**Regular Article**

**Highlighted Paper selected by Editor-in-Chief**

**Generation of Rat Induced Pluripotent Stem Cells Using a Plasmid Vector and Possible Application of a Keratan Sulfate Glycan Recognizing Antibody in Discriminating Teratoma Formation Phenotypes**

Juliet O. Makanga,† Misa Kobayashi,‡ Hiroki Ikeda,‡ Antonius Christiano,† Mitsunori Yamada,§ Toshisuke Kawasaki,∥ and Tetsuya Inazu*∥

∥Laboratory of Functional Genomics, College of Pharmaceutical Sciences, Ritsumeikan University; Kusatsu, Shiga 525–8577, Japan; †Laboratory of Bio-analytical Chemistry, College of Pharmaceutical Sciences, Ritsumeikan University; Kusatsu, Shiga 525–8577, Japan; §Laboratory of Neuropathology, Department of Clinical Research, Saigata Medical Center, NHO; Joetsu, Niigata 949–3193, Japan; and ∥Research Center for Glycobiotechnology, Ritsumeikan University; Kusatsu, Shiga 525–8577, Japan.

Received September 30, 2014; accepted October 20, 2014

Induced pluripotent stem cells (iPSCs) offer an invaluable tool for biological research and regenerative medicine. We report establishment of rat iPSCs (riPSCs) using a plasmid vector encoding four transcription factors, Oct3/4, Sox2, c-Myc and Klf4. Although all riPSC clones were generated and cultured under the same conditions, expressed hallmark pluripotency markers and differentiated successfully in vitro, the expression of a keratan sulfate glycan epitope with unique properties defined by R-10G antibody varied in the riPSC clones. In contrast, tumor rejection antigen (TRA)-1-81 epitope expression was comparable. A clone highly reactive to R-10G antibody formed teratomas in vivo consisting of cells from all three germ layers. However, clones expressing a lower level of the epitope defined by R-10G resulted in tumors with rapid growth consisting of undifferentiated cells. Additionally, riPSCs could be successfully differentiated into a neuronal lineage including glutamate neurons that responded to agonist stimulation. These observations demonstrate a glyco-phenotypic difference that may potentially serve as a useful probe for riPSC evaluation and to study the role of glycans in pluripotency and carcinogenesis in these cells.

**Key words** induced pluripotent stem cell; teratoma; R-10G antibody; glycobiology

The generation of induced pluripotent stem cells (iPSCs) by the forced expression of the exogenous transcription factors, Oct3/4, Sox2, c-Myc and Klf4, in mouse embryonic fibroblasts (MEFs) by Yamanaka’s group paved way for a new era in stem cell research and regenerative medicine.†‡ Using reprogramming factors, iPSC have been successfully generated from humans and other mammalian species such as the rat†± and monkey.‡

The rat is utilized vastly as a model in pharmacology, toxicology, immunology research fields and behavioral science and has been useful in modeling human neural and cardiac system diseases.†‡ Rat iPSC lines have been derived from a variety of somatic cells mainly via viral transduction of reprogramming factors.∥ The successful generation of transgenic rats using riPSC shows great promise in their use.∥

Variation in iPSC properties are widely reported∥ and definitive evaluation systems to assess riPSC pluripotency, differentiation propensity and potential for tumorigenesis still remain a challenge to the field. Glycans are considered to be ideal targets for identifying and analyzing cellular phenotype and cell surface epitopes such as stage-specific embryonic antigens (SSEA)-3/4 and tumor rejection antigen (TRA)-1-60/81 are conventionally used to evaluate pluripotency. However, these epitopes are also expressed in embryonal carcinoma (EC) cells.∥¶ In previous work, we generated the monoclonal antibody, R-10G, which recognizes a keratan sulfate epitope with unique structure on podocalyxin in human iPSC and human embryonal stem (ES) cells.∥∥ Notably, however, the antibody did not react with human EC cells that are known to be of a tumorigenic nature.

Here we describe generation of iPSC from rat adult fibroblasts using a plasmid vector containing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc). Although generated and cultured under the same conditions, we identified distinct clones that were similar in pluripotency marker expression and in vitro differentiation potential but varied in reactivity to R-10G. Contrastingly, TRA-1-81 reactivity was comparable. In teratoma forming assay, clone highly reactive to R-10G efficiently formed teratomas consisting of cells from all three germ layers. In contrast, tumors formed by clones expressing R-10G epitope at lower levels had aggressive growth and consisted only of undifferentiated cells. Furthermore, we differentiated the derived iPSC into neurons including glutamate neurons that were responsive to agonist stimulation.

MATERIALS AND METHODS

**Animals** Wistar rat and Balb-cSLC-ν/ν immune deficient mice were obtained from Japan SLC, Inc. Experimental procedures were carried out in accordance with the guidelines and laws of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University.

**Cell Culture** Adult rat fibroblasts were obtained from 6 week old male Wistar rats. Abdominal skin specimens were grown on tissue culture dishes in Media 1: Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Skin specimens were removed after 7d and cells that migrated out of the skin pieces grown to 100% confluence. Fibroblasts were passaged and used for reprogramming between passage 2 and 4. EmbryoMax primary
mouse embryo fibroblasts (MEFs), Neo resistant (Millipore) were maintained in Media 1 and inactivated by treatment with mitomycin C. Rat iPSC were cultured in riPSC media: high glucose DMEM supplemented with 15% FBS, 1 mM β-mercapto-ethanol, nonessential amino acids, 1000 U/mL leukemia inhibitory factor (LIF) supplemented with signal inhibitors 2i including MEK1/2 inhibitor PD 0325901 (0.5 µM; Cayman Chemical) and GSK3β inhibitor CHIR99021 (3 µM; Axon Medchem). Passage was done using trypsin-based dissociation with a split ratio of 1:4 every 4 to 5 d.

Reprogramming Reprogramming was conducted using pCAG2LMKOsimO vector encoding p53, p21, p16 (kindly provided by Dr. K. Kaji, University of Edinburgh). Eight micrograms of pCAG2LMKOsimO vector was introduced into 10^6 fibroblasts with the Basic Nucleofector Kit for Primary Mammalian Fibroblasts using the Nucleofector II (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013), plated on inactivated MEFs (Lonza) device (program V-013). 1 µg/ml G418 was added for selection. ES cells like colonies (Lonza) device (program V-013) were visible from day 7 post transfection. These were individually picked at days 14–20 and further propagated.

Alkaline Phosphatase Staining and Immunocytochemistry Alkaline phosphatase (AP) staining was performed using the Alkaline Phosphatase Detection Kit (Millipore) according to the manufacturer’s protocol. For Immunocytochemistry, fixation was performed using 4% paraformaldehyde. Cells were washed with phosphate buffered saline (PBS) followed by treatment with 0.2% Triton X-100 in PBS for 15 min at room temperature and blocking done with 3% FBS in PBS for 1 h at room temperature. For R-10G and TRA-1-81 immunocytochemical analysis cells were not treated with Triton X-100. Cells were incubated in primary antibodies overnight at 4°C and incubated with the respective secondary antibodies for one hour at room temperature. Cell nuclei were stained using 4’-6-diamidino-2-phenylindole (DAPI). Cells were imaged with a confocal microscope (Olympus, Japan) or KEYENCE BZ-9000 (KEYENCE, Japan). Quantification of fluorescence intensity was performed using Hybrid cell count BZ-H2C software (KEYENCE). Antibodies used include: Nanog (1:500, Abcam), SSEA1 (1:500, Abcam), OCT4 (1:100, Millipore), R-10G (1:100), TRA-1-81 (1:100, Abcam), Nestin (1:500, Sigma), smooth muscle actin (SMA) (1:250), Albumin (1:250), Class IIIβ-tubulin (1:1000, Millipore), Map2 (1:1000 Sigma), TH (1:200, Sigma). Alexa fluorophores 488 (immunoglobulin G (IgG)), 555 (immunoglobulin M (IgM)) and 555 (IgG) (Invitrogen).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted and 1 µg RNA used for cDNA generated by SuperScript III First-Strand Synthesis System for RT-PCR. PCR was performed using OneTaq DNA polymerase (TaKaRa). PCR conditions were optimized to determine linear amplification range. PCR primers used are shown on Supplementary Table 1.

Bisulfite Genomic Sequencing Genomic DNA for bisulfite sequencing was isolated using the PureGene Genomic DNA isolation kit (Qiagen) and bisulfite treatment performed using Methyl Easy Xceed (human genetic signatures) following the respective manufacturer’s protocols. Rat specific Oct3/4 promoter region (−1861 to −2045 and −2794 to −2953; GenBank: EU419996.1) was amplified using ExTaq HS DNA Polymerase (TaKaRa), products were cloned into pGEM-T Easy (Promega) vector and sequenced 8 samples of each riPSCs clone with M13 forward primer. Primers used are listed on Supplementary Table 1.

Karyotyping riPSCs were treated with 10 µg/mL Colcemid for 3 h. Cells were trypsinized into a single cell suspension and incubated in 75 mM KCl at 37°C for 15 min followed by fixation with ice cold methanol–acetic acid (3:1). Metaphase preparation and chromosome counting were then performed. A total 100 spreads were analyzed for each clone.

In Vitro Differentiation of riPSC riPSC were dissociated into single cells and transferred to non-adherent bacterial culture dishes in riPSC media without LIF and 2i for embryo body (EB) formation. For spontaneous differentiation, EBs were dissociated by Accutase (Wako Pure Chemical Industries, Ltd.), attached on gelatin-coated tissue culture dishes and maintained in Media 1 for an additional 7 d with media change every 2 d.

Teratoma Formation riPSC were tyniposed, counted and injected subcutaneously into BALB/cSLC-nu/nu mice. Tissue masses were harvested after 3–5 weeks, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (H&E) according to standard protocols.

Differentiation of riPSC into Neurons riPSC colonies were dissociated and plated on non-adherent bacterial culture dishes in iPSC media without LIF and 2i with media change every 3 d. After 7 d, EBs were collected by centrifugation and dissociated with Accutase. For induction of differentiation toward neuronal lineage, cells were plated on gelatin-coated tissue culture dishes and maintained in Induction media: DMEM-F12 supplemented with 2% N2 supplement (Wako Pure Chemical Industries, Ltd.) and 1% B27 (Millenti Biotec). At culture day 7, cells were dissociated and plated on poly-l-lysine coated dishes in Induction media containing 10 ng/mL bFGF, 100 ng/mL FGF8 and 200 ng/mL Sonic Hedgehog (SHH) were added for 5 d. Media was changed to Induction media supplemented with 2% (vol/vol) B27, 30 ng/mL brain derived neurotrophic factor (BDNF), 5 ng/mL glia derived neurotrophic factor (GDNF) and 200 mM ascorbic acid for another 7 d for terminal differentiation.

Calcium Influx For Ca^{2+} influx experiments, riPSC derived neurons were cultured in poly-l-lysine coated dishes. Neurons were washed once with recording medium (RM): 129 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 4.2 mM glucose and 10 mM N-(2-hydroxyethyl)perazine-N′-2-ethanesulfonic acid (HEPES) (pH 7.4), then incubated for 1 h at 37°C in RM containing 30 mM Pluronic F-127 (Invitrogen) and 1.5 mM fluo-3 acetoxy methyl ester (Nacalai Tesque). Culture dishes were then triple washed with Mg^{2+}-free RM, then incubated with it and induced fluo-3 Ca^{2+} influx activity visualized at 488 nm with confocal microscope (Olympus). N-Methyl-d-aspartic acid (NMDA) was added directly onto the medium of the dish undergoing microscopy to a final concentration of 200 µM. Fluorescent intensity was estimated by the brightness of images of single cropped neuronal bodies using Image J software.

RESULTS Generation of riPSCs and Analysis of Pluripotency Markers riPSCs were generated from adult rat fibroblasts
as described in Materials and Methods. We manually picked 11 colonies and successfully expanded them on MEF feeder cells. Three clones, riPSC #3 (data not shown), riPSC #6 and riPSC #11, were selected for subsequent studies (Fig. 1A). Immunocytochemical analysis on these morphologically rat ES cell-like cells showed they expressed ES cell marker, Nanog, and surface marker, SSEA1 (Fig. 1A). riPSC also stained positive for AP staining (Fig. 1B). Rat fibroblasts used as source cells were negative for all the above. Expression of endogenous pluripotency marker genes was analyzed by RT-PCR (Fig. 1C). riPSC clones generated expressed transcripts for pivotal pluripotency markers (Nanog, Oct3/4, Sox2, Eras, Lin28, Lefty, Fgf4, Nodal and Esg1) (Fig. 1C). Absence of expression of pluripotency genes in rat adult fibroblasts was confirmed indicating reprogramming had indeed taken place. Another parameter for reprogramming is DNA demethylation of pluripotency gene promoter regions. We performed bisulfite sequencing analysis to investigate the epigenetic state of Oct3/4 promoter region (Fig. 1D). Additionally karyotyping analysis was also conducted. It revealed riPSC#6 was of normal karyotype (2n=42). On the other hand, riPSC#11 had a majority aneuploidy spreads with an increase in the number of chromosomes up to 64 (Supplementary Fig. 1).

R-10G Antibody Reactivity We examined the expression of a keratan sulfate glycan, binding R-10G antibody, which we previously reported to be expressed only in human iPSC and ES cells but not human EC cell lines. Interestingly, in immunocytochemical analysis, riPSC#6 reacted remarkably and staining was detected on the cell surface and boundaries. Contrastingly, riPSC#11 and riPSC#3 (data not shown) had markedly lower binding activity (Fig. 2A). As R-10G shares a common carrier protein with the conventionally used iPS/ES cell marker TRA-1-81, we also investigated TRA-1-81 binding activity in these clones. Immunocytochemical analysis showed TRA-1-81 binding activity was similar in the all the clones assessed (Fig. 2B). Quantification of fluorescence staining indicated no significant difference in staining intensity for TRA-1-81 while R-10G staining intensity was three times lower in riPSC#11 (Fig. 2C). Taken together, these results indicated that the clones differentially expressed the glycan epi-

![Fig. 1. Generation and Characterization of iPSC from Adult Rat Fibroblasts, riPSC](image-url)
tope that binds R-10G antibody while expressing similar levels of the surface antigen defined by TRA-1-81 antibody.

In Vitro and In Vivo Differentiation of riPSC Following the glycophenotypic difference we observed along with our previous findings on R-10G, we investigated in vitro and in vivo differentiation capacities of the clones. For in vitro differentiation, cells were dissociated into a single cell suspension and prompted to differentiate into EBs (Fig. 3A). All clones formed EBs that were dissociated and differentiated spontaneously. Immunocytochemical analysis showed uniform differentiation into Albumin positive cells (endodermal lineage), SMA positive cells (mesodermal lineage) and Tuj1 positive cells (ectodermal lineage) (Fig. 3B). RT-PCR also confirmed mRNA of various marker genes of the three germ layers were also expressed at comparable levels after 8 d of differentiation.

To examine in vivo differentiation, we performed teratoma formation assays. riPSCs were injected subcutaneously into immunodeficient mice. Tumor formation efficiency was 100% and palpable tumor masses developed in all cases. Intriguingly, tumor masses of riPSC#11 and riPSC#3 injected groups had significantly high growth rate in comparison to riPSC#6 injected group and were harvested earlier. Histological examination was performed by a qualified pathologist and riPSC#6 tumors were identified as teratomas. The teratomas contained organized structures of derivatives of three germ layer cells including glands and ciliated epithelium (endoderm), muscle fibers and cartilage (mesoderm), and neural tissue and epidermis (ectoderm). Contrastingly, riPSC#11 and riPSC#3 (data not shown) resulted in tumors consisting only of undifferentiated cells (Figs. 3C–H for riPSC#6, Figs. 3I, J for riPSC#11).

In Vitro Differentiation into Neural Lineage Since we observed that riPSC#6 expressed features of neural tissue in histological analysis, we attempted to examine whether #6 clone could differentiate to functional neurons. We subjected riPSCs to a multistep protocol with modifications for converting iPSCs into neurons (Fig. 4A). We explored the differentiation potential by performing immunocytochemical analysis. Majority of the cells at differentiation day 14 expressed the neural progenitor (NP) cell marker, nestin (Fig. 4B). After bFGF withdrawal and treatment with BDNF, GDNF, ascorbic acid and dbcAMP, cells were abundantly positive for the neuron markers Tuj1 and Map2 (Figs. 4C, D). Immunocytochemical analysis also detected a population of tyrosine hydroxylase/Tuj 1 positive cells. For further characterization, RT-PCR showed expression of Nestin, Pax6 and Bhlp which are typical neural stem cell markers as well as Ascl1, Satb2, Thr1 and NeuroD1, which are involved in neuronal differentiation suggesting neural differentiation had
been initiated (Fig. 4E). RT-PCR analysis revealed expression of vesicular glutamate transporter (VGLUT)-1, N-methyl-d-aspartate receptor (NMDAR) subunits: GluN1, GluN2A and GluN2B, excitatory amino-acid transporter (EAAT)-3, that are typically expressed in excitatory glutamate neurons as well as engrailed (EN)-1 and tyrosine hydroxylase (TH) which are expressed in dopamine neurons (Fig. 4E). Glutamic acid decarboxylase (GAD)-67 (GABAergic neurons), choline acetyltransferase (ChAT) (cholinergic neurons) or tryptophan hydroxylase (TPH) (serotonergic neurons) expression was not detected. Taken together, these data indicated that riPSC had differentiated into neurons with general glutamatergic and dopaminergic features.

As we observed expression of N-methyl-o-aspartate receptor (NMDAR) subunits: GluN1, GluN2A and GluN2B, we tested the capacity of these cells to respond to stimulation. Five minutes following extracellular stimulation with 200µM of NMDAR agonist, NMDA, cells previously loaded with Fluo-3-AM showed an increase of fluorescence intensity up to 1.6 times that of neuronal bodies at resting state (Fig. 4G). Response to NMDA was suppressed by omission of extracellular Ca²⁺ with 10 mM EGTA, suggesting that intracellular Ca²⁺ elevation was as a result of influx from outside of cells (data not shown). Furthermore, NMDAR antagonist, MK-801 also abolished intracellular Ca²⁺ elevation. Taken together, these results suggest that derived cells were functional and reactive to agonist mediated signaling.

**DISCUSSION**

Here we describe establishment of riPSC via a plasmid vector and demonstrate a difference in glycophenotype between teratoma and tumor forming clones with regard to a type of keratan sulfate glycan. riPSC clones established expressed pluripotency markers, differentiated into cells of the three germ layers in vitro as well as neurons similary. Increasing attention has been paid to cell surface glycans as targets for identifying and characterizing ES cells/iPSCs studying pluripotency, self-renewal and differentiation processes. Glycan signatures vary with cell types, stages of development and differentiation. It is probable that changes in glycan surface molecules occur in iPSCs when they acquire pluripotency although much remains to be elucidated in this concern. In this study we assessed a total of three clones and
observed an unexpected differential level of expression of the keratan sulfate glycan epitope bound by R-10G among riPSC clones. Contrastingly, conventionally used TRA-1-81 expression was the same in the clones. TRA-1-81 not only shares a carrier protein, podocalyxin, with the R-10G binding epitope, but is also a keratan sulfate albeit of a different structure. Indeed, Fujitani et al. reported cellular glycomes to be highly cell type specific demonstrating their utility as unique cellular descriptors and the study suggests the presence of stem-cell-specific glycosylation spectra. In this light and based on observations in this study, it is reasonable to consider that a difference in R-10G glycan epitope profile of teratoma and non-teratoma forming riPSC clones exists. Studies reported properties of iPSC clones varying in pluripotent potential between laboratories despite being generated via similar induction systems. This is unlikely as riPSC in this study were generated in the same laboratory and cultured under the same conditions.

Furthermore, we observed only one riPSC clone highly reactive and two clones low reactive with R-10G antibody in this study. However, results we obtained from the highly reactive riPSC clone are consistent with those of our previous work on three different human iPSC lines. This strongly suggests that the results are applicable to other highly reactive clones.

The two riPSCs clones expressing keratan sulfate glycan defined by R-10G at low levels failed the teratoma formation assay forming tumors comprised of undifferentiated cells. Teratoma formation is a criterion for in vivo pluripotency asessment and iPSC are expected to result in benign teratomas that contain remnants of all three germ layers. During reprogramming process, somatic cells gain an embryonic-like state but reprogramming stress may led to genetic mutations and chromosomal aberrations resulting iPSC may be of a tumorigenic nature. The cause of unsuccessful teratoma formation remains unclear but karyotypic abnormality or exogenous gene integration could be speculated. On the other hand, we analyzed rat Oct4 promoter region which partially overlapped a region that Merkl et al. demonstrated as completely demethylated. Although our data was not consistent with previous observations, endogenous rat Oct4 expression determined by immunocytochemistry and RT-PCR analysis were both apparent. The data discrepancy may come from the vector used, difference in species used or as a result of unsuccessful reprogramming. On the contrary, given endogenous Nanog expression was also detected by both immuno-

---

**Fig. 4.** riPSC Differentiation into Neurons

A: Schematic of the method for differentiating riPSC to neurons. B: Expression of nestin (red) at differentiation day 14. Nuclei were stained with ToPro3 (blue). C, D: Immunofluorescence images of day 28 riPSC derived neurons. A population of Tuj1 (green) and Map2 (red) positive cells revealed an abundant population of neurons. Nuclei were stained with ToPro3 (blue). D: Immunocytochemical staining for TH (red) revealed a population of dopaminergic neurons. E: RT-PCR of gene expression in day 7, day 14 and day 28. Representative results of three independent experiments. F: Calcium influx signaling after NMDA receptor stimulation. Representative results of three independent experiments. (The colored figures are available in the electronic version.)

---

**Table:**

| Day 1 | Day 7 | Day 14 | Day 28 |
| --- | --- | --- | --- |
| EB formation | Induction | Differentiation |  |
| bFGF | FGF8 SHH | BDNF, GDNF, Ascorbic acid, dbcAMP |  |

- Nestin
- Pax6
- Blbp
- NeuroD1
- Mash1
- Satb2
- Tbr1
- Gapdh
- VGLUT-1
- GluN1
- GluN2A
- GluN2B
- EAAT3
- Enl
- Th
cytochemistry and RT-PCR, the latter possibility is unlikely. Therefore, we speculate the first two demethylation sites in Oct 4 promoter region (open circle in Fig. 1D) may be sufficient to express Oct4. Further studies will be needed to elucidate which demethylation site(s) in the Oct 4 promoter region are pivotal for Oct4 expression.

We also investigated the capacity of the plasmid vector derived riPSC to differentiate into neural lineage cells. In our hands, derived cells expressed hallmark neural stem cell markers Nestin, Pax6 and Blbp. Upon further differentiation, the cells acquired glutamate and dopamine neuron features, which excitatory glutamate neurons was confirmed by the expression of VGluT1, EAAT3 and NMDAR subunits. Chang et al. reported differentiation of rat iPS cells into neurons of which approximately 10% were dopamine neurons, but the nature of the remaining population was not demonstrated.10) To our knowledge, this is the first work identifying a population of glutamate neurons from riPSCs and furthermore demonstrating the neurons were responsive to agonist mediated stimulation. Indeed, it would be of great interest to study pharmacological glutamate neurotransmitter signaling using riPSCs derived neurons.

CONCLUSION

Although the number of riPSC clones was limited (one R-10G high and two R-10G low clones), the present findings are consistent with our initial work in which R-10G antibody did not bind EC cell lines that are tumorigenic in nature, while strongly reacted to human iPSCs and human ES cell lines. This study provides insight on the nature of glyco-phenotype of rat iPS cells. Epitopes defined by R-10G could potentially offer a tool for evaluation of rat iPS cells in regard to glyco-phenotype. Expression of the R-10G epitope could be useful in investigating glycans involved in pluripotency, tumor formation and cancer biology. Additionally, riPSC derived neurons may be useful in various pharmacological studies of neurotransmitter pathways.

Acknowledgments We thank Dr. K. Kaji for kindly providing the plasmid used in this work. We thank Mr. Koga, Igarashi, and Hirocho, Ms. Karima and Konishi for technical support. This work was supported by Grants-in-Aid for Scientific Research C-24570171 (to T.K.), for Scientific Research on Innovative Areas 24110517 (to T.K.) from the Japan Society for the Promotion of Science, Grants-in-Aid for Adaptable and Seamless Technology Transfer Program through target-driven R&D, AS242Z01520P (to T.K.) and AS251Z01560P (to H.T.), from the Japan Science and Technology Agency, and by the R-GIRO (Ritsumeikan Global Innovation Research Organization) Program (to H.T.).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.