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Ubiquitin ligase CHIP suppresses cancer stem cell properties in a population of breast cancer cells

Mai Tsuchiya [a], Yuka Nakajima [a,b], Naoya Hirata [c], Tamaki Morishita [a], Hiroyuki Kishimoto [a,b], Yasunari Kanda [c], Keiji Kimura [a,*]

[a] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572, Japan
[b] Life Science Center of Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572, Japan
[c] Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagya-ku, Tokyo 158-8501, Japan

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ABSTRACT

Cancer stem cells (CSCs) have several distinctive characteristics, including high metastatic potential, tumor-initiating potential, and properties that resemble normal stem cells such as self-renewal, differentiation, and drug efflux. Because of these characteristics, CSC is regarded to be responsible for cancer progression and patient prognosis. In our previous study, we showed that a ubiquitin E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP) suppressed breast cancer malignancy. Moreover, a recent clinical study reported that CHIP expression levels were associated with favorable prognostic parameters of patients with breast cancer. Here we show that CHIP suppresses CSC properties in a population of breast cancer cells. CHIP depletion resulted in an increased proportion of CSCs among breast cancers when using several assays to assess CSC properties. From our results, we propose that inhibition of CSC properties may be one of the functions of CHIP as a suppressor of cancer progression.

1. Introduction

Breast cancer is the most frequent cause of cancer death among women [1]. Although numerous therapeutic strategies have been developed for breast cancer, many problems with achieving complete remission remain to be resolved.

In a previous study we showed that a ubiquitin E3 ligase carboxyl terminus of Hsc70-interacting protein (CHIP) suppressed breast cancer malignancy [2]. CHIP is a tetratricopeptide repeat (TPR) containing protein that is expressed in a number of organs [3,4]. CHIP ubiquitinates misfolded proteins along with heat shock proteins and induces their degradation through the proteasome pathway, which is involved in protein quality control [5–7]. Moreover, CHIP participates in several biological processes through ubiquitination of specific target proteins [8–10]. In our previous study, we found that CHIP expression levels were negatively correlated with tumor malignancy in human breast tissues and suppressed tumor growth and metastasis of breast cancer cells [2]. In addition, a recent clinical study reported that CHIP expression levels were associated with favorable prognostic parameters of patients with breast cancer [11]. Thus, CHIP may be a novel target for breast cancer therapy. However, many issues remain to be addressed regarding the mechanism(s) of how CHIP is involved in breast cancer progression.

Recent studies suggested the importance of eliminating cancer stem cells (CSCs) to achieve complete remission [12,13]. CSCs have tumor-initiating potential [14] and high metastatic potential [15]. They also have properties that resemble those of normal stem cells, such as self-renewal, differentiation [16], and drug efflux [17]. Because of their self-renewal and differentiation properties, CSCs organize into a hierarchical structure and give rise to cancer heterogeneity [16], which hampers prediction of tumor behavior and clinical outcome [18]. Moreover, these cells' drug efflux capability results in resistance to conventional chemotherapies, which allows CSCs to survive even under anticancer drug treatment conditions [17]. Thus, understanding the biology of CSCs would contribute to the development of novel cancer therapies by overcoming problems encountered clinically, such as therapeutic resistance and relapse.

CSC properties can be assessed using different specific experimental systems; such as a sphere formation assay, a side

Abbreviations: CHIP, carboxyl terminus of Hsc70-interacting protein; CSC, cancer stem cell; SP, side population.

* Corresponding author at: University of Tsukuba, 1-1-1 Tenno-dai, Tsukuba Science City, Ibaraki 305-8577, Japan. Fax: +81 29 853 6439.
E-mail address: kekimura@tara.tsukuba.ac.jp (K. Kimura).

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population (SP) assay, and a limiting dilution xenograft assay. These assays are based on stem cell-like characteristics; including non-adherent growth capability [19] and drug efflux capacity [20], and on tumor-initiating potential [14]. To assess the non-adherent growth capability, the sphere formation assay is conducted [19]. In this assay, cancer cells are cultured under non-adherent conditions and form some spheres [21], after which the numbers of spheres are counted. To examine the drug efflux capacity mediated by transporters, the SP assay has been used [20]. In this assay, the proportions of SP cells are determined by evaluating the efflux capacity for the DNA-binding dye Hoechst 33342 using a fluorescence-activated cell sorter (FACS) [20]. To test the tumor-initiating potential, the limiting dilution xenograft assay is conducted [14,22]. While non-CSCs cannot form tumors by themselves, CSCs can initiate tumors *in vivo* [22]. This tumor-initiating potential is assessed by transplantation of serial diluted cancer cells into immunodeficient mice.

Numerous studies analyzed the CSC properties using breast cancer cell lines [21,23]. Clinically, breast cancer cells with stem cell phenotypes were also detected in bone marrow of breast cancer patients [24]. Thus, the presence of CSCs among breast cancer cells is becoming clear, but the precise regulation and characteristics of CSCs remains to be determined.

Here we show that depleting CHIP increases the proportions of CSCs among breast cancer cells by examining CSC properties using these assays. Thus, CHIP may contribute to a favorable prognosis for patients with breast cancer through its inhibition of CSC properties.

2. Materials and methods

2.1. Cell culture and treatment

MCF-7, MDA-MB-231 and T47D cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin Mixed Solution (Nacalai Tesque).

2.2. RNA interference

RNA interference experiment was performed as described in Kajiro et al. [2], with minor modification. We used a retroviral expression system. The pVSV-G vector and pSINsi-hU6 (Takara) vector containing either the *CHIP* or *LacZ* (control) target sequence were co-transfected into GP2-293 cells (Clontech). MCF-7 and T47D cells were incubated with the retroviral supernatant in the presence of 8 μg/ml polybrene. Twenty-four hours after infection, the viral supernatant was replaced with fresh DMEM containing 10% FBS. The infected cells were selected with 1 mg/ml G418. The target sequences were 5′-gcacgacaagtacatggcgga-3′ for *CHIP*, and 5′-gctacacaaatcagcgatt-3′ for *LacZ*.

2.3. Immunoblotting

Immunoblotting experiment was performed as described in Kajiro et al. [2]. Cells were lysed in 0.5% Triton buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and 0.5% TritonX-100]. Extracted proteins were separated by SDS–PAGE, transferred onto PVDF membranes (Millipore), immunoblotted with the indicated antibodies. The antibodies employed in this study included mouse monoclonal antibodies specific for β-Actin (1:1000; Santa Cruz, clone C4), and rat monoclonal antibodies against human CHIP (1:250; Green Space Biomed, Japan).

2.4. Sphere forming assay

MCF-7 cells were plated onto 6-well ultra-low-attachment plates (Corning Costar) at 1000 cells per well. MCF-7 cells were maintained in DMEM/Ham's F-12 (Nacalai Tesque) supplemented with 0.4% bovine serum albumin, 4 μg/ml bovine insulin (Sigma-Aldrich), 20 ng/ml EGF (Peprotech), and 1 × B27 supplement (Gibco). After 7 days, over 100 μm spheres were counted using BioZero BZ8000 microscope (Keyence). T47D cells were plated onto 6-well ultra-low-attachment plates at 5000 cells per well. T47D cells were maintained in CnT-27 medium (Cellntec). After 10 days culture, over 100 μm spheres were counted. MDA-MB-231 cells were plated onto 6-well ultra-low-attachment plates at 5000 cells per well. MDA-MB-231 cells were maintained in CnT-Prime medium (Cellntec). After 7 days culture, over 100 μm spheres were counted.

2.5. Side population (SP) analysis

Cells were removed from culture dish by Tripsin-EDTA solution. 1×10^6 cells were suspended in 970 μl Hanks' balanced salt solution (HBSS) with 2% fetal bovine serum (FBS) and 10 mM HEPES (pH 7.4). The cells were incubated at 37°C for 90 minutes with 17.5 μg/ml Hoechst 33342 (Sigma-Aldrich). Then, the cells were washed with 2% FBS in PBS, and resuspended in ice-cold HBSS with 2% FBS, 10 mM HEPES (pH 7.4), and 2 μg/ml propidium iodide (Sigma-Aldrich). Fumitremorgin C (FTC, 1 μM) was used to confirm the SP fraction. SP cells were analyzed with FACSAria (BD Biosciences). Collected events were analyzed using FlowJo (Tree Star).

2.6. Real-time RT-PCR

Real-time RT-PCR was performed as described in Kajiro et al. [2], with minor modification. Cells were homogenized in 1 ml of Sepasol-RNA I Super G (Nacalai Tesque) and total RNA was extracted according to the instruction manual and treated with DNase (Promega) for 30 min at 37°C. cDNA was synthesized from total RNA using RevatraAce reverse transcriptase (Toyobo) and random primers. The cDNA was amplified by rea-ltime PCR using Thermal Cycler Dice™ TP800 (Takara) and SYBR® Premix Ex Taq™ (Takara). Samples were normalized by *PPIA* mRNA levels. The primers for real-time PCR are as follows: 5′-acgtggtataaaaggggcgggag-3′ and 5′-tcaccaccctgacacataaaccctg-3′ for *PPIA*, 5′-atgcctgtgatttgtgggcc-3′ and 5′-gccagttgtttttctgccac-3′ for *NANOG*, and 5′-ggacccattggcattctc-3′ and 5′-caggacacagcatagaataatc-3′ for *CD133*.

2.7. Limiting dilution transplantation assay

NOD.CB17-*Prkdc*^{scid}/J (NOD/SCID) female mice at 5 weeks of age were purchased from Charles River Laboratories US. MCF-7 cells were cultured as monolayers, trypsinized, and resuspended in Matrigel (BD Biosciences) at dose ranging from 1.0×10^6 to 5.0×10^2 cells/ml. Female NOD/SCID mice were given bilateral subcutaneous injections of 0.1 ml MCF-7 cells. The mice were kept in a pathogen-free environment. After 6 weeks all of the mice were sacrificed, and the tumor tissues were collected. All animal experiments were in accordance with institutional guidelines.

2.8. Statistical analysis

Student's *t*-test was used to compare number of sphere (Fig. 1C and E) and mRNA levels (Fig. 3). Standard deviation and sample number were abbreviated as "S.D." and "*n*", respectively.

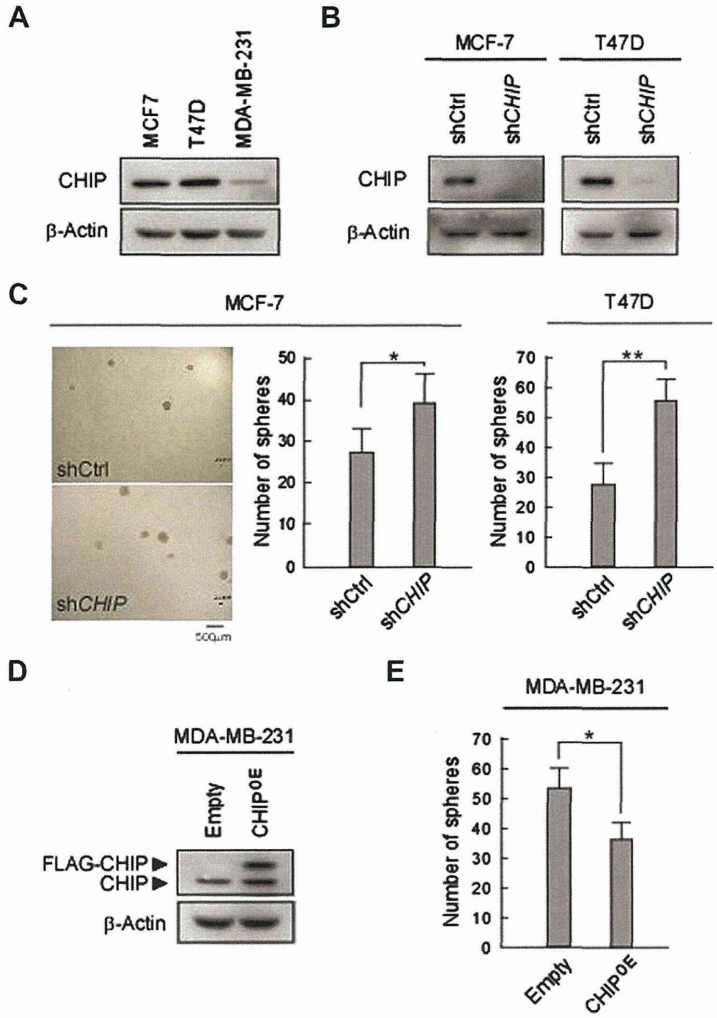

Fig. 1. CHIP inhibits sphere-forming capability. (A) Endogenous CHIP expression levels in MCF-7, T47D, and MDA-MB-231 cells. Total cell lysates of these cells were analyzed using the indicated antibodies. (B) CHIP depletion efficiency by shRNA. CHIP protein levels in MCF-7 and T47D cells infected with a retro virus containing shRNA against *LacZ* (shCtrl) and *CHIP* (sh*CHIP*) were analyzed by immunoblotting using the indicated antibodies. (C) Sphere-forming capabilities of MCF-7 and T47D shCtrl and sh*CHIP* cells. Representative photomicrographs (left panels) and numbers of spheres (right graphs) are shown. (D) CHIP protein levels in MDA-MB-231 cells introduced FLAG-CHIP ($CHIP^{OE}$) or empty vector (empty). Cells were generated as described by Kajiro et al [2]. CHIP expression levels were assessed using the indicated antibodies. (E) Sphere-forming capabilities of MDA-MB-231 empty and $CHIP^{OE}$ cells. Numbers of spheres (right graphs) are shown. Error bars are + S.D.; $n = 3$ for C and E. $^{*}p < 0.05$; $^{**}p < 0.01$.

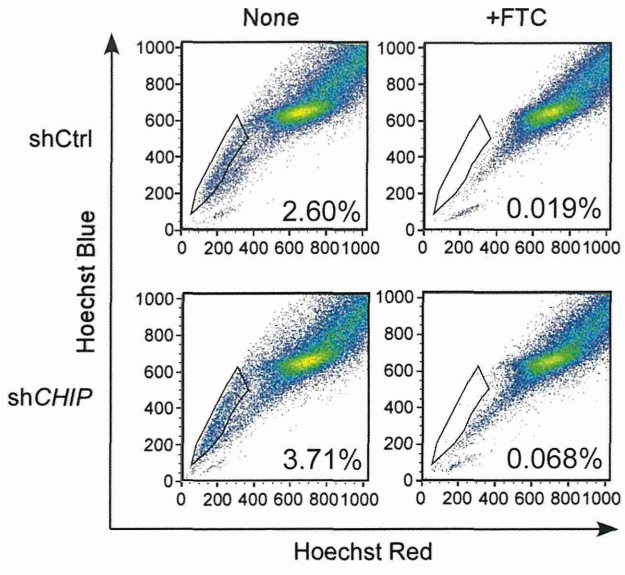

Fig. 2. CHIP depletion increases the proportion of SP cells. Fumitremorgin C (FTC) was used to confirm the SP fraction. SP cells were detected using FACS analysis.

3. Results

CHIP expression levels were previously found to be significantly associated with the prognosis of patients with breast cancer [11]. Consistent with this clinical observation, CHIP expression levels in MCF-7 and T47D cell lines, both non-aggressive human breast cancer cell lines, were much higher than in the MDA-MB-231 cell line, an aggressive triple-negative human breast cancer cell line (Fig. 1A). However, little is known regarding the mechanism(s) of how CHIP is involved in breast cancer progression. Therefore, we investigated the relationship between CHIP expression levels and CSC properties, as recent evidence suggests that CSCs are responsible for cancer progression and recurrence [12,13].

We first analyzed sphere-forming capability, which is one of the major indices used to define CSCs [19], to test the effects of CHIP depletion on CSC properties. When CHIP expression levels were reduced using shRNA for *CHIP* (sh*CHIP*) in MCF-7 and T47D cells (Fig. 1B), sphere formation assay results showed that sh*CHIP* cells more frequently formed spheres than did shCtrl cells both for MCF-7 and T47D cells (Fig. 1C). Alternatively, we used FLAG-tagged CHIP over-expressed MDA-MB-231 cells ($CHIP^{OE}$; Fig. 1D), as

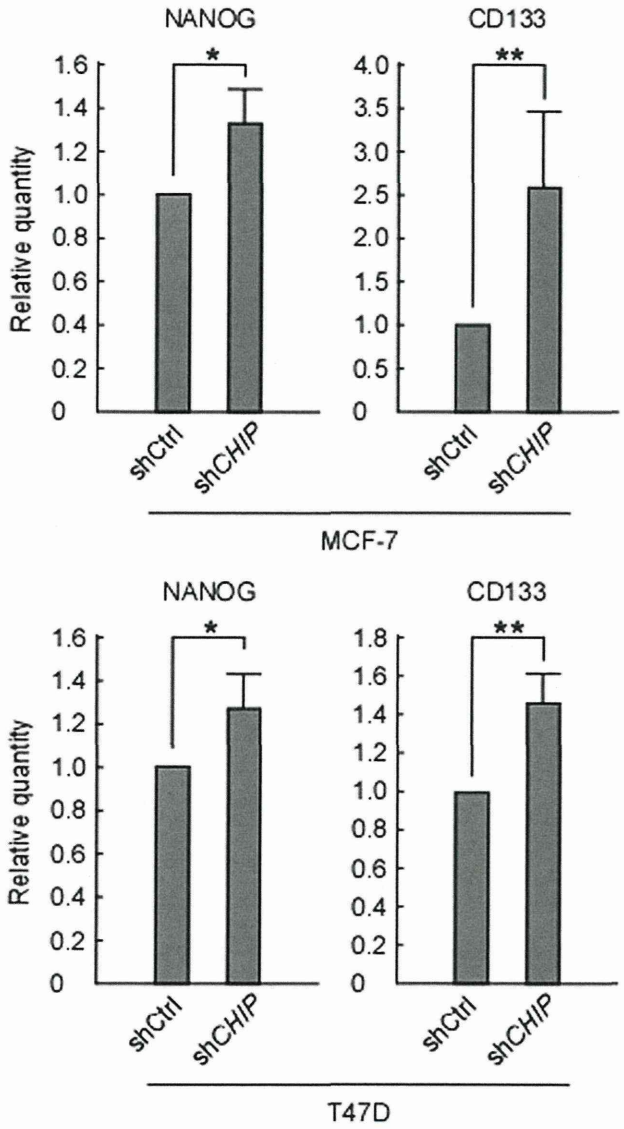

Fig. 3. mRNA levels of CSC related genes are enhanced in spheres after CHIP depletion. Error bars are +S.D.; $n \geqslant 3$. $^{*}p < 0.05$; $^{**}p < 0.01$.

described by Kajiro et al. [2], to further test the effect of CHIP expression on sphere-forming capability. Consistent with the CHIP depletion results, sphere-forming capability was reduced for CHIP^{OE} MDA-MB-231 cells (Fig. 1E). Taken together, these results (Fig. 1) indicated that CHIP expression levels were negatively correlated with sphere-forming capability, which suggested that CHIP suppressed CSC phenotypes.

We next analyzed the proportion of the SP fraction in sh*CHIP* cells to further assess whether CHIP suppressed CSC phenotypes. SP assay is one method used to assess CSC enrichment in several cancers, including breast cancer [25], hepatocellular carcinoma [26], ovarian cancer [27], and lung cancer [28]. SP cells, which have the ability to efflux the DNA-binding dye Hoechst 33342 out of the cell membranes, were detected using FACS analysis [20]. This showed that the proportion of the SP fraction in sh*CHIP* cells was markedly higher than that of shCtrl cells (Fig. 2); thus, CHIP depletion had increased the SP fraction in breast cancer cells.

We then tested whether CHIP depletion affected the expression levels of CSC-related genes. We isolated total RNAs from spheres derived from MCF-7 or T47D shCtrl and sh*CHIP* cells and analyzed the expression levels of some CSC-related genes. As a result, CHIP depletion increased the expression levels of self-renewal gene NANOG [29] and cell surface marker gene CD133 [23,29,30] in spheres (Fig. 3), suggesting that CSC-related genes such as NANOG and CD133 were involved in enhanced CSC properties by CHIP depletion.

Table 1
Tumor initiation capability of MCF-7 cells is enhanced by CHIP depletion.

Number of cell line	Number of cells injected				
	10^5	10^4	10^3	10^2	50
shCtrl	4/4	4/4	3/4	1/4	0/4
sh*CHIP*	4/4	4/4	4/4	4/4	1/4

Finally, we examined whether tumor-initiating potential was affected by CHIP expression levels, as numerous studies have indicated that CSCs have tumor-initiating potential [14]. To examine this, we conducted a limiting dilution xenograft assay using NOD/SCID mice. This showed that tumors were more effectively initiated in MCF-7 sh*CHIP* cells than in shCtrl cells (Table 1), which demonstrated that the tumor-initiating capability was increased in CHIP-depleted cells.

All of these results showed that CHIP depletion increased the CSC population among breast cancer cells (Figs. 1–3 and Table 1). Thus, CHIP may reduce breast cancer malignancy by suppressing CSC properties.

4. Discussion

In our previous study, we found that CHIP suppressed breast cancer metastasis and tumor growth. Moreover, a clinical study reported that CHIP expression levels were associated with a favorable prognosis for patients with breast cancer [11]. All of these findings suggest that CHIP suppresses breast cancer malignancy.

In this study, using several different assays, we found that CHIP depletion increased the CSC population among non-aggressive breast cancer cell lines that had higher CHIP protein levels, whereas CHIP over-expression reduced the CSC population in an aggressive breast cancer cell line that had lower CHIP protein expression. Thus, inhibition of CSC properties by CHIP may account for one aspect of CHIP as a suppressor of cancer progression, because CSCs have high metastatic [15] and tumor-initiating potentials [14]. Thus, it is possible that inhibition of CSC properties by CHIP could result in a favorable prognosis for patients with breast cancer with higher CHIP expression. However, we have not yet determined the molecular mechanism(s) of how CHIP suppresses CSC properties.

We consider that there are two possibilities for inhibition of CSC properties by CHIP, one is by quantity control and the other is quality control of proteins due to its ubiquitinating potential. Numerous studies have identified CSC-related genes, including transcription factors [31,32], receptors [33,34], and signaling factors [35,36]. Thus, the abundance of one or some of these CSC-related factors may be regulated by CHIP (quantity control), as CHIP regulates the quantity of its specific substrates by inducing protein degradation [2,9,10]. Alternatively, the accumulation of misfolded proteins due to CHIP depletion may cause for the changes in the expression of genes that function to maintain CSC properties, as CHIP regulates protein quality by inducing the degradation of misfolded proteins [5–7]. Our next goals will be to identify the target proteins of CHIP and elucidate the molecular mechanisms of how CHIP suppresses CSC properties.

In this study, we showed that CHIP suppressed not only breast cancer metastasis and tumor growth [2], but also CSC properties. Thus, developing methods to enhance CHIP expression levels may provide an efficient therapy for patients with breast cancer.

Therefore, understanding the relationship between CHIP expression and CSC properties and regulating its expression may provide important clues for developing a novel breast cancer therapy.

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Full Paper

Assessment of Testing Methods for Drug-Induced Repolarization Delay and Arrhythmias in an iPS-Derived Cardiomyocyte Sheet: Multi-site Validation Study

Yuji Nakamura[1], Junko Matsuo[1,2,3], Norimasa Miyamoto[4], Atsuko Ojima[4], Kentaro Ando[1], Yasunari Kanda[2], Kohei Sawada[4], Atsushi Sugiyama[1,*a], and Yuko Sekino[2,5,*b]

[1]*Department of Pharmacology, Faculty of Medicine, Toho University, Tokyo 143-8540, Japan*
[2]*Division of Pharmacology, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[3]*Drug Safety Research Laboratories, Shin Nippon Biomedical Laboratories, Ltd., Japan*
[4]*Biopharmaceutical Assessments Core Function Unit, Eisai Product Creation Systems, Eisai Co., Ltd., Japan*
[5]*Department of Neurobiology and Behavior, Gunma University Graduate School of Medicine, Gunma 371-8511, Japan*

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Abstract. A prospective comparison study across 3 independent research laboratories of a pure I_{Kr} blocker E-4031 was conducted by using the same batch of human iPS cell–derived cardiomyocytes in order to verify the utility and reliability of our original standard protocol. Field potential waveforms were recorded with a multi-electrode array system to measure the inter-spike interval and field potential duration. The effects of E-4031 at concentrations of 1 to 100 nM were sequentially examined every 10 min. In each facility, E-4031 significantly prolonged the field potential duration corrected by Fridericia's formula and caused early after-depolarizations occasionally resulting in triggered activities, whereas it tended to decrease the rate of spontaneous contraction. These results were qualitatively and quantitatively consistent with previous non-clinical in vitro and in vivo studies as well as clinical reports. There were inter-facility differences in some absolute values of the results, which were not observed when the values were normalized as percentage change. Information described in this paper may serve as a guide when predicting the drug-induced repolarization delay and arrhythmias with this new technology of stem cells.

Keywords: E-4031, iPS cell–derived cardiomyocyte, multi-site validation, field potential, TdP

Introduction

Drug-induced proarrhythmia has been a major safety concern about the development of new drugs, leading to issuing of ICH E14 and S7B guidelines in May 2005 (1, 2). The guidelines have effectively reduced risks of a new compound causing torsades de pointes, whereas non-clinical and clinical studies in the current approach still remain imperfect because they identify many drugs as being "positive" despite a lack of demonstrable pro-arrhythmic risk (3 – 6). In a recent workshop held in July 2013 by the US Food and Drug Administration (FDA), the Cardiac Safety Research Consortium and the non-profit Health and Environmental Sciences Institute (HESI), a new paradigm was proposed and discussed, focusing on a comprehensive assessment of multi ion channel effects to determine actual proarrhythmic risk of drugs (7). This new approach will include a stem-cell technology that has the potential to improve the currently used assessment of cardiotoxicity; however, more work is required prior to the use of stem cell–derived cardiomyocyte models to accurately predict proarrhythmias in humans (7).

There have been a large number of various studies with stem cell–derived cardiomyocytes examining electrophysiological effects of drugs (8 – 13). In an effort to further improve upon the assay system, this report describes a more simple and reliable protocol of an induced pluripotent stem (iPS) cell–derived, cardio-

Corresponding authors.
*[a]atsushi.sugiyama@med.toho-u.ac.jp, *[b]yukos@nihs.go.jp
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myocyte-sheet model. Extensive preliminary studies have confirmed that the protocol proposed in this paper could be optimal for assessing E-4031-induced repolarization delay and arrhythmias and would qualitatively and quantitatively reflects its electropharmacological profile in humans. This is a critically new finding and a significant improvement over the previous in vitro I_{Kr} assay systems including the hERG potassium channels and the papillary muscle of guinea pigs. In this study, in order to start verifying the reproducibility of our protocol, a prospective comparison study of E-4031 was conducted across 3 independent research laboratories with the same batch of iPS cell–derived cardiomyocytes.

Materials and Methods

Cell culture and plating

Each facility (E, N, T) obtained the same batch (#1089404) of cryopreserved human iPS cell–derived cardiomyocytes [iCells; Cellular Dynamics International (CDI), Madison, WI, USA]. The cells were thawed in specially prepared medium (Plating Media, CDI), which were plated onto 0.1% gelatin-coated, 6-well tissue-culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of $1.3 - 2.6 \times 10^6$ (E: 1.3×10^6, N: 2.0×10^6, T: $2.4 - 2.6 \times 10^6$) of cells per well. Two days after plating, Plating Media was replaced to specially prepared culture medium (Maintenance Media, CDI). Then, the culture medium was changed with fresh one every 2 days. The cells were cultured for 3.7 ± 1.4 days (2 – 7 days) after thawing at 37°C with 5% CO_2 prior to re-plating.

The electrical activity of cardiomyocytes was measured by using our original protocol. Briefly, the recording area of probes with 64 of the recording electrodes (MED probe; MED-P515A, Alpha Med Scientific, Osaka) of the MED64 System (Alpha Med Scientific) was coated with 2 μL of fibronectin (50 μg in 1 mL of distilled water), which was incubated at 37°C for $\geq$ 1 h. The cells cultured in the 6-well tissue-culture plates were dispersed with 0.25% trypsin-EDTA or TrypLE Select, which were re-plated onto the MED probes at a density of 3×10^4 cells in a 2 μL of the culture medium. The cells were incubated at 37°C with 5% CO_2 for 2 – 18 h (E: 4 – 12 h, N: 2 – 18 h, T: 12 – 18 h) in moisture condition prior to filling each probe with 1 mL of the culture medium. The half volume or all of the culture medium of the probes was changed with the culture medium, which had been warmed to 37°C, every 2 days thereafter. The cells were cultured for 5.2 ± 1.6 days (3 – 7 days) to obtain a sheet of cardiomyocytes with spontaneous and synchronous electrical automaticity.

Field potentials (FPs) assay

Maintenance Media was used as a culture medium throughout the experiment. Prior to the measurement of FPs, cardiomyocyte sheets were equilibrated for $\geq$ 30 min in the CO_2 incubator in 1 or 2 mL of fresh culture media. After equilibration, the probes were kept at 36°C – 37°C with thermo-control systems and covered with a lid, through which aeration of 95% O_2 / 5% CO_2 gas was provided. FPs from spontaneously beating cardiomyocyte sheets were recorded and digitized at 20 kHz by using the MED64 System. The stability and constancy of the waveforms, inter-spike interval, and field potential duration (FPD) were confirmed for $\geq$ 20 min. FPD was defined as an interval from the initial sharp deflection to the peak of the dome (8). Using the information obtained in this observation period, we selected 3 – 6 electrodes, which would be suitable for continuous monitoring of the FP configuration consisting of spike and dome. After recording the basal control state, the effects of 1, 3, 10, 30, and 100 nM of E-4031 were assessed by adding stock solution cumulatively to the culture medium to obtain target concentrations. The final concentration of DMSO was limited to be $< 0.6\%$, since DMSO at a concentration of $< 0.6\%$ has been reported to hardly affect any of the variables assessed in this study (8). At each concentration, the FP was recorded for $\geq$ 10 min and the last 30 beats were extracted as a dataset to analyze waveforms, inter-spike interval, and FPDs according to the previous report (8). The datasets of concentrations were excluded from the statistical analysis, when early after-depolarization and/or triggered activity were observed. Early after-depolarization was defined as deflection occurring at the plateau of the dome, and sharp deflection originating from early after-depolarization was judged as a triggered activity. FPD was corrected with Fridericia's formula, which was defined as the primary method of correction in this study [$FPDcF = FPD / (\text{inter-spike interval} / 1000)^{1/3}$] (14). The values of inter-spike interval and FPDcF from the last 30 waves at each concentration were averaged.

Drugs and chemicals

E-4031 was obtained from WAKO (Osaka) or synthetized at Eisai Co., Ltd. (Tsukuba). Gelatin was obtained from Sigma (St. Louis, MO, USA). Fibronectin was obtained from Becton Dickinson or Invitrogen (Carlsbad, CA, USA). Trypsin-EDTA and TrypLE Select were obtained from Invitrogen.

Data analyses and statistical assessment

In each experiment, one electrode that satisfied the following two conditions was chosen: 1) FP was recorded whole through the experiment, and 2) The amplitude of

the dome was the largest. The data were expressed as the mean ± S.E.M. The effects of the drug on inter-spike interval and FPDcF obtained in each facility were evaluated with the paired *t*-test or one-way repeated-measures analysis of variance (ANOVA) followed by Contrasts for mean values comparison between the baseline value (0 nM) and others. Meanwhile, inter-facility variability was assessed with one-way factorial ANOVA followed by Fisher's test or unpaired *t*-test. A P value < 0.05 was considered statistically significant.

Results

The effects of E-4031 in concentrations of 0, 1, 3, 10, 30, and 100 nM were examined in each facility, except that 1 nM was not performed in facility N. The number of preparations that can be used for the assessment of inter-spike interval, field-potential duration, and categorical analysis decreased due to the onset of early after-depolarization and/or triggered activity as the concentration of drug increased.

Inter-spike interval

The effects of the drug on the inter-spike interval (ms) are summarized in Fig. 1 (upper panel). The baseline values (0 nM) were 926 ± 44 ms in facility E, 1,216 ± 56 ms in facility N, and 956 ± 22 ms in facility T. Inter-facility difference was observed between N and E besides between N and T, which was not detected between E and T. No significant change from the respective baseline values was detected at 1, 3, or 10 nM in E and T and at 3 nM in N. Inter-facility difference was observed at 3 nM between N and E besides between N and T, which was not detected at any concentration between E and T. Meanwhile, the effects of the drug on the inter-spike interval (%) are summarized in Fig. 1 (lower panel). The significant increase was observed at 3 nM in N, which was not detected at 1, 3, or 10 nM in E or T, although the similar trend was observed. Inter-facility difference was not detected at any concentration.

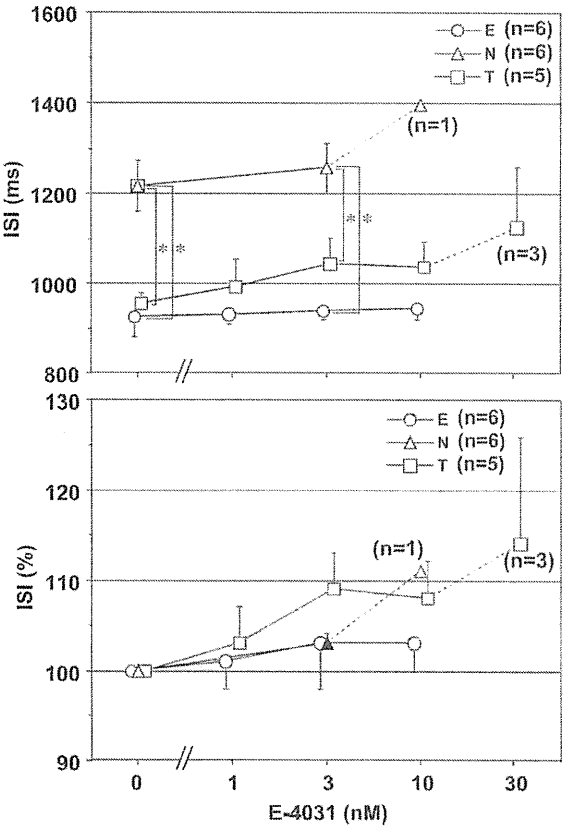

Fig. 1. Summary of the results showing the actual measurement values (upper) and their percentage changes (lower) in inter-spike interval (ISI) of E-4031 in human iPS cell-derived cardiomyocytes in each facility. Each value represents the mean ± S.E.M. of 6 preparations for facility E and facility N and 5 preparations for facility T. Values in parentheses represent the number of the preparations. An asterisk indicates significant difference between the facilities, whereas a closed symbol represents significant change from the respective baseline value.

Prolongation of field-potential duration

The effects of the drug on the FPDcF (ms) are summarized in Fig. 2 (upper panel) and typical tracings of field potential before and after the drug treatment are depicted in Fig. 3. The baseline values (0 nM) were 430 ± 12 ms in E, 443 ± 5 ms in N, and 320 ± 15 ms in T. Inter-facility difference was detected between T and E besides between T and N, which was not detected between E and N. FPDcF was prolonged at 3 and 10 nM in E and at 10 nM in T, which tended to be prolonged at 3 nM in N without statistical significance. Inter-facility difference was detected at 1, 3, and 10 nM between E and T and at 3 nM between N and T. Inter-facility difference was not detected at any concentration between E and N. Meanwhile, the effects of the drug on the FPDcF (%) are summarized in Fig. 2 (lower panel). FPDcF was prolonged at 3 and 10 nM in E and at 10 nM in T, whereas it tended to be prolonged at 3 nM in N without statistical significance. Inter-facility difference was not detected at any concentration.

Incidence of early after-depolarization or triggered activity

The incidence of early after-depolarization or triggered activity is summarized in Table 1 and typical tracing of field potential with triggered activity is depicted in

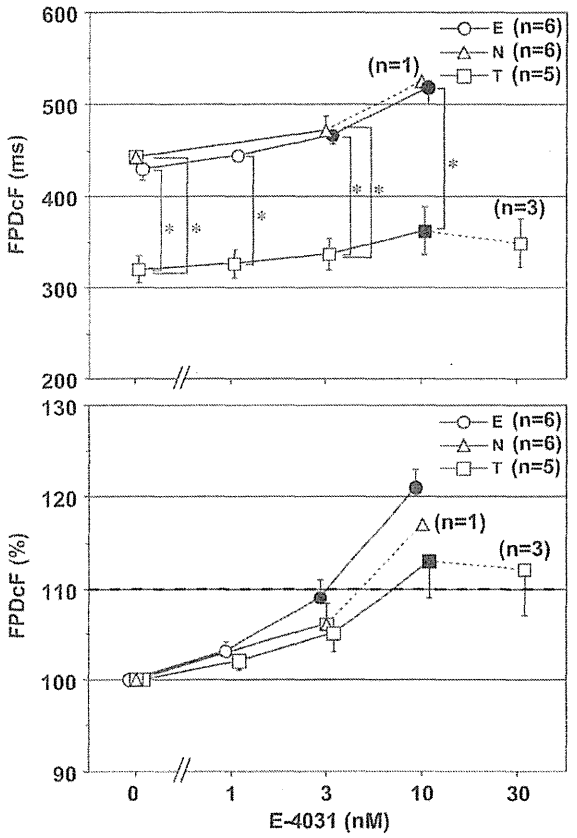

Fig. 2. Summary of the results showing the actual measurement values (upper) and their percentage changes (lower) in FPDcF of E-4031 in human iPS cell-derived cardiomyocytes in each facility. Each value represents the mean ± S.E.M. of 6 preparations for facility E and facility N and 5 preparations for facility T. Values in parentheses represent the number of the preparations. An asterisk indicates significant difference between facilities, whereas a closed symbol represents significant change from the respective baseline value.

Fig. 3. Early after-depolarization or triggered activity was induced at ≥ 10 nM in N and at ≥ 30 nM in E and T.

Categorical analysis of FPDcF

The results of categorical analysis of absolute FPDcF (ms) are summarized in Table 2. At the baseline and 1 nM, all FPDcF in each facility were categorized in ≤ 480 ms, whereas FPDcF of ≥ 500 ms was observed at ≥ 3 nM in N and at ≥ 10 nM in E, which was not observed in T.

The results of categorical analysis of ⊿FPDcF are summarized in Table 3. At 1 nM, all ⊿FPDcF in E and T were categorized in ≤ 60 ms. ⊿FPDcF of > 60 ms was observed at ≥ 3 nM in E and N and at ≥ 10 nM in T.

Discussion

In this study, a prospective comparison study of E-4031 was conducted with the same batch of human iPS cell-derived cardiomyocytes in order to start verifying the reproducibility of our original standard protocol across 3 independent research laboratories. We demonstrated that the protocol can be reliable in detecting the drug-induced repolarization delay and arrhythmias with high reproducibility.

E-4031 tended to show a negative chronotropic effect at concentrations of ≥ 3 nM; however, a significant change was detected only at 3 nM in facility N when assessed by percentage change (Fig. 1). A more potent negative chronotropic effect was observed by higher concentrations of E-4031 in each facility, although we did not perform the statistical analyses on inter-spike interval at concentrations of ≥ 10 nM in facility N and ≥ 30 nM in facilities E and T because of the limited number of experiments (n = 0 – 3). These results are in good accordance with a previous observation in patients with supraventricular tachyarrhythmias (15), in which E-4031 at a plasma concentration of 4.85 ± 1.35 ng/ml (11 nM) modestly prolonged RR interval, but it did not achieve statistical significance. Meanwhile in the single sinoatrial nodal cells of rabbits, E-4031 at a concentration of 100 nM suppressed or blocked the spontaneous activity (16), and moreover in the Langendorff-perfused whole hearts of guinea pig, E-4031 at concentrations of 30 – 300 nM or 5 μM significantly reduced the heart rate (17, 18). Thus, our testing method using the human iPS cell-derived cardiomyocytes can be considered to be more sensitive than currently available in vitro non-clinical models in detecting the E-4031-induced negative chronotropic effect.

E-4031 caused early after-depolarization and/or triggered activity in a concentration-related manner as shown in Fig. 3 and Table 1. In previous studies using the Langendorff-perfused rabbit heart, 0.5 μM of E-4031 induced early after-depolarization and triggered activity (19, 20). Also, in human embryonic stem cell-derived cardiomyocyte clusters, 1 μM of E-4031 induced early after-depolarization in half of the clusters (11). Meanwhile, in the human engineered heart tissue sheet made of the human embryonic stem cells, 10 nM of E-4031 was reported to induce arrhythmias (12). Thus, our testing method as well as the previous human engineered heart tissue sheet is considered to have higher sensitivities than the human cardiomyocyte clusters or the Langendorff-perfused rabbit heart in detecting E-4031-induced early after-depolarization and/or triggered activity.

E-4031 prolonged the FPDcF in a concentration-related manner as shown in Figs. 2 and 3. A wide variety

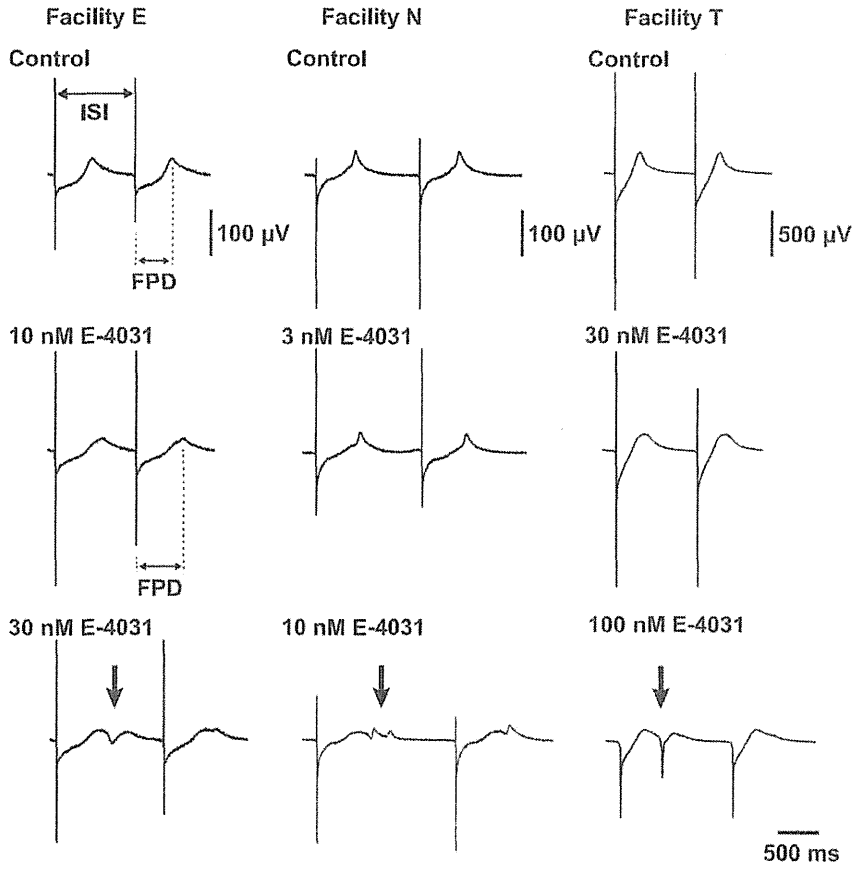

Fig. 3. Typical tracings of the field potential from each facility. Upper traces show the field potential at control (Control), middle traces indicate the prolongation of field potential duration (FPD) with 3 – 30 nM of E-4031, and lower traces represent the onset of early after-depolarization or triggered activity with 10 – 100 nM E-4031 (arrow). ISI: inter-spike interval.

Table 1. Incidence of E-4031-induced early afterdepolarization or triggered activity

Concentration (nM)	Facility			All
	E	N	T	
0	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
1	0% (0/6)	NT	0% (0/5)	0% (0/11)
3	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
10	0% (0/6)	83% (5/6)	0% (0/5)	29% (5/17)
30	100% (6/6)	100% (6/6)	40% (2/5)	82% (14/17)
100	100% (6/6)	100% (6/6)	100% (5/5)	100% (17/17)

Note: The numerator in parentheses shows the number of preparations exerting early after-depolarization or triggered activity, whereas the denominator indicates the total number of preparations assessed. NT: Not tested.

of analyses have been performed to clarify the effects of E-4031 on the repolarization process as summarized in Table 4 (12, 15, 17 – 26). The effects of E-4031 in these previous reports are directionally the same as the currently observed results, although their potency varied greatly. Thus, the present findings suggest that the sensitivity of current testing method to detect drug-induced repolarization delay can be considered to be comparable to hERG assay and human subjects, but it may be higher than those of in vivo and in vitro animal models.

Table 2. Summary of categorical analysis of absolute FPDcF values

Concentration of E-4031 (nM)	FPDcF (ms)	Facility			All
		E	N	T	
0	≤ 450	67% (4/6)	83% (5/6)	100% (5/5)	82% (14/17)
	> 450	33% (2/6)	17% (1/6)	0% (0/5)	18% (3/17)
	> 480	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
	> 500	0% (0/6)	0% (0/6)	0% (0/5)	0% (0/17)
1	≤ 450	50% (3/6)	NT	100% (5/5)	73% (8/11)
	> 450	50% (3/6)		0% (0/5)	27% (3/11)
	> 480	0% (0/6)		0% (0/5)	0% (0/11)
	> 500	0% (0/6)		0% (0/5)	0% (0/11)
3	≤ 450	17% (1/6)	17% (1/6)	100% (5/5)	41% (7/17)
	> 450	50% (3/6)	50% (3/6)	0% (0/5)	35% (6/17)
	> 480	33% (2/6)	17% (1/6)	0% (0/5)	18% (3/17)
	> 500	0% (0/6)	17% (1/6)	0% (0/5)	6% (1/17)
10	≤ 450	17% (1/6)	0% (0/1)	80% (4/5)	42% (5/12)
	> 450	0% (0/6)	0% (0/1)	20% (1/5)	8% (1/12)
	> 480	17% (1/6)	0% (0/1)	0% (0/5)	8% (1/12)
	> 500	67% (4/6)	100% (1/1)	0% (0/5)	42% (5/12)
30	≤ 450	— (0/0)	— (0/0)	100% (3/3)	100% (3/3)
	> 450	— (0/0)	— (0/0)	0% (0/3)	0% (0/3)
	> 480	— (0/0)	— (0/0)	0% (0/3)	0% (0/3)
	> 500	— (0/0)	— (0/0)	0% (0/3)	0% (0/3)

Note: The numerator in parentheses shows the number of preparations exerting respective FPDc values in each category, whereas the denominator indicates the total number of preparations assessed. FPDcF = FPD / (inter-spike interval / 1000)$^{1/3}$. NT: Not tested.

Table 3. Summary of categorical analysis of ΔFPDcF values

Concentration of E-4031 (nM)	ΔFPDcF (ms)	Facility			All
		E	N	T	
1	≤ 30	83% (5/6)	NT	100% (5/5)	91% (10/11)
	> 30	17% (1/6)		0% (0/5)	9% (1/11)
	> 60	0% (0/6)		0% (0/5)	0% (0/11)
3	≤ 30	67% (4/6)	67% (4/6)	100% (5/5)	76% (13/17)
	> 30	0% (0/6)	17% (1/6)	0% (0/5)	6% (1/17)
	> 60	33% (2/6)	17% (1/6)	0% (0/5)	18% (3/17)
10	≤ 30	0% (0/6)	0% (0/1)	60% (3/5)	25% (3/12)
	> 30	17% (1/6)	0% (0/1)	20% (1/5)	17% (2/12)
	> 60	83% (5/6)	100% (1/1)	20% (1/5)	58% (7/12)
30	≤ 30	— (0/0)	— (0/0)	33% (1/3)	33% (1/3)
	> 30	— (0/0)	— (0/0)	33% (1/3)	33% (1/3)
	> 60	— (0/0)	— (0/0)	33% (1/3)	33% (1/3)

Note: The numerator in parentheses shows the number of preparations exerting respective ΔFPDc values in each category, whereas the denominator indicates the total number of preparations assessed. ΔFPDcF: Increase from baseline in FPDcF. NT: Not tested.

Since 82% of the basal FPDcF was ≤ 450 ms, which is the upper limit of the normal range of QTc in human subjects, we examined the repolarization delays with the categorical analysis described in the ICH E14 guideline. FPDcF > 500 ms and/or ΔFPDcF > 60 ms were detected at concentrations of ≥ 3 nM in some preparations (Tables 2 and 3), indicating that 3 nM of E-4031 will be a critical concentration for inducing the excessive QT-interval prolongation by this testing method.

The major purpose of this study was to clarify the

Table 4. Summary of nonclinical and clinical reports regarding the effects of E-4031 on the repolarization markers

Model	Method	Marker	Change (%)	Concentration (nM)	References
hERG		IC_{50}		7.7	21
Guinea pig	Ventricular myocytes	APD_{90}	26	5,000	22
		APD_{90}	71	100	23
	Papillary muscles	APD_{90}	9 – 68	30 – 300	23
		APD_{90}	10	20	24
		$APD_{90(0.5Hz)}$	10	7	24
	Langendorff heart	QTc	5 – 27	3 – 300	18
		MAP_{90}	3 – 18	3 – 300	18
		QTc	26	5,000	17
Rabbit	Langendorff heart	QT	51	500	19
		MAP_{90}	50	500	19
Dog	In vivo (Anesthetized)	QT	57	6.2	25
		QTc	10	5.1	24
	In vivo (Conscious)	QTc	10	19.2	24
Monkey	In vivo (Conscious)	QTc	10	3.1	24
Human		QTc	14	12.1	15
		QTc	4.7 – 15	5.1 – 27	26
iPS cell	Single cell	APD_{90}	40 – 70	30 – 100	20
		APD_{90}	5 – 11	10 – 1,000	12
		$APD_{90(0.5Hz)}$	15 – 29	10 – 1,000	12

extent of the inter-facility difference in sensitivity and reliability of this new testing method. The concentrations of E-4031 that caused early after-depolarization and/or triggered activity were close to each other among the 3 facilities; however, there were some variations in the basal absolute values of inter-spike interval and FPDcF. Since we used the same batch of the cardiomyocytes, these differences might be induced by small inter-facility variability in the net culture period, the cell density on recording electrodes and/or experimental temperature. It should be noted that there was no difference in inter-spike interval or FPDcF among the 3 facilities when compared using percentage change.

In conclusion, we demonstrated that the use of the standardized protocol for the iPS cell–derived cardiomyocyte sheets can minimize inter-facility difference in detecting the drug-induced repolarization delay and arrhythmia. While further studies are needed to establish the currently proposed protocol including the assessment of variability across batches, more reference compounds and clinical predictability, information described in this paper may help predict the potential of the drug-induced repolarization delay and arrhythmias with this new technology. Also, methodological work for high-throughput evaluation is now ongoing.

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Conflicts of Interest

The authors declare no conflicts of interest.

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セッション2-2　ICH S7Bに関する今後の展望—新しいパラダイムの主な方向
(心臓安全性薬理学研究のためのiPS細胞由来心筋細胞)

ヒトiPS細胞を用いた成熟心筋細胞の開発

諫田泰成

Ⅰ．はじめに

ヒトiPS細胞の医療分野への応用として，大きく分けて「再生医療」と「創薬」があげられる[1]．再生医療への注目度は非常に高く，2013年にヒトiPS細胞から作製した網膜色素上皮細胞を移植する臨床研究が承認され，造腫瘍性など様々な観点から検証される予定である[2]．一方，創薬応用は，モデルとなる分化細胞を作製して*in vitro*のアッセイ系で使用するため，ウイルスなど様々な加工が可能であるが，医薬品の安全性や有効性を評価するためには，ヒト成体組織を反映した分化細胞が必要不可欠である．

ヒトiPS細胞は2007年の樹立から多くの知見が蓄積され，未分化ヒトiPS細胞には株間の差や継代数の差，研究室間における差などのバリエーションが存在することが明らかになり[3]，国際幹細胞イニシアティブなどによるプロジェクトにおいても標準化作業が進められているが，実用化において最も重要な分化細胞の標準化に関しては，思うように進んでいない．今後，分化誘導の標準プロトコールを整備して，分化細胞の品質基準などを定めることにより，実用化が加速すると思われる．

本稿では，ヒトiPS細胞から心筋細胞への分化誘導技術ならびに分化心筋細胞の薬理学的な特性を概説し，医薬品による催不整脈作用に対する応用可能性，さらにはICHガイドラインへの展望について議論したい．

Ⅱ．医薬品による催不整脈作用

医薬品によって発生する副作用の中で，torsade de pointes (TdP)とよばれる重篤な不整脈は重要である[4]．発生頻度は極めて少ないものの，心室細動に移行し突然死に至る症例が報告されている．TdPはQT間隔の延長を伴うことから，現在TdP誘発リスクは，非臨床試験として*in vitro*でカリウム電流(hERGチャネル)阻害作用を検討している．次いで*in vivo*で動物のQT延長作用を評価し，その後，臨床においてThorough QT/QTc試験により厳密にヒトのQT間隔に対する作用を調べることで，総合的にTdP誘発リスクを評価している．これらのガイドラインが整備された後は，薬剤性の心毒性に関して大きな問題は起きていないことから，一定の評価ができていると考えられる．しかしながら，hERG試験で有用な医薬品候補化合物を見落としてしまう可能性，TdP≠QT延長であること，hERG試験だけではわからない不整脈作用があることなど，問題点がいまだ残されている．iPS心筋細胞はヒトの細胞であり，さらにマルチチャネルを発現し

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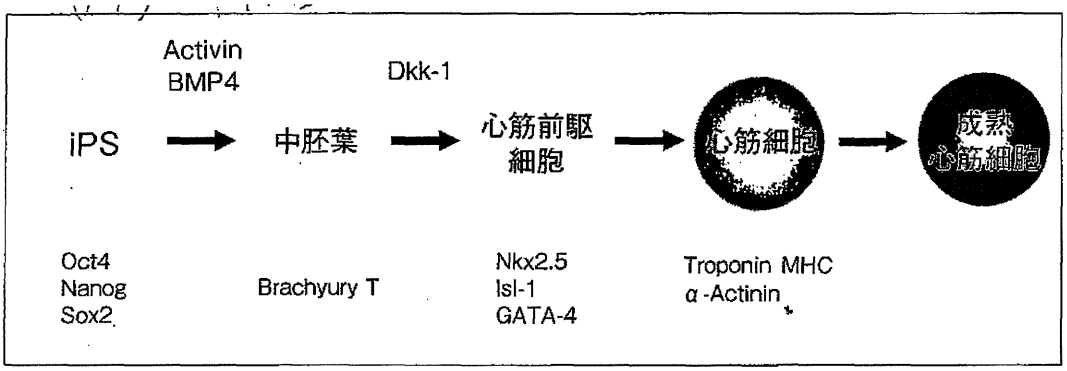

図1　ヒトiPS細胞から成熟心筋細胞の分化誘導法

ており総合的な評価が可能であることから[5]，hERG試験よりも予測性が向上するのではないか，と期待は大きい．

Ⅲ．ヒトiPS細胞を用いた心筋分化誘導法

ヒトiPS細胞の心筋分化誘導法は，EB形成法と定方向分化誘導法の2種類に大きく分類できる．ここでは，定方向分化誘導法に絞って述べる[6]．ヒトiPS細胞にサイトカインや増殖因子などを用いることにより，中胚葉，心筋前駆細胞，心筋細胞と段階的に分化誘導を行うことができる(図1)．具体的には，ヒトiPS細胞をマトリゲルでコートしたディッシュに高密度で播種して数日間培養をした後，ActivinとBMP-4により中胚葉へ分化誘導する．次に，Wnt antagonistであるDickkopf-1(Dkk-1)など，Wntシグナル修飾剤を用いて心筋前駆細胞に分化させる．さらに，心筋前駆細胞はVEGFなどの増殖因子の存在下で培養することにより，心筋細胞に分化して自律的な拍動が観察される．

このように作製されたiPS心筋細胞は，未成熟であると考えられている．パッチクランプにより電気生理学的特性を検討すると，静止膜電位は通常-90 mV程度であるのに対して，iPS心筋細胞はいずれも-50 mV程度と浅い．米国Cellular Dynamics International社(CDI)から販売されているiCell心筋細胞も同様の傾向を示したことから，元のiPS細胞株や分化誘導法に依存せず，iPS心筋細胞に共通の課題であると考えられる．

医薬品の作用を評価する上で，成熟した心筋が必要であるのかはわかっていない．すでに心筋の成熟化を促進するためのアプローチとして，電気刺激など様々な手法が検討されている[7]．われわれは成体心室筋と比較して，iPS心筋細胞において不足している因子に着目し，心筋細胞の成熟化を誘導できる方法を明らかにしている(論文投稿中)．われわれが検討した範囲では，アデノウイルスの感染効率は，未分化よりも分化が進んだiPS細胞のほうが高かったことから，iPS心筋細胞にアデノウイルスを用いて遺伝子導入することにより，成熟化を誘導している．図2の模式図に示したように，E-4031によるAPD延長は，成熟心筋細胞のほうが有用であるという予備的な結果を得ており(図2)，一定レベルの成熟した心筋細胞が必要であると考えられる．今後，薬剤評価用にさらなる改良を行い，予測性の高いモデルへと発展させたい．

Ⅳ．iPS心筋細胞を用いた薬理試験

薬理試験に用いる分化心筋細胞の形状としては，個々の細胞，EBなどの細胞塊，シート状の細胞などがあげられる．個々の細胞の場合は，パッチクランプで解析が可能である．一つ一つの細胞の活動電位の波形をもとにQT間隔を評価するため，心筋細胞のサブタイプの情報も同時に得られるが，スループット性は極めて低い．さらには，個々の細胞のばらつきの問題が残されている．

一方，細胞塊やシート状の標本に関しては，多点

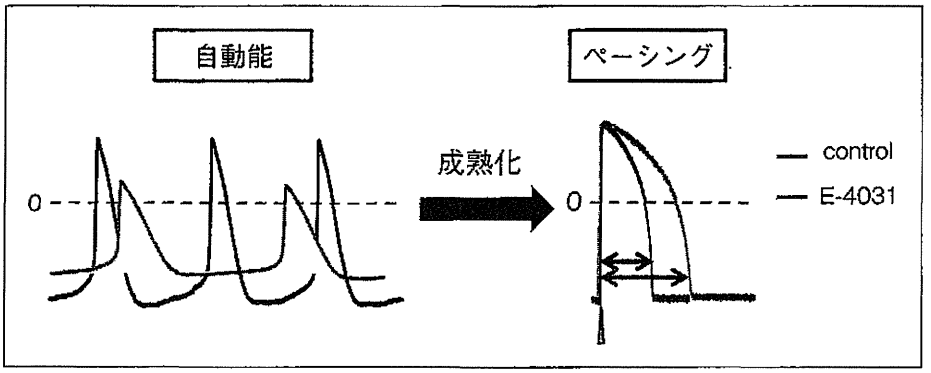

図2
成熟心筋細胞におけるE-4031の作用

電極システムを用いて，電極を埋め込んだディッシュに細胞塊やシートを接着させると，QT間隔に相当する細胞外電位(field potential：FP)の測定が可能である．個々の心筋細胞のばらつきが平均化されるため，比較的安定したデータが得られることが期待される．また，侵襲がないため，医薬品候補化合物の長時間曝露による薬理作用が調べられ，電気生理学特有の敷居の高さはなく，比較的簡便である．ただし，多点電極にしても，1日で解析できる数には限界があり，大規模なスクリーニングには向いていないので，改良が必要ではないかと思われる．

われわれは，iPS心筋細胞を用いた安全性薬理試験の確立を目指して，iCell心筋細胞をモデル細胞として用いて細胞外電位装置による評価を行った[8]．その結果，hERG阻害剤E-4031の添加により，濃度依存的にFP延長が観察された．さらに，E-4031により，triggered activityやEADなどの異常な波形を検出できることが明らかになった．こちらはFPD (FP duration)とは異なり，既存の*in vitro*評価系では検出できないような不整脈のリスク評価につながると考えられ，非常に興味深い．現在，われわれは成熟した心筋細胞を用いて，同様の検討を行っている．

V．ガイドラインに向けて

上述のように，iPS心筋細胞を使った安全性薬理試験系が確立し，催不整脈作用の総合的な理解につながることが明らかになれば，ICHのガイドラインへの追加も期待される．そのためには，多くの陽性対照物質や陰性対照物質を用いて，多施設間で検証作業を行い，科学的根拠を得る必要がある．日本としても明確なデータを用意しなければならない．

このような背景の下，2013年7月から，FDA/HESI/CSRCにおいてCiPA (Comprehensive *in vitro* Proarrhythmia Assay)の枠組みが発足し，薬剤による不整脈誘発リスク評価に関する国際的な議論も開始された[9),10)]．この動きはヒトiPS細胞に限定されたわけではないが，ヒト幹細胞由来心筋細胞のワーキンググループが本格的に活動を開始しており，iPS心筋細胞への期待がうかがえる．CiPAの議論を受けて，現行のhERG阻害/QT延長に基づく催不整脈リスク評価ガイドライン(S7B)とTQT試験(E14)の改訂につながる可能性があり，今後，国際協調を図っていく必要がある．

Ⅵ．まとめ

ヒトiPS細胞の分化誘導技術などの研究が進展し，またCiPAなどの国際的な議論も開始されたことにより，iPS心筋細胞を心毒性試験に応用する機運が高まってきている．実用化に向けては，ヒトiPS細胞の元の株間の差，iPS心筋細胞の特性解析や大量かつ安定した供給体制の確立，安全性薬理評価法の開発などを，一つ一つ丹念に地道に検証する作業が不可欠である．また，多施設間による協力体制も必要不可欠である．将来的には，日本発のヒトiPS細胞技術を用いて心毒性の発生を回避すること

が可能となり，より安全な医薬品が提供されることを期待したい．

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Metallomics

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Tributyltin induces mitochondrial fission through NAD-IDH dependent mitofusin degradation in human embryonic carcinoma cells

Shigeru Yamada,[a] Yaichiro Kotake,[b] Mizuho Nakano,[a] Yuko Sekino[a] and Yasunari Kanda*[a]

Organotin compounds, such as tributyltin (TBT), are well-known endocrine disruptors. TBT acts at the nanomolar level through genomic pathways *via* the peroxisome proliferator activated receptor (PPAR)/retinoid X receptor (RXR). We recently reported that TBT inhibits cell growth and the ATP content in the human embryonic carcinoma cell line NT2/D1 *via* a non-genomic pathway involving NAD^+-dependent isocitrate dehydrogenase (NAD-IDH), which metabolizes isocitrate to α-ketoglutarate. However, the molecular mechanisms by which NAD-IDH mediates TBT toxicity remain unclear. In the present study, we evaluated the effects of TBT on mitochondrial NAD-IDH and energy production. Staining with MitoTracker revealed that nanomolar TBT levels induced mitochondrial fragmentation. TBT also degraded the mitochondrial fusion proteins, mitofusins 1 and 2. Interestingly, apigenin, an inhibitor of NAD-IDH, mimicked the effects of TBT. Incubation with an α-ketoglutarate analogue partially recovered TBT-induced mitochondrial dysfunction, supporting the involvement of NAD-IDH. Our data suggest that nanomolar TBT levels impair mitochondrial quality control *via* NAD-IDH in NT2/D1 cells. Thus, mitochondrial function in embryonic cells could be used to assess cytotoxicity associated with metal exposure.

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Introduction

Growing evidence suggests that environmental organometals contribute to the observed increase in neurodevelopmental disorders, such as learning disabilities, autism spectrum disorder, behavioral abnormalities and teratogenicity.[1–3] Since the developing brain is more vulnerable to injury than the adult brain, exposure to these organometals during early fetal development can cause permanent or delayed neural disorders at much lower doses than in adults.[4–7] Therefore, it is necessary to elucidate the cytotoxic effects of organometals at low levels during development.

Organotin compounds, such as TBT, are well known to cause various types of cytotoxicity *via* genomic and non-genomic pathways. In the genomic pathway, nanomolar concentrations of TBT activate the retinoid X receptor (RXR) and/or peroxisome proliferator-activated receptor γ (PPARγ) and result in neurodevelopmental defects in mammals.[8,9] Conversely, many reports have shown that TBT at micromolar levels causes mitochondorial toxicity in the non-genomic pathway. For example, micromolar TBT and dibutyltin (DBT) levels have been shown to prevent mitochondrial respiration by inhibiting the electron transfer from complexes I and III, and Mg-ATPase activity.[10–12] The non-genomic effect of TBT mediates cell death in rat neurons. TBT induces neuronal death *via* AMPK activation and the phosphorylation of the mammalian target of rapamycin (mTOR) in rat cortical neurons.[13,14] TBT also induces neuronal degeneration *via* mitochondria-mediated ROS generation in rat neurons.[15]

We studied nanomolar TBT toxicity using neuronal precursor NT2/D1 cells as a model of the neurodevelopmental stage[16] and found that nanomolar TBT levels inhibit intracellular energy metabolism, including ATP production, *via* mitochondrial NAD^+-dependent isocitrate dehydrogenase (NAD-IDH), which catalyzes the irreversible conversion of isocitrate to α-ketoglutarate in the tricarboxylic acid (TCA) cycle.[17,18] Based on these observations, we hypothesized that nanomolar TBT levels affect mitochondrial functions, thereby altering the energy metabolism of neuronal precursor cells.[19]

Mitochondria continuously change their morphology through fission and fusion. These mitochondrial dynamics are an important quality control mechanism that maintains mitochondrial function, such as ATP production.[20] Mitochondrial fission and fusion are regulated by several GTPases. In mitochondrial fusion, mitofusins 1 and 2 (Mfn1, 2) and optic atrophy 1 (Opa1) induce the fusion of the outer and inner mitochondrial membranes,

[a] *Division of Pharmacology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku 158-8501, Japan. E-mail: kanda@nihs.go.jp; Fax: +81-3-3700-9704; Tel: +81-3-3700-9704*

[b] *Department of Xenobiotic Metabolism and Molecular Toxicology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Japan*

respectively.[21,22] The deletion of Mfn1 and Mfn2 in mice is embryonically lethal, and cells from these embryos contain fragmented and dysfunctional mitochondria.[23] In contrast, dynamin-related protein 1 (Drp1) is a cytoplasmic protein that assembles into rings surrounding the outer mitochondrial membrane, where it interacts with fission protein 1 (Fis1) to promote fission.[24,25]

In the present study, we have investigated the effect of TBT on mitochondrial quality control in NT2/D1 cells. We found that exposure to 100 nM TBT induced proteasomal degradation of Mfn and mitochondrial fragmentation through an NAD-IDH-dependent mechanism. Thus, impaired mitochondrial quality control is a novel mechanism of nanomolar level TBT-induced toxicity in human embryonic carcinoma cells.

Methods

Cell culture

NT2/D1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Ashrat, Israel) and 0.05 mg ml^{-1} of the penicillin–streptomycin mixture (Life Technologies, Carlsbad, CA, USA) at 37 °C in 5% CO_2.

Assessment of mitochondrial fusion

After treatment with TBT (100 nM, 24 h), the cells were fixed with 4% paraformaldehyde and stained with 50 nM Mito-Tracker Red CMXRos (Cell Signaling Technology, Danvers, MA, USA) and 0.1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI; Dojin, Kumamoto, Japan). Changes in the mitochondrial morphology were observed using confocal laser microscopy (Nicon A1). Images (n = 3–7) of random fields were obtained, and the number of cells displaying mitochondrial fusion (<10% punctiform) was counted in each image, as previously reported.[26]

Real-time PCR

Total RNA was isolated from NT2/D1 cells using the TRIzol reagent (Life Technologies), and quantitative real-time reverse transcription (RT)-PCR using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Valencia, CA, USA) was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), as previously reported.[27] The relative change in the amount of transcript was normalized to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primer sequences were used for real-time PCR analysis: human Drp1: forward, 5-TGGGCGCCGACATCA-3, reverse, 5-GCT CTGCGTTCCCACTACGA-3; human Fis1: forward, 5-TACGTCCG CGGGTTGCT-3, reverse, 5-CCAGTTCCTTGGCCTGGTT-3; human Mfn1: forward, 5-GGCATCTGTGGCCGAGTT-3, reverse, 5-ATTAT GCTAAGTCTCCGCTCCAA-3; human Mfn2: forward, 5-GCTCG GAGGCACATGAAAGT-3, reverse, 5-ATCACGGTGCTCTTCCCATT-3; human GAPDH: forward, 5-GTCTCCTCTGACTTCAACAGCG-3, reverse, 5-ACCACCCTGTTGCTGTAGCCAA-3.

Western blot analysis

Western blot analysis was performed as previously reported.[28] Briefly, the cells were lysed with cell lysis buffer (Cell Signaling Technology). The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Immobilon-P (Millipore, Billerica, MA, USA). The membranes were probed using the following antibodies: an anti-Mfn1 polyclonal antibody (1 : 1000; Cell Signaling Technology), an anti-Mfn2 monoclonal antibody (1 : 1000; Cell Signaling Technology), an anti-cytochrome c oxidase subunit IV (COX IV) monoclonal antibody (1 : 1000; Cell Signaling Technology), and an anti-β-actin monoclonal antibody (1 : 5000; Sigma-Aldrich). The membranes were then incubated with secondary antibodies against rabbit or mouse IgG conjugated to horseradish peroxidase (Cell Signaling Technology). The bands were visualized using the ECL western blotting analysis system (GE Healthcare, Buckinghamshire, UK), and images were acquired using a LAS-3000 imager (FUJIFILM UK Ltd., Systems, Bedford, UK).

Chemicals and reagents

Tributyltin chloride was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tin acetate (TA), rosiglitazone (RGZ), CD3254,

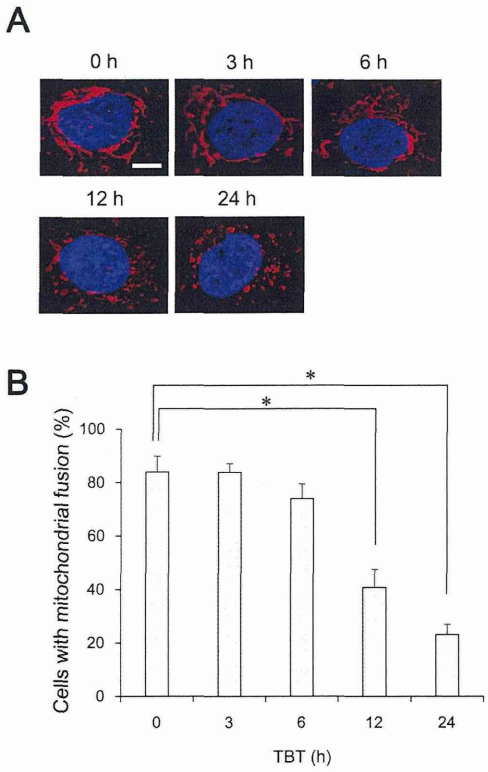

Fig. 1 Effect of TBT on the mitochondrial morphology in NT2/D1 cells. Cells were exposed to 100 nM TBT for 3, 6, 12, or 24 h. (A) The cells were stained with MitoTracker Red CMXRos and DAPI. Mitochondrial morphology was observed by confocal laser microscopy. Bar = 10 μm. (B) The number of cells undergoing mitochondrial fusion (<10% punctiform) was counted in each image. Data represent mean ± s.d. (n = 5). *P < 0.05.

apigenin, cycloheximide (CHX), carbonylcyanide m-chlorophenylhydrazone (CCCP), and MG132 were obtained from Sigma-Aldrich.

Statistical analysis

All data were presented as means ± S.D. ANOVA followed by a *post hoc* Tukey' test was used to analyze data in Fig. 1B, 2B, 3C, 4C, 5B, and 5C. Student's *t*-test was used to analyze data in Fig. 3A and 4B. *P*-values less than 0.05 were considered to be statistically significant.

Results and discussion

Effects of TBT on mitochondrial morphology

We have previously examined the effect of TBT (30–300 nM) on cell growth in NT2/D1 cells and found that TBT levels at the concentrations of 100 nM or more induced growth arrest in the cells.[17] Here we investigated whether 100 nM TBT affects mitochondrial dynamics in the cells. After exposure to 100 nM TBT for 12 h, we observed the increase in the number of cells with fragmented mitochondria, as compared to untreated control cells (Fig. 1A, B). After 24 h, the proportion of cells with mitochondrial fusion was nearly 80%. As a positive control, we used CCCP, which induces mitochondrial uncoupling and mitochondrial fission in other cells.[29] As expected, fragmented mitochondria were also observed following CCCP treatment for 24 h (Fig. 2A and B). In contrast, exposure to tin acetate (TA), which is less toxic, did not affect the mitochondrial morphology. To investigate whether TBT-induced mitochondrial fission was caused by changes in transcription, we treated the cells with the protein synthesis inhibitor cycloheximide. Treatment with cycloheximide did not alter the effects of TBT on the mitochondrial morphology (Fig. 2A and B). Moreover, rosiglitazone, an agonist of the TBT genomic target PPARγ, did not induce mitochondrial fragmentation. These results suggest that TBT induces mitochondrial fission through a non-genomic pathway in NT2/D1 cells.

TBT exposure induces proteasomal degradation of Mfn1 and 2

To examine the molecular mechanism by which TBT induces mitochondrial fragmentation, we assessed the effect of TBT on

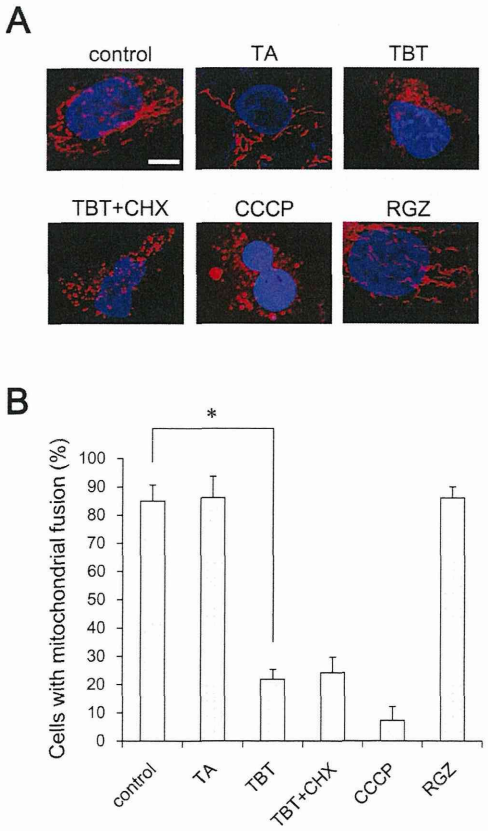

Fig. 2 Non-genomic effect of TBT-induced mitochondrial fission. Cells were exposed to 100 nM TA, 100 nM TBT, 100 nM TBT + 10 μg ml^{-1} cycloheximide (CHX), 1 μM CCCP or 100 nM rosiglitazone (RGZ) for 24 h. (A) The cells were stained with MitoTracker Red CMXRos and DAPI. Mitochondrial morphology was observed by confocal laser microscopy. Bar = 10 μm. (B) The number of cells undergoing mitochondrial fusion (<10% punctiform) was counted in each image. Data represent mean ± s.d. (n = 5). *P < 0.05.

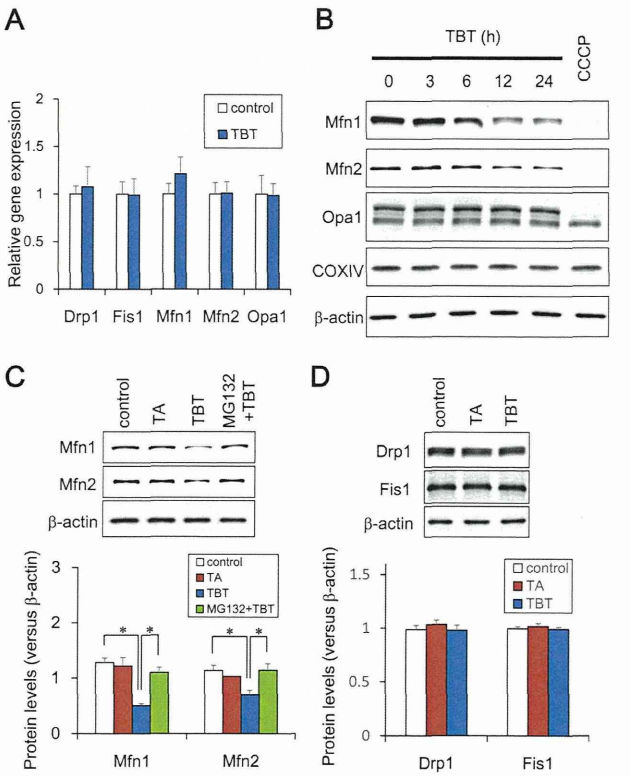

Fig. 3 Effect of TBT on mitochondrial protein levels in NT2/D1 cells. (A) After 24 h TBT exposure, the expression of mitochondrial genes was analyzed by real time PCR. The gene expression was not significantly altered by TBT exposure. (B) After TBT exposure for 3, 6, 12, or 24 h, mitochondrial proteins were analyzed by western blot using anti-Mfn1, Mfn2, Opa1, COXIV, or β-actin antibodies. (C) Cells were exposed to 100 nM TA, 100 nM TBT, or 100 nM TBT + 3 μM MG132 for 6 h. Mitochondrial proteins were analyzed by western blot using anti-Mfn1 or Mfn2 antibodies. (D) After 6 h TBT exposure, other mitochondrial proteins were analyzed by western blot using anti-Drp1, Fis1, or β-actin antibodies. Data represent mean ± s.d. (n = 3). *P < 0.05.