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Terahertz pulse-altered gene networks in human induced pluripotent stem cells

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Terahertz (THz) irradiation has been exploited in biomedical applications involving non-invasive manipulation of living cells. We developed an apparatus for studying the effects of THz pulse irradiation on living human induced pluripotent stem cells. The THz pulse of the maximum electric field reached 0.5 MV/cm and was applied for one hour with 1 kHz repetition to the entire cell–culture area, a diameter of 1 mm. RNA sequencing of global gene-expression revealed that many THz-regulated genes were driven by zinc–finger transcription factors. Combined with a consideration of the interactions of metal ions and a THz electric field, these results imply that the local intracellular concentration of metal ions, such as Zn^{2+}, was changed by the effective electrical force of our THz pulse.

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The ability to generate bright, intense terahertz (THz) pulses from high-intensity lasers has ushered in a new era of material laser spectroscopy, and is a novel way to control material properties [1–3]. Besides its use in studies on nonlinear optical phenomena in solid-state materials, THz pulse excitation has excellent potential to manipulate living cells in a non-invasive manner, because it drives the intra- and intermolecular vibrational modes of biological molecules, such as DNA, RNA, or proteins [4–9], movements of charged particles [2,10], and the generation of thermal effects [11,12], without physically disrupting the cell membrane. These effects are believed to cause changes in intracellular ions, such as metal ions and pH, gene expression, protein folding, biomolecule interactions, electron transfer, and enzymatic activity. In addition, because of the small photon energy (∼meV) of THz pulses, their use as an electric field or thermal effect for noncontact stimulation has advantages over conventional laser-based cell–manipulation methods that use higher energy ultra-violet or infrared ranges (∼eV), and cause catastrophic damage to DNA and other biological molecules by breaking covalent bonds [13].

Several studies have been conducted on the effects of THz pulses on living cells, and contradictory results have been reported [14–17]. One reason for this discrepancy could be the conditions of irradiation of the cultured cells. In most studies, a general cell culture dish is used; the area of the dish is much larger than that of THz irradiation. This difference often requires a scanning stage to expose the entire dish, but doing so introduces variability and reduces the total THz intensity.

Alternatively, the use of an unfocused and expanded THz light beam has been shown to result in the production of very weak electric fields. In contrast to the continuous THz wave sources obtained from a large facility, such as synchrotron radiation or free electron laser, single-cycle THz pulses with a unipolar temporal shape [1,18] generated from a femtosecond laser may allow the electric field effects on the cells to remain, even after the pulse period, because the field strength averaged over the temporal cycle does not vanish. However, it is still unclear how THz pulse irradiation affects living cells.

We investigated the effect on gene expression networks of THz pulse irradiation. In this Letter, intense THz pulses at an electric field strength of 0.5 MV/cm enabled homogeneous irradiation of cells cultured in microwells designed to have the same spot area as that of THz pulse irradiation. As a proof-of-concept, we used human induced pluripotent stem cells (hiPSCs) [19]. Besides the vital importance of medical applications, the use of highly environmentally sensitive hiPSCs [20] can provide new insights into the underlying mechanisms through which high-field THz pulses affect cultured cells.

Schematic diagrams of the THz pulse irradiation apparatus are shown in Figs. 1 and S1. The temporal shape of the THz pulse, measured using electro-optic (EO) sampling after passing through glass of 170 μm thickness on a glass-bottom dish [Fig. 1], is a quasi-unipolar shape [Fig. 2(a), red solid line]. The peak maximum electric field centered at around 0.8 THz is estimated to be 0.5 MV/cm [Figs. 2(a) and 2(b)]. Although the cell sample contains liquid water which shows strong absorption (about 200 cm^{-1}) at the THz frequency [21], the THz properties are almost the same as those inside the cell [Fig. 2(a), black dashed line], because only a few layers of cells, of a thickness per

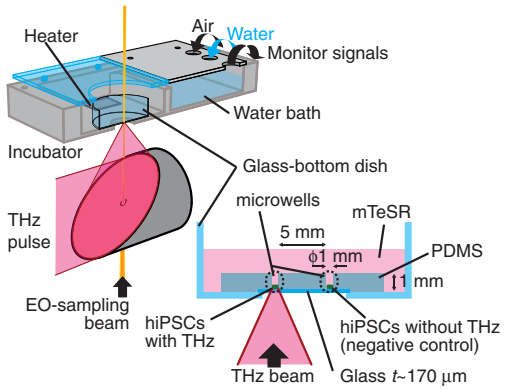

Fig. 1. Schematic diagram of the THz irradiation apparatus. The cells were kept in a microwell of polydimethylsiloxane (PDMS) in a glass-bottom dish in a handmade incubator. The generated THz pulses are focused by a parabolic mirror and irradiated onto the microwell. During the irradiation experiment, fresh air and water were supplied to the water bath in the incubator at 37 ± 0.3°C, and humidity of higher than 95% was maintained at all times by a feedback control. The CO_2 concentration was not controlled, and the pH in cell culture media was maintained using 25 mM of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid. The inset shows a magnified cross section of the culture dish.

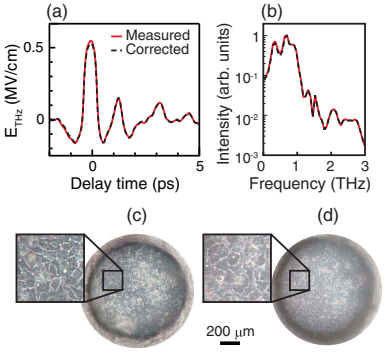

Fig. 2. (a) Measured THz electric field in the time domain (red solid line). Assuming that a few layers of cells in the microwell correspond to a 3 μm thickness of liquid water, the THz pulse is corrected by taking into account the absorption coefficient of water (black dashed line). (b) THz pulse spectrum. (c), (d) Micrographs of 585A1 hiPSCs after (c) 1 h of THz irradiation and (d) no irradiation cultured in microwells. The insets show 200 × 200 μm images.

cell 1 μm, cover the bottom of the microwell. The maximum increase in the temperature by THz pulses focused onto a sample through the glass bottom with a repetition rate of 1 kHz, and a fluence of 9 mW/cm^2 was estimated to be ∼3 mK. This measurement was estimated using a time-dependent finite-element simulation (COMSOL Multiphysics 5.3a), assuming that the heat capacity c_P, thermal conductivity λ, and the absorption coefficient α of the buffer (mTeSR) were the same as those of water (c_P = 4.18 kJ/(kg K), λ = 0.6 W/(m K), α = 200 cm^{-1}, Supplement 1).

Our apparatus allowed us to irradiate cells in an area of 1 mm in diameter (Fig. 1), while typical cell culture dishes are manufactured at millimeter to centimeter scales; therefore, it is quite difficult to use them to harvest only the irradiated cells without

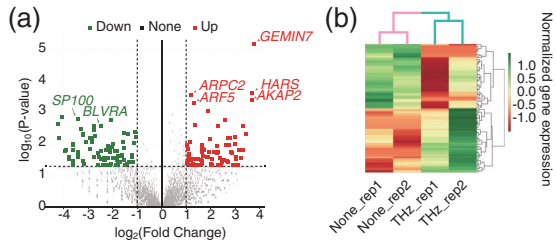

Fig. 3. (a) Volcano plot comparing differentially expressed genes (DEGs) in 585A1 hiPSCs irradiated with THz pulses for 1 h and nontreated cells. The vertical axis corresponds to the mean expression value of log 10 (P-value), and the horizontal axis displays the log 2 fold change value. The red and green dots represent upregulated and downregulated genes, respectively. (b) Clustered heat map showing expression of identified DEGs. "None" and "THz" represent 585A1 hiPSCs with no or 1 h THz irradiation with duplicated samples (_rep1 and 2), respectively.

the sample being contaminated by non-irradiated cells. To solve this problem, we made a simple dish with four microwells of diameter 1 mm for culturing hiPSCs. The microwells were coated with extracellular matrix proteins, as required for cell culture, and all cells in a microwell could be irradiated with THz pulses. After one hour of irradiation, the 585A1 hiPSCs exhibited neither abnormal morphologies, an important parameter for assessing the stem cell phenotype [Figs. 2(c), 2(d) and S2], nor an increased proportion of floating cells. Thus, we confirmed that the apparatus allowed THz irradiation of hiPSCs without causing abnormalities.

To identify intracellular events triggered by THz irradiation, we performed gene expression analysis, since an alteration in gene expression is one of the early outcomes of changes in intracellular signaling pathways, and is directly involved in cell dynamics. RNA samples were extracted from hiPSCs cultured in a microwell 3–24 h after THz irradiation. RNA sequencing was carried out to identify the genes that had altered expression, affected by the THz pulse (Supplement 1, Figs. S3–S6, and Table S1, Dataset 1, Ref. [22] and Dataset 2, Ref. [23]). At a threshold of fold change cutoff ranging from −1 to 1, and P-value cutoff = 0.05 [Fig. 3(a)], we found that irradiation upregulated 92 (Table S2, Dataset 3, Ref. [24]) and downregulated 116 genes (Supplement 1 and Table S3, Dataset 4, Ref. [25]). Hierarchical clustering of these genes showed apparent differences between untreated controls and THz-treated expression patterns [Fig. 3(b)].

Among the upregulated genes [Fig. 3(a)], gem nuclear organelle associated protein 7 (*GEMIN7*), which is involved in pre-mRNA splicing and regulating the survival of motor neurons [26], and spinal muscular atrophy [27], had the largest increase in expression. We also identified enriched gene networks by using gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome databases, which confirmed the pathway to be an "ncRNA metabolic process" (GO:0034660) [Figs. 4(a), S7 and Supplement 1, Table S4, Dataset 5, Ref. [28]]. Although the functions of GEMINs in stem cells have not yet been reported, it would be interesting to study the differentiation of hiPSCs into neuronal lineages following THz irradiation. Another gene, histidyl-tRNA synthetase (*HARS*) is enriched in "mitochondrial translation"

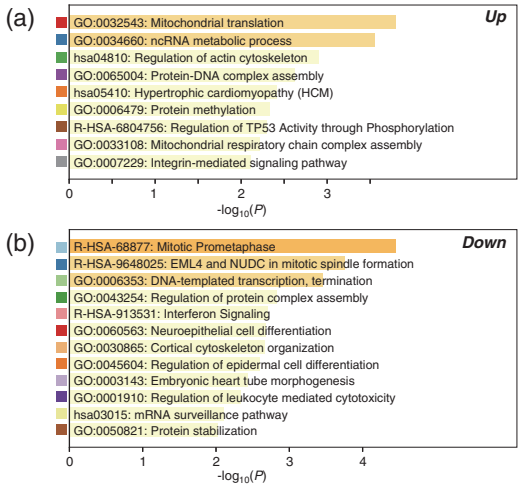

Fig. 4. Enriched GO, KEGG, and Reactome terms of (a) upregulated and (b) downregulated genes in irradiated 585A1 hiPSCs.

(GO:0032543). In addition to the essential function of charging histidine to its cognate tRNA necessary for entire protein synthesis, *HARS* has been reported to restore mitochondrial function in deafness when overexpressed, while its knockdown or inhibition causes neural diseases [29]. It could be informative to investigate the mitochondrial function and global protein synthesis activity of hiPSCs following THz irradiation, which might lead to the development of new approaches to treating certain diseases.

We found significantly upregulated genes, such as a-kinase anchoring protein 2, actin-related protein 2/3 complex subunit 2 (*ARPC2*), and ADP-ribosylation factor 5 (*ARF5*), which play important roles in cell migration and adhesion [30,31]. In light of enrichment analysis, these genes were grouped in "regulation of actin cytoskeleton" (hsa04810), suggesting that THz irradiation modulated cytoskeleton arrangement to facilitate adhesion for hiPSCs. According to the TF2DNA database, which contains details of transcription factors [32], *GEMIN7* is downstream of the transcription factors ZFP37, zinc–finger (ZNF)627, ZNF263, and ZNF275. *ARPC2* and *ARF5* are regulated by ZNF37A and ZNF460, respectively. These factors are all Zn^{2+}-dependent, and the aforementioned gene networks might be activated via changes in intracellular Zn^{2+} concentration ($[Zn^{2+}]_i$) triggered by THz irradiation.

Among the 116 genes downregulated by THz irradiation [Fig. 3(a)], we found *BLVRA* and *SP100*, which are regulated by the transcription factors ZNF263 and ZNF607, respectively, according to the TF2DNA database. This result suggested that some of the downregulated genes were also influenced by $[Zn^{2+}]_i$ changes triggered by THz irradiation. To identify the functions of downregulated genes, we observed enrichment for the groups "mitotic prometaphase" (R-HAS-68877), "EML4 and NUDC in mitotic spindle formation" (R-HAS-9648025), and "DNA-templated transcription and termination" (GO: 0006353) [Figs. 4(b), S8, Supplement 1 and Table S5, Dataset 6, Ref. [33]]. These groups were reportedly involved in the mitotic phase of the cell cycle. This result suggested that THz irradiation might cause G2/M cell cycle arrest of hiPSCs. Some pathways associated with cell differentiation, such as "neuroepithelial cell differentiation" (GO:0060563), "regulation of epidermal cell differentiation" (GO:0045604), and "embryonic heart tube morphogenesis" (GO:0003143), were enriched, suggesting the prevention of cell differentiation of hiPSCs by THz irradiation.

The fact that both upregulated and downregulated genes were identified after THz irradiation raises the possibility that irradiation affects local concentrations of metal ions, such as Zn^{2+}, creating heterogeneity within the cells, and forming distinct domains with increased or decreased ion concentrations. There is a growing awareness that the intracellular environment, including proteins, small molecules, ions, or temperature, is not homogeneous, and local differences in cellular components play a part in controlling cellular responses. Metal ions, including Fe^{2+}, Mg^{2+}, and Cu^{2+} are one group of such components that are precisely regulated in local vicinities, and are essential for proper cellular function, by enhancing enzyme activity or inducing conformational change of certain proteins. In particular, Zn^{2+} plays an important role in regulating the activity of transcription factors and in controlling gene expression [34]. ZNFs are known to be the most abundant protein superfamily in the mammalian genome [35]. The THz electric field allows the acceleration of electrical charges, including those of free electrons in vacuum and metal ions, by providing a large ponderomotive energy (kinetic energy of a charged particle in an oscillating electric field) [2,10]. This suggests a model in which THz pulses drive metal ions in a cell to alter gene expression.

An electric field of strength E accelerates a particle of electric charge e and mass m to eE/m, assuming the ballistic motion of a particle. The temporal integral of acceleration derived the shift of the charged particle: $d = \frac{e}{m}\int E(t)\mathrm{d}t$. Using $m = 1.1 \times 10^{-25}$ kg as the mass of Zn^{2+} and temporal profile of the THz pulse electric field, shown in Fig. 2(a), the Zn^{2+} shift by a THz pulse was calculated as ~0.15 nm in the direction of the electric field. Considering the repeated irradiation of THz pulses at a repetition rate of 1 kHz, the mobility of the Zn^{2+} was estimated to be 150 nm/s. The mobility of the ions was sufficient for the ions to move across proteins (around 1 to 200 nm in diameter) in seconds. $[Zn^{2+}]_i$ were maintained around 10^{-9} to 10^{-11} M, being regulated by Zn^{2+} transporters and the intracellular Zn^{2+} store [36]. Since the reported Kd of Zn^{2+} binding to ZNFs varies between 10^{-6} and 10^{-9} M [37], it is possible that either intracellular accumulation or compartmentalization of Zn^{2+} might result from THz irradiation, which would exceed the ZNF activation threshold. In light of this estimation and our finding that ZNFs could both negatively and positively regulate gene expression, we hypothesize that THz irradiation is involved in a novel mechanism of gene regulation.

Previously, several studies suggested that THz irradiation may strongly drive specific molecular vibrations, therefore affecting gene expression [15]. While a mechanism supposes the unwinding of the double helix and enhancement of subsequent transcription, it cannot adequately explain the existence of both regulations which we observed, since the prominent vibrational modes of DNA do not exist in the frequency region of our THz pulse [38]. There is another possibility: that of thermal effects occurring when water and cells are heated by the absorption of electromagnetic energy from the THz pulses. It is known that temperature activates the expression of certain proteins, such as heat shock proteins (HSPs). However, the temperature raise of

around 3 mK caused by THz pulses cannot trigger the expression of HSPs. Thus, it can be supposed that gene expression was induced nonthermally by the electric field of the THz pulse.

In summary, we have developed a new THz apparatus that irradiates cultured cells with intense THz pulses under culture conditions and utilized it to influence gene expression in hiPSCs. The genes that were strongly affected by THz irradiation were identified to be regulated ZNFs. This suggests that the unipolar THz pulse causes movement of Zn^{2+}. ZNFs are one of the most abundant proteins and play important roles in a range of cellular functions, including physiological and pathological conditions [39]. Further development of THz pulse sources for cell culture will be necessary to investigate the effects of THz irradiation, but we envision that our current apparatus will be useful in both biological research and biomedical applications.

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See Supplement 1 for supporting content.

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