




# Forskolin rapidly enhances neuron-like morphological change of directly induced-neuronal cells from neurofibromatosis type 1 patients

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## Abstract

**Aim:** Neurofibromatosis type 1 (NF1) is a multifaceted disease, and frequently comorbid with neurodevelopmental disorders such as autism spectrum disorder (ASD) and learning disorder. Dysfunction of adenylyl cyclase (AC) is one of the candidate pathways in abnormal development of neuronal cells in the brain of NF1 patients, while its dynamic abnormalities have not been observed. Direct conversion technology can generate induced-neuronal (iN) cells directly from human fibroblasts within 2 weeks. Just recently, we have revealed that forskolin, an AC activator, rescues the gene expression pattern of iN cells derived from NF1 patients (NF1-iN cells). In this microreport, we show the dynamic effect of forskolin on NF1-iN cells.

**Methods:** iN cells derived from healthy control (HC-iN cells) and NF1-iN cells were treated with forskolin (final concentration 10  $\mu$ M), respectively. Morphological changes of iN cells were captured by inverted microscope with CCD camera every 2 minutes for 90 minutes.

**Results:** Prior to forskolin treatment, neuron-like spherical-form cells were observed in HC-iN cells, but most NF1-iN cells were not spherical-form but flatform. Only

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20 minutes after forskolin treatment, the morphology of the iN cells were dramatically changed from flatform to spherical form, especially in NF1-iN cells.

**Conclusion:** The present pilot data indicate that forskolin or AC activators may have therapeutic effects on the growth of neuronal cells in NF1 patients. Further translational research should be conducted to validate our pilot findings for future drug development of ASD.

#### KEYWORDS

adenylyl cyclase, autism spectrum disorder, cyclic adenosine monophosphate, forskolin, induced pluripotent stem cells, induced-neuronal cells, learning disorder, neurofibromatosis type 1

## 1 | INTRODUCTION

Neurofibromatosis type 1 (NF1; also known as von Recklinghausen disease) is a multifaceted disease, which shows a variety of physical symptoms including multiple café-au-lait spots, Lisch nodules, neurofibromas, scoliosis, and vision disorder,<sup>1–3</sup> and also shows a variety of mental symptoms including mental retardation, epilepsy, and cognitive impairment/ learning disorder.<sup>4,5</sup> About 30% of NF1 patients are comorbid with autism spectrum disorder (ASD).<sup>6–10</sup> These clinical reports have suggested some neurodevelopmental pathophysiology in the brains of NF1 patients.

Neurofibromatosis type 1 is a monogenic disease, and its causative gene is the NF1 gene coding “neurofibromin 1”. A strong association between neurofibromin 1 and Ras-GTPase has widely been known.<sup>11</sup> Neurofibromin 1 regulates not only Ras-GTPase but also adenylyl cyclases (ACs) in various cell types.<sup>12</sup> Detailed molecular basis of ACs-mediated neurofibromin 1 has not been well clarified. Interestingly, a recent study using a zebrafish model of NF1 has shown that the AC signaling pathway is involved in learning and that the Ras-GTPase pathway is associated with memory.<sup>13</sup>

Regenerative medicine technologies using induced pluripotent stem (iPS) cells from human tissues have been highlighted to clarify the cellular-level pathophysiology of brain disorders including psychiatric disorders.<sup>14,15</sup> Direct conversion technologies, not using iPS cells, have also been attracting attention as a useful translational research tool.<sup>16,17</sup> Directly converted neuronal cells, called “induced-neuronal (iN) cells,” were developed from mouse fibroblasts transfected with three transcriptional factors: Brn2, Ascl1, and Myt1l (BAM factors), without via iPS cells.<sup>18</sup> Human iN cells have been utilized for neuropsychiatric research,<sup>19,20</sup> and some previous reports have shown that iN cells have the advantage of retaining some of the physiological conditions that are lost in iPS cells.<sup>21,22</sup> We have successfully induced iN cells from adult human fibroblasts using human BAM factors in 2 weeks.<sup>23–27</sup> Recently, we have reported on gene expression analysis of iN cells derived from NF1 patients (NF1-iN cells).<sup>26</sup> Interestingly, forskolin, an activator of AC pathway, rescued the abnormal gene expression in NF1-iN cells to the levels of gene expression in iN cells derived from healthy controls (HC-iN cells).

Therefore, we hypothesize that forskolin could compensate for some of the dynamic neuronal abnormalities in NF1-iN cells. To clarify our hypothesis, we herein performed cytomorphological observations of iN cells in the presence or absence of forskolin.

## 2 | METHODS

All methods of this study were performed in accordance with the Declaration of Helsinki and were approved by the ethics committees of Kyushu University (Fukuoka, Japan).

Human iN cells were generated as reported previously.<sup>26</sup> We used two fibroblasts cell lines from a NF1 patient and a healthy volunteer in the present study, which were already used in our previous report.<sup>26</sup> Briefly, on Day –3, fibroblasts were seeded in 35-mm glass-bottom dishes (Matsunami Glass) at a density of  $1 \times 10^5$  cells/dish. On Day 0, lentiviruses infected to fibroblasts to each of the human BAM factors (human BRN2, ASCL1, MYT1L, MOI = 10 each) in Fibroblast Growth Medium (FGM) that contained 15% fetal bovine serum (Japan Bioserum), 0.1 mmol/L MEM Non-Essential Amino Acids (Thermo Fisher Scientific), and 1% Pen Strep (Thermo Fisher Scientific) in Minimal Essential Medium Eagle (Sigma Chemical) which contained 8 µg/mL of polybrene (Sigma Chemical) for 24 hours. On Day 1, the medium was changed with fresh FGM. After Day 2, the medium was changed every 3 days with iN Medium (10 ng/mL FGF2 [Peprotech], 1 mmol/L valproic acid [Sigma Chemical], 0.8% N2 supplement [Thermo Fisher Scientific], 0.4% B27 supplement [Thermo Fisher Scientific], 1% Pen Strep, 10 µg/mL blasticidin [Thermo Fisher Scientific] in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham [Sigma Chemical]: Neurobasal medium [Thermo Fisher Scientific] = 4:1).

On Day 14, Forskolin (Nacalai Tesque) was added to the cell culture medium (final concentration 10 µmol/L), and images of cell contour were captured every 2 minutes for 90 minutes using an Olympus IX70-22FL inverted microscope and ×20 objective (Olympus).

For cell counting, ImageJ was used to measure the number of cells with an apparently spherical-form in all cells. To preclude bias, the cell measurements were performed blind to cell type and

condition. To determine the differences between the groups, two-way ANOVA followed by Tukey's correction was used by GraphPad Prism 7.04 (GraphPad Software).

### 3 | RESULTS

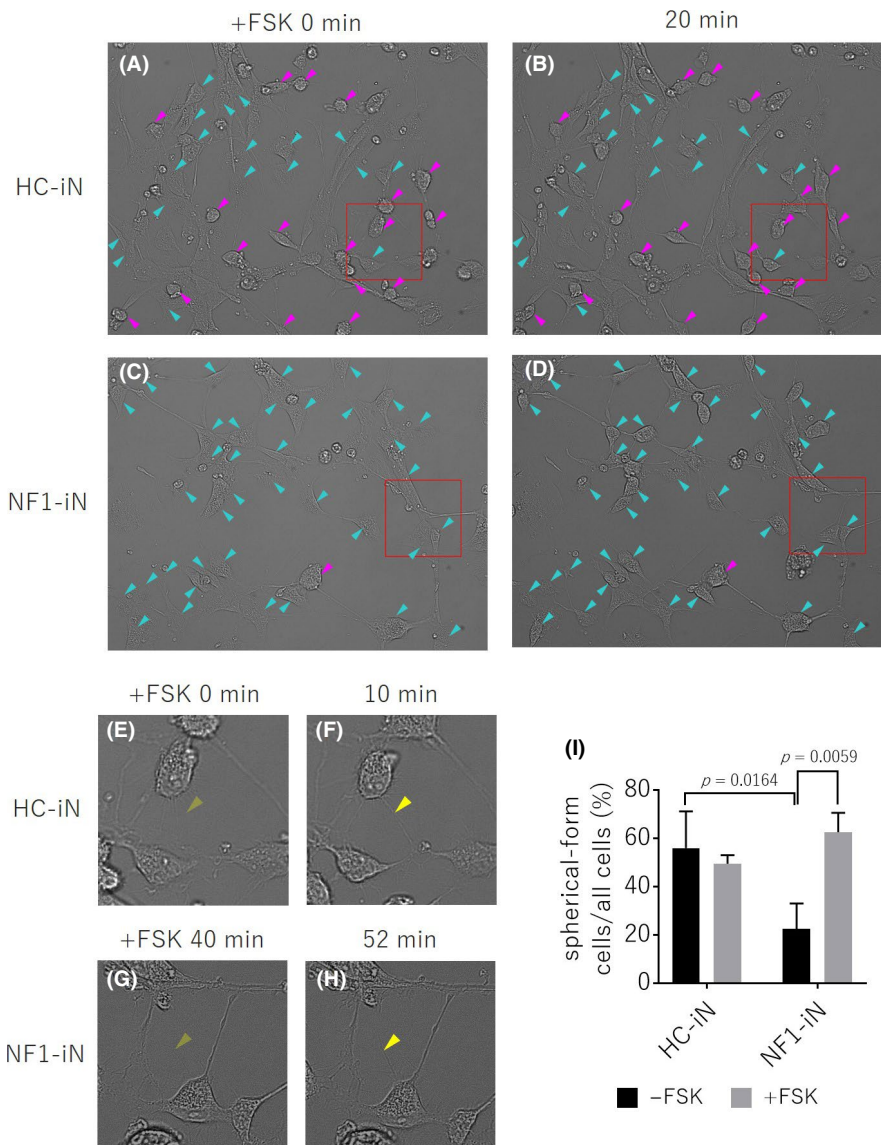
In iN cells from healthy control (HC-iN cells), many cells were neuron-like spherical-form (Figure A, magenta arrow head). On the other hand, most cells of iN cells derived from NF1 patient were thin and flat (NF1-iN cells: Figure C, cyan arrow head). This result suggests that most NF1-iN cells could not form neuron-like spherical-form cell morphology because of deficit of AC ability. Interestingly, only 20 minutes after AC activation by forskolin treatment, most NF1-iN cells began to have a dense cell contour and the cell morphology dramatically changed to neuron-like spherical-form (Figure D, cyan arrow head, Video S1). Furthermore, since forskolin appeared to promote neurite outgrowth of iN cells (Figure E-H, yellow arrow head,

Video S2), quantitative experiments and analysis should be performed on more samples in the near future.

Cell counts showed that NF1-iN cells were significantly flattened compared to HC-iN cells (Figure 1,  $P = .0164$ ), and their cell morphology was significantly recovered by forskolin treatment (Figure 1,  $P = .0059$ ).

### 4 | DISCUSSION

We have previously reported that forskolin improves gene expression abnormalities in NF1-iN cells.<sup>26</sup> Here, we showed that forskolin dramatically changed the cell morphology from flatform to spherical form, especially in NF1-iN cells, and the possibility of enhancing neurite outgrowth of NF1-iN cells to the similar level of HC-iN cells. These results only suggest a possible effect of forskolin, and further experiments will be needed to confirm these results.



**FIGURE (A-D)** Phase difference images of iN cells derived from healthy control (HC-iN, A, B) and NF1 patient (NF1-iN, C, D) after forskolin (FSK) treatment. Neuron-like spherical-form cells mainly appeared in HC-iN cells (magenta arrow head). Flat cells with a thin cell contour obtain a dense cell contour only 20 minutes after forskolin treatment (cyan arrow head). (E-H) Enlarged images of the part surrounded by the red frame on FIGURE A-D. Forskolin appeared to enhanced neurite outgrowth in the iN cells (yellow arrow head). (I) The ratio of the number of neuronal-like spherical-form cells to the total number of cells. NF1-iN cells in the absence of forskolin had a significantly lower percentage of the spherical-form cells compared to HC-iN cells ( $p = 0.0164$ , two-way ANOVA / Tukey's test,  $n = 3$  each group). In the presence of forskolin, the spherical-form cell morphology of NF1-iN cells was significantly higher ( $p = 0.0059$ , two-way ANOVA / Tukey's test,  $n = 3$  each group)



How does forskolin change the cell morphology? Forskolin is known to activate intracellular ACs and increase intracellular cyclic adenosine monophosphate (cAMP) levels, and a previous report has shown that forskolin regulates cytoskeletal formation in mouse adrenal cortex tumor-derived cell line Y1 cells.<sup>28</sup> Elevated intracellular cAMP levels cause dephosphorylation of paxillin at the edge of the cells, and paxillin moves from focal adhesion to cytoplasm.<sup>28</sup> NF1 patients have aberrant gene expression of neurofibromin 1 which is known to regulate AC activity and the intracellular cAMP levels.<sup>12</sup> We have recently shown that gene expression of neurofibromin 1 was also low in NF1-iN cells,<sup>26</sup> suggesting low intracellular cAMP levels in NF1-iN cells. We herein showed that NF1-iN cells tend to have flat cell morphology compared to HC-iN cells and that these cell morphologies were rescued by the application of forskolin. Thus, such morphological abnormalities may be caused by abnormal cytoskeleton development due to lower levels of paxillin dephosphorylation from lower ACs activation and lower intracellular cAMP levels in NF1-iN cells.

Paxillin is known to be involved in neurite outgrowth in experiments using rat adrenal medulla pheochromocytoma-derived cell line PC12 cells.<sup>29</sup> Similarly, the present data have suggested that forskolin alters the phosphorylation state of paxillin and activated neurite outgrowth.

The present pilot experiment has indicated that AC and cAMP activation can normalize neuronal development in the brain of NF1 patients. We propose that the administration of forskolin or forskolin-like AC activators into the brain of NF1 patients during neurodevelopmental periods may contribute to prevent neurodevelopmental disorders including ASD and neuropsychiatric disorders in later life.

#### 4.1 | Limitations

The present data should be validated with multiple further experiments. Further verification is necessary using neurofibromin 1 and/or paxillin gene knockdown/ knockout iN cells or human iPS cell-derived neurons, or in vivo NF1 model animals. Mechanism studies using the other AC activators/ inhibitors, regarding the association between NF1 and ACs, are also needed to validate the present pilot data. Moreover, the present findings based on morphological observation should be validated by additional analysis such as protein level analysis to determine the expression/ localization/ phosphorylation status of paxillin in NF1-iN cells.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### AUTHOR CONTRIBUTIONS

TAK was the principal investigator of the present research. NS was the first author created the conception and design of the project and wrote the protocol. TAK, TN, and M.N-K performed clinical recruitment and sampling collection. TAK, NS, S-IK, MO, SI, YS, HK, and KM involved in the performance of experiments and data analyses/ interpretation. NS wrote the first draft of the manuscript. TAK, SO, MF, AS, and SK made critical revisions of the manuscript. All authors contributed substantially to the scientific process leading up to the writing of the present manuscript and approved this submission in its current form.

#### ETHICAL APPROVAL OF THE RESEARCH PROTOCOL BY AN INSTITUTIONAL REVIEWER BOARD

All methods of this study were performed in accordance with the Declaration of Helsinki and were approved by the ethics committees of Kyushu University (Fukuoka, Japan).

#### INFORMED CONSENT

Informed consent was obtained from all the healthy volunteers and patients before donating skin fibroblasts.

#### REGISTRY AND THE REGISTRATION NO. OF THE STUDY/TRIAL

28-89/2019-541 (the ethics committees of Kyushu University).

#### ANIMAL STUDIES

This study did not include animal experiments (all data are derived from human cell experiments.)

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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