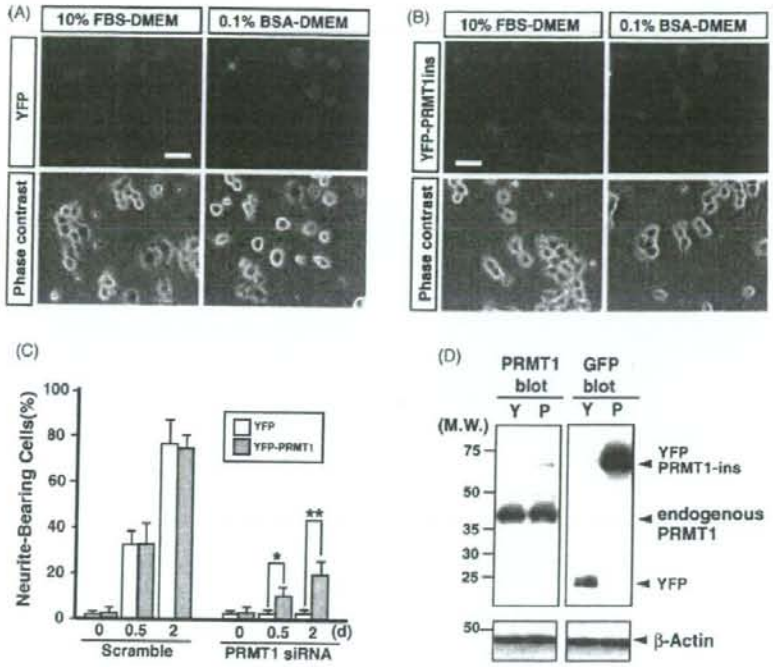

Fig. 2. PRMT1 expression necessary for the neurite outgrowth of Neuro2a cells. (A and B) Fluorescent micrographs of the Neuro2a cells. Depletion of PRMT1 in Neuro2a cells by transfection with 100 pM PRMT1 siRNA and mock YFP (A) or YFP-fused siRNA insensitive PRMT1 (B) for 3 days is shown. Cells were grown in normal growth medium (10% FBS) or differentiation medium (0.1% BSA) for 2 days. Bar, 30 μm. (C) Quantification of the results shown in panel A and B. PRMT1 expression is necessary for neurite outgrowth in the Neuro2a cells. PRMT1 siRNA-insensitive, YFP-fused PRMT1 (yellow) or mock YFP (white) expressed in PRMT1-depleted Neuro2a cells cultured for 2 days. The results are expressed as the mean ± S.E.M. of three independent experiments. The asterisks indicate the significant effects of an increase in the number of neurite-bearing cells (Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$). (D) Checking the expression of YFP-fused siRNA-insensitive PRMT1 in Neuro2a cells. Y, mock YFP-transfected cell lysate; P, YFP-fused siRNA insensitive PRMT1-transfected cell lysate; M.W., molecular weight.

GCTGGAGACGGCCATCACAT-3′. As primers for ribosomal protein L27 (RPL27) primers: forward, 5′-TGTGTGGATATCCCCTTGGA-3′; reverse, 5′-TAAAAGCGAAGCTTCTGGAAA-3′. The fluorescence derived from the incorporation of SYBR Green I into the double-stranded PCR products was performed and measured according to the manufacturer's instructions (Applied Biosystems). In Btg2-targeted siRNA-transfected cells, the optimal induction of Btg2 mRNA after serum withdrawal was reduced to 20% of that in the scrambled siRNA-transfected cells (Fig. 3A), with almost no effect on PRMT1 and CARM1 protein expression (data not shown). At the same time point, the nuclear fractions of the Btg2 siRNA-transfected cells exhibited a significant decrease in the level of arginine-methylated proteins recognized by the ASYM24 antibody (1:500; Upstate Biotech) (scrambled siRNA, 1.00 ± 0.53; Btg2 siRNA, 0.58 ± 0.10) (Fig. 3B). However, the cytoplasmic fraction of the Btg2 siRNA-transfected cells did not show any change in the arginine-methylated protein level (scrambled siRNA, 1.00 ± 0.41; Btg2 siRNA, 1.12 ± 0.79) (Fig. 3B). These results showed that Btg2 might act as one of the regulators of arginine-methylating activity in the nuclear fraction possibly though interaction with PRMT1. Furthermore, the Btg2-targeted siRNA decreased the population of neurite-bearing cells 2 days after serum deprivation (2 days: scrambled siRNA, $78.2\% \pm 8.3\%$; Btg2 siRNA, $32.4\% \pm 9.3\%$) (Fig. 3C). These results raised the possibility that Btg2 expression was one of the regulators of PRMT1 activity in the nucleus through the neurite outgrowth of Neuro2a cells.

The recent investigations and functional analyses of the PRMT family raised the possibility that protein arginine methylation is highly regulated in response to external stimuli and may play different roles in physiological processes; however the functions of the PRMT family in the nervous system remain unknown [3,17]. This report demonstrated that the neurite outgrowth of Neuro2a cells might be regulated by the PRMT1 and Btg2 expressions. This result is in keeping with that of a previous report on the drug-induced inhibition of the protein methylation of PC12 cells that caused the loss of neurite extension in the presence of NGF with no effect on the survival rate [5]. The recovery of YFP-fused PRMT1 in the knockdown cells partly reverted the defective neuritogenesis in the serum-starved condition (Fig. 2), confirming that the knockdown phenotype was attributable to the loss of PRMT1. In this study, we further demonstrated that the Btg2 expression is related to neurite outgrowth (Fig. 3). These results indicate that one of the possible mechanisms of neurite outgrowth is regulated by Btg2 protein induction and PRMT1 activity regulation. It is well known that many RNA-binding proteins modify arginine methylation [20] and that the Btg2 protein can bind to PRMT1, thereby increasing PRMT1 activity [14]. This suggests that the expression of Btg2 and up-regulate PRMT1 activity plays an important role in the function of RNA-binding proteins in arginine methylation and can change the localization of specific mRNAs in neurons; this confirms the fact that arginine methylation is an important event in the formation of neural circuits. Therefore, it is likely that arginine methylation is emerging as a major regulator of protein function.

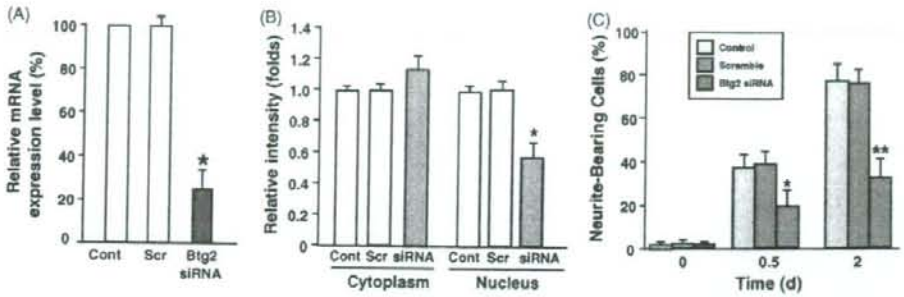

Fig. 3. Knockdown of Btg2 mRNA expression decreases arginine methylation and inhibits neurite outgrowth in Neuro2a cells. Real-time PCR analysis of Btg2 mRNA expression in Neuro2a cells transfected with 200 pM scrambled (Scr) or Btg2 siRNA for 3 days or in a nontransfected control (Cont). The results are expressed as the mean ± S.E.M. of three independent experiments. The asterisks (*) indicate the significant effects of a decrease in Btg2 mRNA expression after Btg2 siRNA transfection ($P < 0.05$). (B) Quantification of the results of the siRNA-transfected Neuro2a cells were fractionated into cytoplasmic and nuclear fractions that were analyzed by western blotting with the anti-ASYM24 antibody. As controls, Neuro2a cell lysates were analyzed using an anti-β-actin and anti-histonH4 antibody. Scr, scrambled siRNA-transfected cells; siRNA, Btg2 siRNA-transfected cells. The data are presented as relative to the level of the expression of every asymmetric dimethylated protein in the lysates of nontransfected cells detected by the ASYM24 antibody at 0 h (set at 1.0). The results are expressed as the mean ± S.E.M. of three independent experiments. The asterisk (*) indicates the significant effects of a decrease in the relative intensities (Student's *t*-test; $^*P < 0.05$). (C) Quantification of the neurite outgrowth levels of the Btg2 depletion Neuro2a cells. The results are expressed as the mean ± S.E.M. of three independent experiments. The asterisks (*) indicate the significant effects of a decrease in the number of neurite-bearing cells (Student's *t*-test; $^*P < 0.05$, $^{**}P < 0.01$).

It is possible that the examination of methylated or demethylated RNA-binding proteins will clarify the candidate molecular mechanisms of neuritogenesis.

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