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ORIGINAL ARTICLE

In utero gene therapy rescues microcephaly caused by Pqbp1-hypospension in neural stem progenitor cells


Human mutations in Pqbp1, a molecule involved in transcription and splicing, result in a reduced but architecturally normal brain. Examination of a conditional Pqbp1-knockout (cKO) mouse with microcephaly failed to reveal either abnormal centosomes or mitotic spindles, increased neurogenesis from the neural stem progenitor cell (NSPC) pool or increased cell death in vivo. Instead, we observed an increase in the length of the cell cycle, particularly for the M phase in NSPCs. Corresponding to the developmental expression of Pqbp1, the stem cell pool in vivo was decreased at E10 and remained at a low level during neurogenesis (E15) in Pqbp1-cKO mice. The expression profiles of NSPCs derived from the cKO mouse revealed significant changes in gene groups that control the M phase, including anaphase-promoting complex genes, via aberrant transcription and RNA splicing. Exogenous Apc4, a hub protein in the network of affected genes, recovered the cell cycle, proliferation, and cell phenotypes of NSPCs caused by Pqbp1-cKO. These data reveal a mechanism of brain size control based on the simple reduction of the NSPC pool by cell cycle time elongation. Finally, we demonstrated that in utero gene therapy for Pqbp1-cKO mice by intraperitoneal injection of the Pqbp1-AAV vector at E10 successfully rescued microcephaly with preserved cortical structures and improved behavioral abnormalities in Pqbp1-cKO mice, opening a new strategy for treating this intractable developmental disorder.

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INTRODUCTION

Microcephaly is a group of multiple disorders including PQBP1 gene mutations and classified by the presence or absence of architectural change of cortex. The mechanism of neuronal production is one of the most critical factors affecting brain size. According to the classical radial unit hypothesis, brain volume (cortical thickness x surface area) is determined by neuronal production from neural stem cells (NSCs) per radial glia unit.6

Neurons in the neocortex are generated over a 6-day neurogenesis interval (E11-E17) that comprises 11 cell cycles of NSC self-renewal and neurogenesis.7 In this model, excessive neurogenesis during cell division depletes the NSC pool, decreases the vertical expansion (radial growth) of the pool as well as the final production of neurons, and leads to microcephaly.8,9 However, lateral expansion (tangential growth), which should be also related to brain size, has not been explained well.

The discovery of new subsets in the neural stem progenitor cell (NSPC) pool has made the story more complex. First, radial glia were shown to be identical to NSCs and apical progenitor cells (AP) in the ventricular zone (VZ).9–12 Next, the Tbr2-positive basal progenitor cell (BP) in the subventricular zone (SVZ) was identified to be the primary source of neurons and assumed to regulate tangential (horizontal) and radial (vertical) expansion of the cortex.12–15 Moreover, basal radial glia (bRG), Pax6-positive cells on the basal side of the SVZ, were discovered to be another source of neuron production.8,16–18 Therefore, the hypothesis was revised to state that the amounts of BPs/bRGs relative to NSCs/APs regulate cortical thickness, although the details of the contribution of the two basal progenitors to corticogenesis remained unknown.

Two recent reports have provided important lines of evidence to this question. Analysis of Trnp1, a DNA-associated protein whose exact molecular function is not yet known, revealed that overexpression of Trnp1 increased the self-renewal of radial glia and expanded the NSPC pool laterally, while knockdown (KD) of Trnp1 decreased the number of BPs/bRGs and increased the radial expansion and gynification of the cortex.19 Another group simultaneously reported that the overexpression of Cdk4 and CyclinD1 (4D) in transgenic mice increased BP/bRG numbers and gynification.20 Importantly, no abnormality was detected in the cortical layer structure of the folded cortex in 4D transgenic

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mice.20 Therefore, the combination of lateral expansion (tangential growth) of the NSPC pool, which is affected by neurogenesis ratio, and vertical expansion (radial growth) to produce BP/bRG and neurons is now believed to determine the surface area, thickness and gyration of the cortex.19–21

In this study, we report that cell cycle elongation of NSPCs by the NSPC-specific depletion of Pqbp1, which simply delays the entire process, finally causes brain downsizing in vivo that mimics the primary microcephaly (PM)-like phenotype of human patients with PQBP1-linked intellectual disability (ID). Although neuron and BP production ratios are unchanged, cell cycle elongation in NSPCs decreases the AP and BP pools equally, and results in the proportional reduction of both tangential and radial expansion. Cell death is not increased in vivo during these processes except in the extreme case of shRNA-mediated KD. The molecular mechanism, elongation of cell cycle time, is due to aberrant transcription and RNA splicing of cell cycle-regulating genes. Moreover, we have developed a new therapy, the peritoneal injection of Pqbp1-adenosine-associated virus (AAV) to pregnant mice, that rescues the microcephaly of the offspring.

MATERIALS AND METHODS

For more detailed information, please refer to ‘Methods’ in Supplementary information.

Generation of conditional knockout of murine Pqbp1

To generate the targeting vector, three Pqbp1 genomic fragments were PCR amplified from a murine bacterial artificial chromosome (BAC) library (ID: RP23-404N15). A 3.6-kb 5′ fragment containing exons 1 and 2 was inserted upstream of a neomycin resistance cassette flanked by Flp recognition target sites. A 3.9-kb fragment containing exons 3 to 7 was inserted between two LoxP sites and a 4.1-kb non-coding fragment was added 3′, with the diphtheria toxin A gene to prevent random insertion. After electroporation into ES cells (C57BL/6), and G418 selection (Sigma, St. Louis, MO, USA), clonal ES cells were obtained by genomic DNA PCR using the following primers: fwd, 5′-AATCTTGGAGGTTAATGCTGCTT-3′; and rev, 5′-AATCTCATGTAATGGACAGACGAG-3′. Selected ES clones were corroborated by Southern blot analyses of genomic DNA digested with EcoRI or EcoRV. Probes for Southern blot analyses were prepared using primers from the BAC clone using the following primers (locations given in Supplementary Fig 2): 5′ probe (408 bp), fwd: AAGTGGAACCTCGTATTAGGAGA-3′; rev: TCAGTGAGATCTGACTTCCACA; 3′ probe (462 bp), fwd: GTCAATAGATCAGATGGACGACT; rev: TACAAGATCTTTGAAGCTCTATA. Chimeric mice were generated by injecting the recombinant ES cells into C57BL/6 blastocysts, subsequently crossed with C57BL6/J mice to generate the targeted allele. The neomycin resistance cassette was removed by crossing with CAG-FPL recombinase transgenic mice.22 The resultant Pqbp1-floxed heterozygous female mice were further crossed with Nestin-Cre transgenic male mice (B6.Cg-Tg(Nestin-Cre)1Kln/J; The Jackson Laboratory, Bar Harbor, ME, USA) and Synapsin1-Cre transgenic male mice (B6.Cg-Tg(Syn1-Cre)671Jxm/J; The Jackson Laboratory) to generate the Pqbp1 conditional knockout.

Cumulative labeling

The method for analyzing cell cycle parameters in the neuroepithelium23 was employed with minor modifications. BrdU (Sigma; 100 mg/kg of body weight) was injected intraperitoneally into pregnant mice at E14. Cumulative labeling was performed by repeated injections (at 3-h intervals) into pregnant mice, which were killed 1, 1.5, 2, 3.5, 6.5 and 24.5 h after the first BrdU injection. Embryonic brains were fixed with 4% paraformaldehyde and paraffin embedded. Sections were made at 3 mm intervals, deparaffinized, rehydrated and then microwaved in 10 mM of citrate buffer, pH 6.0, for 15 min. Antibody incubations were done with the mouse anti-BrdU antibody (1:200, DAKO Biosciences, Franklin Lakes, NJ, USA) and rabbit anti-phospho-histone H3 (pH3) antibody, a marker for M-phase cells (1:500, Millipore, Billerica, MA, USA) at 4 °C overnight. Secondary antibody incubations were done with Alexafluor-488 or Cy3 conjugates (1:500, Invitrogen, Carlsbad, CA, USA). The ratio of BrdU/pH3-double-positive cells to pH3-positive cells in the ventricular zone was calculated at 1, 1.5 and 2 h after a single injection of BrdU to determine the length of the G2/M phase. A straight-line graph of the labeling index values (LIs) at 1, 1.5, 2, 3.5 and 6.5 h allowed us to extrapolate to a y-axis intercept (the LI at 0 h) and calculate the slope. Since the growth fraction (the ratio of proliferating cells) is nearly 1.0 in the ventricular zone of wild mice, the LI at 0 h and slope represent the ratio of S-phase to total cell cycle (Ts/Tc) and the reciprocal of total cell cycle (1/Tc), respectively. Ts and Tc denote the length of the S-phase and total cell cycle, respectively. From these values (Ts/Tc and 1/Tc), Ts and Tc were calculated.

Exon array-based analysis of alternative splicing

We analyzed wild-type (WT) and PQBP1 conditional knock-out (cKO) mouse brain samples using Affymetrix GeneChip Mouse Exon 1.0 ST array (exon array) (http://www.affymetrix.com) to find significantly changed genes in terms of potential alternative splicing. The exon array contains over five million probes representing about 1.4 million probesets that are designed based on the genomic regions of known genes and exons to measure both gene-level and exon-level expression in samples. The sequences of the probes and the probesets were downloaded at the Affymetrix website (http://www.affymetrix.com/support/technical/whitepapers/exon_probeset_trans_clust_whitepaper.pdf). To summarize the probes into the exon-level probesets, the PLIER algorithm (http://www.affymetrix.com/analysis/index.affx) was applied to the probe signals of our samples.

The methodology and statistical hypothesis testing, exon–exon and variance analyses, were performed on the exon-level probesets to detect changes in alternative splicing. Before these tests, the signals of each exon-level probeset in one gene were normalized by their total signals to eliminate the effects of different numbers of exons among compared genes. The exon–exon analysis was performed to compare each exon’s signals between WT and cKO mice to find the significant change in exon level that could be induced by the change of alternatively transcribing or splicing. The difference was examined by Student’s t-test based on the null hypothesis that the means of the two samples are the same. The variance analysis was performed on exon-level probesets in each gene of WT and cKO mice to detect changes in relative expression levels among exons by an F-test for the null hypothesis that these two samples have the same pattern and the variance.

The smallest P-value was selected as the significance level of the gene to find alternatively spliced exons and it was significant with P-value < 0.05. Genes showing significantly different expression between WT and cKO mice in exon–exon and/or variance analysis were listed to apply to PANTHER analysis (http://www.pantherdb.org/). In Panther analysis, statistical overrepresentation test was performed to determine whether the genes were enriched or deprived for specific biological processes.

Three groups of significantly changed genes, (A) Nestin-kO-NSC specific genes, (B) co-occurrence between the NSC and cortex of Nestin-kO mice and (C) co-occurrence genes of the three types of cKO samples were tested individually by PANTHER analysis to be compared with all the genes detected in the assay and then pie-charts of protein function classification were formed.

In vivo rescue experiment of Apc4

In utero electroporation and determination of Pial-to-Apical surface area experiments were performed as described previously.16 Full-length murine Apc4 cDNA (Genbank accession number NM_024213) was obtained by RT-PCR using fwd (5′-GGGGCTCGAGACATGGAATGCTGGTACCTGCAGCTT-3′) and rev (5′-GGCCGATCTTATTATTGTTCGTATCAGC-3′) primers. The product was inserted into Xhol/BamHI cut pIRE2-EGFP (Clontech) to generate pApc4-IRE2-EGFP. For the Apc4 rescue experiments, pApc4-IRE2-EGFP or pIRE2-EGFP was electroporated into the ventricular zone of E13 embryos. Brain tissues of E18 embryos were fixed, embedded in 3% agarose, and sectioned on the rostral-to-caudal axis as 50-μm-thick sections using a vibratome. Brain sections were incubated with mouse anti-green fluorescent protein (GFP) monoclonal antibody (1:500, Millipore) at 4 °C overnight, followed by treatment with Alexafluor-488 labeled secondary antibodies. Sections were analyzed with a confocal fluorescence microscope (Olympus FV10i, Tokyo, Japan) and the ratio of pial-to-apical surface determined as described previously.24

Gene therapy with PQBP1-AAV vector

The AAV vector plasmids contained an expression cassette, consisting of a simian virus 40 polyadenylation signal sequence (SV40 poly (A)) between the AAV vector plasmids contained an expression cassette, consisting of a simian virus 40 polyadenylation signal sequence (SV40 poly (A)) between

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Microcephaly with normal cortical structures in PQBP1 patients and nestin-Cre Pqbp1-cKO (conditional Pqbp1-knockout) mice.

(a–c) Magnetic resonance imaging of a PQBP1-mutated patient showed normal cortical structures with no periventricular heterotopia: (a) horizontal, (b) coronal and (c) sagittal sections. (d) Macroscopic images of the brain at the age of 2 months. Male nestin-Cre Pqbp1-cKO mouse brains (Nes-Cre; X\textsuperscript{FloxY}) were consistently the smallest among the littermates. (e) Pqbp1-cKO mice showed reduction of brain weight at 2 months. The bar graph shows the mean±s.e.m. for each genotype with the number of mice used indicated above. The mean and s.e.m. values are provided in the text. Asterisks indicate significance (P < 0.01) in one-way analysis of variance with post hoc Bonferroni test. (f) Macroscopic images of the brain at the age of 2 months. Male synapsin 1-Cre Pqbp1-cKO mouse brains (synapsin1-Cre; X\textsuperscript{FloxY}) were not different from the littermates in size. (g) The brain weight of the male synapsin-1-Cre Pqbp1-cKO mouse was not different from that of the background control. (h) Coronal sections of adult brains of nestin-Cre Pqbp1-cKO mouse and littermates (2 months, at −1.82 mm from the bregma in background mice). (i) Staining for layer-specific markers, Cux1, Foxp1 and Tbr1, together with GAD67, shows preservation of cortical layers in Pqbp1-cKO mice at 2 months.
the inverted terminal repeats of AAV 3 genome. The recombinant AAV vectors were produced by transient transfection of HEK293 cells using the vector plasmid, an AAV2 rep and AAV1 vp expression plasmid, and an adenoviral helper plasmid, pH helper (Agilent Technologies, Santa Clara, CA, USA). The recombinant viruses were purified by isolation from two sequential CsCl gradients, and the viral titer were determined by qRT-PCR. For in vivo administration of AAV vectors, C57BL/6J pregnant mice (E10) were injected with AAV-PQBP1 (2.0 × 10¹¹ genome copies) by intraperitoneal administration.

RESULTS

Human PQBP1 mutations cause PM

First, we defined the characteristics of PQBP1-linked microcephaly in human patients. Microcephaly is generally defined as an innate non-progressive small brain sized less than 4 standard deviations (s.d.); a milder form of microcephaly (less than 3 or 2 s.d.) has been also reported in some patients. In PQBP1-linked microcephaly, the brain size ranges from less than 6 s.d. to less than 2 s.d. Although one PQBP1-linked microcephaly patient with pterygionization heterotopia (6 s.d.) was reported,24 PH is likely to be a rare clinical feature as it was not observed in an affected sibling with the identical mutation,24 or in a further 13 microcephalic patients with PQBP1 mutations.25 By magnetic resonance imaging, we confirmed the presence of well-preserved cortical architecture in two previously described but unrelated patients with mutated PQBP12,26 (Figures 1a–c). A slight dilation of the ventricles was observed in these patients having mutated PQBP1, as seen in the autosomal recessive PM (MCPH) cases. All these considerations revealed that PQBP1-linked microcephaly is quite similar to PM in morphology.

Pqbp1-cKO in NSCs mimics the microcephaly of human patients

Next, we aimed to generate a model of PQBP1-linked microcephaly in NSCs. Microcephaly is highly expressed in NSPCs (Sox2-positive AP cells and RC2-positive radial glia in the VZ and SVZ) and Sox2 transcriptionally regulates Pqbp1 in NSPCs.27 Pqbp1 is also expressed in differentiated neurons at a lower level.5,27,28 We expected that the expression level of the Pqbp1 protein was remarkably decreased in the total brain tissue of nestinCre-cKO mice, although a faint band was detected due to the non-specific PM (MCPH) cases. All these considerations revealed that PQBP1-linked microcephaly is quite similar to PM in morphology.

Asymmetric cell division is not changed in Pqbp1-cKO mice

At the cell level, we checked pathologies in the NSPCs of nestinCre-cKO mice. The M phase was specifically elongated in NSPCs (Sox2-positive AP cells and RC2-positive radial glia in the VZ and SVZ) and Sox2 transcriptionally regulates Pqbp1 in NSPCs.27 Pqbp1 is also expressed in differentiated neurons at a lower level.5,27,28 We therefore generated two types of conditional KO (cKO) mice using nestin-Cre and synapsin-1-Cre (Figures 1d and e). Surprisingly, the brain size was not changed in synapsin-1-Cre derived Pqbp1-cKO mice (Figures 1f and g). This discrepancy indicated that Pqbp1 function in NSPCs but not in neurons is responsible for microcephaly. The PM-like morphological features of the brain indicated that the nestin-Cre cKO mouse is a good model for PQBP1-linked microcephaly.

Figure 2. Nestin-Cre Pqbp1-cKO (conditional Pqbp1-knockout) delays the cell cycle but does not affect neurogenesis of neural stem progenitor cells (NSPCs). (a) The M phase was specifically elongated in NSPCs of Pqbp1-cKO mice (Nes-Cre; X<sup>−/−</sup>) in vivo. Cumulative labeling of NSPCs at E14 in vivo showed an increase in the total cell cycle length (Tc) of +2.2 h, +12% (upper left panel). G2/M phase time was evaluated using phosphorylated histone H3 (upper right panel). The middle panels show immunostaining data corresponding to the upper panels. pH3/BrDU + cells were reduced in number in upper right panel, indicating elongation of the G2/M phase (middle right panel). The summary of cumulative labeling and G2/M analyses is shown in the lower table. A significant extension of the G2/M phase (+67%) and a slight extension of G1 (+6%) were observed. The crossing point between the plot line and the x-axis indicates the length of G2 phase (upper right panel). Thus, the M phase was remarkably elongated while the length of the G2 phase was unchanged in Pqbp1-cKO mice. (b) Neurogenesis from the stem cell pool was analyzed by co-staining for BrdU and Ki67. At 12 or 72 h after intrauterine injection of BrdU, E15 embryonic brains were analyzed to calculate the numbers of cells after neurogenesis (BrdU+/Ki67−), cells remaining in the stem cell pool (BrdU+/Ki67+) and nonlabeled stem/progenitor cells (BrdU−/Ki67+). The bar graphs show the relative percentages of the three groups (left graph) and the neurogenesis percentage of BrdU-labeled cells (right graph). No difference was detected at 24 and 72 h after BrdU injection by the Student’s t-test or Welch’s t-test. (c) Levels of cell death in the cerebral cortex were evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining at E10, E15, E18, P0 and P60 of Pqbp1-cKO mice (Nes-Cre; X<sup>−/−</sup>) and nestin-Cre mice (Nes-Cre; XY). Quantitative analysis of apoptotic cells did not reveal any differences (Student’s t-test).
(microcephalin, WDR62, CdkSrap2, CEP152, ASPM, CENPJ and STIL) are localized to the centrosome-spindle pole of NSPCs, and their defective function causes abnormal centrosome-spindle pole structures. A recent report showed that shRNA-mediated KD of Cdk5Rap2 actually increased neurogenesis and the BP pool but reduced the AP pool. Intriguingly, despite large amounts of accumulated data, this series of cell cycle abnormalities have not yet been sufficiently integrated into the newest hypothesis of corticogenesis.

Hence, we first evaluated the cell cycle time of NSPCs in vivo using the cumulative labeling method. The data showed that the total cell cycle time (Tc) was increased (+2.2 h, +12%) (Figure 2a).
The S phase (Ts) was unaltered compared to the strict control nestin-Cre XY mice (Figure 2a). The G1 phase was slightly longer (Figure 2a). However, G1 elongation in the Pqbp1-cKO was minimal (5.2%) compared to other reports of cell cycle length regulating brain size.33,34 G2 phase time was not changed (Figure 2a). In contrast to the G1, S, and G2 phases, the M phase was remarkably increased (Figure 2a) and the increase in Tc was principally due to a longer M phase (+1.4 h, +67%). This cell cycle change in Pqbp1-cKO mice is quite unique among various disease models.

Neurogenesis and apoptosis of NSCs are unchanged in Pqbp1-cKO mouse embryos (Figure 2b). We labeled proliferating cells at the S phase with BrdU, and examined the brain after an interval to check whether they exited from the proliferating stem cell pool, by co-staining with the proliferation marker Ki67. At 24 and 72 h after labeling, the ratios of the remaining stem cells to differentiated cells were not largely different between nestin-Cre XY littermates and Pqbp1-cKO mice (Figure 2b, graphs). In addition, in utero electroporation of a GFP-expressing vector into the embryonic brain of the Pqbp1-cKO mouse was performed at E14 and the embryos were dissected at E16. The neurogenesis rate, calculated from the ratio of GFP+/ki67− versus total GFP+ cells, was also unaltered (data not shown).

Further, to test the effect of Pqbp1 on neurogenesis, we performed in vivo KD using short hairpin RNA against Pqbp1 (Pqbp1-shRNA). We generated plasmids expressing Pqbp1-shRNAs (Supplementary Figure 4A) that successfully suppressed Pqbp1 in P19 cells, as evidenced by western blot (Supplementary Figure 4B), and dramatically reduced Pqbp1 in NSPCs in vitro or in vivo (Supplementary Figures 4C–E). In all these cases, Pqbp1 was decreased to nearly 10%. Pqbp1-KD by in utero electroporation of Pqbp1-shRNA-ZsGreen into normal mice delayed the exit of transfected cells from the stem cell layer V2/SVZ (Supplementary Figures 5A and B) and delayed their entrance into the differentiating cell layer VZ (Supplementary Figure 5C). These results supported the conclusion from Pqbp1-cKO mice that neurogenesis is not increased in Pqbp1 deficiency.

Increased apoptosis of NSPCs is another plausible mechanism for PM. Indeed, apoptosis was found to be increased in human microcephalic with preserved brain structure caused by mutations in polynucleotide kinase 3′-phosphatase, which is essential for many DNA repair pathways including base-excision repair and non-homologous end joining.46 However, repeated analyses by terminal deoxynucleotidyl transferase dUTP nick end labeling staining failed to demonstrate elevated apoptosis in embryonic (E10–E18), fetal (P0) and adult brains of Pqbp1-cKO mice (Figure 2c).

Single-cell volume of neuron is unchanged in Pqbp1-cKO

One may suspect that Pqbp1 deficiency causes brain size reduction by decreasing the single-cell volume of neurons. However, this is not plausible because synapsin-1-Cre cKO mice did not show microcephaly, as mentioned previously (Figures 1f and g). In addition, we examined the space occupied by a single neuron (cell volume) and the total dendrite length of a neuron (retrosplenial dysgranular cortex, layer V) in adult nestin-Cre and synapsin-1-Cre Pqbp1 cKO mice (P90) by two-photon microscopy (Supplementary Figures 6A and B). The results revealed no significant changes in total neuronal cell volume in nestin-Cre Pqbp1 cKO mice that had microcephaly, whereas the volume was decreased in synapsin-1-Cre Pqbp1 cKO mice that did not show microcephaly (Supplementary Figures 6A and B), indicating that the single-cell volume does not account for the microcephaly. Unexpectedly, total dendrite length was not changed either in nestin-Cre or in synapsin-1-Cre Pqbp1 cKO mice in comparison to control mice (Supplementary Figures 6A and B). However, the dendrite diameter tended to be thinner than in the control (data not shown).

Our previous collaborative study revealed the localization of Pqbp1 in the cilium of differentiated hippocampal neurons and its essential role in the maintenance of cilia.28 Given that cilia receive and transfer environmental signals like Shh to the cell body47 and that NSPCs also possess cilia, the function of Pqbp1 in cilia might be related to the proliferation of NSPCs.33 However, we could not detect any differences in the morphology of cilia on the ventricular surface of cortex where the cilia of NSPCs are located (Supplementary Figure 6C), and also could not localize the Pqbp1 protein to the centrosome of NSPCs during mitosis (Supplementary Figures 7A and B). These results suggested that the role of PQBP1 in promoting cilia is specific to differentiated neurons but not directly linked to the proliferation of NSPCs. The decreased cell volume of adult neurons in synapsin-1-Cre Pqbp1 cKO mice (Supplementary Figures 6A and B) was consistent with the change in the Pqbp1/cilia-derived signal in differentiated neurons.29 The cell volume change was compensated during development when Pqbp1 was depleted from NSPCs.

A unique mechanism of microcephaly in Pqbp1-cKO mouse embryos revealed that the size of the stem cell pool is already small at E10, when neurogenesis has not yet started (Supplementary Figure 2B). After E11, when neuroepithelial cells start to switch from symmetric proliferative to asymmetric neurogenic division, delayed cell cycle time (Figure 2a) keeps both AP and BP stem cell pools equally reduced at E15 (Supplementary Figures 2C and D), and the reduction of the stem cell pool leads to a decrease in the number of differentiated neurons (Supplementary Figure 2A). These results support a simple hypothesis of microcephaly based on delayed cell proliferation and reduced expansion of the stem cell pool, which is independent of unequal changes in the production of neurons or BPs.

Systems biology analyses reveal the molecular mechanism of microcephaly

Next, we investigated the molecular mechanisms underlying the cell cycle dysregulation of NSPCs. PQBP1 is a factor coupling transcription and splicing through two protein-binding motifs, the WW and C-terminal domains. Currently, mutations of the human POQB1 gene are classified into two types. The first category causes frame shifts that introduce premature stop codons before the C-terminal domain of POQB1.2–4 The second category targets a conserved amino-acid residue in the WW domain.59,60 These defects relate to a failure of PQBP1 to interact with its partner proteins RNA polymerase II61 and WBPI1/SIPPI152,53 via the WW domain, and US-15KD54,55 via the C-terminal domain.

Therefore, mutant PQBP1 is believed to disturb multiple cellular functions through impairment of RNA splicing and/or transcription. Wang et al.35 screened genes affected by aberrant splicing in mouse embryonic neurons and identified neural cell adhesion molecule (NCAM)-140, a splicing isoform of NCAM1 involved in neurite extension, as a target of PQBP1-mediated aberrant
Moreover, the Bonni group recently reported that Pqbp1 interacts with the GTPase dynamin2 and affects ciliary morphogenesis in postmitotic neurons. However, the splicing target of Pqbp1 in proliferating NSPCs might be different from targets in non-dividing neurons, and even in neurons splicing might be influenced by primary culture and could be different in vivo, where neurons are supported by multiple circumstantial factors.

Therefore, we rescreened the target genes of Pqbp1 in NSPCs by a combination of an ordinary mRNA gene chip to evaluate gene expression levels, and an exon array to estimate alternative splicing patterns. Regarding the gene expression levels, we first summarized data from the ordinary gene chip with primary cultured NSPCs prepared from nestin-Cre Pqbp1-cKO mice (E15), and extracted functional gene groups sensitive to Pqbp1 deficiency (reactomes) using Gene Set Enrichment Analysis (Supplementary Figure 8A and Supplementary Table 1). This revealed that, in any combination, nearly a half of the reactive gene groups (reactomes) were related to the M phase (in Supplementary Figure 8A and Supplementary Table 1) or another cell cycle phase (yellow in Supplementary Figure 8A and Supplementary Table 1), and supporting cell cycle genes were the main targets of Pqbp1 in NSPCs at the transcriptional level that are affected by Pqbp1 deficiency. In particular, M phase-related reactomes were significantly downregulated (Supplementary Figure 8B and Supplementary Table 1), consistent with the elongation of the M phase seen in vivo (Figure 2a). Reactomes in a dPqbp1 mutant fly showed related changes, specifically in the M phase (data not shown).

Further, to identify critical genes whose expression was altered by Pqbp1 deficiency in NSPCs, we merged genes selected by Gene Set Enrichment Analysis with nodes in the protein–protein interaction network database around PQBP1 (Supplementary Table 5). By carrying out reverse transcription polymerase chain reaction (RT-PCR) with Southern blot, we further tested whether Pqbp1 affected the splicing of NCAM1 (Figure 3 and Supplementary Table 7). Both splicing and transcription affected the expression of NCAM1 in NSPCs (Supplementary Figure 8). The new results from exon arrays consistently supported this idea. Apc1, which links to Apc4, was significantly affected with respect to splicing in NSPCs and the cortex (Figure 3c, Supplementary Table 7). Both splicing and transcription affected the expression of Apc1 judging from the results of ‘exon–exon comparison’ and ‘variance’ analyses. Apc2 expression was also remarkably changed in NSPC and the cortex, but the effect of splicing on Apc2 did not seem large (Figures 3a and c, and Supplementary Table 7). On the other hand, Apc4 was altered specifically in NSPCs of nestin-Cre cKO mice (Figure 3c and Supplementary Table 7), suggesting a relatively large effect of Apc4 on NSPCs and on microcephaly. Interestingly, Pqbp1 was also affected by itself more strongly through splicing than through transcription.

With regard to NCAM1, which was previously reported as a target of aberrant splicing by Pqbp1 KD, we confirmed aberrant splicing of the NCAM1 gene in the cortical tissue of nestin-Cre and synapsin-1-Cre Pqbp1-cKO mice in exon–exon signal comparison (Figure 3c and Supplementary Table 7). The discrepancy between normal dendrite length in vivo (Supplementary Figure 6) and the aberrant splicing of NCAM1 (Figure 3 and Wang et al.) suggests that certain compensatory mechanism(s) recovered the neurite length in vivo. The aberrant splicing of NCAM1 was not significant in NSPCs under Pqbp1 deficiency (Figure 3c and Supplementary Table 7).

Apc4 is a target gene of Pqbp1 that links to microcephaly. By carrying out reverse transcription polymerase chain reaction (RT-PCR) with Southern blot, we further tested whether Pqbp1 affected the splicing of Apc4. Pqbp1-KD by verified shRNA
Supplementary Figure 4) increased the amount of intron-containing pre-mRNAs of Apc4 in primary NSPCs (Supplementary Figure 9A). In addition, we found that the Pqbp1 protein was essential for the splicing of Apc4 pre-mRNA because the addition of anti-PQBP1 antibodies specifically inhibited the in vitro splicing of Apc4 but not of crystallin (Supplementary Figure 9B). Nonsense
RNA decay dependent on Upf1 was expected to be the mechanism for the decrease of Apc4 in NSPCs induced by Pqbp1-KD, (Supplementary Figure 9C). Co-transfection of Upf1-shRNA into NSPCs increased the number of unspliced introns of Apc4 between exons 22 and 23 or exons 6 and 7 (Supplementary Figure 9C) and supported nonsense RNA decay.

Figure 4. Apc4 plays a main role in the proliferation and horizontal expansion of neural stem progenitor cells (NSPCs). (a) Proliferation of NSPCs from E14 nestin-Cre conditional Pqbp1-knockout (cKO) embryos was retarded in primary culture but rescued by transfection of Apc4 (n = 4). **P < 0.01 in one-way analysis of variance (ANOVA) with post hoc Tukey's test. (b) Fluorescence-activated cell sorting analysis showed G2/M accumulation and a mild increase in the G1 population in NSPCs derived from E14 cKO embryos. Apc4 transfection rescued delayed proliferation and prevented cyclin B accumulation in NSPCs from cKO embryos. (c) The pial-to-apical surface area ratio reflects the proliferation of in-utero transfected EGFP-positive cells after a defined time period. pApc4-IRES-hrGFPII or pIRES-hrGFPII were electroporated into the ventricular zone of E13 embryos (nestin-Cre control and cKO mice), and the brains were analyzed at E18. (d) Pial-to-apical ratio was deduced from 3D reconstruction of the rostral-to-caudal axis serial sections of six embryos in each genotype. The ratio was decreased in Pqbp1-cKO embryos, reflecting the decreased cell cycle times of NSPCs, but it was rescued by Apc4 expression. **P < 0.01 or *P < 0.05 in one-way ANOVA with post hoc Tukey's test.

Figure 3. Analyses of genes affected by aberrant splicing in neurogenesis of neural stem progenitor cells (NSPCs) from nestin-Cre conditional Pqbp1-knockout (cKO) mice. (a) Representative patterns of exon array signals in a gene are shown. The expected results in the two analyses (exon–exon and variance) of exon array data and the contribution of splicing/transcription are correlated. (b) Affected genes (P < 0.05) were selected from the exon array results of NSPCs from nestin-Cre cKO mice, the cortex of nestin-Cre cKO mice and the cortex of synapsin-1-Cre cKO mice by comparison with wild-type mice (B6). The selected genes were further compared among the three genotypes as shown in the Venn diagram. (c) Results from the two analyses (exon–exon and variance) of exon array data are shown for APC1, APC2, APC4, NCAM (variants 1 and 3) and Pqbp1. When a gene possessed multiple exon probes, the lowest P-value was used as the representative. APC1 and Pqbp1 were remarkably affected in both analyses in all genotypes. APC4 was significantly affected in neural stem cells (NSCs) but not strongly in the cortex. From the speculation in (a), the effect of transcription was relatively large on Apc4 while both transcription and splicing affect APC1 and Pqbp1. NCAM1 (especially variant 3) was affected in the cortex of two types of cKO mice, but the change in NSCs was not so significant.
In addition, we confirmed that human APC4 mRNA is normally recognized as a splicing target by a spliceosome complex containing PQBP1, by showing the co-precipitation of PQBP1 and APC4 mRNA in human HEK-293 cells (Supplementary Figures 9D and E). Blocking nonsense-mediated mRNA decay by cycloheximide after PQBP1 KD resulted in the preservation of aberrant isoforms of APC4 pre-mRNA containing intron 23 in human HEK-293 cells (Supplementary Figures 9F–H). The levels of APC4 mRNAs
measured by quantitative PCR were also reduced in PQBP1-KD
HEK-293 cells (Supplementary Figures 9F and G). Taken together
with NSPC-specific suppression of Apc4 (Figure 3c), these results
suggested that Apc4 is a specific target of Pqbp1 deficiency in
NSPCs that represses NSPC proliferation through its aberrant
transcription and splicing.

To test whether Apc4 is a major contributor to microcephaly in
Pqbp1 deficiency, we performed rescue experiments in vitro and
in vivo. First, we transfected the Apc4 expression vector into NSPCs
from nestin-Cre cKO mice (Figures 4a and b). Overexpression of
Apc4 recovered the decreased Apc4 protein level in NSPCs from
cKO mice and normalized the cell growth speed (Figure 4a).
Consistent with this observation, Apc4 recovered the accumula-
tion of NSPCs at the G2/M phase, as seen with fluorescence-
activated cell sorting (Figure 4b). The effect of Apc4 was also
tested in vivo by in utero electroporation of the Apc4 expression
vector into the brains of nestin-Cre cKO mouse embryos (Figures
4c and d). We transfected either a GFP or a GFP-Apc4 plasmid into
control (nestin-Cre) or Pqbp1-cKO (nestin-Cre; Floxed) mouse
embryos at E13 by in utero electroporation, and dissected them at
E18, following a previously reported method to quantify cortical
expansion from NSPCs.33 The ratio of pial-to-apical surface area
was calculated from serial consecutive vibratome sections (50 µm
thick) through the entire rostral-to-caudal axis of E18 brains, as
described.33 The decreased pial-to-apical surface area ratio in
Pqbp1-cKO mice was partially rescued by electroporation of GFP-
Apc4 plasmid but not by GFP in controls (Figures 4c and d). All the
results revealed the cascade from Pqbp1 deficiency to micro-
cephaly by cell cycle elongation via Apc4 and related molecules
that were decreased via aberrant transcription and splicing.

PQBP1 rescues microcephaly of nestin-Cre cKO mice

Finally, we performed therapeutic trials for Pqbp1 deficiency-
induced microcephaly using AAV expressing human PQBP1
(Figure 5). The viral vector was similar to the one used previously
for human gene therapy of a young girl who suffered from
atrophic l'-amino acid decarboxylase deficiency62 and for experi-
mental therapy of a mouse model of spinal and bulbar muscular
atrophy (Kennedy’s disease) with mir-196.64 The viral vector was
supplied to mother mice by peritoneal injection at E10, the earliest
time point at which we could confirm pregnancy.

The effect of the AAV vector is known to be sustained for more
than a year.63 Consistent with this, we found that the expression
level of the Pqbp1/PQBP1 proteins, whose sizes are exactly similar
on western blot due to their high homology, was increased to 2.5-
fold at 10 weeks after birth (Figure 5a) though the expression level
was still far lower than that of the background control mice
(Figure 5a). Interestingly, even this limited recovery of Pqbp1/
PQBP1 expression resulted in a significant increase in brain weight
(Figure 5b). The macroscopic and microscopic brain architectures
of nestin-Cre cKO mice were normal and proportionally enlarged
(Figures 5c and d). Immunohistological analyses with layer

markers also revealed that the cortical layer structure was well
preserved (Figure 5e), and this conclusion was supported by
quantitative analyses of the thickness of each layer (Figure 5f).

Recruitment by PQBP1 supplementation was confirmed not only
for brain morphology but also for behavioral phenotypes (Figures
5g and h). We performed multiple behavior tests with nestin-Cre
and synapsin-1-Cre Pqbp1 cKO mice (Supplementary Figures 10
and 11). Interestingly, while the fear conditioning and rotarod
tests showed similarly abnormal results in both types of cKO mice,
the open field, light-dark box, elevated plus maze, and water maze
tests revealed some differences in the results from the different
mouse models (Supplementary Figures 10 and 11). In brief,
synapsin-1-Cre cKO mice were typically careless and insensitive
to fear. On the other hand, the phenotypes of nestin-Cre cKO mice
seemed more complex, which might be a result of developmental
compensation for Pqbp1 deficiency in this model. Among such
behavior tests, we observed significant recovery of nestin-Cre
Pqbp1 cKO mice in rotarod and fear conditioning tests, which were
abnormal in both types of cKO mice (Supplementary Figures 10
and 11) at 10 weeks after birth, following the intra-peritoneal
injection of PQBP1-AAV to mother mice (Figures 5g and h).

The incomplete recovery in behavioral tests (Supplementary
Figure 10) can be simply explained by the insufficient expression
of the PQBP1/Pqbp1 protein by AAV vector (Figure 5a). However,
it might suggest that brain size recovery is not sufficient for
functional recovery. If this is the case, two explanations are
possible. The first one is that the levels of Pqbp1 protein necessary
for architectural recovery and functional recovery might be
different. A higher level of Pqbp1 might be necessary for synap-
seal dynamism. The second possibility is that the AAV-mediated
delivery of Pqbp1 was too late. There might be a critical period
before E10 for some downstream functions. Further investigations
of these possibilities will be necessary to develop this technique
towards human gene therapy of the ID of PQBP1-mutated

patients.

**DISCUSSION**

This study provides a unique mechanism of brain size regulation
that depends largely on cell cycle elongation of NSPCs but not on
unequally changed neurogenesis or decreased BP production
ratio. Our findings might be also discussed from the viewpoint
of the hypothesis that the cell division times of NSPCs determine
neuronal subtypes.65,66 Given that the cell division number of
NSPCs was expected to decrease roughly from 11 to 10 in Pqbp1-
deficient cKO embryos, our findings might suggest that extrinsic
factors rather than intrinsic determination of NSPCs control the
cortical layer to produce from the NSPC pool at a developmental
time point. However, since the decrease of cell division number is
small, it might also be explained by assuming that cortical layer
formation was compensated in nestin-Cre cKO embryos by

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**Figure 5.** Peritoneal injection of PQBP1-AAV recovers the microcephaly and behaviors of nestin-Cre Pqbp1-cKO (conditional Pqbp1-knockout) mice. (a) Confirmation of the increase of Pqbp1 protein level in nestin-Cre Pqbp1-cKO mice after injection of PQBP1-AAV vector. Western blot analysis revealed a 2.5-fold increase. (b) The brain weight of nestin-Cre Pqbp1-cKO mice was recovered by PQBP1-AAV vector (*P < 0.01 in Student’s t-test). In multiple-group comparison with Tukey’s test, the change was also confirmed (**P < 0.05). (c) Macroscopic comparison of brain morphology between non-treated and AAV-injected male nestin-Cre Pqbp1-cKO mice at 2.5 months (10 weeks). (d) Comparison of coronal sections of the brain at the exactly same position of non-treated and AAV-injected male nestin-Cre Pqbp1-cKO mice at 2.5 months (10 weeks) revealed recovery of brain size by the PQBP1-AAV vector. (e) Staining for layer-specific markers, Cux1, Foxp1, and Tbr1, together with GAD67, shows the preservation of cortical layers in the rescued nestin-Cre Pqbp1-cKO mice by the PQBP1-AAV vector at 2.5 months. (f) Quantitative analysis of the relative thickness of each layer to total thickness of the cortex. No difference was detected between AAV-injected and non-injected nestin-Cre Pqbp1-cKO mice. (g) The decline of nestin-Cre Pqbp1-cKO mice in the rotarod test recovery was observed at 3 months after birth by in utero gene therapy with the PQBP1-AAV vector. (**P < 0.01 in ANOVA with post hoc Tukey’s test). (h) The decline of nestin-Cre Pqbp1-cKO mice in fear-conditioned memory was recovered at 3 months by in utero gene therapy with the PQBP1-AAV vector. (**P < 0.01 or *P < 0.05 in ANOVA with post hoc Tukey’s test).
overlapped time spans of cortical layers. This is a question for further investigation.

The results in this study, especially from a newly developed cKO mouse model that faithfully mimics the magnetic resonance images of human PQBP1-linked microcephaly, elucidated the molecular mechanism of Pqbp1-dependent brain size regulation. Systems biology analyses revealed candidate molecules that are possibly involved in the mechanism of microcephaly. Especially, Apc4 is a critical downstream target of Pqbp1 for microcephaly, and both aberrant splicing and transcription contribute to the downregulation of Apc4 by Pqbp1.

In the other categories of developmental disorders such as enzyme deficiencies or cystic fibrosis, in utero gene therapy has been considered. However, it has not been considered for normalizing brain tissues or whole brains that were impaired by genetic defects in microcephaly. Therefore, this is the first study to prove that brain size disorder could be another objective of in utero gene therapy. This study is just an example, but the method could be applied to the other microcephalies by targeting each causative gene. Future advance in genetic diagnosis will help extremely early initiation of gene therapy especially in the case of pregnancy of high-risk mothers with the patients in her or husband's family.

Technically, gene transfer efficiency, virulence, tumorigenesis and inflammation would be the next issues to tackle in order to adapt this technique to human patients, as shown in the application of gene therapy for treating cystic fibrosis and other diseases. With regard to expression level, this study revealed that low-level PQBP1 expression was still effective for treating microcephaly. Regarding the toxicity of AAV, it has been already used in clinical trials of multiple diseases like cystic fibrosis, hemophilia B, muscular dystrophy, Parkinson’s disease, Canavan’s disease and Alzheimer’s disease, and side effects have rarely been observed. Tumorigenesis or inflammation has not been reported thus far. Therefore, a similar approach would be a potential therapeutic approach to treat human patients with PQBP1-linked microcephaly if further intensive studies in higher animals can exclude such side effects.

Naturally, ethical consideration is also essential before clinical application to human patients. The timing of administration of AAV vector and the route of gene delivery (intravenous, amniotic fluid, or cerebrospinal fluid administration; delivery to mother or embryo) would be the issues in such a case. However, this is the first step for the possibility of gene therapy in microcephaly, and the issues raised above will be cleared in the future by technical advances. It would be worth discussing and investigating on it further.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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