Chapter

Clarifying the Pathophysiological Mechanisms of Neuronal Abnormalities of NF1 by Induced-Neuronal (iN) Cells from Human Fibroblasts

Noriaki Sagata, Yasunari Sakai and Takahiro A. Kato

Abstract

Direct conversion techniques, which generate induced-neuronal (iN) cells from human fibroblasts in less than two weeks, are expected to discover unknown neuronal phenotypes of neuropsychiatric disorders. Here, we present unique gene expression and cell morphology profiles in iN cells derived from neurofibromatosis type 1 (NF1) patients. NF1 is a single-gene multifaceted disorder with relatively high co-occurrence of autism spectrum disorder (ASD). Adenylyl cyclase (AC) dysfunction is one of the candidate pathways in abnormal neuronal development in the brains of NF1 patients. In our study, microarray-based transcriptomic analysis of iN cells from healthy controls (males) and NF1 patients (males) revealed significantly different gene expression of 149 (110 were upregulated and 39 were downregulated). In iN cells derived from NF1 patients (NF1-iN cells), there was a change in the expression level of 90 genes with the addition of forskolin, an AC activator. Furthermore, treatment with forskolin dramatically changed the cell morphology, especially that of NF1-iN cells, from flat-form to spherical-form. Current pilot data indicate the potential therapeutic effect of forskolin or AC activators on neuronal growth in NF1 patients. Further translational research is needed to validate the pilot findings for future drug development of ASD.

Keywords: adenylyl cyclases (ACs), autism spectrum disorder (ASD), forskolin, induced-neuronal (iN) cells, neurofibromatosis type 1 (NF1)

1. Introduction

Neurofibromatosis type 1 (NF1: also called von Recklinghausen disease) is a multifaceted disease with a variety of physical manifestations, including multiple café-au-lait spots, neurofibromas, Lisch nodules, scoliosis, and visual impairment [1–3]. NF1 patients also exhibit a variety of psychiatric symptoms such as mental retardation, epilepsy, and cognitive dysfunction/learning disabilities [4, 5]. Approximately half of NF1 patients exhibit impaired social information processing and disturbed social behavior [6–8]. In addition, about 30% of NF1 patients have comorbid autism spectrum disorder (ASD) [9–13]. These clinical reports suggest some kind of neurodevelopmental pathophysiology in the brains of NF1 patients.

NF1 is a monogenic disease, and the causative gene is the *NF1* gene, which encodes 'neurofibromin'. A recent mouse model study has shown that neurofibromin dysfunction increases the protein level of the anti-apoptotic protein BCL2 (B cell leukemia/lymphoma 2) in neural stem cells (NSCs) [14]. The strong association between neurofibromin and Ras-GTPase is widely known [15]. Some studies have shown that neurofibromin is involved not only in Ras-GTPase pathway, but also adenylyl cyclases (ACs) pathway of various cell types [16]. However, the detailed molecular basis for the ACs-mediated function of neurofibromin has not been elucidated. Interestingly, a recent study using the zebrafish model of NF1 has shown that the AC signaling pathway is involved in learning and the Ras-GTPase pathway is associated with memory (**Figure 1**) [17].

Few studies have shown whether such dysfunction is present in human living neuronal cells of NF1 patients. This is because it is difficult to directly analyze human brain cells containing neuronal cells. Very recently, a study has been published that reveals patient-common and mutation-dependent abnormalities using neural progenitor cells and cerebral organoids derived from induced pluripotent stem (iPS) cells in NF1 patients [18]. Regenerative medicine technologies using iPS cells from human tissues have been attracting attention to clarify the pathophysiology of brain disorders, including psychiatric disorders, at the cellular level [19, 20]. Direct conversion technologies without using iPS cells have also attracted attention as a useful translational research tool [21, 22]. The cells directly converted to neuronal cells are called "induced-neuronal (iN) cells" and were first developed from mouse fibroblasts transfected with the three transcriptional factors *Brn2*, *Ascl1*, and *Myt1l* (BAM factors) [23]. Human iN cells have been utilized in neuropsychiatric research, [24, 25] and several advantages have been reported that iN cells retain some of the aging-related physiological conditions that are lost in iPS cells [26, 27]. Using human-derived BAM factors, we succeeded in inducing iN cells from adult human fibroblasts in 2 weeks [28–32]. Briefly, in our protocol, the transfected fibroblasts were cultured in a medium containing 10 ng/mL FGF2, 1 mM valproic acid, 10 μM forskolin (optional), 0.8% N2 supplement, and 0.4% B27 supplement. And, we performed unbiased microarray analysis using SurePrint G3 Human Gene Expression Microarray 8 × 60 K v2 (Agilent Technologies) to investigate aberrant gene expression in NF1-iN cells. We reported the results of gene expression analysis of NF1 patients-derived iN cells (NF1-iN cells) [31]. Interestingly, forskolin, an

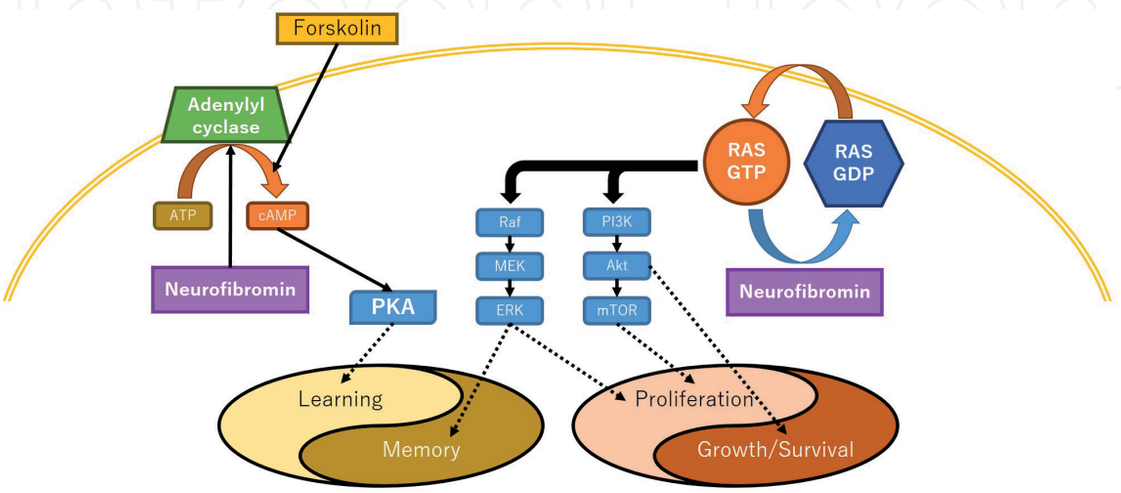

Figure 1.
Schematic diagram of the pathways involved in neurofibromin. Two pathways involving neurofibromin have already been identified. One is the RAS pathway and the other is the AC pathway. Forskolin is an exogenous additive that activates the AC pathway.

activator of AC pathway, restored the aberrant gene expression in NF1-iN cells to the gene expression level of healthy controls-derived iN cells (HC-iN cells). Furthermore, prior to forskolin treatment, many HC-iN cells had neuron-like spherical-form cell morphology, whereas most NF1-iN cells were flat-form rather than spherical-form. After forskolin treatment, iN cells morphology changed rapidly and dramatically from flat-form to spherical-form, especially in NF1-iN cells. These pilot data show that forskolin or AC activators have possible therapeutic effects on neuronal growth in NF1 patients.

In this chapter, we present the results obtained to date on abnormalities in gene expression and cell morphology in iN cells derived from NF1 patients, and describe future prospects.

2. Dysregulated gene expression in the neuronal cells of NF1 patients

Direct conversion methods that generate human induced-neuronal (iN) cells from fibroblasts within two weeks are expected to discover unknown neuronal phenotypes in neuropsychiatric disorders. Here, we introduce unique gene expression profiles in iN cells of neurofibromatosis type 1 (NF1) patients, a single-gene multifaceted disorder with relatively high co-occurrence with autism spectrum disorder (ASD). The association between NF1 and adenylyl cyclases (ACs) activity has been reported in animal model studies, [16, 17] as far as we know, there are no experimental studies using human neuronal cells. To clarify how abnormalities in the ACs pathway affect the gene expression pattern of iN cells derived from NF1 patients (NF1-iN cells), a group treated with forskolin, an ACs activator, was included in the microarray analysis. First, an unbiased microarray analysis was performed to investigate aberrant gene expression in NF1-iN cells (6 male samples including 3 healthy controls (HC) and 3 NF1). Interestingly, in the iN cells, the expression of 149 genes was significantly different in NF1-iN cells compared to HC-iN cells (**Figure 2**). It is strongly suggested that these aberrant gene expressions in NF1 patients are shown only in iN cells and not in fibroblasts. In NF1-iN cells, the expression level of 90 genes was changed by the addition of the AC activator forskolin. Among the above149 genes (HC-iN cells vs. NF1-iN cells) and 90 genes (NF1-iN cells without forskolin vs. NF-iN cells with forskolin), 31 genes were overlapped (**Figure 2**). Interestingly, all of their expression levels in NF1-iN cells were rescued to HC level by the application of forskolin (**Figure 3**). These 31 genes may be strongly dysregulated via the AC pathway in neurons of NF1 patients (especially males).

To confirm the validity of the differences in expression of the 31 gene mentioned above, all samples on hand (3 male HC samples and 3 female NF1 samples) were added and reassessed by real-time PCR analysis. Unfortunately, when we validated these results with real-time PCR analysis using a total of six HC and six NF1 samples, including female samples, we could not reproduce most of the differences in the expression of the 31 genes. Recent epidemiological studies have shown that NF1 patients have a high comorbidity with ASD, and prevalence of ASD is about twice as high in males than females [12, 33]. Further investigation with larger samples may clarify our novel hypothesis about the tendency for neuronal pathologies to develop especially in male NF1 patients, and may lead to a better understanding of gender differences in ASD and other neuropsychiatric disorders.

2.1 *MEX3D* gene expression in the neuronal cells of NF1 patients

Interestingly, in the real-time PCR analysis described above, only the gene expression of the *MEX3D* (Mex-3 RNA Binding Family Member D) was

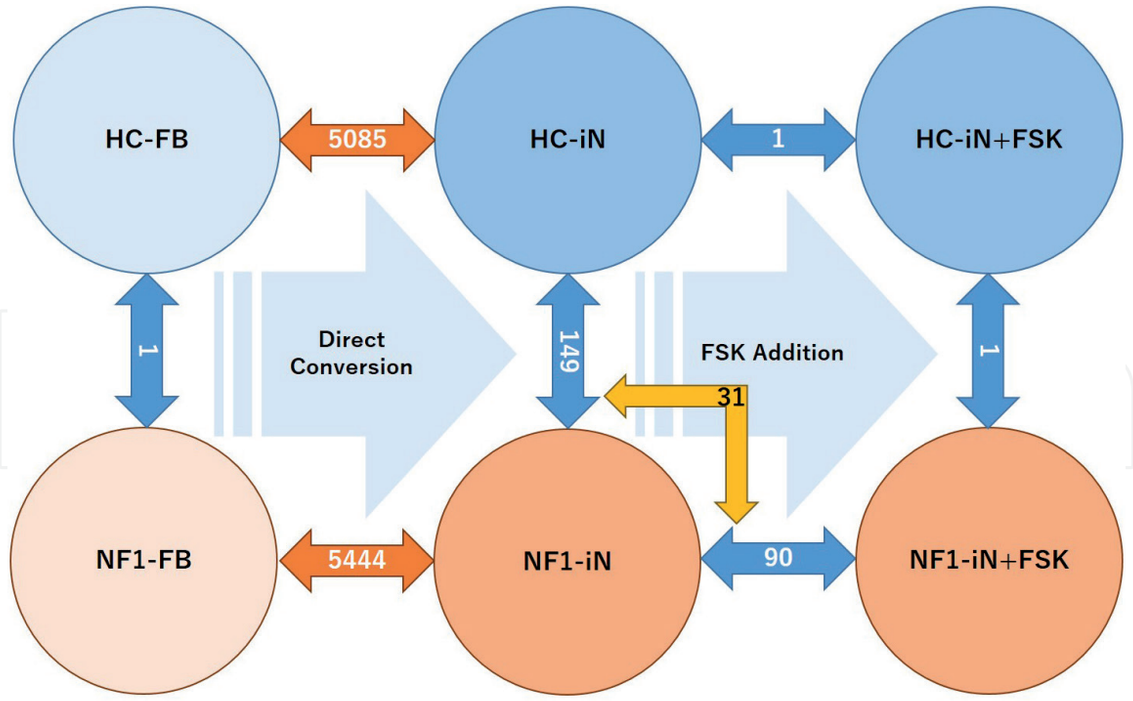

Figure 2.
Simplified schematic of microarray analysis. Circles show 6 samples groups: Healthy control fibroblast (HC-FB), NF1 patient fibroblast (NF1-FB), healthy control iN cells (HC-iN), NF1 patient iN cells (NF1-iN), healthy control iN cells with forskolin (HC-iN+FSK), and NF1 patient iN cells with forskolin (NF1-iN+FSK). Blue and orange double arrows indicate the number of aberrant genes between two groups (circles). A yellow double arrow indicates the number of overlapping genes between two blue double arrows. (modified from Sagata et al. *2017).*

significantly downregulated in NF1-iN cells (p = 0.0040). MEX3D is a member of the RNA-binding protein family with homologous members: MEX3A, MEX3B, MEX3C, and MEX3D [34]. All members of the MEX3 family have two KH (K Homology) RNA-binding domains at the N-terminus, and a RING (Really Interesting New Gene) finger domain with ubiquitin E3 ligase activity at the C-terminus. A previous study has shown that MEX3D promotes the degradation of *BCL2* mRNA by interacting with its AU-rich elements (AREs) [35]. Therefore, we assessed the mRNA level of *BCL2* of the iN cells and found no difference between HC- and NF1-iN cells (p = 0.3134). AREs were initially reported to be present in the 3′-UTR (untranslated region) of the mRNAs of early response genes such as *FOS* (Fos Proto-Oncogene, AP-1 Transcription Factor Subunit), *MYC* (V-MYC Avian Myelocytomatosis Viral Oncogene Homolog), and *JUN* (Jun Proto-Oncogene, AP-1 Transcription Factor Subunit), which code for powerful transcriptional activators, and *CSF2* (Colony Stimulating Factor 2), *IL2* (Interleukin 2), *IL3*, and *IL6*, which code for growth factors and cytokines. These mRNAs are finely controlled in response to external stimuli and have a fast turnover [36, 37]. Therefore, we next assessed whether the reduction of *MEX3D* in NF1-iN cells was associated with expression levels of these mRNAs. The expression level of *FOS* mRNA in NF1-iN cells was significantly higher than that in HC-iN cells (p = 0.0428). Conversely, the mRNA expression level of *JUN* was significantly lower in NF1-iN cells (p = 0.0395). There were no significant differences in the expression levels of other genes (*MYC*, *CSF2*, *IL2*, *IL3*, and *IL6*). To the best our knowledge, there is no report showing a direct interaction between MEX3D and FOS or JUN in human neuronal cells.

To evaluate whether the reduction of *MEX3D* affects the expression levels of *FOS* and *JUN* mRNA in neuronal cells, we performed *Mex3d*-knockdown experiment using the Neuro2A cells, mouse neuroblastoma cell line. Knockdown of *Mex3d* with siRNA significantly increased the mRNA expression levels of both *Fos* and *Jun*

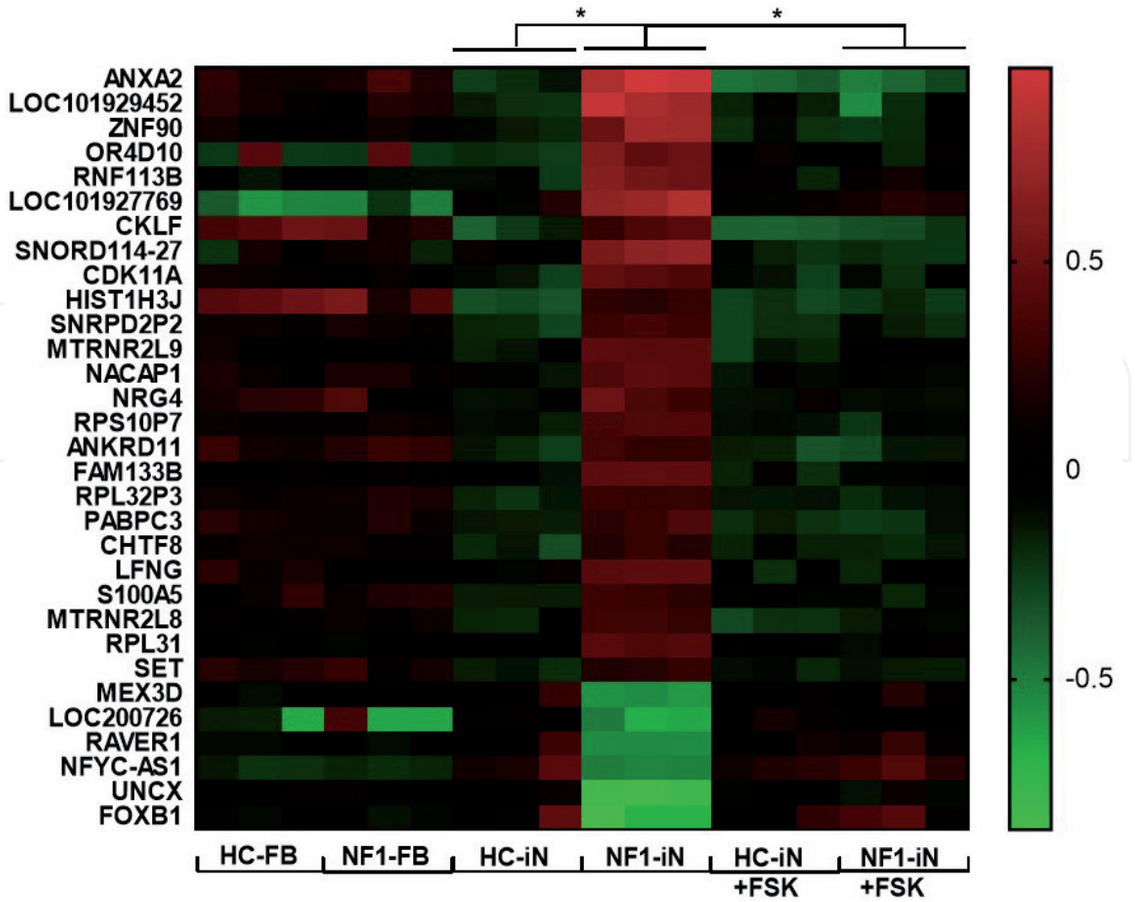

Figure 3.
*Unique gene expression profile in iN cells from patients with NF1. Heatmap of the 31 genes that were revealed as aberrant in microarray analysis. *limma adjusted p-value <0.05. Red indicates higher expression genes, and green indicates lower expression genes. (modified from Sagata* et al. *2017).*

in Neuro2A cells (p = 0.0002, 0.0360, respectively). This result suggests that there is a strong interaction between *MEX3D* (*Mex3d*) and *FOS*/*JUN* (*Fos*/*Jun*) not only in human neuronal cells but also in mouse cells. On the other hand, in Neuro2A cells, *Mex3d* knockdown did not change the *Nf1* mRNA expression level, and *Nf1* knockdown did not change the *Mex3d* mRNA expression level. The low expression level of *MEX3D* mRNA seen in human NF1-iN cells was not reproduced in the mouse neuronal cell line Neuro2A. This result suggests the importance of analyzing human cells in disease models.

2.2 *BCL2* gene expression in the neuronal cells of NF1 patients

A previous study has shown that BCL2, an anti-apoptotic protein, is elevated in neuronal stem cells (NSCs) from *NF1* gene-disrupted mice [14]. To our knowledge, there are no data on BCL2 abnormalities in the mature neurons in *NF1*-disrupted mice or NF1 patients. Our data also showed that elevated *BCL2* mRNA was not observed in Day-14 mature iN cells. Therefore, we hypothesized that upregulation of *BCL2* by *NF1* gene-disruption could be a developmentally specific phenomenon in early-stage neuronal cells.

Treutlein *et al.* showed that the initial transcriptional response of iN cells generation occurs relatively homogeneously among fibroblasts, but during neuronal maturation of iN cells, a portion of the induced cells population takes on an alternative myogenic cell fate [38]. This should also imply that early-stage iN cells after transfection constitute a homogeneous population. In addition, although cell

linage conversion and neuronal maturation are different events, we believe that early-stage iN cells may exhibit some characteristics of pre-mature neuronal cells in early developmental stage.

The morphology of Day-5 iN cells (early-stage iN cells) was not significantly different from that of fibroblasts. Surprisingly, forskolin transformed iN cells from a fibroblast-like shape to a long-branched neuron-like morphology even at Day 5. These Day-5 iN cells showed higher levels of *MAP2* (Microtubule Associated Protein 2: a pan-neuronal marker) than fibroblasts, even in the absence of forskolin ($p < 0.0001$). Day-14 iN cells showed higher expression level of *RBFOX3* (RNA Binding Protein, Fox-1 Homolog 3: a mature neuronal marker), while fibroblasts and Day-5 iN cells with/without forskolin showed no difference in *RBFOX3* expression. From these data, we speculate that Day-5 iN cells may exhibit some of the characteristics of pre-mature neuronal cells compared to Day-14 developed-stage iN cells. Interestingly, similar to the data of NSCs of *NF1*-disrupted mice, the expression level of *BCL2* mRNA in early-stage NF1-iN cells was significantly higher ($p = 0.0002$). These data partially support our hypothesis that high expression of BCL2 is observed only in early-stage neuronal cells in NF1 patients.

BCL2 mRNA and protein are present at relatively high levels during the nervous system development and are reduced in the postnatal brain [39–41]. Abnormalities of apoptosis constitute the pathogenesis of neurodevelopmental disorders [42]. The majority of neurons are immature or premature at the stage of neurodevelopment, and apoptosis of immature / premature neurons needs to be highly controlled in order to form proper neural circuits. Therefore, in the brains of NF1 patients, BCL2-mediated neuronal apoptosis may be disturbed during neurodevelopment, thereby leading to the formation of abnormal neural circuits. Disruption of this pathway may be one of the pathogenic mechanisms underlying the development of ASD and other neurodevelopmental disorders in NF1 patients.

2.3 *MAGEL2* gene expression in the neuronal cells of NF1 patients

Aberrant gene expression in NF1-iN cells has also been discovered from a completely different approach. Akamine *et al*. reported on a 45-year-old woman with NF1, epileptic encephalopathy of infantile onset, and severe developmental delay [32]. Whole genome sequencing confirmed *de novo* pathogenic mutations in *NF1* and *MAGEL2*, a gene associated with Schaaf-Yang syndrome. According to STRING (http://string-db.org/), a protein–protein interaction database, NF1 and MAGEL2 were predicted to be closely linked in this network through a common interacting protein. To test the possibility of a functional interaction between NF1 and MAGEL2, it was examined whether pathological mutations in *NF1* affect the neuronal expression of *MAGEL2*. Interestingly, NF1-iN cells had significantly lower expression of *MAGEL2* than HC-iN cells (54%, $p = 0.0047$) [32]. These data are the first to show that pathogenic mutations of *NF1* regulate the expression of other neurodevelopmental disease-associated genes. *De novo* mutations in multiple genes can cause severe developmental phenotypes due to their cumulative effects or synergistic interactions.

3. Aberrant cell morphology of the neuronal cells of NF1 patients

Adenylyl cyclase (AC) dysfunction is one of the candidate pathways in abnormal neuronal development in the brain of NF1 patients, but its dynamic abnormalities have not been observed. Therefore, we observed the dynamic effects of forskolin on iN cells. In HC-iN cells, most of cells were neuron-like spherical-form. On the other

hand, in NF1-iN cells, most of the cells were thin and flat. Interestingly, after only 20 minutes of AC activation by forskolin treatment, most NF1-iN cells had a dense cell contour and their cell morphology changed dramatically to neuron-like spherical-form. This result suggests that most NF1-iN cells were unable to form neuron-like spherical-form cell morphology due to lack of AC ability. Counting the number of cells, NF1-iN cells had a significantly higher number of flat-form cells than HC-iN cells (**Figure 4**, p = 0.0164), and their cell morphology was significantly restored by forskolin treatment (**Figure 4**, p = 0.0059) [43]. In addition, forskolin appeared to promote neurite outgrowth in iN cells, so quantitative experiments and analysis with more samples should be conducted in the near future.

Forskolin activates intracellular ACs and increases intracellular cyclic adenosine monophosphate (cAMP) levels, and it has previously reported that forskolin regulates cytoskeletal formation in Y1 cells, a cell line derived from mouse adrenocortical tumors [44]. When intracellular cAMP levels increase, dephosphorylation of paxillin occurs at the cell edge, and paxillin moves from the focal adhesion to the cytoplasm [44]. Patients with NF1 have aberrant gene expression of neurofibromin that is known to regulate the activity of ACs and the intracellular cAMP levels [16]. Recently, we have shown that neurofibromin gene expression is also low in NF1-iN cells, [31] suggesting that intracellular cAMP levels are low in NF1-iN cells. As mentioned above, NF1-iN cells tend to have flat-form cell morphology compared to HC-iN cells, and these cell morphologies are restored by application of forskolin [43]. Thus, such morphological abnormalities may be attributed by abnormal cytoskeleton development due to decreased dephosphorylation levels of paxillin due to decreased activation of ACs and decreased intracellular cAMP levels in NF1-iN cells. Paxillin has been shown to be involved in neurite outgrowth in PC12 cells, a cell line derived from rat adrenal medulla pheochromocytoma [45]. Similarly, our findings suggest that forskolin alter the phosphorylation level of paxillin and activated neurite outgrowth.

Our pilot experiment showed that activation of ACs may normalize the development of neuronal cells in the brain of NF1 patients. We propose that administration of forskolin or forskolin-like AC activators into the brain during

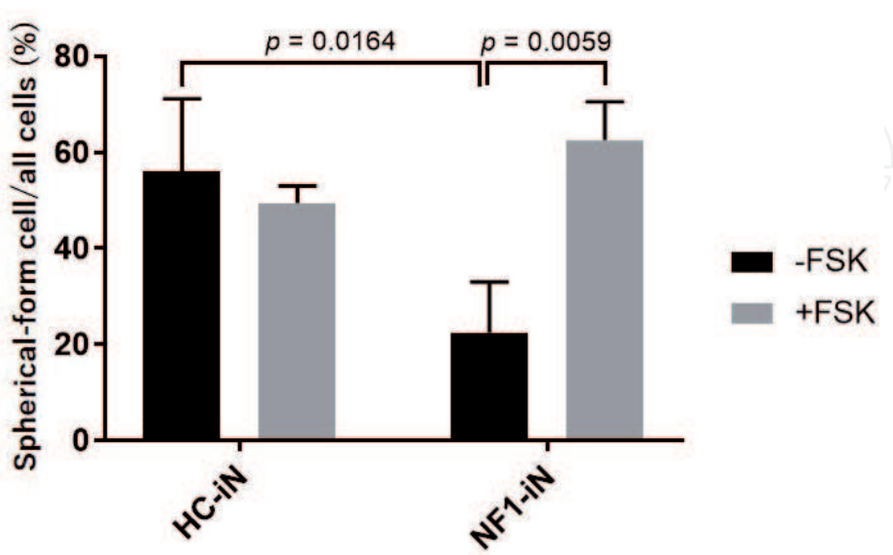

Figure 4.
The ratio of the number of neuronal-like spherical-form cells to the total number of cells. NF1-iN cells in the absence of forskolin had a significantly lower percentage of the spherical-form cells compared to HC-iN cells (p = 0.0164, two-way ANOVA/Tukey's test, n = 3 each group). In the presence 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) (modified from Sagata et al. 2020*).*

neurodevelopmental periods of NF1 patients may contribute to the prevention of neurodevelopmental disorders such as ASD and neuropsychiatric disorders in subsequent life.

4. Conclusions

In this chapter, we have presented unique gene expressions and cell morphology profiles in induced-neuronal (iN) cells of patients with neurofibromatosis type 1 (NF1), a single-gene multifaceted disorder with relatively high co-occurrence of autism spectrum disorder (ASD). Microarray analysis revealed that the expression of 149 genes was abnormal in the neuronal cells of NF1 male patients, and that the expression of 90 genes was altered in the presence of forskolin. Of these, 31 genes in particular were suggested to be normalized by improvement of the AC pathway. These abnormalities of gene expressions may be male-specific and may be related to gender differences in the development of ASD. Further cellular analysis, especially considering gender-specific neuronal dysregulation, should be performed to reveal unknown neurobiological roles of gender underlying the pathophysiology of ASD.

We also introduced that the effects of forskolin shows dramatic changes not only in gene expression but also in the cell morphology of neuronal cells in NF1 patients. We propose that research is needed to prevent the development of ASD and neuropsychiatric disorder later in life by administering forskolin and other AC activators, which are easily introduced in to the brain, to NF1 patients early in their developmental period.

Furthermore, we found that the expression of *FOS* and *BCL2* mRNA, which have anti-apoptotic effects in neuronal cells, were elevated in developed- and early-stage iN cells of NF1 patients, respectively. Therefore, neuronal apoptosis during neurodevelopmental period can be disturbed in NF1 patients.

Moreover, the findings presented here should be validated by additional analyses such as apoptosis analysis, protein level analysis and functional analysis of neurons. On the other hand, more detailed molecular mechanisms, especially the interactions between NF1, MEX3D, FOS, JUN, BCL2, and MAGEL2, will be the subject of future work. In addition, *in vitro* studies using mouse Neuro2A cells did not show some of interactions seen in the gene expression analysis of human NF1-iN cell (e.g., the interaction between *Nf1* and *Mex3d*), suggesting that these interactions may be unique to humans, highlighting the importance of studying human cellular models. Neuron studies derived from human iPS cell are expected to confirm the findings introduced here.

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Conflict of interest

The authors declare no conflict of interest.

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