Low folate induces abnormal neuronal maturation and DNA hypomethylation of neuronal differentiation-related genes in cultured mouse neural stem and progenitor cells

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ABSTRACT

Folate deficiency in a fetus is well known to cause neurodevelopment defects and development disorders. A low level of folate is also thought to be a risk for depression in adults. We have previously shown that post-weaning low folate induces neuronal immaturity in the dentate gyrus in mice, which suggests that low folate causes neuropsychological disorders via inhibition of neuronal maturation. In this study, we examined the effects of low folate on expression and epigenetic modification of genes involved in neuronal differentiation and maturation in primary mouse neural stem/progenitor cells (NSPCs) in vitro. An increase in Nestin (NSPC marker)-positive cells was observed in cells differentiated in a low folate medium for 3 days. An increase in βIII-tubulin (Tuj1: immature neuron marker)-positive cells and a decrease in microtubule-associated protein 2 (MAP2: mature neuron marker)-positive cells were observed in cells differentiated in a low folate medium for 7 days. In these cells, mRNA levels for genes involved in neuronal differentiation and maturation were altered. Hypomethylation of DNA, but not of histone proteins, was also observed at some promoters of these neuronal genes. The level of S-adenosylmethionine (SAM), a methyl donor, was decreased in these cells. The abnormalities in neural maturation and changes in gene expression in culture under low folate conditions were partially normalized by addition of SAM (5 μM). Based on these results, decreased SAM may induce DNA hypomethylation at genes involved in neuronal differentiation and maturation under low folate conditions, and this hypomethylation may be associated with low folate-induced neuronal immaturity.

1. Introduction

Neural stem/progenitor cells (NSPCs) are self-renewing and multipotent cells that can differentiate into cells such as neurons and glia [1]. To form the sophisticated mammalian central nervous system (CNS), maintenance and differentiation of NSPCs are strictly controlled by complex mechanisms, including epigenetic regulation [2, 3, 4, 5, 6]. Differentiation from NSPCs to neurons is most active in the embryonic brain and is required for brain development. This differentiation continues throughout life in specific brain regions, such as the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Newborn neurons from NSPCs in the SGZ are functionally integrated into existing neural circuits in the DG [1, 7], and are required for responses to stress and antidepressant [8, 9]; therefore, abnormalities in newborn neurons are thought to be involved in neuropsychological disorders [10].

Folate is a B vitamin that serves as a source for transfer of one-carbon units in several biosynthetic reactions, including DNA and histone methylation [11]. Since these reactions play crucial roles in regulation of gene expression, folate deficiency induces biological dysfunction throughout the body, including the CNS. A low level of folate during pregnancy has been associated with fetal neural tube defects and developmental disorders such as autism spectrum disorders [12, 13]. Low folate can also lead to an increased risk of depression [14, 15, 16, 17, 18, 19, 20]. We have previously found an increase in immature neurons and a decrease in newborn mature neurons in the DG of mice fed a low folate diet. Furthermore, these mice show a depression-like state, such as...
increased immobility in the forced swim test [21]. These findings suggest that neuronal immaturity of newborn neurons may underlie psychiatric disorders such as depression; however, the mechanisms through which low folate levels cause neuronal immaturity are unclear.

In this study, to investigate these mechanisms, we examined epigenetic modifications such as DNA and histone methylation at genes involved in neuronal differentiation and maturation in cells differentiated from primary NSPCs in a low folate medium.

2. Material and methods

2.1. Cell culture

Primary NSPCs were prepared as previously reported with minor modifications [22]. Briefly, primary NSPCs were isolated from telencephalon of ddY outbred mice at E14.5 and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (Nacalai tesque, Kyoto, Japan) supplemented with 1% (v/v) N-2 supplement (Invitrogen, Carlsbad, CA, USA), 20 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 20 μg/ml fibroblast growth factor 2 (PeproTech) and 2 μg/ml heparin (Nacalai tesque) on non-treated 100 mm dishes. After 7 days in culture, neurospheres that formed were gathered and dissociated into a single cell suspension by pipetting. For differentiation of NSPCs, dissociated cells were plated on laminin/poly-L-ornithine-coated 24-well plates, 35-mm dishes or 100-mm dishes, and cultured in control (DMEM/Ham's F-12, folic acid 2.44 mg/l) or low folate (DMEM without folic acid (Sigma-Aldrich, St. Louis, MO, USA):Ham's F-12 (Nacalai tesque) 1:1, folic acid 0.66 mg/l) medium supplemented with 2% (v/v) B-27 supplement (Invitrogen). For SAM supplementation experiments, SAM folic acid (Sigma-Aldrich, St. Louis, MO, USA):Ham's F-12 (Nacalai tesque) 1:1, folic acid 0.66 mg/l) medium supplemented with 2% (v/v) B-27 supplement (Invitrogen). For SAM supplementation experiments, SAM

Table 1. List of antibodies.

| Immunochemistry | Antigen | Dilution | Host | Source | Identifier |
|-----------------|---------|----------|------|--------|------------|
| Primary antibody | Nestin  | 1:200    | Mouse | Sigma-Aldrich, St. Louis, MO, USA | Cat#: MAB353; RRID: AB_949911 |
| Antigen         | Tuj1    | 1:1,000 | Mouse | BioLegend, San Diego, CA, USA | Cat#: 801202; RRID: AB_10063408 |
| Antigen         | MAP2    | 1:1,000 | Rabbit | Sigma-Aldrich, St. Louis, MO, USA | Cat#: AB5622; RRID: AB_1009939 |
| Antigen         | GFAP    | 1:200    | Rabbit | Agilent, Santa Clara, CA, USA | Cat#: 20334; RRID: AB_10013382 |

| Secondary antibody | Antigen | Dilution | Host | Conjugate | Source | Identifier |
|--------------------|---------|----------|------|-----------|--------|------------|
| Mouse IgG          | 1:1,000 | Donkey   | Alexa Fluor 488 | Thermo Fisher Scientific, Waltham, MA, USA | Cat#: A-11029; RRID: AB_138404 |
| Rabbit IgG         | 1:1,000 | Donkey   | Alexa Fluor 488 | Thermo Fisher Scientific, Waltham, MA, USA | Cat#: A-11008; RRID: AB_143165 |
| Rabbit IgG         | 1:1,000 | Donkey   | Alexa Fluor 594 | Thermo Fisher Scientific, Waltham, MA, USA | Cat#: A-21207; RRID: AB_141637 |

| Dot blot | Antigen | Dilution | Host | Source | Identifier |
|----------|---------|----------|------|--------|------------|
| Mouse IgG | 1:5,000 | Horse | HRP | Cell Signaling Technology, Danvers, MA, USA | Cat#: 7076; RRID: AB_339924 |

| Western blot | Antigen | Dilution | Host | Source | Identifier |
|--------------|---------|----------|------|--------|------------|
| Histone H3   | 1:1,000 | Rabbit   | Cell Signaling Technology, Danvers, MA, USA | Cat#: 9715; RRID: AB_331563 |
| Tri-methyl-histone H3 Lys4 | 1:1,000 | Rabbit | Cell Signaling Technology, Danvers, MA, USA | Cat#: 9751; RRID: AB_2616028 |
| Tri-methyl-histone H3 Lys9 | 1:1,000 | Rabbit | Cell Signaling Technology, Danvers, MA, USA | Cat#: 13969; RRID: AB_22978355 |
| Tri-methyl-histone H3 Lys27 | 1:1,000 | Rabbit | Cell Signaling Technology, Danvers, MA, USA | Cat#: 9733; RRID: AB_2616029 |
| Tri-methyl-histone H3 Lys36 | 1:1,000 | Rabbit | Cell Signaling Technology, Danvers, MA, USA | Cat#: 4909; RRID: AB_1950412 |

| Secondary antibody | Antigen | Dilution | Host | Conjugate | Source | Identifier |
|--------------------|---------|----------|------|-----------|--------|------------|
| Rabbit IgG         | 1:1,000 | Goat | HRP | Cell Signaling Technology, Danvers, MA, USA | Cat#: 7074; RRID: AB_2099233 |

| ChIP assay | Antigen | Dilution | Host | Source | Identifier |
|-----------|---------|----------|------|--------|------------|
| Tri-methyl-histone H3 Lys9 | 1:50 | Rabbit | Cell Signaling Technology, Danvers, MA, USA | Cat#: 9751; RRID: AB_2616028 |
| Tri-methyl-histone H3 Lys27 | 1:50 | Rabbit | Cell Signaling Technology, Danvers, MA, USA | Cat#: 9733; RRID: AB_2616029 |
New England Biolabs, Ipswich, MA, USA) was added to a control or a low-folate medium at a final concentration of 5 μM at the planting of NSPCs. Thereafter, the same amount of SAM was added every 24 h. Immunochemistry was examined at differentiation days 1, 3 or 7 after plating of NSPCs. Quantitative RT-PCR, dot blot, Western blot, methylated-CpG island recovery, chromatin immunoprecipitation (ChIP) assay, and enzyme-linked immunosorbent assay (ELISA) for SAM were performed on day 7. A schematic of the experimental design is shown in Figure 1.

2.2. Immunocytochemistry

Immunocytochemistry was performed as previously reported with minor modifications [22]. Briefly, differentiated NSPCs were fixed with 4% paraformaldehyde at room temperature for 30 min and with 80% methanol at -20 °C for 20 min. To block nonspecific antibody binding, fixed cells were incubated with 1% BSA in Tris-buffered saline with 0.3% Triton X-100 (PBS-T) at 4 °C for 2 h. Then, cells were incubated at 4 °C overnight with a primary antibody, followed by incubation at room temperature for 1 h with a secondary antibody. The primary and secondary antibodies (Table 1) were diluted with 1% BSA in PBS-T. Nuclear counterstaining was performed with DAPI. Images were collected using microscope (BX53; Olympus, Tokyo, Japan) with CCD camera (DP73; Olympus). Five visual fields (0.36 mm²; average number of cells per field of view 40) were captured randomly for each well, and data were obtained by averaging the results of the five fields of view. All images were captured under the same excitation intensity and exposure time. The number of cells immunopositive for Nestin, Tuj1, MAP2, or GFAP was determined relative to the number of DAPI-stained nuclei. The percentage of positive cells was determined out of the total number of counted cells stained by DAPI. The numbers of immunopositive cells were measured by an observer blinded to the treatment conditions.

2.3. Quantitative RT-PCR

Total RNA was isolated from differentiated NSPCs with Sepasol-RNA I Super G (Nacalai tesque) and used (1 μg) in reverse transcription with ReverTra Ace (Toyobo, Osaka, Japan). Quantitative PCR was performed

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**Table 2. List of primers.**

| Quantitative RT-PCR | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|---------------------|---------------------------|---------------------------|
| Pax6                | GAGACTGGTGGCACTCACAG      | CTAGGGCAGGGTGAGGAGAAG     |
| Sox2                | ACGGTTGCGCTTGTTTGTG       | GATCTACATTCCGGAGTGAG      |
| Nrsf                | ACGTCGAGCAAGTTTTTCTTGT    | CTAGGTCAGCTCGAGTTGAG      |
| Bmp4                | TGGCGCCGGTCAGGTAATG       | CTAGGTCAGCTCGAGTTGAG      |
| Slug2               | GGGGACGTGGAGGTACTGCAG      | CTAGGTCAGCTCGAGTTGAG      |
| Hey1                | GCTGAGCTGGCAGGTCACAG      | CTAGGTCAGCTCGAGTTGAG      |
| Acr1                | TGGCTGTCGCTGAGTTGAG       | CTAGGTCAGCTCGAGTTGAG      |
| Neurog1             | AGGGACGGATGTTTGTGAG       | CTAGGTCAGCTCGAGTTGAG      |
| Est3                | TGGTGGGTGCTCTGCTGGT       | CTAGGTCAGCTCGAGTTGAG      |
| Msf2c               | GGGCATCGGTGTGAGTTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Proxl               | CTTGACGCTGGGAGGACTACAG    | CTAGGTCAGCTCGAGTTGAG      |
| Neurod1             | GAGACCGAGATGTTTGTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Msf1                | GAGGAGGATGTTTGTGAG        | CTAGGTCAGCTCGAGTTGAG      |
| Creb1               | GGGTACGCTGGGAGGACTACAG    | CTAGGTCAGCTCGAGTTGAG      |
| Gapdh               | ATGGTGAAGGTCGGTGTG        | CTAGGTCAGCTCGAGTTGAG      |

**Methylated-CpG island recovery assay**

| Quantitative RT-PCR | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|---------------------|---------------------------|---------------------------|
| Pax6                | GAGACCGAGATGTTTGTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Sox2                | ACGGTTGCGCTTGTTTGTG       | GATCTACATTCCGGAGTGAG      |
| Nrsf                | ACGTCGAGCAAGTTTTTCTTGT    | CTAGGTCAGCTCGAGTTGAG      |
| Bmp4                | TGGCGCCGGTCAGGTAATG       | CTAGGTCAGCTCGAGTTGAG      |
| Slug2               | GGGGACGTGGAGGTACTGCAG      | CTAGGTCAGCTCGAGTTGAG      |
| Hey1                | GCTGAGCTGGCAGGTCACAG      | CTAGGTCAGCTCGAGTTGAG      |
| Acr1                | TGGTGGGTGCTCTGCTGGT       | CTAGGTCAGCTCGAGTTGAG      |
| Neurog1             | AGGGACGGATGTTTGTGAG       | CTAGGTCAGCTCGAGTTGAG      |
| Est3                | TGGTGGGTGCTCTGCTGGT       | CTAGGTCAGCTCGAGTTGAG      |
| Msf2c               | GGGCATCGGTGTGAGTTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Proxl               | CTTGACGCTGGGAGGACTACAG    | CTAGGTCAGCTCGAGTTGAG      |
| Neurod1             | GAGACCGAGATGTTTGTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Msf1                | GAGGAGGATGTTTGTGAG        | CTAGGTCAGCTCGAGTTGAG      |
| Creb1               | GGGTACGCTGGGAGGACTACAG    | CTAGGTCAGCTCGAGTTGAG      |
| Gapdh               | ATGGTGAAGGTCGGTGTG        | CTAGGTCAGCTCGAGTTGAG      |

**Chromatin immunoprecipitation assay**

| Quantitative RT-PCR | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|---------------------|---------------------------|---------------------------|
| Pax6                | GAGACCGAGATGTTTGTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Sox2                | ACGGTTGCGCTTGTTTGTG       | GATCTACATTCCGGAGTGAG      |
| Nrsf                | ACGTCGAGCAAGTTTTTCTTGT    | CTAGGTCAGCTCGAGTTGAG      |
| Bmp4                | TGGCGCCGGTCAGGTAATG       | CTAGGTCAGCTCGAGTTGAG      |
| Slug2               | GGGGACGTGGAGGTACTGCAG      | CTAGGTCAGCTCGAGTTGAG      |
| Hey1                | GCTGAGCTGGCAGGTCACAG      | CTAGGTCAGCTCGAGTTGAG      |
| Acr1                | TGGTGGGTGCTCTGCTGGT       | CTAGGTCAGCTCGAGTTGAG      |
| Neurog1             | AGGGACGGATGTTTGTGAG       | CTAGGTCAGCTCGAGTTGAG      |
| Est3                | TGGTGGGTGCTCTGCTGGT       | CTAGGTCAGCTCGAGTTGAG      |
| Msf2c               | GGGCATCGGTGTGAGTTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Proxl               | CTTGACGCTGGGAGGACTACAG    | CTAGGTCAGCTCGAGTTGAG      |
| Neurod1             | GAGACCGAGATGTTTGTGAG      | CTAGGTCAGCTCGAGTTGAG      |
| Msf1                | GAGGAGGATGTTTGTGAG        | CTAGGTCAGCTCGAGTTGAG      |
| Creb1               | GGGTACGCTGGGAGGACTACAG    | CTAGGTCAGCTCGAGTTGAG      |
| Gapdh               | ATGGTGAAGGTCGGTGTG        | CTAGGTCAGCTCGAGTTGAG      |

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with Thunderbird qPCR Mix (Toyobo) and primers (Table 2, using Thermal Cycler Dice Real Time System Single (Takara Bio, Shiga, Japan). Relative expression levels of genes were normalized against the endogenous Gapdh standard. Relative gene expression was calculated using the ΔΔCT method.

2.4. Dot blot

Genomic DNA was extracted from differentiated NSPCs with DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl; pH 8.0, 10 mM EDTA, 0.1% SDS and 100 μg/ml proteinase K), and isolated with Phenol saturated with TE buffer, phenol:chloroform:isoamyl alcohol 25:24:1 (pH 7.9) (both Nacalai tesque), and ethanol. Isolated DNA was measured by BioSpec-nano (Shimadzu, Kyoto, Japan) for concentration, diluted to 10 ng/100 μl in 0.4 mM NaOH/10 mM EDTA, denatured at 99 °C for 10 min, and then placed on ice immediately. The denatured DNA solution (10 ng DNA) was neutralized with 100 μl of 2 M ammonium acetate and spotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using a dot-blotter (Sanplecture, Osaka, Japan). After rinsing with 2×SSC and complete drying, spotted DNA was fixed with UV irradiation (302 nm) using 2UV Transilluminator (UV, Upland, CA, USA). The membrane was then blocked with Blocking one solution (Nacalai tesque) and incubated with a primary antibody to 5-methylcytosine at 4 °C overnight, followed by incubation with a secondary antibody at room temperature for 1 h. The primary and secondary antibodies (Table 1) were diluted with 5% Blocking one solution in Tris-buffered saline with 0.05% Tween-20 (TBS-T). The dot signal was detected with Chemi-Lumi One L (Nacalai tesque) using ChemiDoc MP. The fold changes for methylation levels were calculated as a ratio to the mean of controls.

2.5. Methylation-CpG island recovery assay

Genomic DNA of differentiated NSPCs was isolated using DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl; pH 8.0, 10 mM EDTA, 0.1% SDS and 100 μg/ml proteinase K). To shear the DNA, the isolated genomic DNA was sonicated for 5 s with Handy sonic (UR-21P; Tomy Seiko, Tokyo, Japan) and purified with phenol:chloroform:isoamyl alcohol 25:24:1 (pH 7.9) (Nacalai tesque) and ethanol. The sheared DNA (1 μg) was used for enrichment of methylated DNA with EpiXplore Methylated DNA Enrichment Kit (Takara Bio). Quantitative real-time PCR was performed with KOD qPCR Mix (Toyobo) and primers (Table 2), using Thermal Cycler Dice Real Time System Single (Takara Bio). DNA methylation levels were calculated as methylated DNA/total DNA. The fold changes for methylation levels were calculated as a ratio to the mean of controls.

2.6. Histone extraction and Western blot

Histone extraction was performed as previously reported with minor modifications [23]. Differentiated NSPCs were harvested with 1 ml of homogenization buffer (50 mM Tris-HCl; pH 7.5, 25 mM KCl, 250 mM sucrose, 2 mM sodium butyrate, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM protease (thrombin) cocktail). The harvested cells were homogenized with 12 strokes in a pre-cooled Dounce tissue grinder (Wheaton) with a tight pestle. The homogenate was centrifuged at 2,400 × g for 10 min at 4 °C. ChIP DNA and Input DNA were extracted with phenol:chloroform:isoamyl alcohol 25:24:1 (pH 7.9) (Nacalai tesque) and ethanol. Quantitative real-time PCR was performed with KOD qPCR Mix (Toyobo) and primers (Table 2), using Thermal Cycler Dice Real Time System Single (Takara Bio). Histone methylation levels were calculated as ChIP sample/input sample. The fold changes for methylation levels were calculated as a ratio to the mean of controls.

2.7. ChIP assay

The ChIP assay was performed as previously reported with minor modifications [24]. Briefly, for crosslinking of histone-DNA complexes, differentiated NSPCs were fixed with DMEM/Ham’s F-12 containing 1% formaldehyde at room temperature for 10 min. To stop the crosslinking reaction, 5 ml of glycin solution from ChIP-IT Express Kit (Active Motif, Carlsbad, CA, USA) was added to the fixed cells. The cells were incubated at room temperature for 5 min and harvested with 1 ml of supplied Cell scraping solution containing 0.5 mM PMSF. The cell solution was then centrifuged at 800 × g for 10 min at 4 °C. The cell pellet was suspended in 500 μl of supplied Lysis buffer containing protease inhibitor cocktail and 0.5 mM PMSF, and the solution was incubated for 30 min on ice. For release of nuclei, the cell solution was homogenized with 12 strokes in a pre-cooled Dounce tissue grinder (Wheaton) with a tight pestle. The homogenate was centrifuged at 2,400 × g for 10 min at 4 °C. After removing supernatant, the nuclei pellet was resuspended in 150 μl of supplied Shearing buffer. To shear the chromatin, the nuclei solution was sonicated 10 times for 30 s with Handy sonic (UR-21P; Tomy Seiko). The sheared chromatin solution was centrifuged at 18,000 × g for 10 min at 4 °C and ChIP procedures was performed using the ChIP-IT Express Kit. Immunoprecipitation was performed using anti-trimethylhistone antibodies (Table 1) on a rotator overnight at 4 °C. ChIP DNA and Input DNA were purified with phenol:chloroform:isoamyl alcohol 25:24:1 (pH 7.9) (Nacalai tesque) and ethanol. Quantitative real-time PCR was performed with KOD qPCR Mix (Toyobo) and primers (Table 2), using Thermal Cycler Dice Real Time System Single (Takara Bio). Histone methylation levels were calculated as ChIP sample/input sample. The fold changes for methylation levels were calculated as a ratio to the mean of controls.

2.8. ELISA

ELISA for SAM was performed using Mouse S-Adenosylmethionine ELISA kit (MyBioSource, San Diego, CA, USA). Cells were homogenized with RIPA buffer (Nacalai Tesque) and centrifuged at 10,000 × g for 10

![](image)
min at 4 °C. The supernatant was used for ELISA. The intracellular SAM levels were normalized against the total amount of protein.

2.9. Statistical analysis

All data are expressed as a mean ± standard error of the mean (SEM). Data for Figures 2, 3 and 4A were analyzed by Student t-test. Data in Figures 4B, 5B, 5D, 5F, 5H, 6B, 6D, 6F and 6H were analyzed using one-way ANOVA (Figures 4B and two-way analysis of variance (ANOVA) (Figures 5B, 5D, 5F, 5H, 6B, 6D, 6F, 6H) followed by a Tukey-Kramer post-hoc test. All analyses were performed using Statview 5.0J for Apple Macintosh (SAS Institute Inc., Cary, NC, USA). A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Low folate causes neuronal immaturity in primary NSPCs

To investigate the mechanisms of low folate-induced neuronal immaturity, murine embryonic telencephalon-derived NSPCs, which have a high potential to differentiate into neurons, were differentiated in a low folate medium. Immunofluorescence analyses showed an increase in Nestin (NSPC marker)-positive cells on day 3 of differentiation, and an increase in βIII-tubulin (Tuj1: immature neuron marker)-positive cells and a decrease in microtubule-associated protein 2 (MAP2: mature neuron marker)-positive cells on day 7 of differentiation. On day 1, there was no difference in the number of each type of positive cells between the control and low folate conditions, and there was no difference in the number of glial fibrillary acidic protein (GFAP: astrocyte marker)-positive cells in control and low folate conditions over days 1–7 (Figure 2A,B,C,D,E,F). Thus, as found in the DG of folate deficient mice [21], low folate-induced neuronal immaturity was observed in cultured NSPCs.

3.2. Low folate alters expression and epigenetic modifications in genes involved in neuronal differentiation and maturation

Next, we examined expression patterns of genes involved in neuronal differentiation and maturation. Under low folate conditions, mRNA levels for genes encoding transcription factors involved in neuronal differentiation and maturation (B) were reversed by supplementation of SAM. Values are shown as the mean ± SEM of 6 (A), 3 (B), 9 (C), 6 (D), and 9 cultures (E,F). The fold changes were calculated as a ratio to the mean of controls.

Figure 3. Gene expression and epigenetic modifications of genes encoding transcription factors involved in neuronal differentiation and maturation under low folate conditions. The mRNA levels for genes involved in maintenance or proliferation of NSPCs, such as Pax6, Sox2, Nrsf, Bmp4, Stat3 and Hey1, or in neuronal maturation, such as Prox1, Neurod1, Mib1 and Creb1, were downregulated with low folate on differentiation day 3 or 7. In contrast, mRNA levels for genes involved in neuronal differentiation, such as Ascl1, Neurog1 and Eomes, were upregulated on differentiation day 3 or 7 (A). The 5-methylcytosine detected by dot blot analysis (shown in the photograph above each column; 10 ng DNA were spotted in each) on differentiation day 3 or 7 (B) and DNA methylation levels in CpG islands at promoters of Neurog1 and Eomes on differentiation day 7 (C) were significantly decreased with low folate. H3K4me3, H3K9me3, H3K27me3 or H3K36me3 levels in the global genome were unchanged with low folate on differentiation day 3 or 7 (D). H3K4me3 (E) and H3K27me3 (F) in promoter regions were also unchanged except for Neurod1 on differentiation day 7. Values are shown as the mean ± SEM of 6 (A), 3 (B), 9 (C), 6 (D), and 9 cultures (E,F). The fold changes were calculated as a ratio to the mean of controls. For Figures A,C,E,F, the values of low folate group are shown on log scales. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control. 

Figure 4. Effects of SAM supplementation (5 μM) on abnormalities in mRNA expression in cells differentiated from NSPCs in a low folate medium. On differentiation day 7, intracellular SAM was significantly decreased in cells differentiated from NSPCs in low folate (A). Changes of mRNA expression for genes involved in neuronal differentiation and maturation (B) were reversed by supplementation of SAM. Values are shown as the mean ± SEM of 3. For Figure B, the values of low folate with/without SAM group are shown on log scales. *$p < 0.05$, **$p < 0.01$ vs. control with PBS, †$p < 0.05$, ††$p < 0.01$ vs. low folate with PBS. The results of one-way ANOVA for Day3 in Figure B: $F_{(2,6)} = 0.65$, $p > 0.05$ for Pax6; $F_{(2,6)} = 0.01$, $p > 0.05$ for Sox2; $F_{(2,6)} = 1.14$, $p > 0.05$ for Nrsf; $F_{(2,6)} = 4.23$, $p > 0.05$ for Bmp4; $F_{(2,6)} = 0.09$, $p > 0.05$ for Stat3; $F_{(2,6)} = 2.14$, $p > 0.05$ for Hey1; $F_{(2,6)} = 5.43$, $p < 0.05$ for Ascl1; $F_{(2,6)} = 0.85$, $p > 0.05$ for Neurod1; $F_{(2,6)} = 0.09$, $p < 0.05$ for Eomes; $F_{(2,6)} = 3.61$, $p > 0.05$ for Mef2c; $F_{(2,6)} = 3.16$, $p > 0.05$ for Prox1; $F_{(2,6)} = 2.54$, $p > 0.05$ for Neurod1; $F_{(2,6)} = 6.05$, $p < 0.05$ for Mib1; $F_{(2,6)} = 4.73$, $p > 0.05$ for Creb1; Day7: $F_{(2,6)} = 9.17$, $p < 0.05$ for Pax6; $F_{(2,6)} = 0.01$, $p > 0.05$ for Sox2; $F_{(2,6)} = 0.88$, $p > 0.05$ for Nrsf; $F_{(2,6)} = 1.44$, $p > 0.05$ for Bmp4; $F_{(2,6)} = 25.09$, $p < 0.01$ for Stat3; $F_{(2,6)} = 11.38$, $p < 0.01$ for Hey1; $F_{(2,6)} = 0.53$, $p > 0.05$ for Ascl1; $F_{(2,6)} = 19.97$, $p < 0.01$ for Neurod1; $F_{(2,6)} = 15.39$, $p < 0.05$ for Eomes; $F_{(2,6)} = 4.81$, $p < 0.05$ for Mef2c; $F_{(2,6)} = 32.81$, $p < 0.001$ for Prox1; $F_{(2,6)} = 22.09$, $p < 0.01$ for Neurod1; $F_{(2,6)} = 38.37$, $p < 0.001$ for Mib1; $F_{(2,6)} = 2.48$, $p > 0.05$ for Creb1.
Table 3. Number of cells/visual field (Mean ± SEM).

| Figure 2A | Day 1 |
|-----------|-------|
| Nestin    | Number of DAPI-stained cells | Number of Nestin-immunopositive cells |
| Control   | 81.7 ± 14.4 | 51.5 ± 7.6 |
| Low folate| 107.0 ± 5.0 | 61.8 ± 0.8 |
| Tuj1      | Number of DAPI-stained cells | Number of Tuj1-immunopositive cells |
| Control   | 98.0 ± 5.6  | 8.0 ± 0.8  |
| Low folate| 88.4 ± 3.8  | 5.6 ± 0.8  |
| MAP2      | Number of DAPI-stained cells | Number of MAP2-immunopositive cells |
| Control   | 95.3 ± 3.1  | 4.8 ± 0.8  |
| Low folate| 69.6 ± 3.7  | 4.9 ± 0.7  |
| GFAP      | Number of DAPI-stained cells | Number of GFAP-immunopositive cells |
| Control   | 76.5 ± 17.2 | 23.0 ± 4.2 |
| Low folate| 109.3 ± 9.3 | 32.1 ± 5.8 |

| Figure 2B | Day 3 |
|-----------|-------|
| Nestin    | Number of DAPI-stained cells | Number of Nestin-immunopositive cells |
| Control   | 76.0 ± 3.4  | 18.6 ± 1.7  |
| Low folate| 50.9 ± 2.7  | 18.9 ± 1.9  |
| Tuj1      | Number of DAPI-stained cells | Number of Tuj1-immunopositive cells |
| Control   | 89.2 ± 22.0 | 10.8 ± 1.0  |
| Low folate| 80.0 ± 8.4  | 12.8 ± 2.2  |
| MAP2      | Number of DAPI-stained cells | Number of MAP2-immunopositive cells |
| Control   | 156.3 ± 11.2| 12.4 ± 0.9  |
| Low folate| 113.3 ± 3.7 | 6.1 ± 1.1   |
| GFAP      | Number of DAPI-stained cells | Number of GFAP-immunopositive cells |
| Control   | 89.2 ± 22.0 | 28.8 ± 7.6  |
| Low folate| 80.0 ± 8.4  | 33.6 ± 5.6  |

| Figure 2C | Day 7 |
|-----------|-------|
| Nestin    | Number of DAPI-stained cells | Number of Nestin-immunopositive cells |
| Control   | 85.0 ± 3.8  | 12.0 ± 1.6  |
| Low folate| 69.4 ± 5.0  | 12.6 ± 1.8  |
| Tuj1      | Number of DAPI-stained cells | Number of Tuj1-immunopositive cells |
| Control   | 123.4 ± 8.1 | 20.2 ± 1.4  |
| Low folate| 92.2 ± 6.2  | 19.2 ± 1.4  |
| MAP2      | Number of DAPI-stained cells | Number of MAP2-immunopositive cells |
| Control   | 89.2 ± 2.5  | 10.1 ± 0.3  |
| Low folate| 104.5 ± 1.0 | 6.3 ± 0.7   |
| GFAP      | Number of DAPI-stained cells | Number of GFAP-immunopositive cells |
| Control   | 123.4 ± 8.1 | 50.4 ± 3.0  |
| Low folate| 92.2 ± 6.2  | 39.0 ± 2.2  |

Table 3 (continued)

| Figure 4D | Day 7 |
|-----------|-------|
| Nestin    | Number of DAPI-stained cells | Number of Nestin-immunopositive cells |
| Control   | 83.6 ± 6.8  | 2.0 ± 1.0   |
| Low folate| 78.8 ± 2.8  | 9.2 ± 2.4   |
| Tuj1      | Number of DAPI-stained cells | Number of Tuj1-immunopositive cells |
| Control   | 86.4 ± 3.4  | 10.8 ± 1.4  |
| Low folate| 71.4 ± 4.8  | 8.0 ± 1.2   |
| MAP2      | Number of DAPI-stained cells | Number of MAP2-immunopositive cells |
| Control   | 68.5 ± 2.5  | 20.3 ± 2.3  |
| Low folate| 60.5 ± 7.5  | 28.0 ± 6.5  |

and proliferation of NSPCs such as Pax6, Nrsf, Bmp4, Stat3 and Hey, and for genes involved in neuronal maturation such as Prox1, Neurod1, Mib1 and Creb1 were downregulated on differentiation day 3 or 7, compared with levels under control conditions. In contrast, mRNA levels for genes involved in neuronal differentiation such as Ascl1, Neurog1 and Eomes were upregulated under low folate conditions (Figure 3A).

The molecular basis of the low folate-induced changes in neuronal gene expression was examined by analysis of DNA and histone methylation, which are epigenetic mechanisms in which folate-mediated one-carbon metabolism plays a critical role. DNA methylation commonly occurs at cytosines within 5’-CpG-3’ dinucleotides. Sequences with a higher frequency of CpG dinucleotides than the rest of the genome are referred to as CpG islands and are often located in promoter and regulatory regions
A Day 3  Nestin

B Day 3  Nestin

C Day 3  Tuj1

D Day 3  Tuj1

E Day 3  MAP2

F Day 3  MAP2

G Day 3  GFAP

H Day 3  GFAP

(caption on next page)
1.4.1, P < 0.01) for Nestin; no significant effect of folate (F(1,8) = 0.16, P > 0.05) or SAM (F(1,8) = 0.17, P > 0.05) and no significant interaction between folate and SAM (F(1,8) = 0.08, P > 0.05) for Tuji1; no significant effect of folate (F(1,8) = 2.81, P > 0.05) or SAM (F(1,8) = 0.02, P > 0.05) and no significant interaction between folate and SAM (F(1,8) = 1.60, P > 0.05) for MAP2; and no significant effect of folate (F(1,8) = 0.21, P > 0.05) or SAM (F(1,8) = 1.41, P > 0.05) and no significant interaction between folate and SAM (F(1,8) = 0.01, P > 0.05) for GFAP.

There have been many studies of the effects of folate deficiency on proliferation, survival, differentiation and maturation of NSPCs in vitro and in vivo [29, 30, 31, 32, 33]. However, all of these studies have used complete depletion of folate in the medium or in the diet. Under these conditions, the proliferation, survival and differentiation of NSPCs have been found to be markedly reduced. In contrast, in this study, we assessed the differentiation and maturation of NSPCs using a medium in which folate was reduced, but not completely removed, to mimic the neuronal immaturity that we have previously shown in the DG of mice fed a low folate, but not completely folate-deficient, diet [21]. Under the conditions used in this study, an increased number of immature neurons and a decreased number of mature neurons were observed compared to cells differentiated from NSPCs, as we previously reported in the DG [21]. The projections observed in Tuji1-positive cells and MAP2-positive cells on Day 7 appeared to be extremely short at low folate compared with controls. There were no differences between control and low folate conditions in the number of GFAP-positive astrocytes or DAPI-positive total cells (data not shown). The folate level of the medium had no effect on cell numbers, which is consistent with previous studies [34]. These results suggest that neuronal maturation can be affected under low folate conditions at levels that apparently have no effect on cell survival, unlike the complete folate-deficient conditions used in previous studies.

NSPCs differentiate to neurons and glia through strictly regulated processes. Epigenetic mechanisms such as DNA and histone methylation orchestrate neuronal differentiation [2, 3, 4, 5, 6], and loss of DNA methyltransferases, which catalyze DNA methylation, downregulates expression of neural genes and elicits impaired postnatal neurogenesis [35, 36]. H3K4me3 and H3K27me3 also play important roles in transcriptional regulation of several neuronal differentiation-related genes [37, 38]. In this study, murine embryonic telencephalon-derived NSPCs cultured in a low folate medium had decreased methylation of DNA and altered expression of certain neuronal differentiation and maturation-related genes. Low folate-induced changes in gene expression were observed on day 3, when decreased DNA methylation was found under low folate conditions. These results suggest that gene expression changes and epigenetic changes occur in parallel. Significant changes of mRNA expression and DNA methylation were observed for Neurog1 and Eomes. These two genes control neuronal differentiation and are upregulated during neuronal differentiation and downregulated during neuronal maturation in the DG [39, 40, 41]. Tbr2, a transcription factor encoded by Eomes, is critically required for progression from NSPCs to neurons in fetal and adult neurogenesis [42, 43]. Tbr2 regulates numerous genes associated with neuronal differentiation and maturation, such as Neurod1, which encodes NeuroD1 [44], a basic helix-loop-helix transcription factor that is essential for survival and maturation of newborn neurons and embryonic NSPCs [45, 46, 47] and is downregulated by Tbr2 binding [44].

Similarly to expression of genes involved in neuronal differentiation and maturation, the increased number of Nestin-positive cells on differentiation day 3 (Figure 5A,B,C,D,E,F,G,H) and the increased number of Tuji1-positive cells and the decreased number of MAP2-positive cells on differentiation day 7 (Figure 6A,B,C,D,E,F,G,H) were reversed by SAM supplementation. SAM did not affect the number of GFAP-positive cells.

4. Discussion

There have been many studies of the effects of folate deficiency on proliferation, survival, differentiation and maturation of NSPCs in vitro and in vivo [29, 30, 31, 32, 33]. However, all of these studies have used complete depletion of folate in the medium or in the diet. Under these conditions, the proliferation, survival and differentiation of NSPCs have been found to be markedly reduced. In contrast, in this study, we assessed the differentiation and maturation of NSPCs using a medium in which folate was reduced, but not completely removed, to mimic the neuronal immaturity that we have previously shown in the DG of mice fed a low folate, but not completely folate-deficient, diet [21]. Under the conditions used in this study, an increased number of immature neurons and a decreased number of mature neurons were observed compared to cells differentiated from NSPCs, as we previously reported in the DG [21]. The projections observed in Tuji1-positive cells and MAP2-positive cells on Day 7 appeared to be extremely short at low folate compared with controls. There were no differences between control and low folate conditions in the number of GFAP-positive astrocytes or DAPI-positive total cells (data not shown). The folate level of the medium had no effect on cell numbers, which is consistent with previous studies [34]. These results suggest that neuronal maturation can be affected under low folate conditions at levels that apparently have no effect on cell survival, unlike the complete folate-deficient conditions used in previous studies.

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and maturation is likely to be involved in this process in a low folate model, as in other models.

In addition to DNA hypomethylation of genes involved in neuronal differentiation and maturation, lower intracellular SAM was observed in cells differentiated in a low folate medium. Furthermore, SAM supplementation reversed low folate-induced expression changes of genes involved in neuronal differentiation and maturation. These results suggest that SAM levels under low folate may be inadequate for dynamic DNA methylation changes in neuronal genes that regulate cellular differentiation and maturation of NSPCs. Reduced intracellular SAM has been shown in complete folate-deficient models [30], and a similar hypomethylation process may occur in a low folate model. Whereas SAM supplementation reversed expression changes of genes involved in neuronal differentiation and maturation, it did not reverse those for genes involved in maintenance and proliferation of NSPCs, such as Pax6, Stat3 and Hey. These results suggest that low folate-induced changes in genes involved in maintenance and proliferation of NSPCs may not be caused by lower SAM levels. Immunohistochemistry showed that SAM supplementation improved the neuronal immaturity observed under low folate conditions, which supports the view that low folate-induced abnormalities in neuronal maturation may be due to expression changes of neuronal differentiation and maturation-related genes such as Neurog1, Eomes and Neurod1 caused by low SAM.

In conclusion, the current findings suggest that low folate may induce neuronal immaturity via DNA hypomethylation in genes associated with neuronal differentiation and maturation. This DNA hypomethylation may be due to reduction of intracellular SAM levels. These mechanisms may underlie effects in the DG in folate-deficient patients with depression.

Declarations

Author contribution statement

Ryota Araki: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shoji Nishida: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yuki Nakajima, Arimi Iwakumo, Hayato Tachioka, Ayami Kita: Performed the experiments.

Takeshi Yabe: Conceived and designed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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