GPR137 promotes cell cycle exit and neuronal differentiation in the neuro2A cells

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Research Article

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Abstract

The orphan receptor, G protein-coupled receptor 137 (GPR137), is an integral membrane protein involved in several types of cancer. GPR137 is expressed ubiquitously, including in the central nervous system (CNS). We established a GPR137 knockout (KO) neuro2A cell line to analyze GPR137 function in neuronal cells. KO cells were generated by genome editing using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and cultured as single cells by limited dilution. Rescue cells were then constructed to re-express GPR137 in GPR137 KO neuro2A cells using an expression vector with an EF1α promoter. GPR137 KO cells increased cellular proliferation and decreased neurite outgrowth (i.e., a lower level of neuronal differentiation). Furthermore, GPR137 KO cells exhibited increased expression of a cell cycle regulator, cyclin D1, and decreased expression of a neuronal differentiation marker, Neuro D1. Additionally, GPR137 KO cells exhibited lower expression levels of the neurite outgrowth markers STAT3 and GAP43. These phenotypes were all abrogated in the rescue cells. In conclusion, GPR137 deletion increased cellular proliferation and decreased neuronal differentiation, suggesting that GPR137 promotes cell cycle exit and neuronal differentiation in neuro2A cells. Regulation of neuronal differentiation by GPR137 could be vital to constructing neuronal structure during brain development.

Introduction

G protein-coupled receptor 137 (GPR137), discovered by searching the Genbank genomic database [1], is an orphan GPCR-encoding gene [2]. GPR137 is also known as transmembrane 7 superfamily member 1-like protein, C11orf4 or GPR137A, and is an integral membrane protein [3]. It is involved in the proliferation of tumor cells in several cancers, including ovarian [4], gastric [5], pancreatic [6], hepatoma [7], urinary/bladder [8], and prostate cancers [9], as well as medulloblastoma [10], malignant glioma [11], osteosarcoma [12], and leukemia [13]. RNA interference (RNAi)-mediated downregulation of GPR137 inhibits tumor cell growth [5–7, 9–13]. These results indicate that GPR137 plays a role in tumor cell proliferation and could be a potential therapeutic target for several types of cancers. In addition to tumor cells, GPR137 is expressed ubiquitously, including in the central nervous system (CNS) [1]. However, the function of GPR137 and its associated ligands in neuronal cells remains unknown.

Neuro2A cells are widely used as a neurite outgrowth model during neuronal differentiation [14] and share similar properties as neuronal progenitor cells (NPCs) [15, 16]. NPCs can proliferate a limited number of times and differentiate into neurons. The proliferative NPCs initially exist in an undifferentiated immature state and subsequently cease to proliferate and differentiate into mature neurons [17]. Cell cycle regulators and transcription factors are related to the differentiation of NPCs. Cyclin D1, a cell cycle regulator, promotes the transition from G1 to S phase and the progression of the cell cycle to maintain NPCs in an immature state [18]. The transcription factor, prospero homeobox protein-1 (PROX1), downregulates cyclin D1 expression [19]. Neurogenic differentiation factor 1 (Neuro D1) is a member of the basic helix-loop-helix (bHLH) protein family and plays a critical role in neuronal progenitors to neuronal differentiation [20]. Neurite outgrowth is a primary marker associated with neuronal differentiation, which is a crucial process in the development of neuronal functions. STAT3 is
another critical transcription factor that promotes neurite outgrowth [21]. Growth-associated protein 43 (GAP43) is a neurite outgrowth marker and is usually expressed in differentiated neurons [22]. Signaling pathways such as cAMP response element-binding protein (CREB), protein kinase B (AKT), and extracellular signal-regulated kinase (ERK) play a vital role in NPC proliferation and differentiation [23, 24].

To evaluate GPR137 function in neuronal differentiation, we established GPR137 knockout (KO) neuro2A cells and investigated its role in neuronal differentiation.

**Materials And Methods**

**Cell culture**

A mouse neuroblastoma cell line, Neuro2A cells (IFO50081) were obtained from the JCRB Cell Bank (Osaka, Japan). The cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO₂ at 37 °C.

**GPR137 KO neuro2A cell generation and GPR137 genetic rescue**

Experimental protocols were approved by the DNA experiment safety committee of Saitama Medical University. GPR137 KO neuro2A cells were generated using the Guide-it™ CRISPR/Cas9 systems (Takara Bio Inc., Shiga, Japan). GPR137-specific gRNAs (No.1 Forward: 5′-CCGGCTCTGGCCGACGCTTCCCT3′ Reverse: 5′-AAACAGGCGAAGCGTCGGCCAGAG-3′; protospacer adjacent motif (PAM) sequence; TGG: No.2 Forward: 5′-CCGGAGGCATCTAGCCGGCTCCGA-3′ Reverse: 5′-AAACTCGGAGCCGGCTAGATGCCT-3′; PAM sequence; GGG) were designed using CRISPR direct [25] and synthetic oligos were ligated into Guide-it-ZsGreen1 vector. The plasmid vectors were transfected into neuro2A cells with Lipofectamine 3000 (Invitrogen). Neuro2A cells expressing ZsGreen were selected and cultured as single cells by limited dilution. A Guide-it genotype confirmation kit (Takara Bio Inc.) was used to identify the homozygous mutants. In-del detection and cloning of targeting sites were performed using a Guide-it Indel Identification kit (Takara Bio Inc.). The colonies for KO were identified by the changes in their DNA sequences.

Rescue cells were then constructed to re-express GPR137 in GPR137 KO neuro2A (KO R) cells. The full open reading frame of murine GPR137 complementary DNA (cDNA) was obtained by PCR with Pfu DNA polymerase (Promega, Madison, WI) from a cDNA library synthesized from murine mRNA using oligonucleotide primers (Forward: 5′-GAGGAAGAAGCCTCCCAATC-3′ Reverse: 5′-CACCTGGGAGAAGGCGTCCAG-3′). The PCR product was then ligated into pEF6/V5-His vector (Invitrogen). The rescue plasmid vectors were subsequently transfected into GPR137 KO neuro2A cells with lipofectamine (Invitrogen). KO R cells stably expressing GPR137 were selected and cultured as single cells by limited dilution.
RNA extraction and reverse transcription (RT)-PCR

Total RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. Total RNA was reverse-transcribed using a PrimeScript RT reagent kit (Takara Bio Inc.). The following primer sequences were used for RT-PCR: GPR137 (No. 1 Forward: 5'-TGCTTCTGTATGGGCACAAG-3' and Reverse: 5'-CCCTATAGCAGCTGCCTGAC-3', No. 2 Forward: 5'-ATGCCAGCGGGGCTTGTTAC-3' and Reverse: 5'-AGCAGATCACGTCTGTGGTG-3').

Cell growth assay

Microculture tetrazolium technique (MTT) assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolically active cells. Cells (1 × 10^5 cells/well) in 24-well plates were treated and 50 µl of MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (FUJIFILM, Osaka, Japan) (5 mg/ml in PBS) was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for 4 h. After removing the medium, formazan crystals were dissolved in 200 µl isopropanol /HCl (100 : 0.34), and the absorbance was measured using a micro plate reader (Bio-Tek, Redmond, WA) at 570 nm relative to 630 nm.

Measurement of neurite outgrowth

Cells (3 × 10^5 cells/well) were seeded in 6-well plates and incubated for 24 h. The medium was then replaced by serum-free fresh medium with or without retinoic acid (10 nM, RA, FUJIFILM). Cells were incubated for 24h and photographed at 10 × magnification, and images were captures using a KEYENCE BZ-X710 microscope (Keyence Corporation, Osaka, Japan). Differentiated cells were defined as cells with neurites longer than twice the cell body diameter [26].

Western Blotting

Cells were homogenized on ice in RIPA buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% DOC] containing a protease inhibitor cocktail (Calbiochem, San Diego, CA) (1 : 1000 dilution) with a tissue homogenizer (Brinkmann Instruments, Westbury, NY). Protein concentrations were determined using a BCA protein assay kit (Nacalai Tesque, Tokyo, Japan). Proteins (10 µg /lane) in lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Redmond, WA). After blocking with 5% skim milk (MEGMILK SNOW BRAND Co Ltd, Tokyo, Japan) in PBS containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate, Nacalai Tesque) (PBS-T), the membranes were incubated with primary antibodies overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) and then washing thrice with PBS-T. The membranes were then incubated with chemiluminescence reagent (Chemi-Lumi One Super, Nacalai Tesque; ImmunoStar LD, FUJIFILM). Images of the membranes were captured using a C-DiGit blot scanner (LI-COR, Lincoln, NE) and subjected to ImageJ analysis. Each membrane was probed with anti-GAPDH antibody (1 : 1000, ABS16, Millipore,
Billerica, MA), and the bands were used as loading controls. A pre-stained molecular weight marker was used to confirm expected sizes of the target proteins.

The primary antibody is anti-GPR137 (1 : 1000, 11929-1-Ab, Proteintech, Chicago, MA), anti-Cyclin D1 (1 : 1000, ab134175, abcam, Cambridge, MA), anti-PROX1 (1 : 1000, ab199359, abcam), anti-NeuroD1 (1 : 1000, ab213725, abcam), anti-STAT3 (1 : 1000, MAB1799, R&B Systems, Minneapolis, MN), anti-GAP43 (1 : 1000, ab9674, abcam), anti-CREB (1 : 1000, ab32515, abcam) anti-CREB1 (1 : 1000, ab9674, abcam), anti-AKT (1 : 1000, #587F11, Cell Signaling Technology), anti-p-AKT (1 : 1000, #9271S, Cell Signaling Technology), anti-ERK (1 : 1000, #9102, Cell Signaling Technology) dilution, anti-p-ERK (1 : 1000, sc-7383, Santa Cruz, Dallas, TX).

Statistics

Multiple comparisons were performed by one-way ANOVA followed by Newman-Keuls post-hoc test or two-way ANOVA followed by post-hoc Tukey test. All data were analyzed using Graph Pad Prism Ver. 5.01 (Graph Pad Software, Inc., San Diego, CA) and expressed as mean ± SEM. p values < 0.05 were considered statistically significant.

Results

GPR137 KO neuro2A cells were generated using the CRISPR/Cas9 system with two gRNAs, and single cells were cloned. Targeting site cloning suggests that KO cells were homozygous mutants. Sequencing revealed a 5- and 37-base deficiency accompanying a frameshift in two strains (Fig. 1A). The amino acid changes were observed at positions 127 and 234 in KO1 and KO2, respectively (Fig. 1B). Premature terminations, i.e., the introduction of a stop codon, was observed at amino acid positions 257 and 235 in KO1 and KO2, respectively (Fig. 1B). We tested the mRNA expression of GPR137 using reverse transcriptase polymerase chain reaction (RT-PCR) with primers specific to the deleted region. The amplification product of GPR137 was observed in the wild type (WT) but not in KO1 and KO2 cells (Fig. 1C). These data confirmed that KO cells were successfully generated. Genetic rescue experiments were conducted by constructing the cells rescued to re-express GPR137 in GPR137 KO neuro2A (KO R) cells. The western blotting analysis confirmed that GPR137 protein was not expressed in KO1 and KO2 cells, whereas it was expressed in WT, KO1 R, and KO2 R cells (Fig. 1D).

We investigated the effect of GPR137 deletion on cellular proliferation using the 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. KO1 and KO2 cells exhibited increased cell numbers compared to that in the WT (Fig. 2A). KO1 R and KO2 R cell numbers were comparable to that of the WT cells (Fig. 2B and C). To evaluate the effect of GPR137 deletion on neuronal differentiation, we investigated the neurite outgrowth of neuro2A cells. Neuro2A cells respond quickly to serum deprivation, which induces neurite outgrowth [27]. Differentiated cells were characterized by neurites that were twice as long as the diameter of the cell (Fig. 3A). WT cells exhibited normal neurite outgrowth, whereas KO1 and KO2 cells exhibited decreased neurite outgrowth (Fig. 3A and B). The neurite outgrowth levels in rescue cells, KO1 R and KO2 R, were similar to that in the WT cells (Fig. 3A and B). The WT cells induced approximately 30%
differentiation. Differentiated cells were decreased in KO1 and KO2 cells compared to that in the WT cells (Fig. 3A and C). Differentiated cells were increased in KO1 R and KO2 R, similar to the level of WT cells (Fig. 3A and C). Retinoic acid (RA) is a common inducer of neuronal differentiation [28]. We investigated neuronal outgrowth in the presence of RA. RA increased differentiation by approximately 45% (Fig. 4A and B). KO1 and KO2 cells exhibited lower neurite outgrowth and differentiation rates, which were restored in KO1 R and KO2 cells (Fig. 4A–C).

The effect of GPR137 deletion on the expression of neuronal differentiation-related marker proteins, cyclin D1, PROX1, and Neuro D1, were investigated. Cyclin D1 expression levels were upregulated in KO1 and KO2 cells and were restored in KO1 R and KO2 R cells (Fig. 5A and B). PROX1 is a transcriptional factor that downregulates cyclin D1 [29], which was decreased in KO1 and KO2 cells, and restored in KO1 R and KO2 R cells (Fig. 5A and C). Neuro D1 expression was decreased in KO1 and KO2 cells and recovered in KO1 R and KO2 R cells (Fig. 5A and D). STAT3 and GAP43 were downregulated in KO1 and KO2 cells and were restored in KO1 R and KO2 R cells (Fig. 5A, E, and F). Phosphorylated CREB (Fig. 5A and G), AKT (Fig. 5A and H), and ERK (Fig. 5A and I) are upregulated in KO1 and KO2 cells and were suppressed in KO1 R and KO2 R cells.

Discussion

To investigate the neuronal function of GPR137, we established GPR137 KO neuro2A cells by CRISPR/Cas9-mediated genome editing. GPR137 KO cells exhibited increased cellular proliferation and decreased neurite outgrowth, suggesting that GPR137 has a role in cell cycle exit and neuronal differentiation in neuro2A cells. Moreover, these phenotypes were reversed in cells that were rescued to re-express GPR137. These data provide convincing evidence to support the function of GPR137.

We demonstrated that the cyclin D1 protein expression was increased in GPR137 KO cells, accompanied by a decrease of PROX1, a transcription factor that downregulates cyclin D1. Additionally, GPR137 deletion decreased the neuronal differentiation marker, Neuro D1. Concordantly, cyclin D1 directly regulates immature state maintenance and cell cycle acceleration and proliferation in NPCs [19, 30], whereas PROX1 suppresses neuro2A cell proliferation [29]. Neuro D1 (also known as BETA2) plays a critical role in neuronal differentiation of NPCs [20] and induces cell cycle exit [31]. These results indicate that GPR137 promotes cell cycle exit via cyclin D1 downregulation and neuronal differentiation, simultaneously upregulating Neuro D1.

GPR137 involvement in neuronal differentiation was also revealed by decreased neurite outgrowth in GPR137 KO cells. Moreover, the STAT3 and GAP43 protein levels were decreased in GPR137 KO cells. STAT3 is a key transcription factor that regulates neurite outgrowth in neuro2A cells [21]. GAP43 is expressed in the neurite growth cone and is a major determinant of neurite outgrowth [32]. Reduced neurite outgrowth and low marker protein levels suggest that neuronal differentiation is suppressed in GPR137 KO cells. Therefore, these data also confirmed the role of GPR137 in regulating neuronal differentiation.
Intracellular signaling, such as CREB, AKT, and ERK signalling, are involved in neuronal proliferation [33–35] and differentiation [36–38]. Our results indicate that GPR137 deletion increases the phosphorylation of CREB, AKT, and ERK, suggesting that GPR137 downregulated phosphorylation of these signaling pathways. Consequently, downregulated phosphorylation of CREB, AKT, and ERK would act downstream of GPR137 to influence neuronal differentiation in neuro2A cells.

Furthermore, RA induces neuronal differentiation by activating the transcription of genes related to cell signaling, structure protein, enzymes, and receptors [39]. In this study, GPR137 deletion did not alter the phenotypes in the presence or absence of RA. We considered that the mechanism of GPR137-mediated neuronal differentiation was independent of RA signaling cascades.

NPC proliferation is vital in maintaining the NPC pools during neurogenesis [40]. Subsequently, NPCs must halt their proliferation, accelerate cell cycle exit, and differentiate into neurons during brain development [17]. Regulation of these events by GPR137 may be crucial in the formation of the neuronal structure.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

KI performed the majority of experiments. AY performed experiments, contributed data. KY designed the research study. KI and KY wrote the first draft of the manuscript. SY, CH, and KM contributed to the writing of the manuscript. KM supervised the entire project and reviewed the manuscript.

Data availability

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All studies were approved by the DNA experiment Safety Committee of Saitama Medical University.

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References

suppressing the p53/p21 axis. Cancer Sci. 111:175-185


Figures
Figure 1

GPR137 KO neuro2A cell establishment using CRISPR/Cas9 system. (A) Nucleotide sequences corresponding to GPR137 and direct sequencing results of KO cells with their corresponding primers (indicated with blue arrows) and PAM sequences. (B) The amino acid sequence of the WT and CRISPR/Cas9-mediated GPR137 genome editing. The frameshift mutation and premature termination
observed in KO1 and KO2. (C) Gel electrophoresis analysis of the RT-PCR of *GPR137*. (D) Western blot analysis of the GPR137 protein for the WT, KO1, KO1 R, KO2, and KO2 R groups.

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**Figure 2**

Cell growth of WT, *GPR137* KO neuro2A (K0) cells, and *GPR137* KO neuro2A + *GPR137* transfected (K0 R) cells. (A) Cell growth of the WT and KO cells. (B) Cell growth of WT, KO1, and KO1 R cells. (C) Cell Growth
of WT, KO2, and KO2 R cells. Data are mean ± SEM, n = 5 per group. Statistical analysis was performed using two-way ANOVA followed by post-hoc Tukey test (***, ###p < 0.001).

Figure 3

Cellular differentiation rates of the WT, GPR137KO neuro2A (KO) cells, and GPR137KO neuro2A + GPR137 transfected (KO R) cells evaluated by neurite outgrowth. (A) Light micrographs of the
differentiated cells. White arrows indicate clearly differentiated cells. (B) Neurite length of WT, KO, and KO R cells. (C) The cellular differentiation rates of WT, KO, and KO R cells. Data are mean ± SEM, n = 5 per group. Statistical analysis was performed using one-way ANOVA followed by post-hoc Newman-Keuls test (*p < 0.05; **p < 0.001).
Cellular differentiation rates of WT, *GPR137* KO neuro2A (KO) cells, and *GPR137* KO neuro2A + *GPR137* transfected (KO R) cells evaluated by neurite outgrowth in the presence of retinoic acid. (A) Light microscopic photographs of the differentiated cells. Clearly differentiated cells are indicated by white arrows. (B) Neurite length of WT, KO, and KO R cells. (C) Cellular differentiation rates of WT, KO, and KO R cells. Data are mean ± SEM, n = 5 per group. Statistical analysis was performed using one-way ANOVA followed by post-hoc Newman-Keuls test (*p < 0.05; **p < 0.01).

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**Figure 5**
Figure 5

Intracellular signaling of WT, GPR137 KO neuro2A (K0) cells, and GPR137 KO neuro2A + GPR137 transfected (K0 R) cells. (A) Protein expression levels were determined by western blot analysis. Protein levels of Cyclin D1 (B), PROX1 (C), Neuro D1 (D), STAT3 (E), GAP43 (F), p-CREB / CREB (G), p-AKT / AKT (H), and p-ERK / ERK (I). Data are mean ± SEM, n = 5 per group. Statistical analysis was performed using two-way ANOVA followed by post-hoc Tukey test (*p < 0.05; **p < 0.01; ***p < 0.001).

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