotency, suggesting that TADs might affect cell reprogramming. From a disease perspective, the loss of insulated neighborhoods has been associated with the activation of oncogenes (Hnisz et al., 2016), which themselves have extraordinarily large super-enhancers. These findings could provide an important clue as to why cancer cells have proven especially resistant to reprogramming and might also be useful in drug discovery.

Emerging technologies

As significant as iPSCs have been for the development of new cell therapies, the pace of this progress can be accelerated by complementing them with other biotechnologies. One of the most exciting new technologies to emerge recently has been CRISPR-Cas9 gene editing. Sangsu Bae (Hanyang University, South Korea) described Cas-OFFinder, which searches for possible off-target sites, and Digenome-seq, which is an *in vitro* tool to sequence a Cas9-digested genome (Bae et al., 2014; Kim et al., 2015). He then proceeded to show how these technologies can be used to modify the DNA of *Chlamydomonas*, an alga that shows promise as a producer of biofuel, for better translation efficiency. Hirohide Saito (CiRA, Japan) spoke about a synthetic biology tool that his group is working on called miRNA switch, which can be used to selectively isolate and purify distinct cell populations. Although surface receptors are commonly used to purify cells, several cell types do not have unique surface receptors, whereas, as Saito showed, they do harbor unique signature miRNA sequences in some cases. Taking advantage of this, miRNA switches can target specific cells that would otherwise appear as a heterogeneous population. A single miRNA switch consists of synthetic RNA that includes two types of mRNA modules: one complementary to the miRNA sequence located in the 5′UTR and one that codes for a protein of

Development (2016) 143, 2039-2043 doi:10.1242/dev.138172

interest. As an example, Saito showed how an miRNA switch can be designed to express apoptotic genes in undesired cell types, thus producing a relatively homogeneous cell population (Endo et al., 2016; Miki et al., 2015).

Cynthia Dunbar (National Heart, Lung, and Blood Institute, USA) described her efforts in producing a non-human primate model for PSC-based cell therapies. As a proof-of-principle for tissue regeneration, she presented data for bone formation (Hong et al., 2014). The autologous transplant of iPSCs led to teratomas and an inflammatory reaction, whereas the transplant of mesodermal stromal-like cells derived from the iPSCs did not. Furthermore, she showed how using CRISPR-Cas9 technology to insert marker genes into the *AAVS1* locus enabled stable expression in both undifferentiated and differentiated cells, and provides the opportunity for non-invasive tracing of iPSC-derived tissues post-delivery.

Mouse PSCs can be used to produce chimeras, whereas human PSCs cannot. This is attributed to differences in pluripotency: mouse PSCs are considered to have naïve pluripotency, whereas human PSCs are said to have primed pluripotency, which is also the pluripotent state of mouse EpiSCs. Hiromitsu Nakauchi (Stanford University, USA) provided new insight into why this is the case. In an outcome he attributes to raw luck, Nakauchi described a mouse EpiSC line that can form chimeras, which he postulated was the result of inhibited apoptosis. Accordingly, he showed how preventing apoptosis in other mouse EpiSC lines allowed them to contribute to chimera formation. Because mouse EpiSCs and human PSCs are both thought to exist in the primed pluripotent state, there is hope that this EpiSC line will provide clues on how to acquire chimeras using human cells. Nakauchi also reported on his group's work using xenochimeras, in which a mouse pancreas was made in rat, and a rat pancreas in mouse. Interestingly, the size of the organ depended on the species of the transplanted cells, not on the host. In another example of xenochimeras made from cells other than PSCs, Rudolf Jaenisch (Whitehead Institute for Biomedical Research, USA) described work in his lab on neural crest cells (NCCs) (Cohen et al., 2016). Human NCCs, being highly migratory, were introduced into mouse embryos. These committed stem cells, in contrast to PSCs, may be better tolerated by the host embryo as they contribute only to a subset of mouse tissues. He showed that injecting human NCCs into postimplantation mouse embryos could indeed generate chimeras with contributions to several neural crest-derived lineages, but that the proportion of human NCCs that contributed to the chimera was less than that of NCCs from mouse or rat.

Concluding remarks

The CiRA/ISSCR symposium 'Pluripotency: From Basic Science to Therapeutic Applications' brought together an inspiring mix of speakers (Fig. 2) and covered a broad range of topics. Despite the diversity, the underlying theme of understanding and controlling pluripotency and the application of this knowledge to study and treat disease was consistently present throughout the meeting. To this end, it is clear that there is still much work to be done in elucidating the developmental mechanisms that regulate different pluripotent states, as well as how to better control directed differentiation in order to produce the correct mature cell type. Although there has been great progress in this area, particularly in the last decade, it is nonetheless clear that unresolved issues, such as culture-to-culture variation, low efficiency and the formation of immature cell types, still limit the application of PSC-derived therapies. Emerging technologies in gene editing, xenochimerism and *in vitro* organogenesis will no doubt help to overcome some of these hurdles and accelerate clinically available therapies. Indeed, there are already several promising lines of clinical or near-clinical research ongoing. It is extremely encouraging to see the research that is taking place on a global scale in the field of PSC- and iPSC-derived technologies and, as evidenced by the enthusiasm of the participants at the meeting, there is great anticipation with regard to what the future holds for this fast-moving field.

Fig. 2. Invited speakers.

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