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Role of plant homeodomain finger protein 8 in P19 embryonic carcinoma cells revealed by genome editing and specific inhibitor

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ABSTRACT

Plant homeodomain finger protein 8 (PHF8) is a histone demethylase that regulates the expression of various genes. PHF8 targets repressor histone markers and activates gene expression. Although PHF8 has been involved in X-linked mental retardation and certain types of cancers, the role of PHF8 remains largely unknown, and its relevance to the pathogenesis of these diseases is also uncertain. In the present study, we aimed to clarify the cellular function of PHF8 in P19 cells using *Phf8* knockout (KO) cells generated via the CRISPR-Cas9 system and by performing PHF8 specific inhibitor experiments, instead of using *PHF8* small interfering RNA transfection. After establishing *Phf8* KO cells, we analyzed the effects of PHF8 on neuronal differentiation and cell proliferation. Both PHF8 deficiency and inhibition of its activity did not considerably affect neuronal differentiation, however, they showed an increased trend of promoted neurite outgrowth. Moreover, we found that PHF8 regulated cell proliferation via the MEK/ERK pathway. PHF8 deficiency and activity inhibition reduced the phosphorylation of ERK and MEK. The MEK expression level was associated with PHF8 expression, as revealed by chromatin immunoprecipitation analysis. These results suggested that PHF8 regulates cell proliferation via the MEK/ERK pathway in P19 embryonic carcinoma cells.

1. Introduction

Histone methylation represents one of the most important histone modifications and regulates gene expression, DNA replication, and DNA repair. The methylation of either histone 3 lysine 4 (H3K4) or histone 3 lysine 36 (H3K36) activates gene expression, and the methylation of histone 3 lysine 9 (H3K9), histone 3 lysine 27 (H3K27), and histone 4 lysine 20 (H4K20) represses gene expression [1,2].

In addition, certain epigenetic defects caused by mutations in genes related to histone methylation can cause neurodevelopmental disorders, lack of neuroplasticity, and mental retardation [3–5]. Plant homeodomain finger protein 8 (PHF8) is a histone demethylase encoded on the X chromosome [6]. PHF8 has two functional domains: a PHD finger domain that recognizes lysine-methylated histones and a JmjC domain that catalyzes the demethylation of lysine [6,7]. PHF8 demethylates H3K9me1/2, H3K27me2, and H4K20me1 revealed by *in vitro* demethylation assays [8–11]. However, the role of PHF8 remains unclear.

Phf8 has been identified as the causative gene of Siderius X-linked mental retardation syndrome (MRXSSD), which was discovered by Siderius in 1999 [6,12].

PHF8 has also been implicated in many carcinomas, such as prostate [13], non-small cell lung [14], and breast cancer [15]. Björkman et al. reported that PHF8 is involved in cell migration and invasion as revealed by cell motility and 3-D invasion assay, as well as cell proliferation in prostate cancer. Shen Y et al. found that PHF8 is an oncogenic protein upregulated in non-small cell lung cancer. Increased expression of PHF8 correlates with poor survival. Furthermore, they observed that PHF8 regulates not only cell proliferation and cell transformation, but also DNA damage and apoptosis. PHF8 also regulates miR-21 expression involved in proliferation and apoptosis in non-small cell lung cancer. Wang et al. found that PHF8 and ubiquitin-specific protease 7 (USP7)

Abbreviations: PHF8, plant homeodomain finger protein 8; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; RT-PCR, reverse transcription polymerase chain reaction; TSS, transcription start site; NeuN, RNA binding fox-1 homolog 3; GFAP, glial fibrillary acidic protein.

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physically interact with each other in breast cancer. USP7 promotes deubiquitination and stabilization of PHF8, leading to the upregulation of genes, including cell cycle factor cyclin A2. Although the mechanism by which PHF8 regulates cell growth through miR-21 expression and USP7 in some cancers has been widely researched, the common mechanism by which PHF8 affects growth remains largely obscure.

The siRNA transfection method is commonly used and convenient [16–18]. However, we established *Phf8* knockout (KO) P19 embryonic carcinoma cells (P19 cells) using the CRISPR-Cas9 system, a genome-editing technology [19], and we further used a PHF8-specific inhibitor to evaluate the cellular function of PHF8 in the present study.

Neuronal differentiation is induced in P19 cells via the formation of aggregates called embryoid bodies (EB) in the presence of retinoic acid (RA). In addition, P19 cells can differentiate not only into neurons but also into astroglia and cardiomyocytes upon adding drugs and modulating culture conditions [20–22]. Therefore, P19 cells are useful for analyses related to neuronal or developmental studies and are suitable for achieving the aim of this study.

2. Materials and methods

2.1. Materials

The PHF8 activity inhibitor NCDM-64a (PHF8i) was developed and provided by Drs. Suzuki and Miyata [23] and was later obtained from Tokyo Chemical Industry (Tokyo, Japan). P19 cells were provided by Dr. Miura (Nagoya City University).

2.2. Cell culture and differentiation of P19 cells

P19 cells were cultured in Minimum Essential Medium Eagle, Alpha Modification (α-MEM; Wako, Osaka, Japan) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan). The protocol for cell passages and differentiation was described previously [24]. P19 cells were cultured in medium containing 0.5 μM all-trans retinoic acid (ATRA) (Wako) on 10 cm dishes (IWAKI, Shizuoka, Japan) from suspension culture to EB formation. After 2 d, cells were collected via centrifugation at 1200 rpm for 5 min to change medium and ATRA. Then, cells were re-suspended onto new dishes containing ATRA in the medium. After 2 d, the cells were recovered via centrifugation, and trypsin was used to separate the cell clumps to collect individual cells. Thereafter, 1×10^5 or 1×10^6 cells were plated onto poly-L-lysine-coated six-well plates (Nippon Genetics, Tokyo, Japan) in monolayer culture. These cells were cultured in α-MEM without ATRA, and with or without PHF8i, respectively, for either 2 d to induce neural differentiation or for 4 d to induce astrocyte differentiation.

2.3. RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously [25]. cDNA was synthesized from 2 μg of total RNA using oligo dT primers and ReverTra ace enzyme (Toyobo, Osaka, Japan). The primers used for RT-PCR were *Phf8*, forward, 5′-CCATCCAGGGCATGTTGTGTA-3′ and reverse, 5′-CTTGGTAGG GTTGGAGTCAC-3′, with a PCR product length of 401 bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified using the following primers: 5′-TGCCACTTCAACAGCAACCT-3′ (forward) and 5′-ATGTAGGCCATGAGGTCCAC-3′ (reverse), and PCR products were 253 bp in size. Thermal cycling comprised 35 cycles of 96 °C, 10 s denaturation; 60 °C, 10 s annealing; 72 °C, 1 min extension. This was followed by a final extension step at 72 °C for 7 min.

2.4. Western blotting analysis

Western blotting was performed as previously described [24]. Cultured cells were collected in 1 mL of sodium dodecyl sulfate (SDS) sample buffer lacking protease and phosphatase inhibitors, added to a 1.5 mL microtube, and immediately boiled at 100 °C for 3 min. Finally, the protein concentration was determined using a bicinchoninic acid assay (BCA) protein assay kit (Takara, Shiga, Japan) with bovine serum albumin as the standard. Ten micrograms of protein sample was used for SDS polyacrylamide gel electrophoresis and western blotting. The following primary antibodies were used: PHF8 (ab36068; Abcam, Cambridge, United Kingdom; 1:1000), RNA binding fox-1 homolog 3 (NeuN) (24307; Cell Signaling Technology, Danvers, MA, USA; 1:1000), glial fibrillary acidic protein (GFAP) (3670, Cell Signaling Technology; 1:1000), p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [Erk1/2]) (4696, Cell Signaling Technology; 1:1000), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (9101, Cell Signaling Technology; 1:1000), MEK1/2 (8727, Cell Signaling Technology; 1:1000), Phospho-MEK1/2 (Ser217/221) (9154, Cell Signaling Technology; 1:1000), and b-tubulin (FUJIFILM Wako Pure Chemical Corporation, Japan; 1:1000).

2.5. Generation of Phf8 KO P19 cells using CRISPR-Cas9 system

To generate *Phf8* KO P19 cells, the PrecisonX^{TM} Cas9 SmartNikase System (System Biosciences, Palo Alto, CA, USA) was used. Annealed oligo pairs to the targeting (+) strand (5′-ATCCGATCGAGTGTGACATGTGCC-3′ and 5′-AAACGGCACATGTCACACTCGATC-3′) and targeting (−) strand (5′-ATCCATGAAGCGGGTCACATCATA-3′ and 5′-AAACTATGATGTGACCCGCTTCAT-3′) were cloned into the CMV-hspCas9(D10A)-T2A-GFP-H1-gRNA vector. The experimental procedures, such as selection and isolation of cells, were performed as described previously [24]. Finally, the cells were screened using PCR, direct Sanger sequencing, and western blotting.

2.6. Cell proliferation analysis

P19 cells (1×10^5 cells) were plated in 10 cm dishes with or without PHF8i and cultured for 1, 2, or 3 d under adhesion conditions. After culturing, the cells were peeled off the plate with trypsin, dispersed, and collected. The number of cells was measured using counting chambers in a box (HIRSCHMANN, Stuttgart, Germany).

2.7. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the Pierce^{TM} Magnetic ChIP Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Each DNA-protein complex was immunoprecipitated using 2 μg of PHF8 antibody (93801, Cell Signaling Technology) and normal rabbit IgG as the negative control. The bound DNA fragments were subjected to quantitative PCR using the following primers targeting the transcription start site (TSS) of *Mek1*: 5′-GCGGCGTCTCGGAGCG-3′ and 5′-CTCGGG CTCGGACGGC-3′, with a PCR product length of 100 bp. The thermal cycling stage comprised 40 cycles of 95 °C, 3 s denaturation, 60 °C, 30 s annealing, and a melt curve stage of 95 °C for 30 s, 60 °C for 1 min, and 95 °C for 15 s. The % of input was calculated as $2^{\{(Ct_{input} - \log_2(X)) - Ct_{Chip\ sample}\}} \times 100$ (https://diagenode.co.jp/chip-school/chip-qpcr-calculations). Ct_{input} is the threshold cycle of the input sample in real-time PCR, $Ct_{Chip\ sample}$ is the threshold cycle of the Chip sample in real-time PCR, and X is the dilution factor of the input sample.

2.8. Immunocytochemistry

Briefly, cells were fixed in 4% paraformaldehyde, washed twice with phosphate-buffered saline (PBS), and treated with 0.1% Triton-X in PBS for 5 min at room temperature (RT). Cells were blocked with 1% bovine serum albumin in PBS for 20 min at RT and then incubated with β3-tubulin (5568, Cell Signaling Technology, 1:200) for 2 h at RT, followed by Alexa Fluor 488-conjugated anti-rabbit IgG (4412, Cell Signaling

Technology, 1:1000) for 2 h at RT. Cell nuclei were stained with 4-6-diamidino-2-phenylindole (DOJINDO, Kumamoto, Japan). Fluorescence images were obtained using a BZ-X710 microscope (KEYENCE, Osaka, Japan). Neurite length was measured using an Analysis Measurement Module (BZ-H3M, KEYENCE). In total, 100 neurites were measured.

2.9. Statistical analysis

The data were analyzed with a one-way ANOVA followed by Dunnett's multiple comparisons test. Quantitative data were obtained from three independent experiments, except for those presented in Fig. 4F, and are expressed as the mean ± standard deviation. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Generation of *Phf8* KO cell lines

First, to confirm whether PHF8 was expressed in P19 cells, we analyzed the mRNA expression of PHF8 under three culture conditions. PHF8 was expressed at the mRNA level in adherent cultures, EB, and neurons, respectively (Fig. 1A). To determine the role of PHF8 in P19 cells, we knocked out *Phf8* using the CRISPR-Cas9 system [19]. The small guide RNAs targeted exon 4, which includes the start codon (Fig. 1B). After genome editing, three independent clones were obtained. Gene deletion was confirmed using Sanger sequencing and western blotting. One clone (KO1) had a 29-base-pair insertion, another clone (KO2) had a 15-base-pair deletion, and one other clone (KO3) had a 19-base-pair deletion (Fig. 1C). All of these mutants were predicted to have a premature stop codon due to frameshifting (Fig. 1D). PHF8 deficiency in all mutants was confirmed via western blotting using the PHF8 antibody, which recognizes the C-terminal of PHF8 (Fig. 1E). The PHF8 protein presented two bands (Fig. 1E) because *Phf8* has two splicing variants (GeneBank Accession No. NM_177201 and NM_001113354) or that PHF8 underwent post-translational modifications [15].

3.2. Effect of *Phf8* KO on neuronal differentiation

Phf8 has been identified as the causative gene of MRXSSD [6] and is involved in neuronal differentiation [16]. Therefore, to clarify the function of PHF8 in neuronal differentiation, we analyzed the expressions of NeuN, a marker of mature neurons, and GFAP, an astrocyte marker, and we measured the length of neurites. The loss of PHF8 had little effect on NeuN and GFAP expression (Supplementary Figs. 1A and B). Similar results were obtained upon using a PHF8 inhibitor (Supplementary Figs. 1C and D). The inhibition of PHF8 significantly promoted neurite outgrowth (Fig. 2C and D), however, the effect of the PHF8 deficiency was not significant (Fig. 2A and B). These results show that PHF8 mildly affects neuronal differentiation.

3.3. *Phf8* KO and PHF8 inhibitor treatment suppressed cell proliferation

PHF8 is implicated in various cancers [13–15,17], and abnormal cell proliferation is a characteristic feature of cancer. Therefore, we examined the difference in cell proliferation between wild-type and *Phf8* KO cells. *Phf8* KO P19 and wild-type P19 cells treated with PHF8 inhibitor showed a reduced number of proliferating cells on day 3 under adherent culture conditions (Fig. 3A and B).

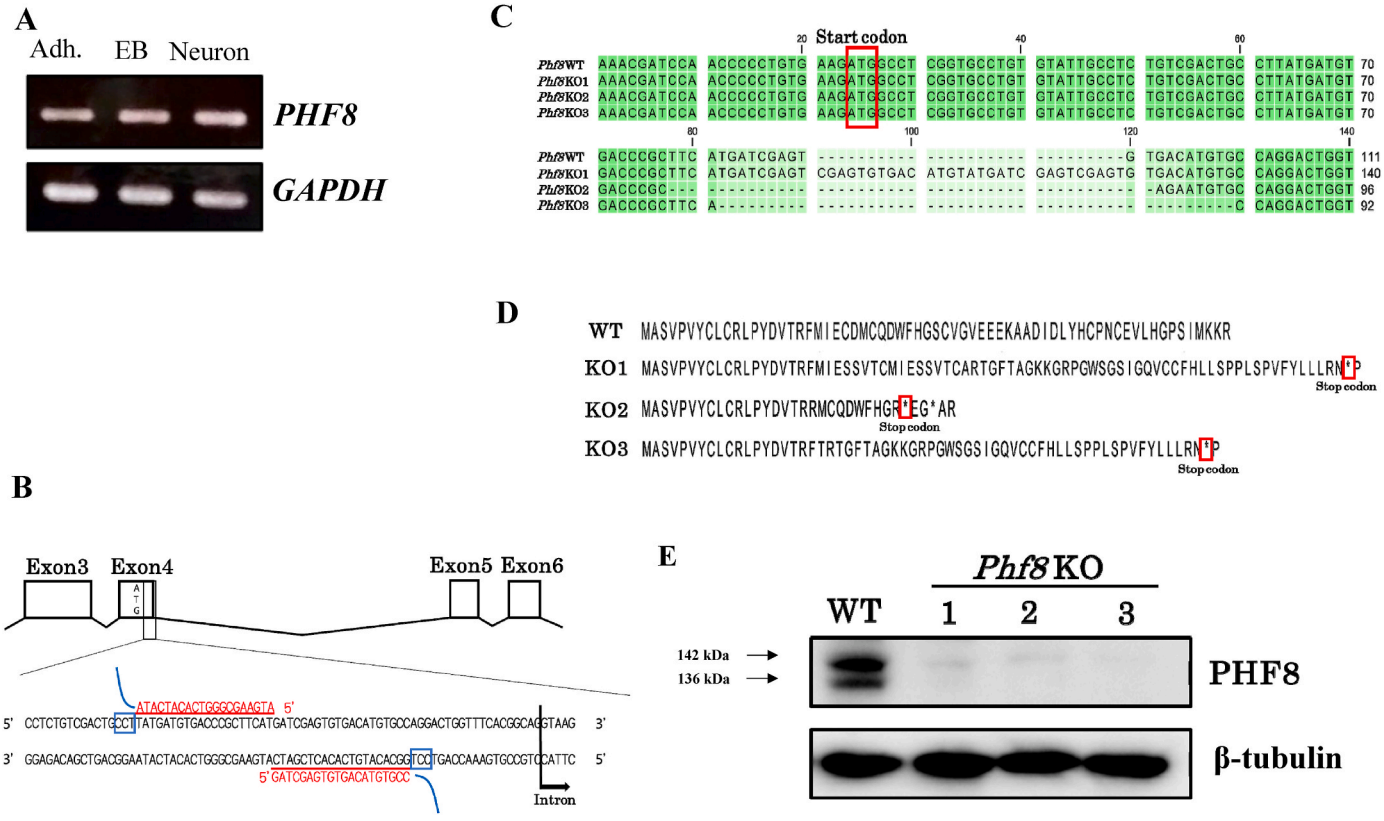

Fig. 1. Generation of *Phf8* KO cell lines. A) *Phf8* mRNA expression in P19 cells in adherent cultures, EB, and neurons. Reverse transcription-polymerase chain reaction was performed using 5 μg total RNA. *Phf8* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression patterns are shown. B) Schematic diagram of PHF8-specific gRNA using CRISPR direct (https://crispr.dbcls.jp). C) Nucleic acid sequence of *Phf8*. Light, green-shaded bases indicate insertion or deletion sequences. D) Predicted amino acid sequence of PHF8. The asterisk surrounded by a red frame indicates the stop codon. E) PHF8 pattern analyzed by western blotting. Ten micrograms of protein samples are used for each lane. PHF8, plant homeodomain finger protein 8; KO, knockout; EB, embryoid bodies; WT, wild type. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

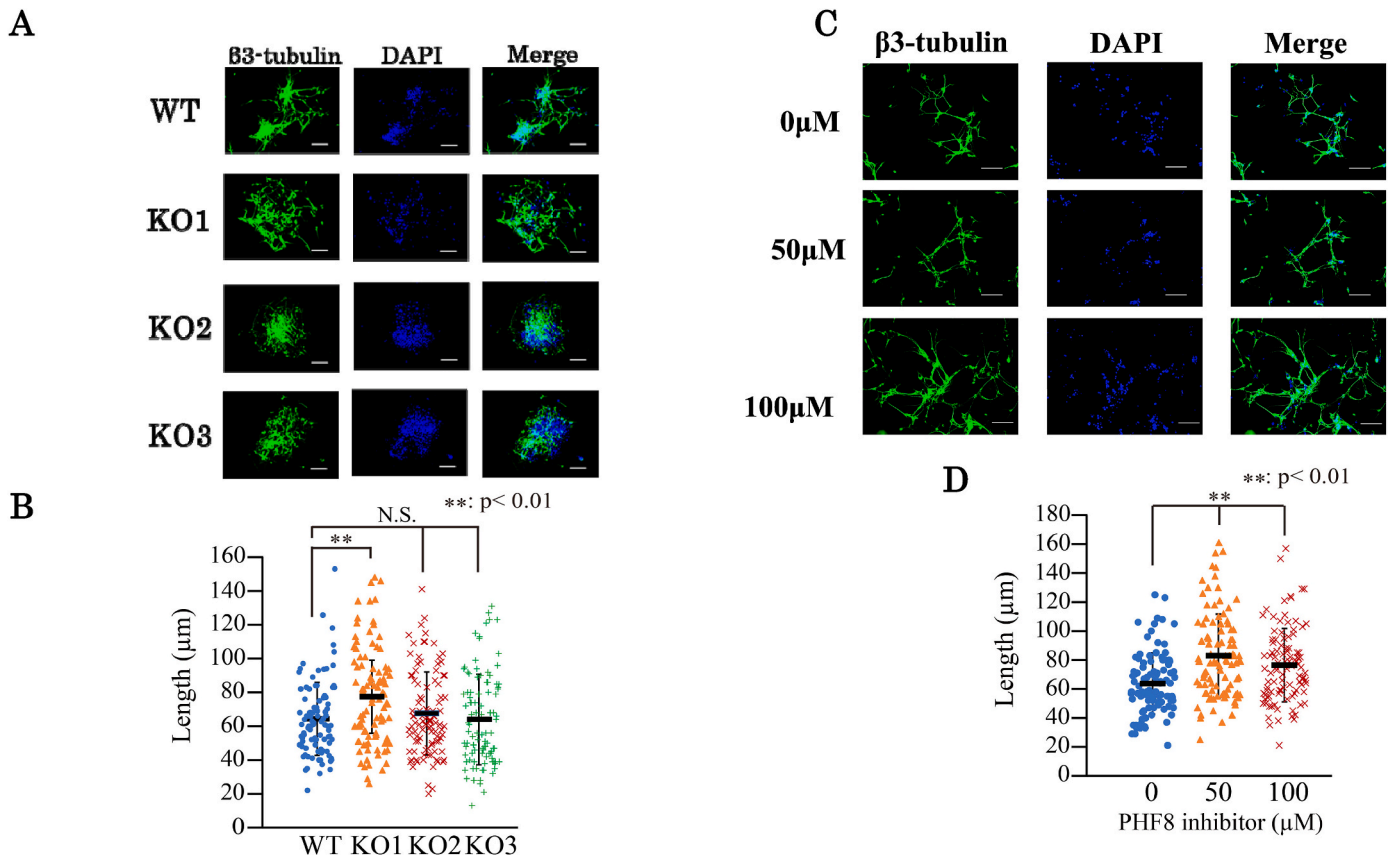

Fig. 2. Effects of *Phf8* KO on neuronal differentiation. A, B) Neurite lengths were measured from β3-tubulin fluorescence images using *Phf8* KO cells. A total of 100 neurites were measured. Scale bar = 100 μm. Effects of PHF8 inhibitor treatment on neuronal differentiation in P19 cells. C, D) Neurite lengths were measured from β3-tubulin fluorescence images after treatment with PHF8 inhibitor. A total of 100 neurites were measured. Scale bar = 100 μm. PHF8, plant homeodomain finger protein 8; KO, knockout; β3-tubulin, beta-tubulin; WT, wild type. Data are presented as the mean ± SD. SD, standard deviation. * *: $p < 0.01$, N.S., not significant.

3.4. PHF8 regulated MEK/ERK signaling pathway with decreasing MEK expression

The MEK/ERK pathway is a pivotal signaling pathway involved in cancer cell proliferation and apoptosis. We found that *Phf8* KO suppressed ERK phosphorylation (Fig. 4A). However, PHF8 deficiency did not affect ERK expression level. Therefore, we investigated the phosphorylation and protein expression levels of MEK, an upstream regulator of ERK. *Phf8* KO decreased MEK expression level but did not affect the phosphorylation level of MEK (Fig. 4B). Similar results were obtained after using the PHF8 inhibitor (Fig. 4C and D). We performed ChIP experiments to further investigate the mechanism via which PHF8 regulates MEK expression. We designed several ChIP primers for the promoter or TSS of *Mek1*. ChIP primers designed around the TSS of *Mek1* showed a desirable function (Fig. 4E). PHF8 binding with the TSS of *Mek1* was efficient and higher than that with the control. PHF8 deficiency decreased the binding of *Mek1* TSS (Fig. 4F). These data suggest that PHF8 regulates the MEK/ERK signaling pathway by directly decreasing MEK expression.

4. Discussion

In this study, to evaluate the cellular function of PHF8, we analyzed whether PHF8 deficiency and PHF8 activity inhibition regulate neuronal differentiation and cell proliferation in P19 cells. We demonstrated that PHF8 regulates growth in P19 cells but is not involved in neuronal differentiation, namely neurons and glia, in P19 cells. The effect of PHF8 deficiency on neuronal differentiation was unexpectedly subtle compared with previous findings [16,18]. However, Jihui et al. demonstrated that PHF8 is involved in neuronal differentiation in P19 cells using *PHF8* siRNA transfection [16]. Further, Iacobucci et al. showed that PHF8 regulates astrocyte differentiation and function using primary neural stem cell culture and *PHF8* siRNA transfection [18]. In this study, we used both *PHF8* KO cells and PHF8-specific inhibitor to evaluate neuronal differentiation and cell proliferation in P19 cells instead of the *PHF8* siRNA method. Our results were different from the previous findings [16,18]. The discrepancy between these results and ours may be because our method generated cells that are completely deficient of PHF8 throughout, from the cell growth stage to the neuronal differentiation process; in contrast, *Phf8* knockdown caused by siRNA creates a partial deficiency of PHF8 only effective in cell differentiation conditions. Thus, complete or incomplete PHF deficiency may affect cell differentiation. Since the PHF8 protein level gradually decreases as differentiation progresses in embryonic stem cells [26], PHF8 may not play a major role in neurogenesis.

MRXSSD is characterized by mental retardation, cleft lip, and cleft palate, with Phf8 as its causative gene [12]. In zebrafish, PHF8 partially regulates jaw development [8]. In mice, PHF8 deficiency causes cognitive impairment via the mTOR pathway [27]. Furthermore, PHF8 and two other X-linked mental retardation genes, *ZNF11* and *JARID1C*, function synergistically to develop complex phenotypes observed in patients developing mental retardation [10]. Thus, loss of PHF8 signal transmission to mTOR and loss of PHF8 interaction with ZNF711 and JARID1C may cause mental retardation. In this study, we could not demonstrate the relationship between PHF8 and other molecules; therefore, the effect of PHF8 deficiency in P19 KO cells still needs further investigation.

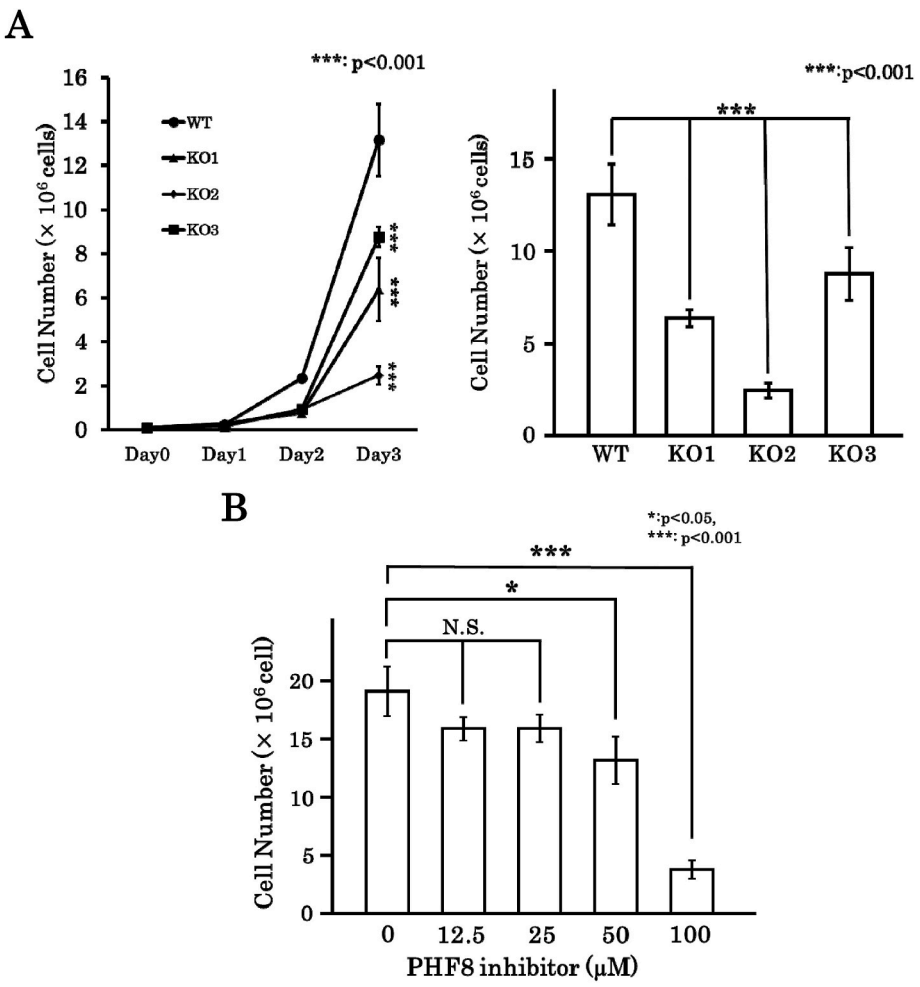

Fig. 3. *Phf8* KO and PHF8 inhibitor treatment suppressed cell proliferation. A) A line graph of cell number in *Phf8* KO cells. The number of cells was measured at 1, 2, or 3 d after culturing. Bar graph shown at 3 d after culturing. B) Cell number at 3 d after culturing with PHF8 inhibitor. PHF8, plant homeodomain finger protein 8; KO, knockout; WT, wild type. Data are presented as the mean ± SD of three independent experiments. SD, standard deviation. *:$p < 0.05$, ***:$p < 0.001$, N.S., not significant.

We found that MEK expression and phosphorylated ERK expression levels were decreased; however, the level of phosphorylated MEK did not change. We also found that PHF8 regulated MEK expression, as evidenced by ChIP analysis. However, we observed that MEK phosphorylation levels were unchanged. Fu et al. found that *PHF8* siRNA transfection decreases MEK expression in acute lymphoblastic leukemia cells [17]. Consistent with their findings, we also found that PHF8 deficiency and inhibition of its activity decreased MEK expression level.

MEK activation occurs via phosphorylation of Ser 218 and Ser 222 residues by Raf-1 kinase [28,29]. This double-phosphorylated form of MEK is sufficient for mediating full activation, suggesting that Raf-1 kinase activity towards these residues on MEK was not supposed to be decreased in this study. These findings suggest that PHF8 upregulates MEK, but is not involved in MEK phosphorylation. Finally, the decreased level of MEK expression may not be sufficient for mediating full activation of ERK and can be attributed to cell proliferation defects. As we observed that the level of phosphorylated ERK (MAPK) was decreased (Fig. 4A and C), we cannot deny the possibility that MAPK (MAPK-ERK) phosphatase activity towards phosphorylated ERK may be increased.

In general, the MEK/ERK (MAPK) pathway plays a pivotal role in the G1/S and G2/M phase transitions [30]. Liu et al. also demonstrated that PHF8 controls the G1-S transition in conjunction with E2F1, HCF-1, and SET1A [9]. Thus, PHF8 controls cell cycle progression via the MEK/ERK signaling pathway in cooperation with E2F1, HCF-1, and SET1A.

Using ChIP experiments, we observed that PHF8 binds to the TSS of *Mek1* (Fig. 4E and F). However, Qi et al. and Liu et al. independently reported that PHF8 can bind to many regions in the genome based on comprehensive ChIP-Seq analysis in HeLa cells and they identified approximately 14,000 binding sites in the genome [8,9], in which most of the PHF8-binding sites are promoters. However, *MEK* promoters were not included. Fu et al. and our study found that PHF8 can bind to the promoter or TSS of *Mek* [17]. These discrepancies may arise owing to the specificity of the PHF8 antibody used, the type of cells, and the cell culture conditions that were suitable for the growth stage but not the differentiation stage.

Taken together, these findings suggest that PHF8 is involved in cell growth. Moreover, although we found weak evidence that PHF8 may be involved in neuronal development, we found that *Phf8* knockout promoted neurite outgrowth, but not completely. Future studies on epigenetic regulation of cell proliferation and neurodevelopment using *Phf8* KO cells will further elucidate the basic mechanism by which epigenomic modification affects cell proliferation and development.

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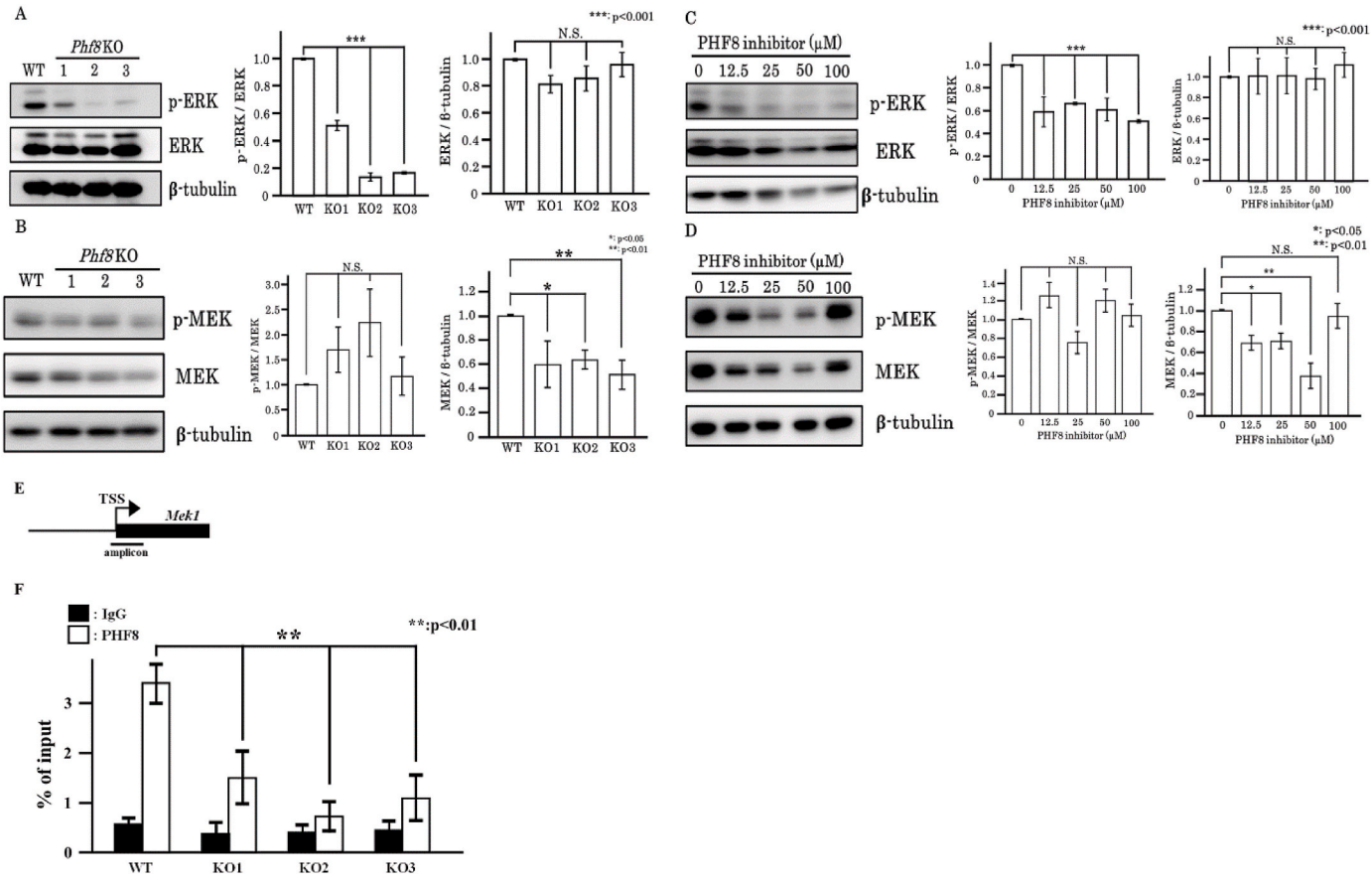

Fig. 4. PHF8 regulates the MEK/ERK pathway with decreasing MEK expression. A) ERK and phospho-ERK expression pattern after *Phf8* knockout. B) MEK and phospho-MEK expression pattern with *Phf8* knockout. C) ERK and phospho-ERK expression pattern after treatment with PHF8 inhibitor. D) MEK and phospho-MEK expression pattern after treatment with PHF8 inhibitor. E) An amplicon for quantitative-polymerase chain reaction is illustrated for *Mek1* around the TSS. F) ChIP assay for the binding region of PHF8 to the *Mek1* TSS. PHF8, plant homeodomain finger protein 8; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; TSS, transcription start site, WT, wild type. Data are presented as the mean ± SD of two independent experiments.SD, standard deviation. *:p < 0.05, **:p < 0.01, ***:p < 0.001, N.S., not significant.

CRediT authorship contribution statement

Shusuke Doi: Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Takayoshi Suzuki:** Resources, Funding acquisition. **Shuhei Soeda:** Methodology, Formal analysis. **Naoki Miyata:** Resources. **Tetsuya Inazu:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101670.

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