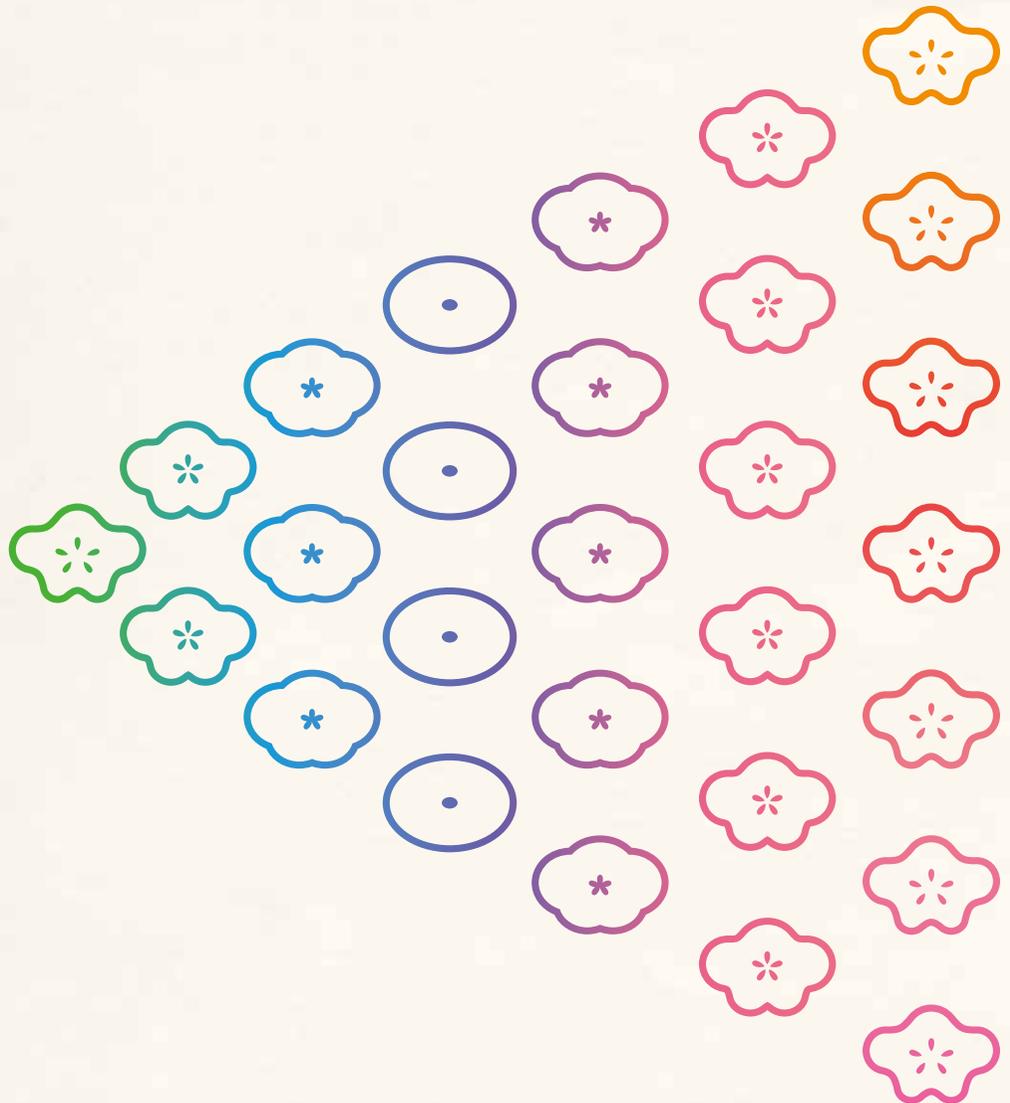


# CiRA

# Reporter

Center for iPS Cell Research and Application,  
Kyoto University



- Gene Therapy for Muscular Dystrophy
- New Cell Sheets for Cardiac Treatment
- CiRA goes to the Vatican

**Vol. 1**  
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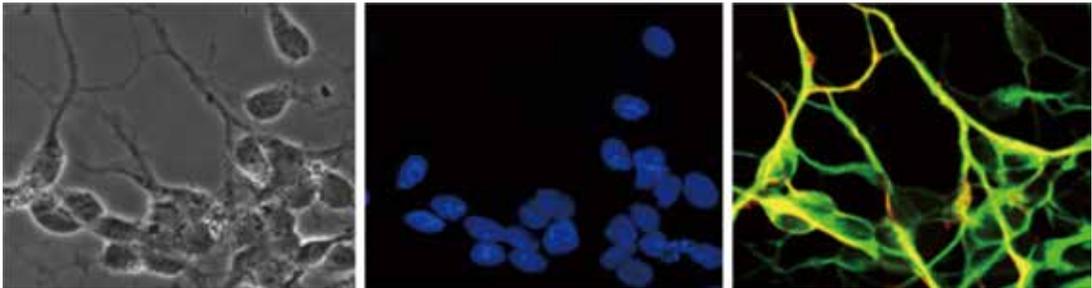
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## Protocol to efficiently induce mesenchymal stromal cells



Differentiated NCCs imaged with different markers and microscopy

Human pluripotent stem cells (hPSCs) can be differentiated into any type of cell, however, storing them long-term without contamination is a great challenge. One strategy is to differentiate hPSCs into multipotent progenitors that can differentiate into fewer cell types. Mesenchymal stromal cells (MSCs) are one option, but their limited proliferative ability calls for an intermediate between them and PSCs. Cranial neural crest cells (NCC) have the potential to be differentiated into cells responsible for the craniofacial skeleton, cornea, peripheral nervous system, and skin pigmentation, and are therefore an ideal choice as a multipotent progenitor. “We know that PSCs make teratoma, and MSCs from adults do not. So, in theory, NCCs make a safer intermediate,” explains Associate Professor Makoto Ikeya of the Dept. of Cell Growth and Differentiation and one of the corresponding authors of a new study that shows the benefits of this strategy.

An important component to minimize contaminations is the use of a chemically defined medium and feeder-free culture. Culturing protocols for NCCs exist, but are inefficient and complex. On the other hand, Ikeya, Professor Junya Toguchida and CiRA colleagues found small molecules that inhibit GSK3 $\beta$  and TGF $\beta$  can induce 70-80% of hPSCs from various cell lines into NCCs.

Importantly, freezing the NCCs did not compromise their growth or differentiation ability, which means they can be kept long term. Moreover, the NCCs were differentiated into an assortment of cell lineages, including neurons, cornea, and MSCs, which were further differentiated into osteogenic, chondrogenic and adipogenic lineages.

Interestingly, there was no need to inhibit BMP during the NCC induction. BMP is considered an important signal in NCC development, but past research has been controversial regarding whether it must be controlled. Although the findings here suggest BMP is not essential, Ikeya feels that the present results may argue for BMP temporal dynamics, where BMP is active in the first few days of NCC induction, but inhibited thereafter. “It is controversial.” Regardless, he is optimistic about how this simple protocol will invite more opportunities for cell therapies.

### Reference

Fukuta, Nakai, Kirino et al. (2014) Derivation of Mesenchymal Stromal Cells from Pluripotent Stem Cells through a Neural Crest Lineage using Small Molecule Compounds with Defined Media. *PLOS ONE* 9(12):e112291.  
Online publication: Dec. 2, 2014

## iPS cells used to correct genetic mutations that cause muscular dystrophy

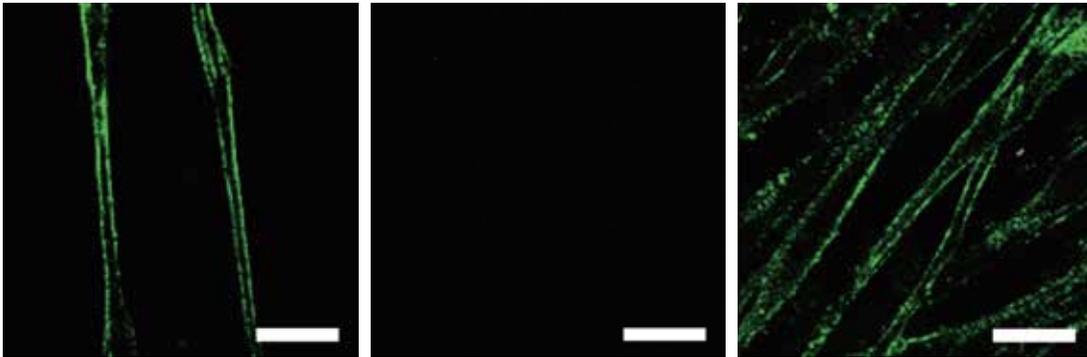


Akitsu Hotta (left) and Hongmei Lisa Li

Duchenne muscular dystrophy (DMD) is a severe muscular degenerative disease caused by a loss-of-function mutation in the *dystrophin* gene. It inflicts 1 in 3500 boys and normally leads to death by early adulthood. Currently, very little is available in terms of treatment for patients outside palliative care. One option gaining interest is genomic editing by TALEN and CRISPR, which have quickly become invaluable tools in molecular biology. These enzymes allow scientists to cleave genes at specific locations and then modify the remnants to produce a genomic sequence to their liking. However, programmable nucleases are not pristine and often mistakenly edit similar sequences that vary a few base pairs from the target sequence, making them unreliable for clinical use because of the potential for undesired mutations.

For this reason, iPS cells are ideal models, because they provide researchers an abundance of patient cells on which to test the programmable nucleases and find optimal conditions that minimize off-target modifications. Assistant Professor Akitsu Hotta and members of his lab at the Dept. of Reprogramming Science took advantage of this feature by generating iPS cells from a DMD patient. They used several different TALEN and CRISPR to modify the gene of the iPS cells, which were then differentiated into skeletal muscle cells. In all cases dystrophin protein expression was convalesced, and in some cases the *dystrophin* gene was fully corrected.

One key to the success was the development of a computational protocol that minimized the risk of off-target editing. The team built a database that included all possible permutations of sequences up to 16 base pairs long. Among these, they extracted those that only appear once in the human genome, i.e. unique sequences. DMD can be caused by several different mutations; in the case of the patient used in this



Immunofluorescence staining of dystrophin in WT cells, DMD cells, and DMD cells after exon knock-in (left to right). Scale bars, 50  $\mu$ m.

study, it was the result of the deletion of exon 44. The researchers therefore built a histogram of unique sequences that appeared in a genomic region that contained this exon and found a stack of unique sequences in exon 45. According to Hotta, “Nearly half the human genome consists of repeated sequences. So even if we found one unique sequence, a change of one or two base pairs may result in these other repeated sequences, which risks TALEN or CRISPR editing an incorrect region. To avoid this problem, we sought a region that hit high in the histogram.”

With this target, the team considered three strategies to modify the frame-shift mutation of the *dystrophin* gene: exon skipping by connecting exons 43 and 46 to restore the reading frame, frame shifting by incorporating insertion or deletion (indel) mutations, and exon knock-in by inserting exon 44 before exon 45. All three strategies effectively increased dystrophin synthesis in differentiated skeletal cells, but only the exon knock-in approach recovered the gene to its natural state. Importantly, editing showed very high specificity, suggesting that their computational approach can be used to minimize off-target editing by the programming nucleases.

Moreover, the paper provides a proof-of-principle for using iPS cell technology to treat DMD in combination with TALEN or CRISPR. The group now aims to expand this protocol to other diseases. First author Hongmei Lisa Li explains, “We show that TALEN and CRISPR can be used to correct the mutation of the DMD gene. I want to apply the nucleases to correct mutations for other genetic-based diseases like point mutations.”

#### Reference

Li et al. (2015) Precise Correction of the Dystrophin Gene in Duchenne Muscular Dystrophy Patient Induced Pluripotent Stem Cells by TALEN and CRISPR-Cas9. *Stem Cell Reports* 4(1):143-54.  
Online Publication: Nov. 26, 2014

## Human iPSC-derived cell sheets with defined cardiovascular populations recover heart failure in rat

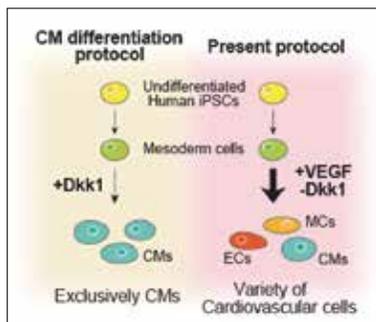


Jun K. Yamashita

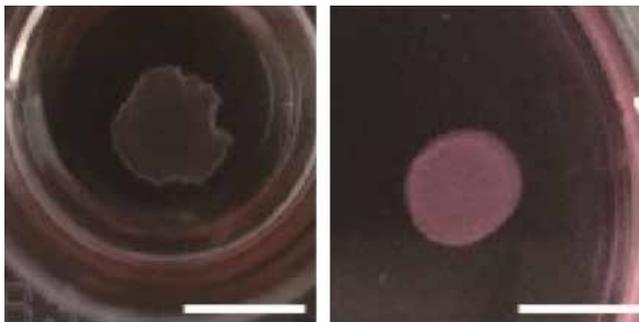
Promising preliminary results in cardiac cell therapy for heart failure have sparked hope to both researchers and patients in search of options beyond heart transplantation. Most important to the outcome of cardiac cell therapy is the functional restoration and engraft rate of the introduced cells, which depend on the cell type and cell delivery. Because they have unlimited proliferation potential and are able to differentiate into all cardiac cells, human induced pluripotent stem cells (hiPSCs) are regarded as a very attractive cell type. At the same time, the cell-sheet method is currently the most effective heart delivery method, because of its relatively high engraftment rate, although improvement is needed.

One issue compromising the quality of a cell sheet is the constituency of its cells. Previous studies have made sheets exclusively of cardiomyocytes with encouraging but nevertheless disappointing results. A population that includes cardiomyocytes and also endothelial and mural cells appears to significantly improve the generation of viable cell sheets, but generating such a heterogeneous population from hiPSCs has proven to be a challenge. The Jun K. Yamashita team of the Dept. of Cell Growth and Differentiation now reports a method that differentiates hiPSCs into different cell types. Mixing the cell types resulted in the synthesis of cell sheets that when stacked and transplanted into a myocardial infarction rat model showed high graft survival and positive effects in the heart.

This work grew from the lab's previous study that used mouse embryonic stem cells. In the current work, they changed the culture



Flow chart shows VEGF differentiates hiPSCs into a heterogeneous population



Cardiac tissue sheets. Left, 1 layer; Right, 3 layers stacked. Scale bars, 1 cm.

conditions such that VEGF, an angiogenic cytokine, was included. “We had a method to exclusively induce cardiomyocytes from mesoderm precursors. For the new approach, we needed vascular cells from mesoderm in addition to cardiomyocytes,” says Professor Yamashita. Consequently, hiPSCs were differentiated into a heterogeneous population, with more than 75% cardiomyocytes.

Transplantation of the sheets into rat caused significant improvement in a number of cardiac parameters including left ventricle systolic function, fractional shortening and systolic thickening among others, indicating that remodeling of the left ventricle, a common outcome of myocardial infarction, was suppressed.

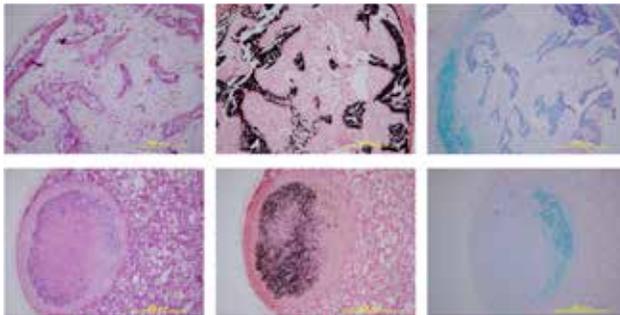
Although a number of studies have reported the benefits of cardiac stem cell therapy, there is still uncertainty whether the observed benefits are directly due to the graft or whether they are the result of paracrine effects from growth factors secreted by the engrafted cells. “At this stage, we can say cellular interactions increased the secretion of growth factors, including angiogenic factors.” Clarifying the optimal proportion of different cell types will be crucial for efficient and long-term survival of the grafts in the treatment of heart failure.

#### Reference

Masumoto et al. (2014) Human iPS cell-engineered cardiac tissue sheets with cardiomyocytes and vascular cells for cardiac regeneration. *Scientific Reports* 4:6716.

Online Publication: Oct. 22, 2014

## A new mechanism to explain the onset of NOMID



iPSC-derived chondrocytes transplanted into mice. Top row, mice with the *NLRP3* mutation; Bottom row, wild type mice. Columns show different staining.

Neonatal-onset multisystem inflammatory disease (NOMID) is an extremely rare autoimmune disease that causes persistent inflammation and tissue damage, especially in nerves, joints and skin. It is thought the disease emanates from a mutation in *NLRP3* that leads to the activation of the NLRP3 inflammasome even in the absence of regulating ligands. Treatments that target IL-1 $\beta$  have been shown to depress the inflammation, however, they are ineffective against other NOMID symptoms including epiphyseal overgrowth, which leads to skeletal abnormalities and low quality of life.

Indeed, studies have indicated that the primary pathophysiology of NOMID is disorganization of cartilage cell columns that lead to lesions. If true, mechanisms independent of activating the NLRP3 inflammasome may be involved in the disease progression. The Junya Toguchida lab of the Dept. of Cell Growth and Differentiation, which specializes in the regeneration of mesenchymal tissues, provides new insight on such a mechanism.

Chondrocytes differentiated from iPS cells that carried the *NLRP3* mutation grew to be much larger than chondrocytes differentiated from those that did not have the mutation. Most of the additional mass, however, was not due to an increase in chondrocytes, but rather because of more extracellular matrix (ECM) proteins secreted by the chondrocytes. Consistent with this result, when the chondrocytes were transplanted into mice, dysregulated ossification occurred. Surprisingly, these pathologies were not accompanied by the expression of molecules that would suggest activation of the NLRP3 inflammasome, such as IL-1 $\beta$ . Instead, the authors found that *SOX9*, which is the master regulator of chondrocyte differentiation, was significantly upregulated in mutant chondrocyte progenitor cells. This finding agreed with the observation that chondrocyte differentiation, but not proliferation, was enhanced in an NLRP3 inflammasome-independent manner. Further investigation suggested that the *SOX9* effects operate via the cAMP/PKA/CREB pathway, which could guide new drug targets for NOMID.

### Reference

Yokoyama et al. (2015) Enhanced Chondrogenesis of Induced Pluripotent Stem Cells From Patients With Neonatal-Onset Multisystem Inflammatory Disease Occurs via the Caspase 1-Independent cAMP/Protein Kinase A/CREB Pathway. *Arthritis & Rheumatology* 67(1):302-14.  
Online publication: Dec. 27, 2014

## iPS Cells for Regenerative Medicine

Every year CiRA organizes an international symposium. Last year, in partnership with the Takeda Science Foundation (TSF), we held, “iPS Cells for Drug Discovery”, in Suita, Osaka. Building on that success, we worked with the TSF again to host the “iPS Cells for Regenerative Medicine” symposium January 15-17. The event welcomed 692 attendees, including representatives of institutes from over a dozen nations.

To demonstrate the potential of iPS cell technology in a comprehensive number of organ systems, 26 speakers, including 6 from CiRA, were invited to discuss their latest research on neurology, cardiology, hematology, nephrology, immunology, and ophthalmology, the last of which included Dr. Masayo Takahashi of RIKEN Center for Developmental Biology, who presented her iPS cell-derived retinal sheet transplantation work from last year. CiRA Professors Jun Takahashi and Koji Eto spoke about their respective research on Parkinson’s Disease and blood transfusion, detailing why they hope to be joining Masayo Takahashi as scientists with iPS cell-based research that will enter clinical researches in the next few years. A session was also devoted to regulatory laws where speakers explained the challenges of preparing clinical grade stem cells for patient care.

At the closing ceremonies, Professor Shinya Yamanaka announced best posters among the 108 submissions. CiRA members Akihiro Yamashita, Kaneyasu Nishimura, Hideyuki Nakanishi and Takafumi Toyohara were all awarded for their contributions.

CiRA will hold its next international meeting in Kyoto in March of next year.



Symposium speakers

## From Kyoto to Tokyo

### A lesson in stem cells

Each year, CiRA, in partnership with iCeMS, a collaborating institute at Kyoto University, hosts a select group of high school students across Japan to visit the institute and learn about stem cells. On November 29th, approximately 80 students participated in the 2014 iCeMS/CiRA Classroom. To avoid the pedantic learning style of the classroom, no lectures were given. Students were instead given interactive activities that taught them about pluripotency and differentiation and ended with them discussing how they could prove iPS cells have pluripotency.



iCeMS/CiRA classroom

### CiRA Café (Science Café)

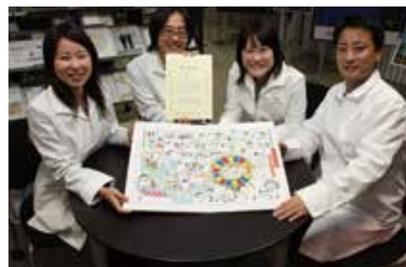
Last month 22 people came to listen to Associate Professor Knut Woltjen of the Dept. of Reprogramming Science at the most recent CiRA Café. It was the first time Woltjen spoke to the general public as a CiRA representative in Japanese, but something he thinks is a responsibility of any government-funded scientist in order to sustain support. “That’s very important. I want to understand the level of their understanding.” The Café went for almost two hours, and Woltjen was impressed by the audience’s ability to soak in erudite topics like genome editing, nucleases, and reporter genes. It also gave him the confidence to do it again. “It was fun actually.”



Knut Woltjen at CiRA Café

### Science Agora 2014

To promote science to the public, Japan Science and Technology Agency, a public funding body, has been hosting Science Agora in Tokyo, a 3-day event that was inaugurated in 2006. This year, more than 10,000 people attended in November, making it the most popular yet. To share the world of iPS cell research with those living in or near the nation’s capital, CiRA participated as one of the 176 exhibitors. CiRA was delighted to hear that they were judged best exhibit based on ballots made by the visiting public.



CiRA celebrates its Science Agora 2014 Participants Special Award

## National & International Distinctions

### A Visit to the Vatican

Last October, Professor Shinya Yamanaka spoke to the Pontifical Academy of Sciences (PAS), which is a scientific body that is funded by but runs independently of the Vatican. The PAS is assembled to bring together what it considers the most prominent minds in all the sciences and mathematics. At the same time, it is an exclusive club, as the academy cannot hold more than 80 living members, which as of last year includes Yamanaka. He was invited to talk about iPS cell technology at the symposium *Evolving Concepts of Nature*. Part of the ceremonies involved a meeting with Pope Francis, which would have been Yamanaka's second time to be greeted by the church's highest magistrate. The first time was shortly after his invention of iPS cells, when he met Pope Benedict XVI. That was a nervous moment. "I wasn't even sure what language he was speaking."



### Awards

Professor Shinya Yamanaka received the degree of Doctor of Science *honoris causa* from the University of Hong Kong on Oct. 18th for his discovery of iPS cells. The university bestows these honorary degrees as its highest recognition to individuals who have made great contributions to the world or humanity.



Shinya Yamanaka in Hong Kong

Professor Hirohide Saito of the Dept. of Reprogramming Science was selected as a recipient of the JSPS (Japan Society for the Promotion of Science) Prize on Dec. 19th for his design of a RNA-based system that can control cell fate. The annual award is given to young researchers who show exceptional creativity and productivity in the sciences.



Hirohide Saito

## The laws, they are a changin’

Part of the great excitement surrounding iPS cell research is the potential for cell therapy. However, as seen in the pharmaceutical industry, medical treatment has a high barrier of entry before reaching clinical care, and iPS cell-based therapies are no exception. On top of this, some argue that cell therapy is unique from other medical products and that current regulatory systems are inappropriate for its evaluation. Listening to these concerns, the Japanese government has responded by changing the Pharmaceuticals and Medical Devices Act last November. The official intention of these changes is to give patients faster access to experimental therapeutics.

The major change in the law is the implementation of conditional and time-limited approval. Normally, for a medical product to be approved for reimbursement from the national health insurance it needs to be proven both safe and effective based on the results of clinical trials. Japan has adjusted its demands to require only the proof of safety and prediction of efficacy. The owners of the product will then be given an additional 7 years to prove efficacy, otherwise the product is revoked.

Every country has its own regulatory body and is ultimately independent in its decision making to provide or not provide a medically related product. However, although laws may only be applicable within borders, their effects reverberate beyond. Harmonizing laws allows data from one country to be considered by the regulatory body of another. The new law in Japan changes the conditions under which data are collected, such that ethically it will be impossible to have randomized trials following conditional approval. It remains unclear then if the data used to approve therapies in Japan will be transferable to other countries.

Seeing that the new law will not reach its first anniversary until November and that no therapy has been approved under it yet, any opinions now on the impact of the law are merely speculative. One thing that is certain though is that many countries are closely watching Japan and considering if it will be a future model.

## Greetings from The Yasuhiro Yamada Lab Dept. of Reprogramming Science

The invention of iPS cells has opened the door for us to see changes in the cell that switch it from a terminally differentiated state to one in a proliferative state. Cancer can be described similarly, as it involves a cell losing control of its proliferation, thus tipping into a pathological state. My lab is therefore applying iPS technology to understand the critical molecular events that must occur for a healthy cell to take the cancerous state and figuring out ways to reverse this process. While the general theory of cancer assumes genetic mutations as the cause of this switch, my lab is exploring the responsibility of epigenetic changes. We are therefore using next-generation sequencers to identify key epigenetic marks to build a comprehensive list of candidates that we will then target with iPS techniques to manipulate the fate of cancer cells. Our ultimate aim is to create a comprehensive map that distinguishes healthy and cancer cells and use iPS technology to reprogram the cells accordingly. To achieve this goal, the group consists of members who have an innate interest in the molecular and cellular biology of cancer and those who are strong in biotechnology and data analysis.



Professor Yasuhiro Yamada



## Charity running



After running, before beer

The people of Osaka are reputed the comedians of Japan, and the annual Osaka marathon is just another way to express their unique culture. Among the approximately 30,000 runners at the 4th annual Osaka Marathon, which took place last October 26th, were the Power Rangers and Japan's most famous Italian siblings, Mario and Luigi. Also included were Professor Kenji Osafune and Assistant Professor Akira Watanabe, who slipped out of their lab coats and headed to Kyoto's neighboring city to represent CiRA. Although in prior years other CiRA members had run the marathon, this was the first time CiRA had entered as an official charity organization.

It is not only the runners that are a little unusual in Osaka. The marathon distinguishes itself from others in the country by being the only one where rather than receiving bananas or other foods known to maintain runner stamina, participants are instead given takoyaki, famous Osaka street snacks that are far from a runner's ideal diet. Even observers from the streets were sure to showcase the uniqueness of Osaka. Normally, the Japanese cheer their brethren with calls of "Gambare!" In Osaka, however, when the run was at its most grueling, Osafune and Watanabe could hear, "Take a break from your walk and run a little!"

After the run, the two CiRA marathoners were joined by a number of colleagues who came to support them. Despite his exhaustion, Watanabe was more than happy to go for drinks. "I am never too tired for a beer." On the other hand, Osafune admitted that, "it took 4-5 days before I was fully recovered." That long recovery was slightly assuaged by the fact that Osafune had accumulated the most donations of any participating charity runner, surpassing 1 million yen.

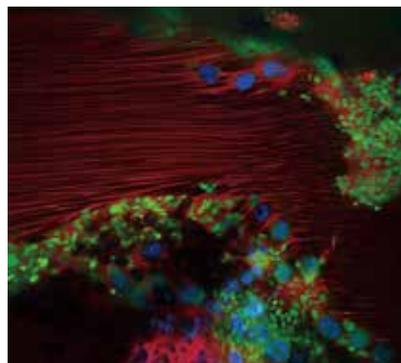
## Philanthropy & Photography

CiRA is extremely fortunate to receive strong support from the public. Every year, donations pour in at amounts extraordinary for Japanese institutes. 2013 saw the highest total yet, reaching over 1.2 billion yen. In gratitude, CiRA annually invites donors to the institute, where they can learn about the research to which their generosity is making a big difference. However, public support is nationwide, which is why last November 26th, CiRA went to Tokyo to hold a separate event. Professors Shinya Yamanaka and Junya Toguchida made the trip from Kyoto to report on CiRA's latest research.

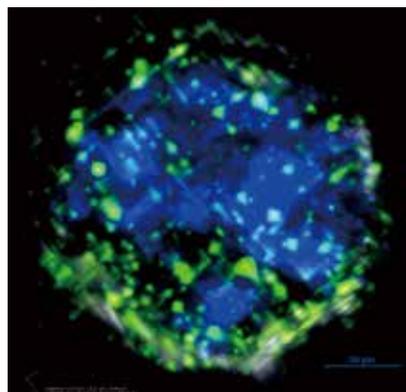
The gacco project, hosted by NTT Knowledge Square Inc., is part of a nationwide free online education initiative. CiRA is participating by offering 23 lectures (about 10 minutes each) over 4 weeks on iPS cells, including cell reprogramming science and applications in medical care and drug discovery. All lectures are in Japanese.

Posters could be seen on trains for months. Professor Shinya Yamanaka was to give a free talk to the public on iPS cells Dec. 21st. The venue in downtown Osaka could host 1700 people, but there were more than 9000 applicants. Therefore, a lottery was held. Moreover, people were lining up well over an hour to the general seating event to hear Yamanaka talk about his research career and the impact iPS technology will have on medical care.

The annual CiRA Science Photo Contest was held last month. Winning art is shown on this page.



“Blood vessel burst is beautiful”, Koji Eto



“Jewel”, Shunsuke Kihara



“Autumn shine”, Yutaka Yasui



*Winter, in the lab*  
*The cells will not reprogram*  
*Suddenly snowflakes*

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