ANTICANCER RESEARCH *39*: 4811-4816 (2019)
doi:10.21873/anticanres.13666

Blockade of γ-Glutamylcyclotransferase Enhances Docetaxel Growth Inhibition of Prostate Cancer Cells

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Abstract. *Background/Aim: γ-Glutamylcyclotransferase (GGCT) is highly expressed in many forms of cancer, and is a promising therapeutic target. The present study investigated whether inhibition of GGCT enhanced the antiproliferative effects of the drug docetaxel in prostate cancer cells. Materials and Methods: Immunohistochemistry and western blot analysis were conducted to measure GGCT expression in prostate cancer tissue samples and cell lines. GGCT was inhibited using RNAi and a novel enzymatic inhibitor, pro-GA, and cell proliferation was evaluated with single and combination treatments of GGCT inhibitors and docetaxel. Results: GGCT was highly expressed in cultured prostate cancer cells and patient samples. GGCT inhibition alone inhibited prostate cancer cell line proliferation and induced cellular senescence. GGCT inhibition in combination with apoptosis-inducing docetaxel had more potent antiproliferative effects than either drug used alone. Conclusion: GGCT inhibition may potentiate anticancer drug efficacy.*

Prostate cancer is the third most common malignancy worldwide after lung and breast cancer, and is a leading cause of cancer-related death (1). Although most prostate cancers respond well to androgen deprivation therapy (ADT) initially, resistance to ADT occurs after 1 to 3 years. Therefore, treatments for castration-resistant prostate cancer (CRPC) are an urgent clinical need (2).

GGCT was originally identified by a proteomics approach as a chromosome 7 open-reading frame 24 (C7orf24), which was highly expressed in urothelial cancer (3). GGCT activity, which generates 5-oxoproline and amino acids from the γ-glutamyl peptide, is involved in glutathione homeostasis (4). GGCT is overexpressed in a wide variety of malignancies, including cervical, lung, colon, and breast cancer (5). Interestingly, in various cancer cells, GGCT knockdown inhibits cell proliferation, that is associated with phenotypes other than apoptosis. Kageyama *et al.* first demonstrated that GGCT knockdown inhibits bladder cancer cell proliferation (6). GGCT depletion also suppresses MCF-7 and MDA-MB-231 breast cancer cells, inducing cellular senescence associated with p21 and/or p16 up-regulation (7).

We identified prohibitin-2 as a binding partner of GGCT, which plays a pivotal role in p21 induction (8). GGCT depletion induces autophagy, while GGCT overexpression promotes proliferation, in NIH-3T3 cells (9). Localized or systemic administration of siRNA against GGCT *in vivo* has anti-tumor effects (10-11). Moreover, we developed the GGCT enzymatic inhibitor, pro-GA, from a compound library screen, which has anti-tumor effects (12). Pro-GA effectively suppresses bladder cancer cell proliferation (13).

In the present study, we investigated whether GGCT inhibition would enhance the chemotherapeutic effects of the apoptosis inducer docetaxel, as GGCT inhibition suppresses proliferation through apoptosis-independent mechanisms, including induction of cellular senescence.

Materials and Methods

Cell culture. Human normal prostate epithelial cells were purchased and maintained in optimized media from Lonza (Basel, Switzerland). LNCaP, PC-3, and HL-60 cells (RIKEN BRC, Japan) were maintained in RPMI1640, and DU-145 cells (ATCC, USA) were maintained in DMEM (Wako, Osaka, Japan), both supplemented with 10% FBS. Docetaxel (Wako) was dissolved in ethanol/DMSO. Pro-GA (Funakoshi, Tokyo, Japan) was dissolved in DMSO.

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Key Words: γ-glutamylcyclotransferase, prostate cancer cells, senescence, docetaxel, pro-GA.

Antibodies. The following antibodies were used: GGCT [6-1E, Cosmo, Tokyo, Japan for Western blot and R&D Systems for tissue microarray (TMA)], Caspase-3 and -8 (Cell Signaling Technology, Danvers, MA, USA), PARP-1 (Enzo, Farmingdale, NY, USA), and β-actin and GAPDH (Wako).

TMAs and immunohistochemical analyses. TMAs were purchased from SUPER BIO CHIPS (Seoul, Republic of Korea). Standard immunohistochemical staining using DAB counterstained with chromogen (Nichirei Bioscience, Tokyo, Japan) was performed. Scoring parameters included evaluation of staining intensity (negative, weak, moderate, or strong) and approximate percentage of stained cells (rare, <25%, 25–75%, or >75%).

siRNA transfection. PC-3 cells were transfected with non-targeting or *GGCT* siRNAs at a final concentration of 5 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). siRNA sequences have been previously described in (7), and will be provided by request.

Western blot analysis. Proteins solubilized in 1% SDS lysis buffer were separated by SDS-PAGE and transferred to membranes. After blocking with 5% fat-free milk in PBS-T, membranes were incubated with antibodies in 3% BSA/PBS-T. Targets were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA).

Measurement of proliferation. A WST-8 assay was performed using a cell count reagent SF kit (Nacalai Tesque, Kyoto, Japan). Cell numbers were counted using a standard trypan blue dye exclusion test.

DNA ladder formation assay. Double-stranded DNA breaks forming a ladder were assessed using standard agarose gel electrophoresis.

Cell cycle analysis. Cells were washed and fixed with 70% ethanol at –20˚C, and stained with propidium iodide (PI) at 100 μg/ml in the presence of 100 μg/ml RNase A. DNA content was analyzed using a BD LSRFortessa X-20 cell analyzer (BD Biosciences, Franklin Lakes, NJ, USA). At least 10,000 cells per sample were analyzed.

Detection of cellular senescence. β-galactosidase staining was performed using a Cellular Senescence Kit (OZ BioScience, San Diego, CA, USA), and β-galactosidase activity was measured using a 96-Well Cellular Senescence Assay kit (Cell Biolabs, San Diego, CA, USA).

Statistical analyses. The results of TMA IHC scoring were analyzed using a Fisher's exact test. A two-tailed Student's *t*-test was used for comparison of two groups, and $p<0.05$ was considered significant. Combination effects of 5-80 μM pro-GA and 0.025-0.5 ng/ml docetaxel were assessed by combination index (CI) using CalcuSyn 2.11 software (Biosoft, Cambridge, UK). A CI value of less than 0.9, between 0.9 and 1.1, or greater than 1.1 was considered as synergy, additivity, or antagonism, respectively.

Results

GGCT expression in prostate cancer tissues and cell lines. We examined GGCT expression using a TMA containing nine normal prostate tissues and 40 prostate cancer tissues (Figure 1A). Moderate or strong expression of GGCT was observed in two of nine normal prostate tissues (22%) and in 35 of 40 prostate cancer tissues (78%). GGCT expression was higher in prostate cancer tissue than in normal prostate tissue (Fisher's exact test, $p<0.01$). However, expression differences by Gleason score were not observed. Western blot analysis revealed overexpression of GGCT in prostate cancer cell lines relative to normal prostate epithelial cells (Figure 1B).

Apoptotic signaling was not activated by GGCT knockdown. Next, we confirmed efficient siRNA knockdown of GGCT in PC-3 cells (Figure 2A). While etoposide (VP-16) induced DNA ladder formation indicative of apoptotic DNA fragmentation, GGCT knockdown did not induce DNA ladder formation (Figure 2B). Western blot analysis confirmed that GGCT knockdown did not induce apoptotic signaling (Figure 2C).

GGCT inhibition induced cellular senescence in PC-3 cells. We confirmed that the GGCT inhibitor pro-GA inhibited proliferation of PC-3 cells (Figure 3A and B), and examined whether GGCT inhibition induced senescence. Both β-galactosidase staining (Figure 3C) and β-galactosidase activity measurement (Figure 3D) confirmed that pro-GA induced senescence, consistent with prior findings that GGCT knockdown induces senescence (7).

GGCT inhibition enhances the antiproliferative effects of docetaxel. Assessments of cell growth demonstrated that the combination of GGCT knockdown with docetaxel had greater antiproliferative effects than either treatment alone (Figure 4A). Cell cycle analysis revealed an increase in the sub-G_1 population with docetaxel, which was not affected by GGCT knockdown alone (Figure 4B). Cell viability was assessed in cells treated with pro-GA, docetaxel, or combined treatment. Combination treatment with 50 μM pro-GA and 0.5 ng/ml docetaxel additively suppressed cell viability in PC-3 cells (Figure 4C, CI=0.916), while in LNCaP cells, a synergistic effect was observed with 40 μM pro-GA and 0.2 ng/ml docetaxel (Figure 4D, CI=0.774).

Discussion

Docetaxel is one of the most frequently applied chemotherapeutic agents for CRPC, and is considered a standard treatment. However, the prognosis for patients with advanced CRPC remains poor (14). In the present study, we identified that GGCT inhibition enhanced the antiproliferative effects of docetaxel in prostate cancer cells. Accordingly, GGCT expression was increased in prostate cancer tissues and cell lines compared with normal prostate tissues and cell lines. siRNA knockdown of GGCT enhanced the antiproliferative effects of docetaxel, indicating that GGCT expression may promote cell

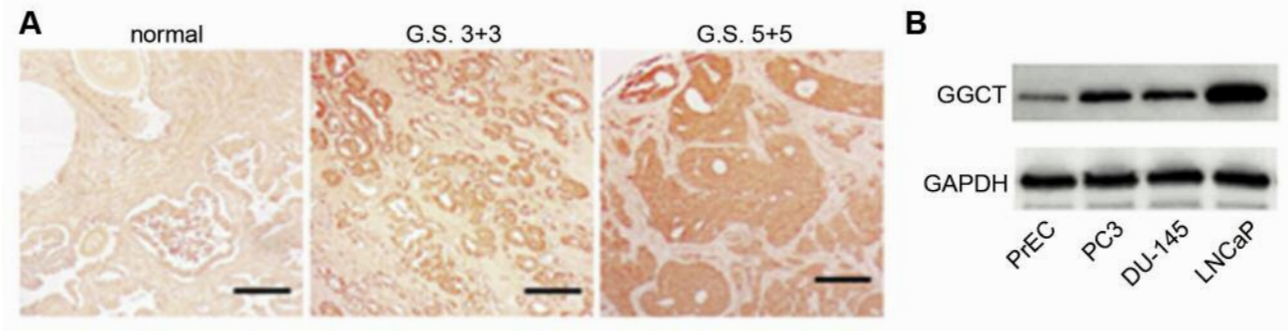

Figure 1. *GGCT is overexpressed in prostate cancer cells. (A) Representative images of immunohistochemical staining for GGCT in normal human prostate tissue and prostate cancer tissues (G.S., Gleason score). Scale bars, 200 μm. (B) Western blot analysis of GGCT and GAPDH in normal prostate epithelial cells (PrECs) and PC-3, DU-145, and LNCaP prostate cancer cells.*

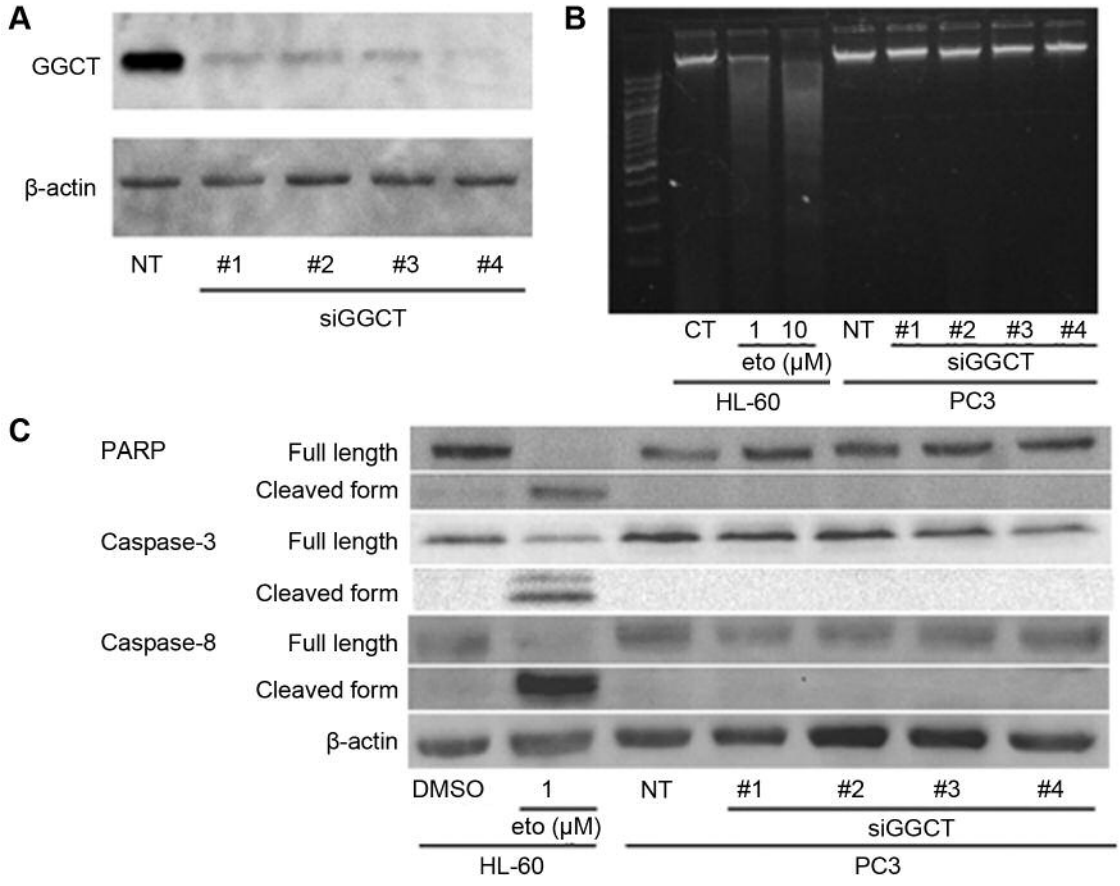

Figure 2. *GGCT knockdown did not activate apoptotic signaling. (A) Western blot analysis confirming efficient knockdown of GGCT. (B) Etoposide (eto)-treated HL-60 cells, but not PC-3 cells with GGCT knockdown, induced DNA ladder formation. (C) Western blot analysis of PARP, caspase-3, and caspase-8.*

survival in the context of docetaxel treatment. Overexpression of GGCT is known to increase cell growth under stress-induced microenvironments such as serum starvation. Further, GGCT knockdown induces autophagy signaling including AMPK-ULK1 in cancer cells, suggesting that GGCT depletion induces metabolic distress (9). Indeed, we recently confirmed that GGCT inhibition enhances the antiproliferative effects of mitomycin c in bladder cancer cells (13). Given the pivotal role

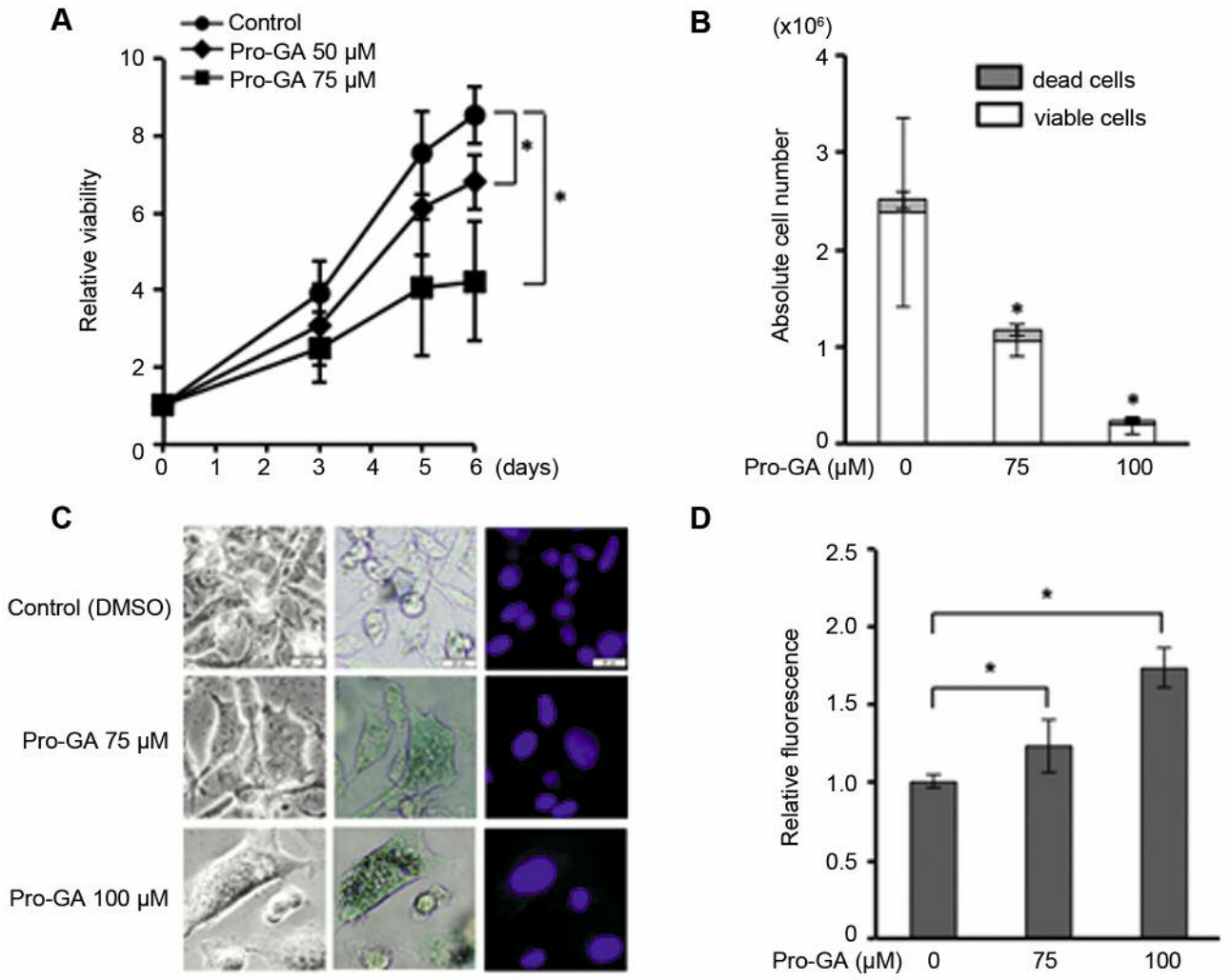

Figure 3. *The GGCT inhibitor pro-GA induced cellular senescence in PC-3 cells. (A, B) Assessment of cell growth by (A) cell-viability assay and (B) cell counting. (C) Representative images of β-galactosidase staining, and (D) measurement of β-galactosidase activity, in cells treated with pro-GA. Scale bars: 20 μm. *p<0.05.*

of GGCT in amino acid metabolism and glutathione homeostasis, it is conceivable that GGCT depletion causes metabolic distress, sensitizing cancer cells to chemotherapeutics.

Interestingly, GGCT is highly expressed in glioma tissues, and GGCT knockdown suppresses Notch-Akt in glioma cells (15). Similarly, GGCT is overexpressed in high-grade serous ovarian cancer, and GGCT knockdown suppresses the PI3K-Akt-mTOR pathway (16). Our group also reported that GGCT knockdown in PC-3 cells decreases AKT activation (9). Because the Akt pathway plays a critical role in resistance to docetaxel (17), these findings suggest that GGCT knockdown may potentiate the effects of docetaxel by inhibiting Akt signaling, although further investigation is required to elucidate the detailed mechanisms.

Pro-GA is a potent cell-permeable inhibitor of GGCT, which is effective *in vivo* (12). We first demonstrated that treatment with pro-GA induced cellular senescence in PC-3 cells, consistent with the GGCT knockdown findings. Taken together, to enhance the anticancer therapeutic effects and minimize enhancement of adverse effects, combined treatment with a GGCT inhibitor and an apoptosis inducer may improve the outcome of CRPC management.

Conflicts of Interest

The Authors declare no conflicts of interest pertaining to the present study.

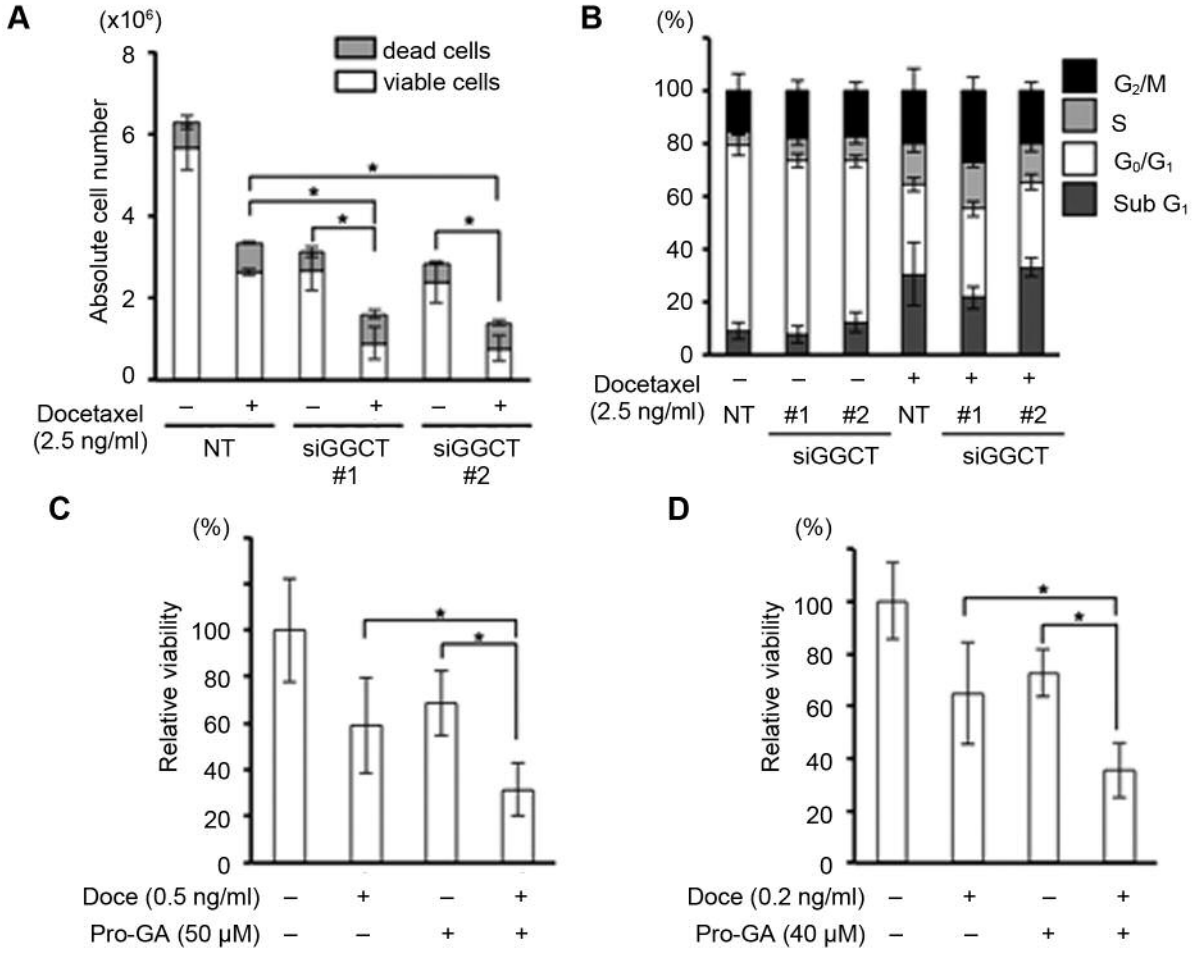

Figure 4. *GGCT inhibition enhanced the anticancer effects of docetaxel. (A) Cell count, and (B) cell-cycle distribution, of PC-3 cells with GGCT knockdown and/or docetaxel treatment. C, D: Cell viability of (C) PC-3 cells and (D) LNCaP cells treated with pro-GA and/or docetaxel (Doce). *p<0.05.*

Authors' Contributions

HT, KT, EH, and HI performed the experiments and drafted the manuscript. TY, SK, TC, and AK designed experiments. SN designed and supervised the study, and wrote the manuscript.

Acknowledgements

The Authors would like to thank the late Dr. Tatsuhiro Yoshiki for providing scientific support. This work was supported by JSPS grants 15K20084, 16K08722, and 18K09192, the MEXT Program for the Strategic Research Foundation at Private Universities 2015-2019, the Takeda Science Foundation, a Scholarship for Young/Women Researchers of the PMAC, and the KPU Fund for the Promotion of Collaborative Research.

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Received July 18, 2019
Revised July 23, 2019
Accepted July 24, 2019