

PRMT1 regulates astrocytic differentiation of embryonic neural stem/precursor cells

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Title

PRMT1 regulates astrocytic differentiation of embryonic neural stem/precursor cells

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Keywords: PRMT, Arginine methylation, Astrocyte, STAT

Abbreviations used: PRMT, protein arginine methyltransferase; NS/PC, neural stem precursor

cells; *Gfap*, glial fibrillary acidic protein; STAT, signal transducer and activator of transcription;

LIF, leukemia inhibitory factor; GF1L, reporter plasmid containing the luciferase gene under the

control of the 2.5-kb *Gfap* promoter

Abstract

Arginine methylation is a posttranslational modification which is catalyzed by protein arginine methyltransferases (PRMTs). Here, we report that PRMT1 is highly expressed in neural stem/precursor cells (NS/PCs) of mouse embryos, and knockdown of PRMT1 in NS/PCs suppresses the generation of astrocytes. The luciferase assay demonstrated that knockdown of PRMT1 inhibits activation of the promoter of a typical astrocytic marker gene, *glial fibrillary acidic protein (Gfap)*, in NS/PCs. The transcription factor signal transducer and activator of transcription 3 (STAT3) is known to generally be critical for astrocytic differentiation of NS/PCs. We found that PRMT1 methylates arginine residue(s) of STAT3 to regulate its activity positively, resulting in the promotion of astrocytic differentiation of NS/PCs.

Introduction

One of the prevalent post-translational modifications of proteins, arginine methylation, is catalyzed by PRMTs and participates in many cellular functions. To date, nine PRMT family members with distinct tissue expression patterns and substrate specificities have been identified in mammals. Of them, PRMT1, which is localized both in the nucleus and the cytoplasm of cells, is one of the best characterized PRMTs, and contributes to over 90% of cellular arginine methylation. However, its function in NS/PCs has yet to be extensively analyzed.

It has been reported that PRMT1 and PRMT5 knockout mice die shortly after implantation and before embryonic day (E)6.5, respectively (Pawlak *et al.* 2000, Tee *et al.* 2010), suggesting that these PRMTs play pivotal roles in early development. A previous study showed that PRMT5 regulates proliferation and differentiation of NS/PCs and oligodendrocytic differentiation of oligodendrocyte progenitor cells (OPCs) (Huang *et al.* 2011). PRMT1 has also been reported to play important roles in the nervous system. For example, a recent study suggested that PRMT1 regulates retinoic acid dependent neuronal differentiation of mouse embryonic stem cells (Simandi *et al.* 2015), yet the methylation targets of PRMT1 and how PRMT1 exerts its effects on embryonic NS/PCs is not well understood.

In the present study, we found that PRMT1 is highly expressed in mouse embryonic NS/PCs and regulates astrocytic differentiation associated with enhancement of the expression of *Gfap*. In contrast, knockdown of PRMT4 or PRMT5 did not affect astrocytic differentiation of NS/PCs. Mounting evidence has suggested that the activation of STAT3 induces astrocytic differentiation of NS/PCs (Nakashima *et al.* 1999). *in vitro* methylation assays we performed here demonstrated that PRMT1 methylates STAT3 directly, enhancing STAT3's activity. Thus, we report for the first time that STAT3 is a novel methylation target of PRMT1 regulating astrocytic differentiation of NS/PCs.

Materials and Methods

Cell culture

We obtained embryonic NS/PCs from time-pregnant ICR mice (RRID:IMSR_CRL:22) as previously described (Takizawa *et al.* 2001). All experiments were performed according to the animal experimentation guideline of Kyushu University. Adult rat hippocampal neural stem cells (HCN) were provide by Dr. Fred H. Gage, and were cultured as described previously (Hsieh *et al.* 2004).

quantitative RT-PCR (qRT-PCR)

Total RNAs were extracted with Sepasol-RNA I Super G (Nacalai Tesque) from E11.5, E14.5, E17.5 derived telencephalons, E14.5 derived NS/PCs, E17.5 derived neurons, or P1 derived astrocytes. Reverse transcription was performed from 500 ng of total RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen) and quantitative real-time PCR (qPCR) was performed with the Stratagene Mx300p (Agilent Technologies) using a KAPA SYBR FAST qPCR Kit (KAPA Biosystems). qPCR primers are provided upon requests.

Lentivirus constructs, generation of lentivirus, and infection with lentivirus

The lentivirus vector (pLLX) used to express short hairpin RNA was provide by Drs. Z.

Zhou and M. E. Greenberg. Oligonucleotide sequences are provided upon requests.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and processed for immunostaining as described (Takizawa et al. 2001). The following primary antibodies were used: anti-GFP (Aves Labs, GFP-1010, AB_2307313), anti-Map2ab (Sigma-Aldrich, M1406, AB_477171), anti-GFAP (Sigma-Aldrich, G9269, AB_477035). The secondary antibodies used were: CF488 donkey anti-chicken IgY (IgG) (H+L) highly cross-adsorbed (Biotium), CF555 donkey anti-mouse IgG (H+L) highly cross-adsorbed (Biotium) or CF647 donkey anti-rabbit IgG (H+L) highly cross-adsorbed (Biotium). Nuclei were stained using bisbenzimidide H33258 fluorochrome trihydrochloride (Nacalai Tesque.).

Western blot analysis

Tissues were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 15 mM MgCl₂, 1% (v/v) NP-40, 0.5% sodium deoxycholate, 1 mM DTT, 1x Protease Inhibitor Cocktail; Nacalai Tesque). Lysates were subjected to SDS-PAGE and subsequent immunoblotting with antibodies against anti-PRMT1 (Abcam, ab73246, AB_1640800), anti-Sox2 (Santa Cruz Biotechnology, sc-17320, AB_2286684), anti-Map2ab,

anti-GAPDH (Millipore, MAB374, AB_2107445), anti-ADMA (Cell Signaling, #13522, AB_2665370), anti-STAT3 (Santa Cruz, sc-482, AB_632440), anti-Flag (Sigma-Aldrich, F3165, AB_259529) and anti-HA (MBL, M180-3, AB_10951811). Detection was performed using the ECL detection system (GE Healthcare Ltd).

Co-immunoprecipitation

Cells were lysed in lysis buffer (10 mM HEPES, pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.1% (v/v) NP-40, 0.5 mM DTT, 1x protease inhibitor Cocktail). Lysates were immunoprecipitated with 2 µg of antibody (anti-HA, anti-STAT3, normal mouse IgG (Santa Cruz, sc-2025, AB_737182) or normal rabbit IgG (Santa Cruz, sc-2027, AB_737197). The immunoprecipitated samples were analyzed by western blotting.

Luciferase assay

The luciferase assay was performed as previously described (Nakashima et al. 1999, Takizawa et al. 2001).

***In vitro* arginine methylation assay**

1 µg of recombinant STAT3 (Active motif) was incubated with or without 1 µg of

PRMT1 (Active motif) in the presence of 0.55 μ Ci of ^3H -SAM (PerkinElmer) at 37°C for 1 hour.

The reaction was stopped by addition of SDS sample buffer and the mixture was subjected to SDS-PAGE, blotting and autoradiography. For detection of ^3H signals, PVDF membranes were treated with EN3HANCE spray (PerkinElmer), and then exposed to X-ray film for 1 month.

Statistical analysis

Statistical analyses were performed using either Student's t test (for comparisons between two groups) or one-way ANOVA with Tukey's multiple comparison test (for multiple groups comparison). All experiments were independently replicated at least three times. Difference were considered statistically significant at $p < 0.05$.

Results

PRMT1 is highly expressed in embryonic NS/PCs

To explore the importance of arginine methylation in brain development, we first analyzed the expression of eight PRMT family genes (*Prmt9* was not examined because there is very limited knowledge about its biological functions compared with the other family members) in mouse fetal cerebral cortex (cortex). Using RT-PCR, we could detect the expression of these genes in the cortex at E11.5, E14.5 and E17.5, with *Prmt1* expression being the highest among

them (Fig. 1a). We further examined PRMT1 protein expression using cortex lysates at the same developmental stages and found that it was highly expressed at E11.5 but then its expression gradually decreased as development proceeded (Fig. 1b). This expression pattern of PRMT1 was correlated with that of NS/PC marker Sex determining region Y box2 (Sox2) and inversely correlated with that of neuronal marker Microtubule Associated Protein-2ab (Map2ab), implying that PRMT1 is abundantly expressed in NS/PCs but not in differentiated cells during brain development (Fig. 1b). To confirm this, we isolated RNA from NS/PCs, neurons, and astrocytes of E14.5, E17.5, and P1 cerebral cortex and subjected them to qRT-PCR. As expected, we observed higher expression of *Prmt1* in NS/PCs compared to neurons and astrocytes (Fig. 1c). The high expression levels of *Hes family bHLH transcription factor 5 (Hes5)* specifically in NS/PCs, *β III-tubulin (Tubb3)* in neurons, and *Gfap* in astrocytes ensured that appropriate cell isolation procedures had been performed for each neural cell type.

Knockdown of PRMT1 inhibits astrocytic differentiation of NS/PCs

Given that high expression of PRMT1 in NS/PCs was observed, we speculated that PRMT1 has some role in the regulation of NS/PC differentiation. To test this idea, we constructed lentiviral vectors carrying a distinct shRNA sequence for *Prmt1* (sh1, 2, or 3) together with the reporter *green fluorescent protein (GFP)* gene. We first co-transfected HEK293T cells

(CVCL_0063) with either of these constructs with Flag-PRMT1-expressing plasmid to check the knockdown efficiency for PRMT1. We found that all three *Prmt1*-shRNA sequences reduced the Flag-PRMT1 protein expression level (Fig. 2a). When we infected E14.5 NS/PCs with these shRNA-expressing lentiviruses, reduced expression of endogenous *Prmt1* was observed by qRT-PCR analysis (Fig. 2b, left). We then examined the effect of PRMT1-knockdown on NS/PC differentiation. NS/PCs were infected with each *Prmt1*-shRNA-expressing lentivirus, and induced to differentiate for 4 days in the presence of 0.5 % fetal bovine serum (FBS), and the cell types generated were evaluated by qRT-PCR or immunostaining. Knockdown of PRMT1 in NS/PCs reduced *Gfap* expression (Fig. 2b, right) whereas no drastic change of *Tubb3* expression was observed (Fig. 2b, middle). Immunostaining also showed a reduction of GFAP-positive cells in GFP-labeled PRMT1-knocked down cells (Fig. 2c). These knockdown effects were specific to PRMT1, since no such changes were observed when we knocked-down PRMT4 or PRMT5 (Fig. 2c).

In light of the above results, we decided to focus further on the PRMT1 function in astrocytic differentiation of NS/PCs. Since it is well known that leukemia inhibitory factor (LIF) effectively induces astrocytic differentiation of NS/PCs through the activation of the Janus Kinase (JAK)/STAT3 signaling pathway (He *et al.* 2005), we examined the effect of PRMT1 knockdown on LIF stimulation of astrocytic differentiation of NS/PCs. As shown in Fig. 2d and 2e, we found

that knockdown of PRMT1 suppressed astrocytic differentiation of NS/PCs. It is noteworthy that PRMT1 knockdown in NS/PCs affected not only *Gfap* expression but also the expression of another astrocyte-specific gene, *aquaporin4* (Fig. 2e). Taken together, these findings indicated that PRMT1 promotes astrocytic differentiation of NS/PCs by enhancing the expression of astrocyte-specific genes.

PRMT1 promotes the activation of the *Gfap* promoter

To gain insight into the molecular mechanism by which PRMT1 regulates the astrocytic differentiation of NS/PCs, we transfected E14.5 NS/PCs with a reporter plasmid containing the *luciferase* gene under the control of the 2.5-kb *Gfap* promoter (GF1L) (Takizawa et al. 2001) together with an shControl or shPRMT1-expressing construct. Forty-eight hours after transfection, NS/PCs were stimulated with LIF for 8 hours and the GF1L activity was measured. In accord with our qRT-PCR results, the GF1L activity was dramatically decreased in shPRMT1-expressing NS/PCs compared to the shControl (Fig. 2f, left). These results indicated that PRMT1 promotes astrocytic differentiation of NS/PCs by enhancing the transcriptional activity of the astrocytic gene *Gfap*. Importantly, knockdown of PRMT1 did not affect a GF1L mutant (2.5-kb *Gfap* promoter with mutation in the STAT3 recognition sequence) (Takizawa et al. 2001) transcriptional activity (Fig. 2f, right). These results emphasize the role of PRMT1 in the regulation of STAT3

activation.

PRMT1 arginine-methylates STAT3

It has been reported that PRMT1 methylates STAT1 and this methylation enhances STAT1's transcriptional activity, probably by inhibiting association between STAT1 and an inhibitory protein of STATs, protein inhibitor of activated STATs (PIAS) (Mowen *et al.* 2001). This finding suggested the possibility that PRMT1 is also involved in STAT3 activation. To investigate whether PRMT1 methylates STAT3, we co-transfected HEK293T cells with HA-tagged STAT3 and empty or Flag-PRMT1-expressing constructs, and immunoprecipitated HA-tagged STAT3. When blots were treated with anti-Asymmetric Di-methyl arginine antibody (ADMA), we observed that the arginine methylation level of STAT3 was significantly increased in the STAT3 and PRMT1 co-transfected sample compared to that of STAT3 and empty constructs (Fig. 3a). Unfortunately, we could not detect any direct interaction of PRMT1 and STAT3 in our immunoprecipitation assay (data not shown), probably due to the transient and unstable association of these proteins. Alternatively, we performed *in vitro* arginine methylation assays to confirm that PRMT1 indeed methylates STAT3. We found that when recombinant STAT3 was incubated with a solution containing recombinant PRMT1 and ³H-S-adenosyl L-methionine (SAM), ³H-labeled STAT3 was detected by autoradiography (Fig. 3b). We further confirmed the

STAT3 methylation by PRMT1 in NS/PCs by assessing the methylation levels of STAT3 in PRMT1-knockdown NS/PCs. We found that the methylation levels of STAT3 were markedly decreased in PRMT1 knockdown NS/PCs (Fig. 3c), suggesting that PRMT1 methylates endogenous STAT3 in NS/PCs.

STAT3 and PRMT1 cooperatively activate the astrocytic *Gfap* promoter

To elucidate the potential transcriptional regulatory function of PRMT1 for STAT3, PRMT1-expressing construct was transfected with or without STAT3 together with GF1L reporter construct in HCN cells. Forty-eight hours after transfection, these cells were stimulated with LIF for 8 hours, and the luciferase activity was measured. When STAT3 was overexpressed in HCN cells, activation of LIF-induced GF1L was significantly increased compared to the control (Fig. 4a). Moreover, overexpression of STAT3 and PRMT1 further activated GF1L compared to overexpression of STAT3 alone (Fig. 4a), suggesting that PRMT1 enhances the transcriptional activity of STAT3.

DISCUSSION

In this study, we have shown that PRMT1 is expressed abundantly in embryonic NS/PCs and promotes astrocytic differentiation of NS/PCs. We found that knockdown of PRMT1 in NS/PCs causes the generation of fewer GFAP-positive astrocytes and reduces GF1L activation

upon LIF stimulation (Fig. 1 and 2). The JAK-STAT signaling pathway has been implicated in astrocytic differentiation of NS/PCs. Although the activation of STAT3 by its tyrosine-phosphorylation is widely accepted, the possible importance of STAT3-arginine methylation has remained elusive. Here, we demonstrated that PRMT1 methylates STAT3 and co-expression of STAT3 and PRMT1 enhances the LIF-induced GF1L activation (Fig. 3 and 4). These results indicated that the arginine methylation of STAT3 by PRMT1 enhances transcription of an astrocyte-specific gene, consequently promoting astrocyte differentiation of NS/PCs (Fig. 4b).

Arginine methylation of STAT proteins was previously described for STAT1 at residue R31, and this methylation site interacts with PIAS1. It was previously reported that interferon-induced STAT1 DNA binding ability was impaired when arginine methylation of STAT1 was inhibited by PRMT inhibitor (Mowen et al. 2001). Since we noted that the N-terminus region of STAT family genes is well conserved, we generated STAT3 R31 mutants (R to A or K substitution). However, both STAT3 R31A and R31K mutants were extremely unstable. Therefore, we could not perform any quantitative experiments (our unpublished observations). The fact that members of the PIAS family act as SUMO ligases (Schmidt & Muller 2002) and that mutations of STAT1 as well as STAT3 in which R31 was substituted by K led to protein destabilization (Mowen et al. 2001, Komyod *et al.* 2005) support our idea that PRMT1 regulates STAT3 activity and enhances transcription of numerous cytokine-induced genes, including *Gfap*. However, Komyod et al.

reported that PRMT1 did not methylate STATs. Although we do not know the exact reasons for the difference between our and their results, the difference of the antibodies used could be one of them. However, further investigation will be needed to precisely determine the cause of the discrepancy.

Recently, CNS-specific PRMT1 conditional knockout mice were generated by crossing *Nestin-Cre* with floxed-*Prmt1* mice. These mice die within two weeks after birth, suggesting the importance of PRMT1 in CNS development for survival (Hashimoto *et al.* 2016). Unexpectedly, these CNS-specific PRMT1 conditional knockout mice showed no dramatic changes in the astrocyte population, and instead showed significantly inhibited oligodendrocytic differentiation (Hashimoto *et al.* 2016). Although we checked an oligodendrocytic marker, myelin basic protein-positive oligodendrocyte generation, in our PRMT1-knockdown experimental setting, no such drastic change was observed (our personal observation). These discrepancies might be in part explained by the timing of knockout or knockdown of PRMT1 in NS/PCs. In addition, our knockdown experiments were performed in NS/PCs, which were prepared from E14.5 mouse telencephalon followed by 4 days expansion with a mitogen, bFGF. Thus, it is possible that during this *in vitro* culture period, we eliminated OPCs that might be more sensitive to PRMT1 expression. Nevertheless, we are convinced that our *in vitro* NS/PCs differentiation procedure is sensitive enough to evaluate slight changes in astrocytic differentiation properties, and we report

here for the first time that STAT3 arginine methylation by PRMT1 promotes astrocyte differentiation of NS/PCs by facilitating transcriptional activity of STAT3-targeted astrocyte-specific genes.

Author contributions and conflict of interest disclosure

M.H. performed all of the experiments with support from S.K., M.H. and S.K. conceived and designed the research, and K.N. coordinated and supervised the study. M.H., S.K., and K.N. wrote the manuscript. All authors reviewed and approved the final version of the manuscript. The authors declare that they have no conflicts of interest. All experiments were conducted in compliance with the ARRIVE guidelines.

Acknowledgments

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Figure legends

Fig 1. PRMT1 is highly expressed in mouse embryonic NS/PCs

(a, b) qRT-PCR analysis of *Prmt* family genes (n=3 of individual embryos) (a), and western blot analysis of PRMT1 (b) in cerebral cortex at various developmental stages. (c) qRT-PCR analysis of *Prmt1*, *Hes5*, *Tubb3* and *Gfap* in NS/PCs, neurons and astrocytes. (One-way ANOVA : Tukey's MC test, n=3 dishes, *p<0.05, ***p<0.001)

Fig 2. Knockdown of PRMT1 inhibits astrocytic differentiation associated with a reduction of *Gfap* expression

(a) Western blot analysis of Flag-PRMT1 expression in HEK293T cells transfected with shRNA vector targeting *Prmt1* (shPRMT1-sh1, sh2 or sh3). (b, c) Each type of shRNA virus infected-E14.5 NS/PCs was induced to differentiate under 0.5% FBS-containing medium for 4 days. qRT-PCR analyses were performed for *Prmt1*, *Tubb3* or *Gfap* (One-way ANOVA : Tukey's MC test, n=3 dishes, **p<0.01, ***p<0.001) (b) Immunostaining was performed with antibodies against GFP, GFAP and Map2ab. (c) The histogram depicts the percentage of marker-positive cells among total GFP-positive cells (One-way ANOVA : Tukey's MC test, n=3 dishes, *p<0.05).

(d-e) Control or shPRMT1 virus infected-E14.5 NS/PCs were stimulated with 50 ng/ml of

leukemia inhibitory factor (LIF) for 4 days to induce astrocyte differentiation. The cells were stained with antibodies against GFP and GFAP. Scale bar=50 μ m. (d) Histogram depicts the percentage of marker-positive cells among total GFP-positive cells (Student t-test, n=3 dishes, *p<0.05). (e) qRT-PCR analysis of *Gfap* or *Aquaporin4* expression (One-way ANOVA : Tukey's MC test, n=3 dishes, ***p<0.001) (f) E14.5 NS/PCs were transfected with Control or shPRMT1 construct together with GF1L or GF1L mutation reporters. The cells were stimulated with LIF (50 ng/ml) for 8 hours, and luciferase activity was measured using a luminometer (One-way ANOVA : Tukey's MC test, n=3 dishes, ***p<0.001).

Fig 3. STAT3 arginine-methylation by PRMT1

(a) HA-STAT3-expressing construct was transfected into HEK293T cells with Flag-PRMT1. To detect methylated STAT3, lysates were immunoprecipitated with anti-HA, followed by western blotting with anti-ADMA. (b) Recombinant STAT3 was incubated with or without recombinant PRMT1 in methylation buffer with 3 H-SAM. 3 H-SAM incorporation was detected by exposure to X-ray film. Ponceau staining was used to confirm the expression of STAT3 and PRMT1. (c) Proteins were extracted from shControl or shPRMT1 virus-infected E14.5 NS/PCs. To detect methylated STAT3, lysates were immunoprecipitated with anti-STAT3 antibody, followed by western blotting with anti-ADMA.

Fig 4. Cooperative promotion of astrocytic differentiation of NS/PCs by STAT3 and PRMT1

(a) HCN cells were transfected with PRMT1 construct with or without STAT3 construct together with GF1L reporter construct. The cells were stimulated with LIF (10 ng/ml) for 8 hours. Luciferase activity was measured using a luminometer (One-way ANOVA: Tukey's MC test, n=3 dishes, **p<0.01, ***p<0.001). (b) Schematic model for PRMT1/STAT3-mediated astrocytic differentiation of NS/PCs.

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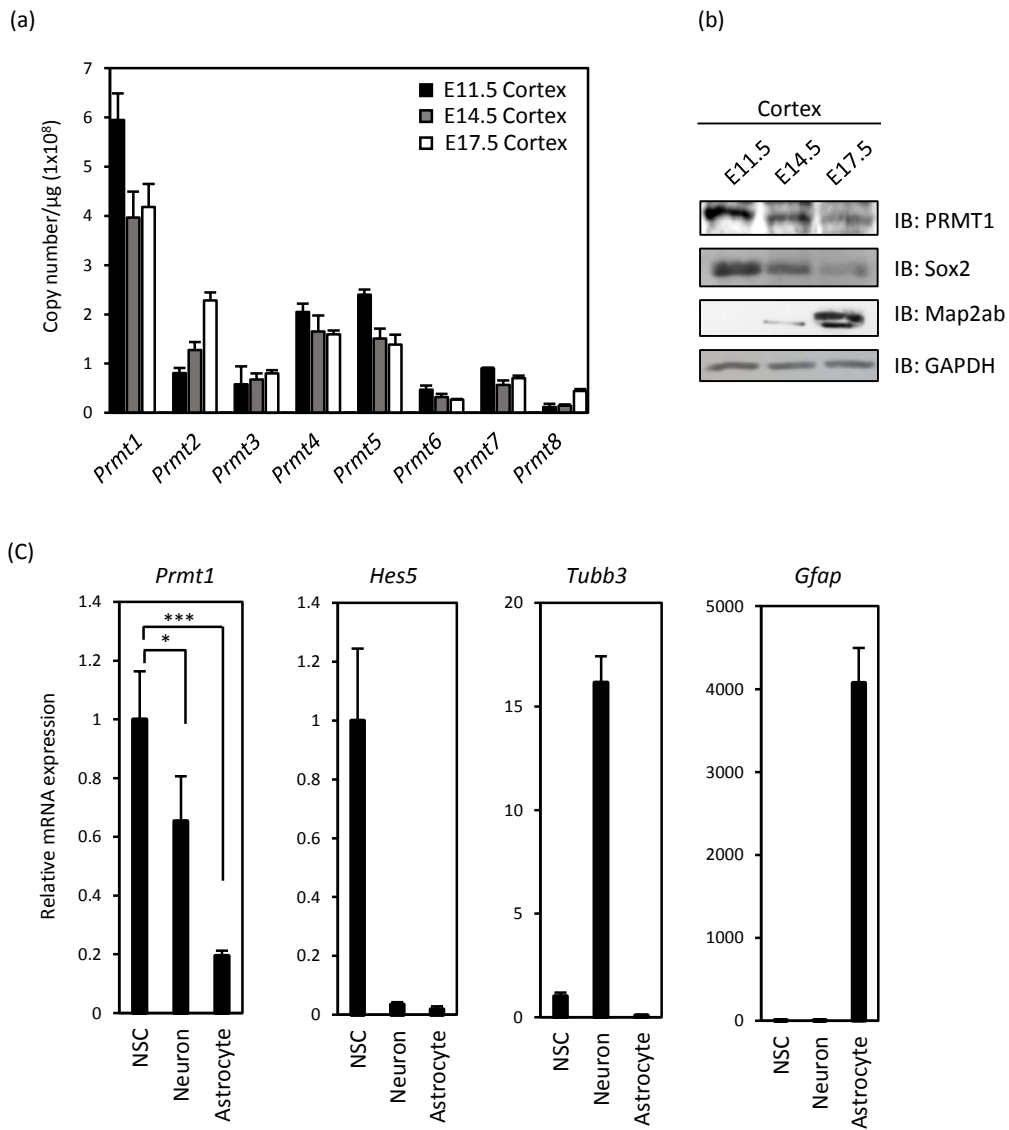


Fig 1. PRMT1 is highly expressed in mouse embryonic NS/PCs

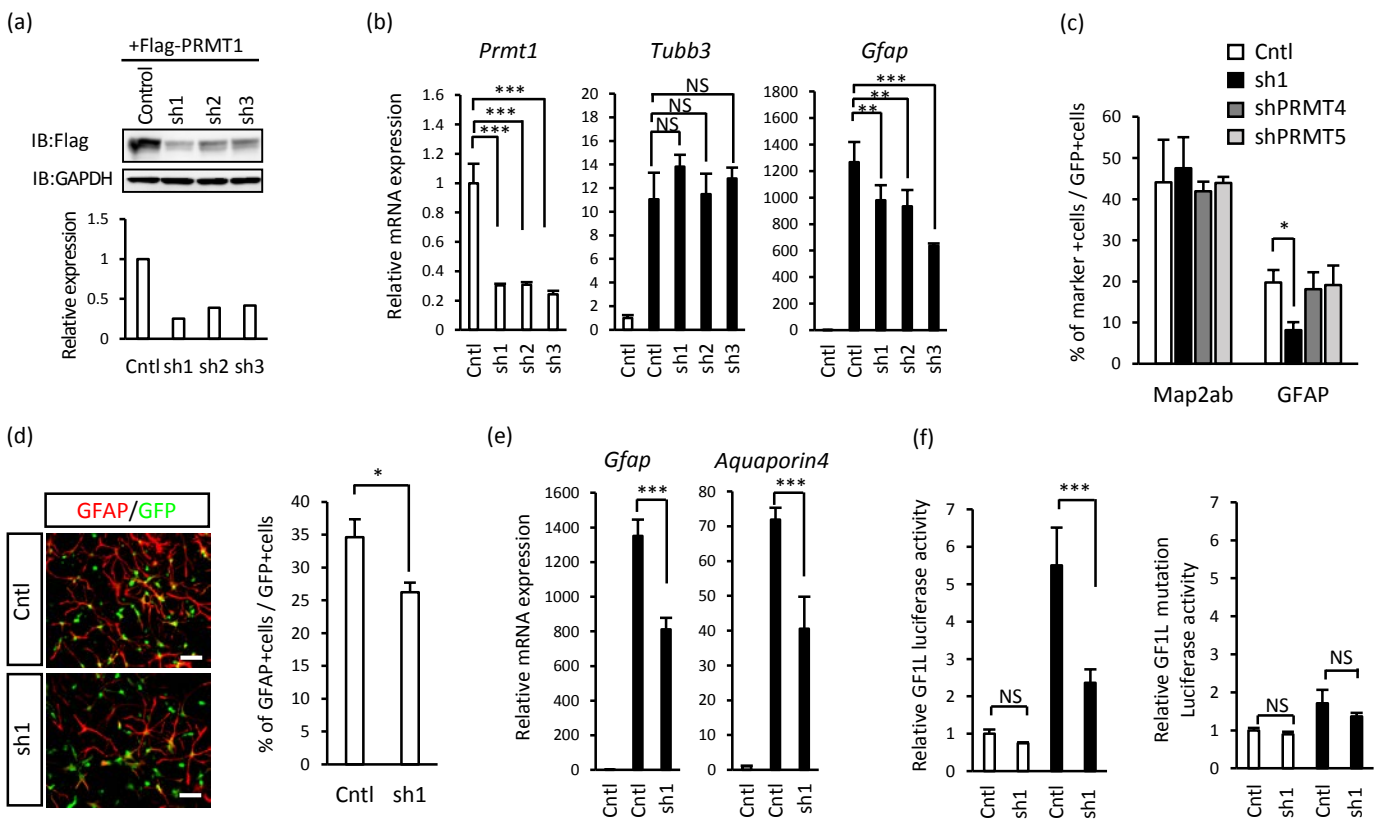


Fig 2. Knockdown of PRMT1 inhibits astrocytic differentiation associated with a reduction of *Gfap* expression

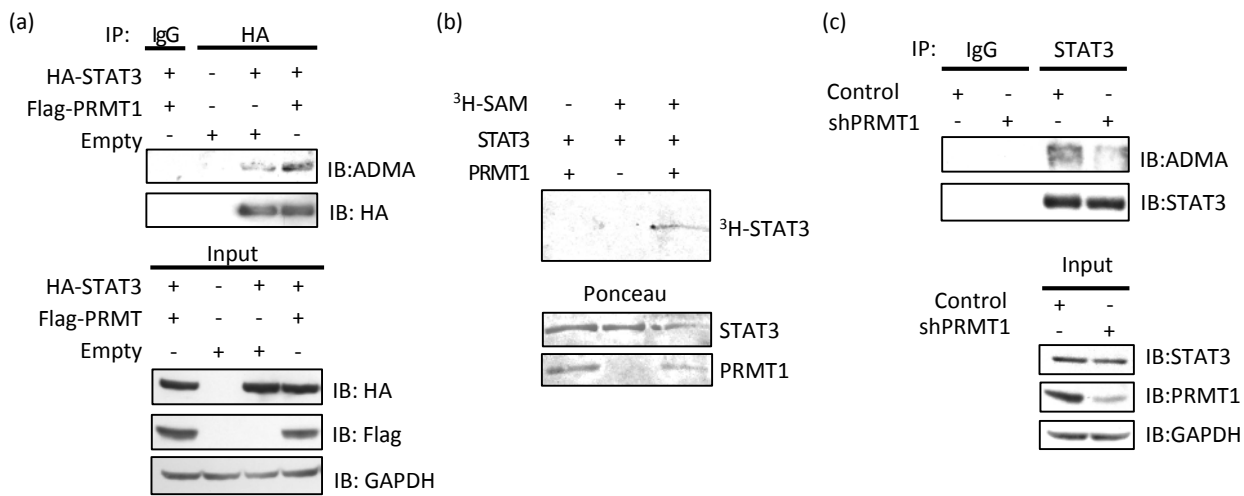


Fig 3. STAT3 arginine methylation by PRMT1

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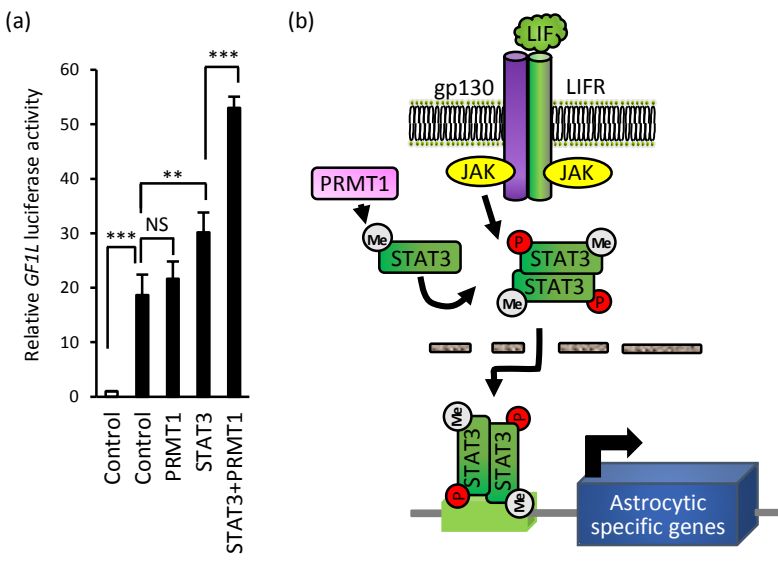


Fig 4. Cooperative promotion of astrocytic differentiation of NS/PCs by STAT3 and PRMT1

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Honda *et al.* Supplemental figure1

Primer		sequence (5'–3')
PRMT1	F	GCTTCTCCACCAGTCCTGAG
	R	CTCACACAGCTGACCCTTGA
PRMT2	F	AGGCTGCTGTGGTTACATCC
	R	GTCAGCCAACATTTCCAGGT
PRMT3	F	GTGCCTTGGGAAAAAGATGA
	R	TCAACAACAGATGCGCTTTC
PRMT4	F	ACCACACGGACTTCAAGGAC
	R	CTCTTCACCAGGACCTCTGC
PRMT5	F	CTGAGTGTCTGGATGGAGCA
	R	CCGAACCACATAAGGCATCT
PRMT6	F	AGCGAGTGGATGGGCTACGGA
	R	GCTGCTTCACCTGGCTCCAGA
PRMT7	F	GCTGCTGTGAAGATTGTGGA
	R	CAAACAGCTCCGTGATCAGA
PRMT8	F	TGGTGACCAATGCCTGTTTA
	R	CCCATTTTCTTGTGGCACTT
Tubb3	F	GGCCTCCTCTCACAAGTATG
	R	TTGTTGCCAGCACCCTCTG
Hes5	F	CTCCGCTCCGCTCCGCTCGCTAAT
	R	GGGGCCGCTGGAAGTGGTAAAGCA
GFAP	F	AAAACAAGGCGCTGGCAGCTGAACTGAA
	R	TTGTTCTCTGCCTCCAGCCTCAGGTTGGT
Aquaporin4	F	ACGGTTCATGGAAACCTCACCG
	R	CCAGTGGTTTGCCAGTTTCCC
be-ta actin	F	GGGGTGTTGAAGGTCTCAA
	R	TGTTACCAACTGGGACGACA

Details

Figure S1. Information of qPCR primers

Title

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Primer	sequence (5'–3')	
P m t1	F	G C T T C T C C A C C A G T C C T G A G
	R	C T C A C A C A G C T G A C C C T T G A
P m t2	F	A G G C T G C T G T G G T T A C A T C C
	R	G T C A G C C A A C A T T T C C A G G T
P m t3	F	G T G C C T T G G G A A A A A G A T G A
	R	T C A A C A A C A G A T G C G C T T T C
P m t4	F	A C C A C A C G G A C T T C A A G G A C
	R	C T C T T C A C C A G G A C C T C T G C
P m t5	F	C T G A G T G T C T G G A T G G A G C A
	R	C C G A A C C A C A T A A G G C A T C T
P m t6	F	A G C G A G T G G A T G G G C T A C G G A
	R	G C T G C T T C A C C T G G C T C C A G A
P m t7	F	G C T G C T G T G A A G A T T G T G G A
	R	C A A A C A G C T C C G T G A T C A G A
P m t8	F	T G G T G A C C A A T G C C T G T T T A
	R	C C C A T T T T C T T G T G G C A C T T
Tubb3	F	G G C C T C C T C T C A C A A G T A T G
	R	T T G T T G C C A G C A C C A C T C T G
Hes5	F	C T C C G C T C C G C T C C G C T C G C T A A T
	R	G G G G C C G C T G G A A G T G G T A A A G C A
G fap	F	A A A A C A A G G C G C T G G C A G C T G A A C T G A A
	R	T T G T T C T C T G C C T C C A G C C T C A G G T T G G T
Aquaporin4	F	A C G G T T C A T G G A A C C T C A C C G
	R	C C A G T G G T T T G C C C A G T T T C C C
be-ta actin	F	G G G G T G T T G A A G G T C T C A A A
	R	T G T T A C C A A C T G G G A C G A C A

Table S1. Primer sequences used for qPCR