

ORIGINAL ARTICLE

Epigenetic silencing of *prostaglandin E receptor 2 (PTGER2)* is associated with progression of neuroblastomasY Sugino^{1,2,3}, A Misawa^{1,2}, J Inoue^{1,2}, M Kitagawa⁴, H Hosoi³, T Sugimoto³, I Imoto^{1,2,5} and J Inazawa^{1,2,5,6}

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We previously identified a cluster of prostanoid receptor genes, *prostaglandin D2 receptor (PTGDR)* and *prostaglandin E receptor 2 (PTGER2)*, as possible targets for DNA methylation in advanced types of neuroblastoma (NB) using bacterial artificial chromosome array-based methylated CpG island amplification method. Among them, in this study, we found that *PTGER2* was frequently silenced in NB cell lines, especially in those with *MYCN* amplification, through epigenetic mechanisms. In NB cell lines, DNA methylation pattern within a part of CpG island was inversely correlated with *PTGER2* expression, and histone H3 and H4 deacetylation and histone H3 lysine 9 methylation within the putative promoter region were more directly correlated with silencing of this gene. Methylation of *PTGER2* was observed more frequently in advanced-type of primary NBs compared with early-stage tumors. Growth of NB cells lacking endogenous *PTGER2* expression was inhibited by restoration of the gene product by transient and stable transfection. A *PTGER2*-selective agonist, butaprost, increased intracellular cyclic adenosine monophosphate (cAMP) level, inhibited cell growth and induced apoptosis of NB cells stably expressing exogenous *PTGER2*. 8-Bromo-cAMP also inhibited growth of NB cells lacking *PTGER2* expression, but not cells expressing this gene. Taken together, it is suggested that NB cells may lose responsiveness to *PTGER2*-mediated growth inhibition/apoptosis through epigenetic silencing of *PTGER2* and/or disruption of downstream cAMP-dependent pathway during the neuroblastomagenesis.

Oncogene advance online publication, 28 May 2007; doi:10.1038/sj.onc.1210550

Keywords: neuroblastoma; *PTGER2*; tumor suppressor; DNA methylation; histone modification; cAMP

Introduction

Neuroblastoma (NB) is one of the most common pediatric solid tumors of neural-crest origin. The outcome of this disease is highly heterogeneous: older patients frequently develop metastatic disease with extremely aggressive progression, whereas spontaneous regression is common in infants and in early-stage NB tumors (Westermann and Schwab, 2002; Brodeur, 2003). Although numerous genetic abnormalities, including the *MYCN* amplification, are involved in development and/or progression of NB, the molecular mechanisms responsible for the pathogenesis of aggressive NB remain unclear. Epigenetic alterations such as hypermethylation of promoter sequences, with consequent silencing of tumor-suppressor genes, such as *CASP8*, *RASSF1A*, *CD44* and *TSP-1* can play important roles in the pathogenesis of NB (Teitz *et al.*, 2000; Yan *et al.*, 2003; Yang *et al.*, 2003, 2004). Therefore, exploration of hypermethylated CpG-rich sequences in NB cell genomes could accelerate identification of unknown tumor suppressors whose loss contributes to progression of this disease.

Recently, we developed 'bacterial artificial chromosome (BAC) array-based methylated CpG-island amplification (MCA)' (BAMCA; Inazawa *et al.*, 2004), which incorporates our custom-made BAC-based genomic DNA array (Inazawa *et al.*, 2004) in combination with MCA (Toyota *et al.*, 1999), as a method for detecting aberrantly methylated sequences in the human genome. When we applied BAMCA to NB genomes using aggressive NB cell lines and stage I 'nonaggressive' primary NB tumors as test and reference samples, respectively, followed by expression and methylation analyses of candidate targets, we successfully identified *NR1I2* as a novel tumor-suppressor candidate that is often silenced by DNA methylation in advanced type of this disease (Misawa *et al.*, 2005). In the process, we also identified several possible targets other than *NR1I2* for methylation-mediated silencing in advanced NB. Since identification of additional epigenetic abnormalities in

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Received 20 February 2007; revised 23 March 2007; accepted 26 March 2007

NB should lead not only to better understanding of the pathogenesis of this disease, but also to development of new diagnostic markers and/or therapeutic strategies (Abe *et al.*, 2005), determining the significance of each candidate tumor-suppressor gene inactivated by epigenetic mechanisms in NB will be highly valuable. Among possible targets, in the study reported here we analysed two genes, *prostaglandin E receptor 2 (PTGER2)* and *prostaglandin D2 receptor (PTGDR)*, both are located within the same BAC and encode receptors for subtypes of prostaglandin E2 (PGE2) and D2 respectively, and identified *PTGER2* as a candidate tumor suppressor for NB.

Results

Analysis of PTGDR and PTGER2 expression in NB cell lines

In our previous report (Misawa *et al.*, 2005), we described a strategy for identifying epigenetically silenced tumor-suppressor genes through exploration of aberrantly methylated sequences in the NB genome. During that program, *PTGDR* and *PTGER2*, within one BAC clone (RP11-262M8) at 14q22.1, were identified as possible targets for inactivation through DNA methylation (Figure 1a). To determine whether these genes might be silenced in NB, we examined expression of *PTGDR* and *PTGER2* mRNAs in a panel of 20 NB cell lines by reverse transcription (RT)-PCR. Expression of *PTGDR* was lost or decreased in most of the cell lines compared with normal adrenal gland and brain, whereas *PTGER2* expression was lost or decreased in eight lines and detected in 12 lines (Figure 1b). All eight lines that lacked expression of *PTGER2* showed amplification of *MYCN*, whereas only five of the 12 *PTGER2*-expressing lines did ($P = 0.015$, Fisher's exact test).

Restoration of PTGDR and PTGER2 expression by 5-aza-dCyd and TSA

To investigate whether DNA demethylation could restore expression of *PTGDR* and *PTGER2* mRNAs in NB cells lacking them, we treated NB cells with 1 or 5 μM of 5-aza-2'-deoxycytidine (5-aza-dCyd), a methyltransferase inhibitor, for 5 days and/or 100 ng/ml of trichostatin A (TSA), a histone deacetylase inhibitor, for the last 12 h. Induction of *PTGDR* and *PTGER2* mRNAs occurred after treatment with 5-aza-dCyd in cells lacking expression of the genes (Figure 1c). Restoration of mRNA expression was also observed for both genes, especially *PTGER2*, after treatment with TSA alone. However, a greater elevation in expression was observed in cells treated with 5-aza-dCyd and TSA compared with those treated with 5-aza-dCyd alone, suggesting that DNA methylation and histone modification, including deacetylation of histones, may be cooperatively involved in silencing these two genes.

Methylation of PTGDR and PTGER2 CpG islands in NB cell lines

We examined the methylation status of CpG islands of the *PTGDR* and *PTGER2* genes predicted by the

CpG PLOT program (<http://www.ebi.ac.uk/emboss/cpgplot/>, Figure 2a). Bisulfite-sequencing revealed aberrant DNA hypermethylation throughout the CpG island of *PTGDR* in four NB cell lines, regardless of expression levels (Figure 2b). On the other hand, aberrant DNA hypermethylation was observed within Region 3 and part of Region 2 of the *PTGER2* CpG island in three cell lines (IMR32, GOTO and SJ-N-CG) that lack expression of *PTGER2*, while this island was hypomethylated in SH-SY5Y expressing the gene. We performed combined bisulfite restriction analysis (COBRA) in a larger set of NB cell lines to confirm the relationship between expression and methylation status within the whole CpG island of *PTGDR* and within Region 3 or part of Region 3 (Region 3-A) of the *PTGER2* CpG island (Figure 2b and Supplementary Figure S1). Methylated alleles were predominant in most of the NB lines lacking *PTGER2* expression, and unmethylated alleles were always detected in cells that expressed this gene. For *PTGDR*, however, no clear relationship was observed between gene expression and methylation status of the CpG island. These results prompted us to characterize further the *PTGER2* gene as a candidate tumor suppressor inactivated through epigenetic mechanism.

Promoter activity of the CpG island of PTGER2

We performed reporter assays to determine whether the CpG island of *PTGER2*, whose methylation status was inversely correlated with gene expression, possessed promoter activity. Unexpectedly, fragment 3, where methylation was observed in NB cell lines lacking expression of *PTGER2*, revealed little promoter activity, whereas upstream fragment 1 showed remarkable activity (Figure 2c), suggesting that methylation within Region 3 of the *PTGER2* CpG island may not directly inhibit a promoter activity for gene expression, but indirectly contribute to silencing of *PTGER2* through additional epigenetic mechanisms.

Chromatin immunoprecipitation assay

Since the results of our promoter assays suggested that epigenetic mechanisms other than DNA methylation might directly regulate transcription of *PTGER2*, we postulated that histone acetylation and/or methylation might determine expression of *PTGER2* (Wolffe and Matzke, 1999; Schubeler *et al.*, 2000; Jones and Baylin, 2002; Kondo *et al.*, 2003; Nakagawachi *et al.*, 2003; Lorincz *et al.*, 2004; Stirzaker *et al.*, 2004; Strunnikova *et al.*, 2005; Azuara *et al.*, 2006; Frigola *et al.*, 2006; Yamada *et al.*, 2006), and examined status of histone modification by means of chromatin immunoprecipitation (ChIP) assays using primers designed within the putative promoter region (Figure 2a). Acetylated histone H3- and H4-binding fragments were decreased in gene-nonexpressing CHP134, SJ-N-CG and GOTO cell lines, as compared with gene-expressing SK-N-AS and SH-SY5Y cell lines

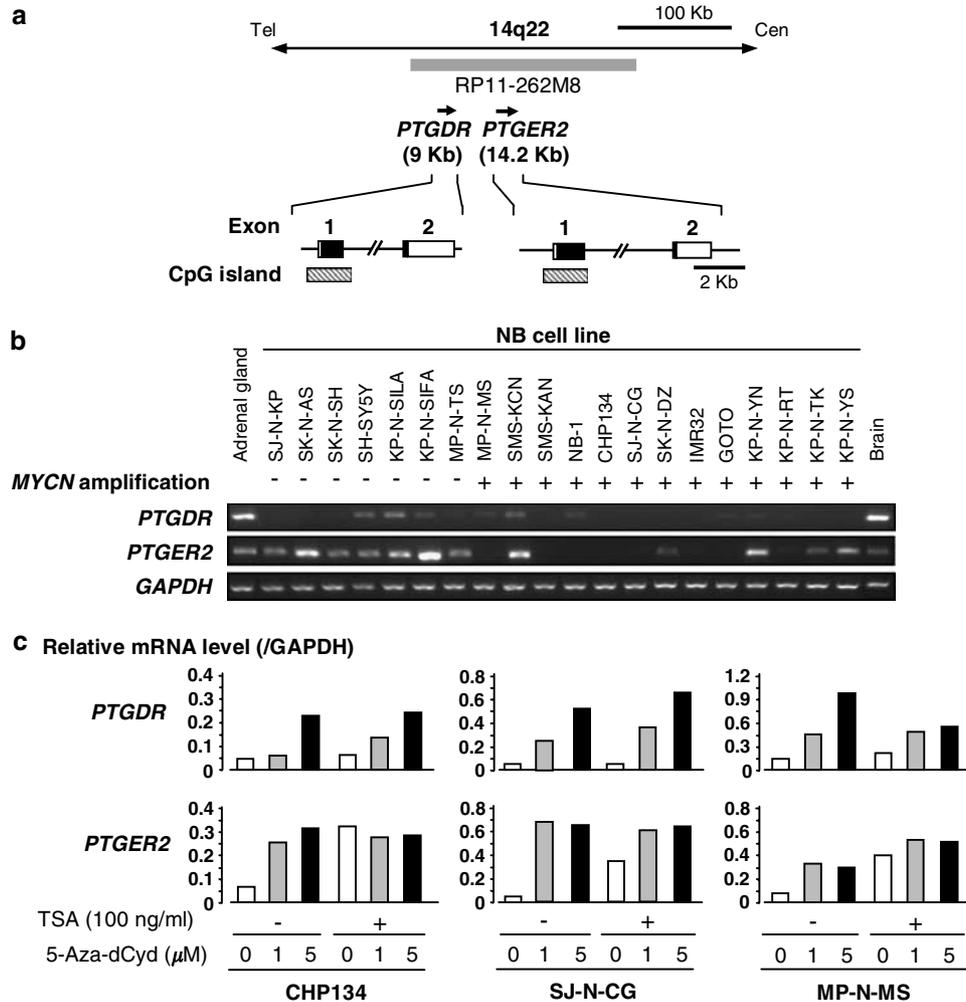


Figure 1 *PTGER2* and *PTGDR* are candidate targets for silencing in NB cells through DNA methylation. (a) Genomic structure of the *PTGDR* and *PTGER2* genes within the RP11-262M8 BAC clone, which was detected as one of BAC clones containing abnormally methylated sequences by BAMCA using two NB cell lines (GOTO and IMR32) as test samples and stage 1 NB tumors as control samples (Misawa *et al.*, 2005). Open and filled boxes represent untranslated and coding exonic sequences respectively; hatched boxes indicate 1479 and 1482 bp CpG islands that exist around exon 1 of each gene, respectively. (b) RT-PCR analysis of *PTGDR* and *PTGER2* mRNAs in normal adrenal gland, normal brain and NB cell lines with (+) or without (-) amplification of *MYCN* (Saito-Ohara *et al.*, 2003). Expression of *GAPDH* served as an internal control. (c) Representative results of RT-PCR analysis to reveal *PTGDR* and *PTGER2* mRNA expression in NB cell lines with (+) and without (-) treatment with 5-aza-dCyd and/or TSA, with *GAPDH* expression as an internal control. PCR products were electrophoresed in 3% agarose gel, and the band quantification was done with LAS-3000 (Fujifilm). Expression levels of *PTGDR* and *PTGER2* mRNA were normalized by that of *GAPDH* amplified at the same time. Experiments were repeated two times.

(Figure 2d). On the other hand, the di- and tri-methylated histone H3 lysine 9- (H3K9-) binding fragment was increased in the CHP134, SJ-N-CG and GOTO cell lines compared with SK-N-AS and SH-SY5Y cell lines (Figure 2d), suggesting that around the promoter region of *PTGER2*, histones H3 and H4 are hypoacetylated and histone H3K9 is di- and tri-methylated in NB cells lacking expression of this gene and those status of histone modification may be associated with DNA methylation within Region 3 of the *PTGER2* CpG island. This observation is consistent with the restoration of *PTGER2* expression by TSA and the observed synergistic effect of TSA together with 5-aza-dCyd (Figure 1c).

Methylation and expression of *PTGER2* in primary NB tumors

To determine whether aberrant methylation of *PTGER2* also takes place in primary NBs, we performed methylation analyses in a panel of surgical samples using bisulfite sequencing, COBRA and methylation-specific PCR (MSP). Several stage 4 tumors showed more or less aberrant methylation within Region 3 of the *PTGER2* CpG island (Figure 3a and Supplementary Figure S1), whereas the control normal adrenal gland or stage 1 and 4S tumors did not. Using primers designed within the Region 3 (Figure 3a) based on the localization pattern of methylation, we investigated the methylation status of the Region 3 within the *PTGER2*

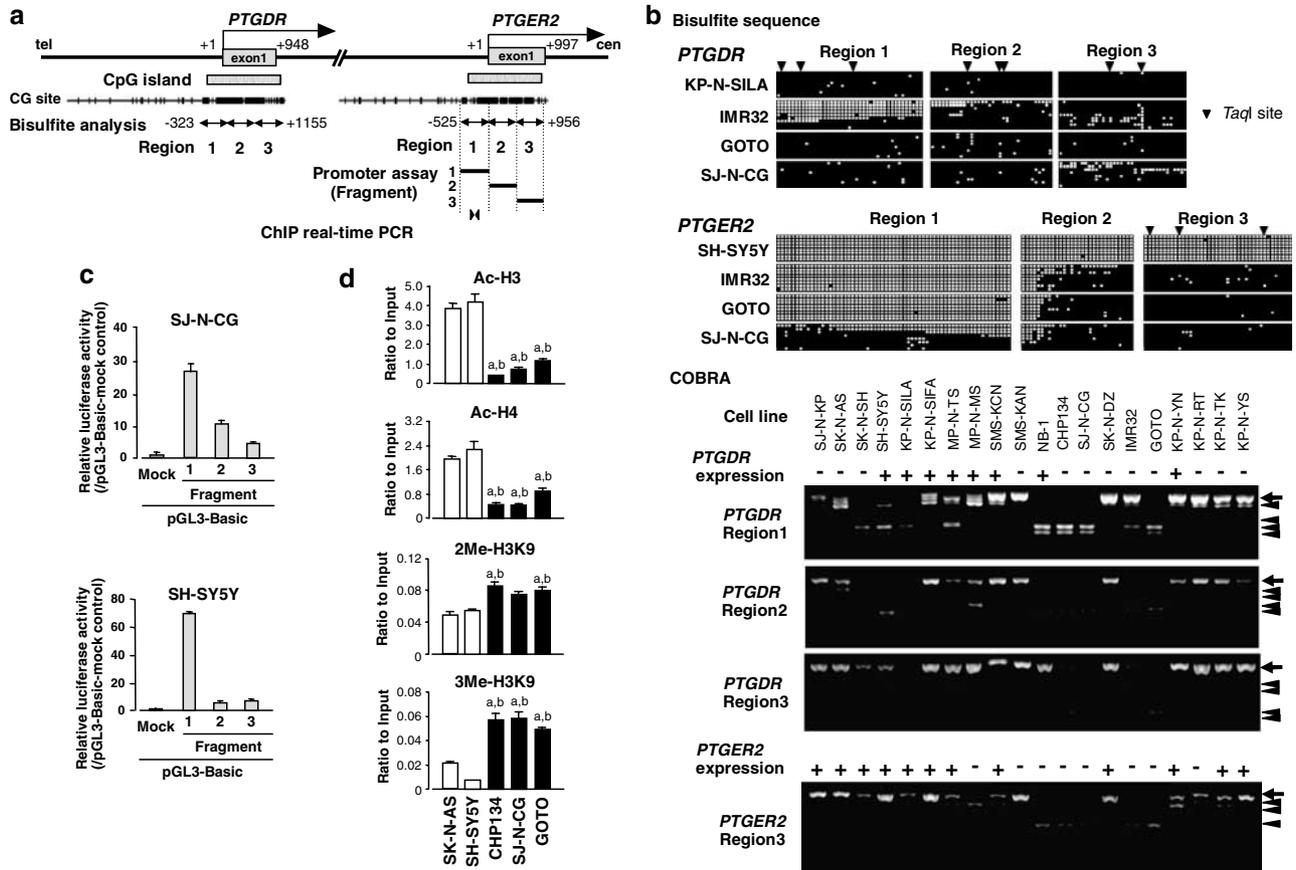


Figure 2 Association of DNA methylation and histone modification with expression status of *PTGER2*. (a) Map of the CpG islands (stippled bars) around the first exons of *PTGDR* and *PTGER2*. CpG sites are represented by vertical marks. Regions of each gene (Regions 1–3) examined for methylation by COBRA and bisulfite sequencing, are indicated by closed arrows. Fragments of *PTGER2* examined in promoter assays (Fragments 1–3) are indicated by horizontal bars. The region analysed by ChIP-PCR is indicated below the map. (b) Left: Results of bisulfite sequencing of the CpG islands of *PTGDR* or *PTGER2* in *PTGDR* and *PTGER2* nonexpressing NB cell lines (IMR32, GOTO and SJ-N-CG) and expressing cell lines (KP-N-SILA or SH-SY5Y). Open and filled squares represent unmethylated and methylated CpG sites, respectively, and each row represents a single clone. *TaqI* restriction sites are indicated by black arrowheads. Right: Results of COBRA experiments involving Region 1–3 of the CpG island of *PTGDR* and Region 3 of the CpG island of *PTGER2* in NB cell lines with (+) or without (–) expression of each gene. PCR products were restricted by *TaqI*. Arrows indicate unmethylated alleles; arrowheads, methylated alleles. (c) Promoter activity of the *PTGER2* CpG island. pGL3 basic empty vectors (mock) and reporter constructs, each containing one of three different sequences within CpG island (Fragments 1–3 in Figure 2a), were transfected into a *PTGER2*-expressing cell line (SH-SY5Y) and a nonexpressing cell line (SJ-N-CG). Luciferase activities were normalized versus an internal control. The data presented are the means \pm s.d. of three separate experiments, each performed in triplicate. (d) ChIP-real time PCR assay quantitatively showing the status of histone acetylation and methylation of the *PTGER2* promoter region in NB cells *in vivo*. ChIP was performed using antibodies against acetylated histone H3 (Ac-H3), acetylated histone H4 (Ac-H4), dimethylated histone H3-lysine 9 (2Me-H3K9) and trimethylated histone H3-lysine 9 (3Me-H3K9). Experiments were performed using crosslinked extracts from *PTGER2*-expressing cell lines SK-N-AS and SH-SY5Y (open bars) and from *PTGER2*-nonexpressing cell lines CHP134, SJ-N-CG and GOTO (closed bars); immunoprecipitated samples containing the *PTGER2* promoter region were amplified by a quantitative real-time PCR (Figure 2b). A portion of the sonicated chromatin before immunoprecipitation (input) was served as a positive control for normalization, and the relative ratio to input was calculated. Differences among multiple comparisons were analysed by one-way ANOVA with subsequent Scheffé’s tests: (a) versus SK-N-AS; (b) versus SH-SY5Y. All, $P < 0.05$.

CpG island in all 49 surgically resected primary NBs and two ganglioneuromas. Methylation of Region 3 was detected in 12 of the 49 tumors (24.5%, Figure 3a), but not in ganglioneuromas. Of those 12 cases, four were in stage 1, 2, 3 and 4S tumors, whereas eight cases were in stage 4 ($P = 0.0004$, Fisher’s exact test; Figure 3b). The results of the MSP experiments were consistent with those from bisulfite sequencing (Figure 2b). Notably, eight of the nine tumors (88.9%) with *MYCN* amplification showed methylation, whereas only four of 40 tumors (10%) without amplification

showed methylation ($P < 0.0001$, Fisher’s exact test; Figure 3b).

Expression levels of *PTGER2* mRNA in 39 primary NB tumors were evaluated by real-time quantitative RT-PCR. No significant difference was also observed between methylated and unmethylated NB cases as well as between *MYCN*-amplified and *MYCN*-unamplified NB cases ($P = 0.6932$ and 0.7003 , respectively, Student’s *t*-test; Figure 3c). Since we conjectured that nontumorous cells such as leukocytes and endothelial cells might disturb accurate evaluation of expression levels of

PTGER2 in primary NBs, we performed immunohistochemical analysis using PTGER2-specific antibody to evaluate expression patterns in more detail. Matured ganglion cells in ganglioneuroma (Figure 3d) and NB cells differentiating to ganglion-like cells, seen mainly in stage 1 tumors with good prognosis (Figure 3e), were strongly stained with PTGER2, while the undifferentiated small round cells filling stage 4 tumors were stained weakly or not at all (Figures 3f and g). Endothelial cells and infiltrating cells, such as lymphocytes and macrophages, were also strongly stained (Figures 3f and g), suggesting that evaluation of *PTGER2* mRNA expression in whole NB samples might be affected by contamination with those normal tissue components.

Suppression of NB cell growth after restoration of PTGER2 expression

To gain further insight into the potential role of *PTGER2* loss in NB carcinogenesis, we investigated whether restoration of the gene product would suppress growth of NB cells lacking endogenous PTGER2. We used two kinds of PTGER2-expression constructs, a Myc-tagged full coding sequence of *PTGER2* (pcDNA-*PTGER2*-Myc) and one without epitope tag (pcDNA-*PTGER2*), with a mock construct (pcDNA-mock) as a control. Two (SJ-N-CG) or three (GOTO) weeks after transfection and subsequent selection of drug-resistant colonies, the number of large colonies produced by *PTGER2*-transfected SJ-N-CG and GOTO cells decreased markedly compared to cells containing empty vector, regardless of the existence of myc-epitope in the C-terminus (Figure 4a and Supplementary Figure S2). After transient transfection, typical apoptotic changes, such as condensation or fragmentation of nuclear chromatin, were observed more frequently in PTGER2-Myc-positive NB cells compared with either PTGER2-Myc-negative cells or control GFP-Myc-transfected cells (Figure 4b). Furthermore, stably *PTGER2*-transfected NB cells established from the SJ-N-CG and GOTO cell lines (Supplementary Figure S3a), showed a lower growth rate possibly in a PTGER2-expression level-dependent manner compared to cells transfected with control empty vector alone (mock, Figure 4c and Supplementary Figure S3b).

Effects of PGE2, butaprost and 8-Bromo-cAMP (8-Br-cAMP) on growth of NB cells

Since PTGER2 mediates a part of PGE2 signaling (Narumiya *et al.*, 1999), we examined whether PGE2 would affect growth of NB cells expressing PTGER2, using stable *PTGER2* transfectants of SN-J-CG and their control counterparts (empty-vector clones). Since transfectants established from GOTO cells were not resistant to culture with serum concentrations lowered to $\leq 1\%$ to reduce the effect of native PGE2 in serum, only SN-J-CG transfectants were available for the experiment. In mock transfectants, as in the DLD-1 colon-cancer cell line in which butaprost promoted cell growth (Castellone *et al.*, 2005), treatment with PGE2

for 72 h induced an increase in cell growth compared to treatment with vehicle alone (Figure 5a). In cells stably expressing PTGER2, on the other hand, almost no increase in cell growth was observed after treatment with PGE2 compared with vehicle alone, suggesting that growth of NB cells might be accelerated by signaling mediated through receptors for PGE2 other than PTGER2, but inhibited by signaling through PTGER2.

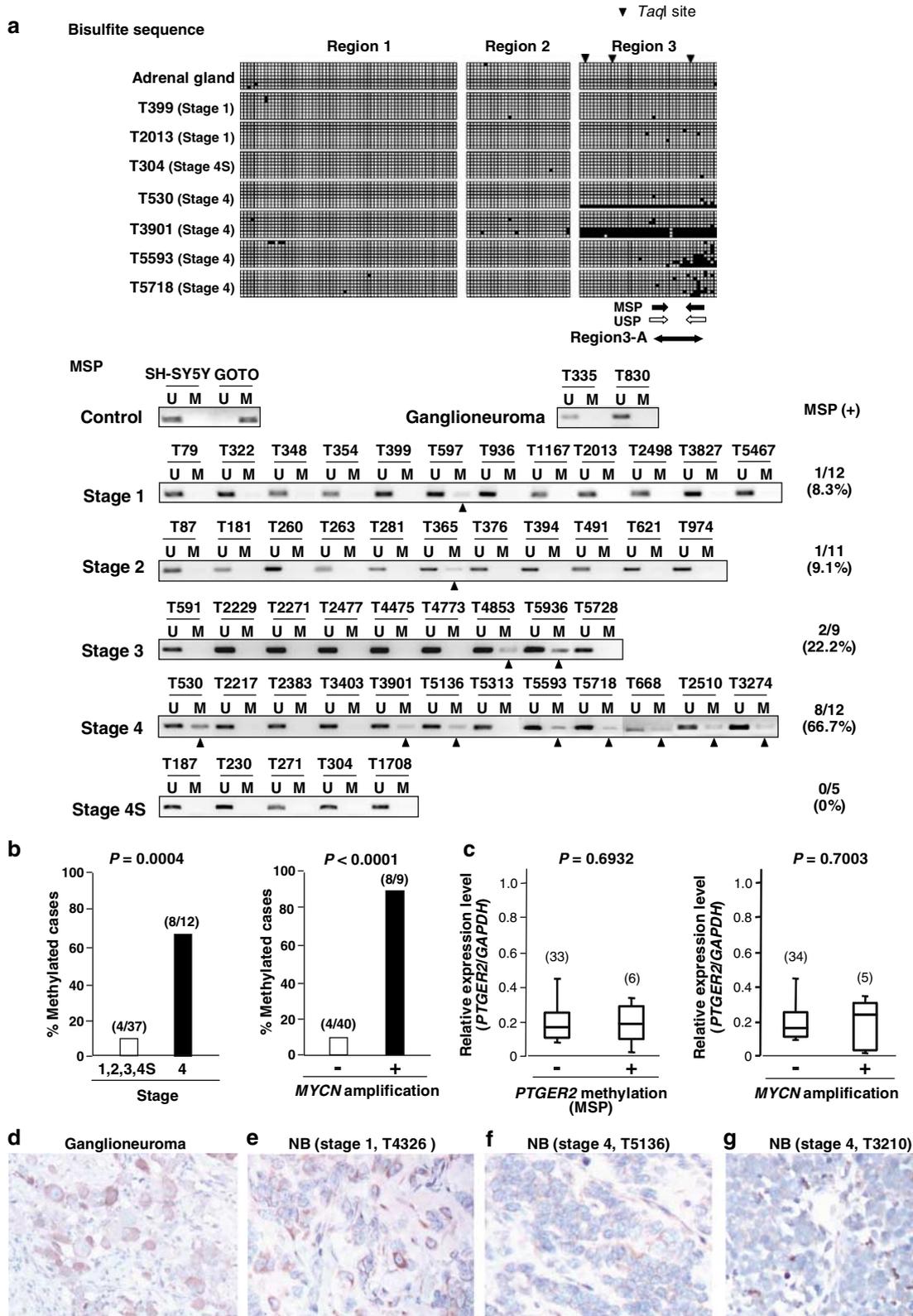
To confirm this hypothesis we examined growth of stable PTGER2 transfectants after treatment with a PTGER2-specific agonist, butaprost. The number of SJ-N-CG cells stably expressing PTGER2 was dramatically decreased after 72-h incubation with butaprost compared with vehicle alone, while the number of mock-transfected cells showed almost no change (Figure 5b), suggesting that signaling mediated by PTGER2, but not by other subtypes of PGE2 receptors, might specifically inhibit growth of NB cells. The same doses of butaprost promoted growth of the DLD-1 cell line. We performed fluorescence-activated cell sorting (FACS) analysis to analyse further the mechanism behind the antiproliferative effect of butaprost on NB cells. Butaprost treatment resulted in accumulation in the sub-G₁ phase of SJ-N-CG cells stably expressing PTGER2 (Figure 5c), suggesting that butaprost exerts its growth-inhibitory effect at least partly through induction of apoptosis mediated by PTGER2. Butaprost-induced apoptotic changes in SJ-N-CG cells stably expressing PTGER2 were also confirmed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay (Figure 5d). Under low-serum condition without butaprost treatment, stable PTGER2 transfectants resulted in an accumulation of cells in G₀-G₁ and a decrease in S and G₂/M phase cells compared with control counterparts (Figure 5c), suggesting that PTGER2 protein may arrest NB cells at the G₁-S checkpoint (G₀-G₁ arrest). However, no significant increase in the sub-G₁ phase was observed in stable PTGER2 transfectants compared with control counterparts (Figures 5c and d).

Since PTGER2 couples to G proteins and increases the intracellular concentration of cAMP (Narumiya *et al.*, 1999), we postulated that increased intracellular cAMP might play a role in inhibiting growth of NB cells. To test this hypothesis, we examined whether stimulation of PTGER2 would induce elevation of intracellular cAMP content in stable PTGER2 transfectants of the SJ-N-CG cell line. After treatment with butaprost, a distinct increase in cAMP was observed in these transfectants, whereas no increase in cAMP occurred in mock transfectants (Figure 5b). We further examined whether increasing intracellular cAMP using a cAMP analog, 8-Br-cAMP, would mimic the effect of butaprost on growth of NB cells. Growth of GOTO and SJ-N-CG cells, which lack expression of PTGER2, was inhibited by 8-Br-cAMP, whereas growth of SJ-N-KP and KP-N-SIFA, which express PTGER2, showed almost no change (Figure 6a). FACS analysis showed accumulation of GOTO and SJ-N-CG cells in the sub-G₁ phase after treatment with 8-Br-cAMP (Figure 6a).

Discussion

In a previous study (Misawa *et al.*, 2005), we identified the *PTGDR* and *PTGER2* genes as possible targets for

DNA methylation in advanced types of NB. In the work presented here, we have demonstrated that expression of *PTGER2* was often silenced in NB cell lines through epigenetic mechanisms such as DNA methylation and



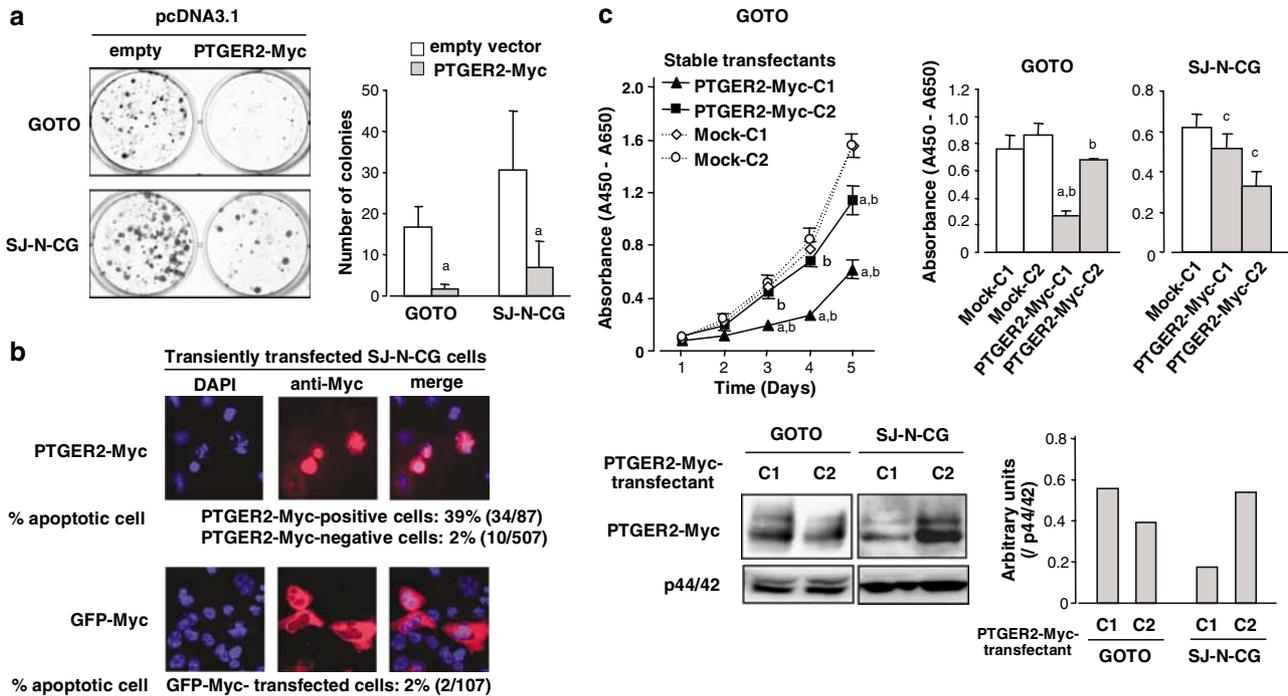


Figure 4 Effect of restoration of *PTGER2* expression on growth of NB cells. **(a)** Colony-formation assays using two NB cell lines without *PTGER2* expression (GOTO and SJ-N-CG). The cells were transiently transfected with a Myc-tagged construct containing *PTGER2* (pcDNA3.1-*PTGER2*-Myc), or empty vector (pcDNA3.1-empty) and selected for 2–3 weeks with appropriate concentrations of G418. Left: Two (SJ-N-CG) or three (GOTO) weeks after transfection and subsequent selection of drug-resistant colonies, the colonies formed by *PTGER2*-transfected cells were less numerous than those formed by empty vector-transfected cells. Right: Quantitative analysis of colony formation. Colonies larger than 2 mm were counted, and results are presented as means \pm s.d. of representative of three separate experiments, each performed in triplicate. Statistical analysis used the Mann–Whitney *U*-test: **(a)** $P < 0.05$ versus empty vector-transfected cells. **(b)** Fluorescent immunocytochemical analysis of SJ-N-CG cells transiently transfected with Myc-tagged *PTGER2* and stained with anti-Myc-tag antibody. Notably, cells with typical apoptotic changes, such as condensation or fragmentation of nuclear chromatin, were observed more frequently in *PTGER2*-Myc-positive NB cells compared with either *PTGER2*-Myc-negative cells or control GFP-Myc-transfected cells. **(c)** Effect of stable *PTGER2* expression on the growth of GOTO and SJ-N-CG cells. Viability was determined by WST assay at the indicated times. The data presented are the means \pm s.d. of three separate experiments. Statistical analysis used one-way ANOVA with subsequent Scheffé's tests: **(a)** *PTGER2*-transfected clone versus mock control clone C1 established from GOTO cells; **(b)** *PTGER2*-transfected clone versus mock control clone C2 established from GOTO cells; **(c)** *PTGER2*-transfected clone versus mock control clone C1 established from SJ-N-CG cells. All, $P < 0.05$. Upper left: Time course of growth of stably mock- (mock-C1 and C2) or *PTGER2*- (*PTGER2*-Myc-C1 and C2) transfected clones established from GOTO cells (Supplementary Figure S3a). Upper right: Comparison of the number of viable cells at day 4 among stable transfectants established from GOTO and SJ-N-CG cells. Lower: Expression level of transfected *PTGER2* protein in each stable transfectant was determined by western blotting using anti-Myc-tag antibody. The quantification determined by densitometer is provided.

Figure 3 Methylation and expression status of *PTGER2* in primary NB tumors. **(a)** Upper, Representative results of bisulfite sequencing of the CpG islands of *PTGER2* in primary NB tumors and the normal adrenal gland as a control. See legend for Figure 2b for interpretation. Black (MSP) and white (USP) arrows indicate the positions of primer sequences designed to amplify methylated and unmethylated alleles, respectively, in MSP experiments. Closed arrow (Region 3-A) indicates commonly methylated region in cell lines and primary tumors of NB (Supplementary Figure S1). Lower, Results of MSP experiments for 49 primary NB tumors and two ganglioneuromas. DNAs from SH-SY5Y and GOTO cell lines were used for unmethylated and methylated controls, respectively. Arrowheads indicate methylated alleles. **(b)** *PTGER2* methylation status of primary NB tumors, compared with tumor stage (left) and *MYCN* amplification status (right). Methylation status was determined by MSP. Left: The *PTGER2* CpG island was methylated in four of 37 stage 1, 2, 3 or 4S tumors (10.8%), while 8 of 12 (66.7%) stage 4 tumors showed methylated alleles ($P = 0.0004$, Fisher's exact test). Right: Methylation of the *PTGER2* CpG island in eight of the nine tumors (88.9%) with *MYCN* amplification; only four of 40 (10%) tumors without *MYCN* amplification show methylated alleles ($P < 0.0001$, Fisher's exact test). **(c)** Expression of *PTGER2* mRNA in 39 primary NB tumors, compared with methylation status of the *PTGER2* Region 3 (left) and *MYCN* amplification status (right). The levels of *PTGER2* mRNA expression were determined by real-time quantitative RT-PCR experiments. Median values are indicated with horizontal bars in the boxes. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles. The number of cases in each group is in parentheses. Statistical analysis used the Student's *t*-test. The number of cases in each group is in parentheses. **(d–g)** Representative immunohistochemical analysis of *PTGER2* protein using specific antibody. **(d)** Ganglioneuroma. Matured ganglion cells showed strong *PTGER2* immunoreactivity. **(e)** Stage 1 tumors of NB with good prognosis. NB cells differentiating to ganglion-like cells showed strongly *PTGER2* immunoreactivity, whereas undifferentiated small round cells showed weak *PTGER2* immunoreactivity. **(f and g)** Stage 4 NB tumors with *MYCN* amplification. Undifferentiated small round cells showed very weak or no *PTGER2* immunoreactivity. Note that nontumorous mesenchymal cells, including endothelial cells and infiltrating cells such as lymphocytes and macrophages, were strongly stained for *PTGER2*.

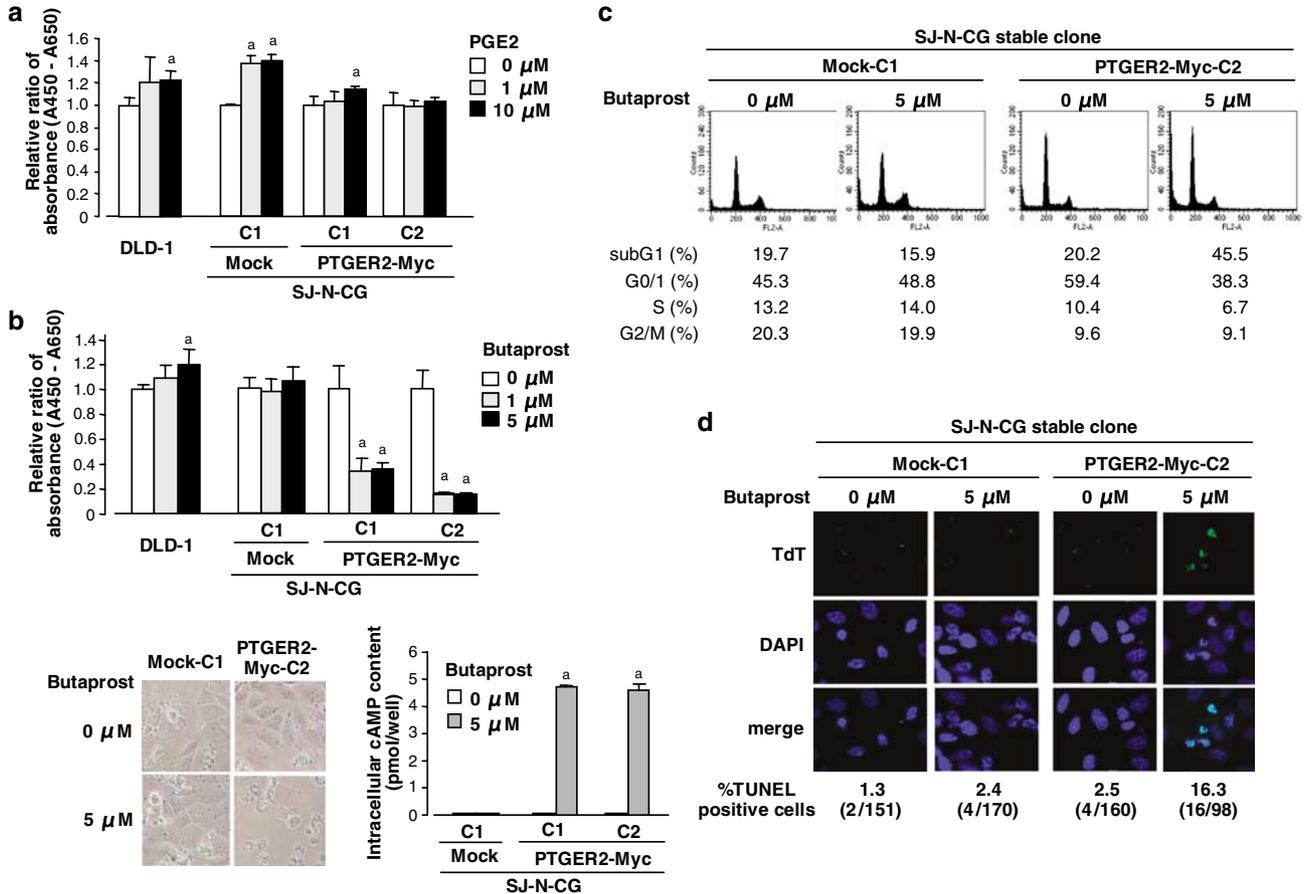


Figure 5 Inhibition of NB cell growth by PTGER2-mediated signaling. (a) Effect of PGE2 on growth of NB cells stably expressing PTGER2. After preculture for 24 h in medium containing 1% FBS, stable mock (mock-C1) or PTGER2 (PTGER2-Myc-C1 and C2) transfectants established from SJ-N-CG cells were treated with 0, 1, or 10 μM of PGE2 for 3 days and cell viability was determined by WST assay. The effect of PGE2 on cell growth was shown by absorbance of PGE2-treated cells relative to that of vehicle-treated cells (relative ratio). Statistical analysis used one-way ANOVA with subsequent Scheffé's tests: (a) $P < 0.05$ versus cells treated with vehicle alone. Note that DLD-1, a colorectal-cancer cell line whose growth is promoted by PGE2 through a PTGER2-mediated signaling pathway other than a cAMP-dependent one, showed an increase in cell numbers after treatment with PGE2 for 72 h. (b) Effect of a specific PTGER2 agonist, butaprost, on growth and levels of intracellular cAMP in NB cells stably expressing PTGER2. Left, after preculture for 24 h in medium containing 1% FBS, stable mock (mock-C1) or PTGER2 (PTGER2-Myc-C1 and C2) transfectants established from SJ-N-CG cells were treated with 0, 1, or 5 μM of butaprost for 3 days and cell viability was determined by WST assay. The effect of butaprost on cell growth is shown as a relative ratio, as described in (a). Statistical analysis used one-way ANOVA with subsequent Scheffé's tests: (a) $P < 0.05$ versus cells treated with vehicle alone. Note that DLD-1 showed an increase in cell numbers after treatment with butaprost for 72 h. Right: after 24-h preculture in medium containing 1% FBS, stable mock (mock-C1) or PTGER2 (PTGER2-Myc-C1 and C2) transfectants established from SJ-N-CG cells were treated with 0 or 5 μM of butaprost for 20 min. Levels of intracellular cAMP were determined by a cAMP EIA system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's protocol. Statistical analysis used the Mann-Whitney U -test; (a) $P < 0.05$ versus vehicle-treated control cells. (c) FACS analysis of PTGER2-stable transfectant (PTGER2-Myc-C2) and control clones (mock-C1). PTGER2-stable transfectants established from SJ-N-CG cells accumulated in G_0 - G_1 phase in comparison with control clones. Notably, an increase in the sub- G_1 fraction and a decrease in the G_2 /M fraction were observed in butaprost-treated PTGER2 transfectants. (d) Representative image of TUNEL staining in stable mock (mock-C1) and PTGER2 (PTGER2-Myc-C2) transfectants of SJ-N-CG with or without butaprost treatment. More TUNEL-positive cells were detected in stable PTGER2 transfectants treated with 5 μM of butaprost (16.3%) than in transfectants treated with vehicle alone (2.5%); the number of TUNEL-positive cells in mock-transfectant cultures was not affected by butaprost. Magnifications are $\times 400$.

histone modification, especially in *MYCN*-amplified cells. Methylation of *PTGER2* was also observed in advanced primary NB tumors, although its direct correlation with expression status in primary NBs remains unclear due to high expression levels of *PTGER2* in unaviable nontumor cells. Growth of NB cell lines lacking expression of *PTGER2* was inhibited by exogenous restoration of the gene product. Moreover, a *PTGER2*-specific agonist inhibited growth

of transfected NB cell lines that were stably expressing exogenous *PTGER2*, at least in part through production of cAMP as a second messenger in those cells.

PTGER2 locates in 14q22.1, a chromosomal region that is involved in loss of heterozygosity (LOH) or copy-number losses in 20–25% of NBs, although the smallest region of overlapping LOH at 14q is more distal (14q23-*qter*; Thompson *et al.*, 2001). Indeed, most of the cell lines we used in this study showed normal copy numbers

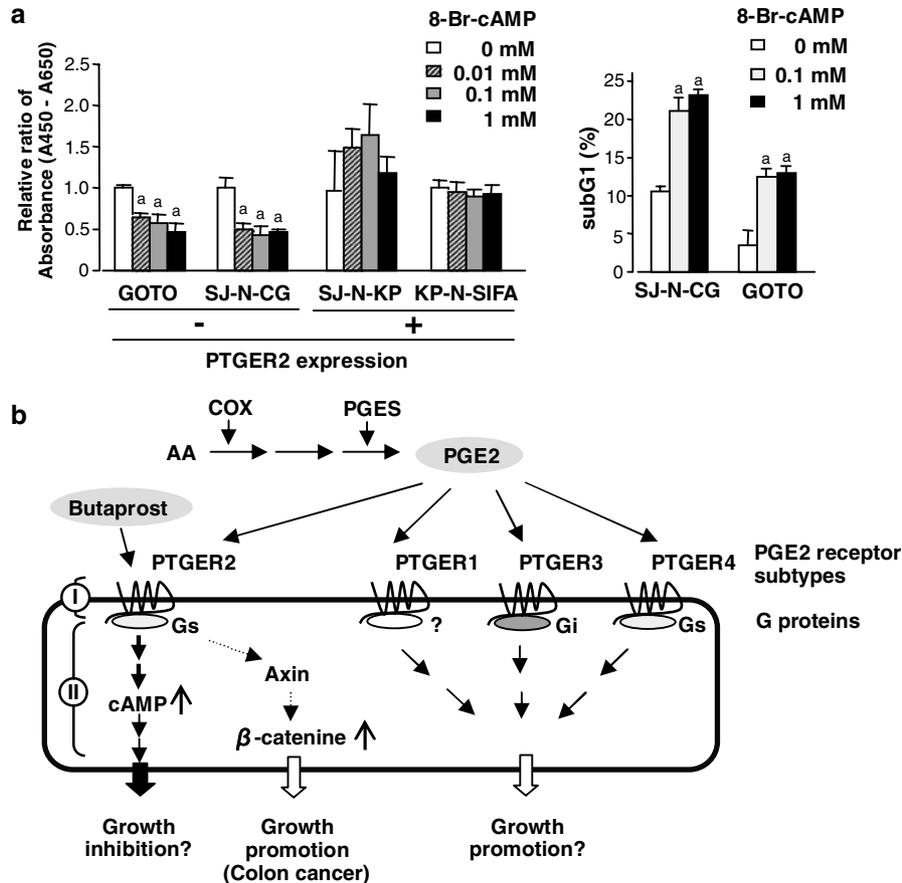


Figure 6 (a) Left: Effect of a cell-permeable cAMP analog, 8-Br-cAMP, on growth of NB cells. Wild-type GOTO and SJ-N-CG cells, which lack expression of *PTGER2*, and SJ-N-KP and KP-N-SIFA, which do express the gene, were cultured for 72 h with various concentrations of 8-Br-cAMP or no 8-Br-cAMP, and cell viability was determined by WST assay. The effect of 8-Br-cAMP on cell growth is shown as a relative ratio, as described for (a). Statistical analysis used one-way ANOVA with subsequent Scheffé's tests: (a) $P < 0.05$ versus cells treated with vehicle alone. Notably, 8-Br-cAMP inhibited cell growth in GOTO and SJ-N-CG cells, but not in SJ-N-KP and KP-N-SIFA. Right: An increase in sub-G₁ fraction was observed after treatment with 8-Br-cAMP in GOTO and SJ-N-CG cells. Wild-type GOTO and SJ-N-CG cells, which lack expression of *PTGER2*, were cultured with 0, 0.1, or 1.0 mM of 8-Br-cAMP for 72 h. The data presented are the means \pm s.d. of three separate experiments. Statistical analysis used one-way ANOVA with subsequent Scheffé's tests: (a) $P < 0.05$ versus 0 mM of 8-Br-cAMP treated cells. (b) Schematic representation of interactions among *PTGER2* signals and cell growth. COX, cyclo-oxygenase; PGES, prostaglandin E synthase. The *PTGER2* signaling pathway may be disrupted in NB cells through epigenetic silencing of *PTGRE2* (I). Alternatively or in addition, disruption of downstream signaling, including unresponsiveness to cAMP (II), may contribute to loss of the tumor-suppressive activity of normal PGE₂-*PTGER2* signaling.

at 14q22 in conventional (Saito-Ohara *et al.*, 2003) and BAC array-based (unpublished data) comparative genomic hybridization (CGH) experiments, suggesting that homozygous inactivation of *PTGER2* might occur through biallelic DNA methylation/histone modification in NB cells.

The putative 'core promoter region' of *PTGER2*, which showed high promoter activity in our reporter assay, was not methylated in some of the NB cell lines we analysed regardless of the gene's expression status, although aberrant DNA methylation in exonic region (Region 3) of *PTGER2* was inversely correlated with expression in NB cell lines. On the other hand, within the putative 'core promoter region' the acetylation status of H3 and H4 histones, and the di- and trimethylation status of H3K9, were positively and inversely correlated with *PTGER2* expression, respectively. In view of these observations, we speculate that

silencing of *PTGER2* in NB cells depends on integrated epigenetic events such as DNA methylation and histone modification. This phenomenon has been observed in other genes, including tumor-suppressor genes among a variety of solid tumors (Nakagawachi *et al.*, 2003; Stirzaker *et al.*, 2004; Strunnikova *et al.*, 2005; Frigola *et al.*, 2006; Yamada *et al.*, 2006).

Epigenetic mechanisms of tumor-suppressor-gene silencing in cancer remains incompletely understood (Jones and Baylin, 2002). It is believed that DNA sequences in entire CpG islands with promoter activity are methylated, H3 and H4 are unacetylated and H3K9 is methylated. However, it was also reported that hypermethylation of nonpromoter exonic sequences affects heterochromatinization and local gene silencing (Frigola *et al.*, 2006). In some cancer-associated gene silencing, moreover, sequences outside the promoter region may be methylated first (Jones and Baylin, 2002;

Nakagawachi *et al.*, 2003; Stirzaker *et al.*, 2004; Strunnikova *et al.*, 2005), and this methylation could induce histone modifications such as deacetylation of lysine residues by histone deacetylase and H3K9 di- and tri-methylation by histone methyltransferase (Kondo *et al.*, 2003; Stirzaker *et al.*, 2004; Strunnikova *et al.*, 2005), mediated by methyl-CpG-binding proteins. These processes lead to condensation of the chromatin, which propagates into a transcription factor-binding region, making target sequences inaccessible to transcription factors; finally gene transcription is shut off (Jones and Baylin, 2002; Nakagawachi *et al.*, 2003). Consequently, methylation outside the promoter region could lead gene silencing indirectly, although histone modification within the critical promoter region may contribute to silencing directly. This scenario is consistent with our results showing that TSA alone can partially restore expression of *PTGER2* and that TSA and 5-aza-dCyd exert a synergistic effect on restoring expression of this gene.

Using primary NB tumors, we showed that *PTGER2* methylation within Region 3 occurred more frequently in aggressive NBs such as stage 4 tumors than early-stage tumors, and in tumors with *MYCN* amplification than tumors without its amplification. Therefore the methylation status of this gene might be useful for predicting the aggressiveness of NB tumors, although our set of tumors was not suitable for survival analysis because few of the patients died during the follow-up period. Since *PTGER2* is expressed in nontumorous cells, such as endothelial cells, and also in infiltrating lymphocytes and macrophages, it will be difficult to evaluate correlation between silenced *PTGER2* expression and methylation status of NB cells in clinical samples. To clarify the clinical significance of the *PTGER2* gene in NB, further examination of methylation status, not expression status, of this gene in a larger set of primary cases of NB will be required.

One of the most striking findings in the study reported here is that restoration of *PTGER2* protein by either transient or stable transfection of expression constructs inhibited growth of NB cells that lacked endogenous *PTGER2*. Moreover, a *PTGER2*-specific agonist (butaprost) inhibited growth of NB cells stably expressing exogenous *PTGER2*, at least partly by inducing apoptosis. Those results suggested that negative regulation of cell growth, including induction of apoptosis, was a significant consequence of ectopic expression of *PTGER2* in NB cells lacking expression of this gene.

PGE2 is biosynthesized from arachidonic acid as a major cyclo-oxygenase product in a number of physiological settings, and produces a broad range of biologic actions in diverse tissues (Narumiya *et al.*, 1999). It was shown that cyclo-oxygenase-2 (COX-2) is expressed in NB, and COX-inhibiting nonsteroidal anti-inflammatory drugs inhibit growth of NB cells *in vitro* and *in vivo* (Johnsen *et al.*, 2004). In some tumors, including colon cancer, PGE2 has been implicated in promotion of tumor growth, promotion of angiogenesis, inhibition of apoptosis, stimulation of invasion, or suppression of immune responses (Hoshino *et al.*, 2003; Hata and

Breyer, 2004; Castellone *et al.*, 2005; Wang *et al.*, 2005a, 2006; Wang and DuBois, 2006). Those activities indicate that genes encoding receptors for PGE2 and/or its downstream targets can act as oncogenes. However, PGE2 can also act anti-proliferatively in some tumors, inducing apoptosis or differentiation (Santoro *et al.*, 1977; Fulton *et al.*, 1989; Fedyk *et al.*, 1996; Okuyama *et al.*, 2002), indicating that receptors for PGE2 and/or its downstream targets also can function as tumor suppressors.

Signaling of PGE2 is mediated by four receptors (PTGRE 1–4); the physiological/pathophysiological behavior of PGE2 appears to depend on the type of its receptors and on downstream pathways, accounting for completely opposite effects (Narumiya *et al.*, 1999). Although an antiapoptotic effect of *PTGER2* in gastric mucosal cells (Hoshino *et al.*, 2003) and a growth-promoting effect in colon-cancers via the *PTGER2*-G_s-axin- β -catenin signaling pathway (Castellone *et al.*, 2005) have been reported, several lines of evidence show that *PTGER2* also can negatively regulate cell growth and induce apoptosis and/or differentiation in various types of cells (Fedyk *et al.*, 1996; Suda *et al.*, 1996; Okuyama *et al.*, 2002). Thus, the role played by the PGE2-*PTGER2* signaling pathway may vary considerably from one neoplasm to another, probably in a tissue- or cell lineage-dependent manner. This hypothesis is consistent with our results demonstrating that butaprost promoted cell growth in colon-cancer cell line DLD-1, as expected on the basis of published data (Castellone *et al.*, 2005), but inhibited growth of NB cells stably expressing *PTGER2*. In NB, disruption of the *PTGER2*-mediated growth-suppressive pathway may contribute to tumor progression.

The question of how the cellular context is able to determine the action of PGE2-*PTGER2* signaling in NB cells is extremely interesting and deserves further investigation. PGE2 increases intracellular concentrations of cAMP through *PTGER2* and *PTGER4* and some splice variants of *PTGER3*, coupling to G proteins (Narumiya *et al.*, 1999). Among PGE2 receptors, *PTGER2* has the most potential for elevating intracellular cAMP concentrations (Regan, 2003). Since elevated levels of cAMP are associated with decreased proliferation and increased differentiation or apoptosis in several types of cells including glioblastoma cells (Chen *et al.*, 1998), esophageal squamous cell carcinomas (Wang *et al.*, 2005b), and hippocampal cells (Takadera *et al.*, 2004), we speculate that *PTGER2*-mediated production of intracellular cAMP may contribute to the growth-inhibitory effect of restored *PTGER2* in NB cells lacking endogenous expression of the gene. In our experiments a *PTGER2*-specific agonist increased intracellular cAMP levels and exerted growth inhibitory effects, at least partly by inducing apoptosis, in stable *PTGER2* transfectants established from the SJ-N-CG cell line, although G₀-G₁ arrest but not apoptosis was predominantly observed in *PTGER2* transfectants under low-serum condition without butaprost treatment. A similar effect of butaprost in hippocampal cells has been reported (Takadera *et al.*,

2004), even though those cells were nontumorous. The fact that a cAMP analog, 8-Br-cAMP, which directly increases intracellular cAMP, inhibited cell growth and induced apoptosis in NB cells lacking endogenous PTGER2, but not in those expressing PTGER2, suggested that NB cells may lose responsiveness to PTGER2-mediated growth inhibition/apoptosis through epigenetic silencing of PTGER2 (I in Figure 6b) or by disruption of a downstream cAMP-dependent signaling pathway (II in Figure 6b). Further examination will be needed to clarify the biological and clinical significance of the PGE2-PTGER2-cAMP signaling pathway in the pathogenesis of NB, especially with regard to the resistance of advanced NB tumors to apoptosis (del Carmen Mejia *et al.*, 2002).

Materials and methods

Cell culture and primary tissue samples

All 20 human NB cell lines we used (SJ-N-KP, SK-N-AS, SK-N-SH, SH-SY5Y, KP-N-SILA, KP-N-SIFA, MP-N-TS, MP-N-MS, SMS-KCN, SMS-KAN, NB-1, CHP134, SJ-N-CG, SK-N-DZ, IMR32, GOTO, KP-N-YN, KP-N-RT, KP-N-TK, KP-N-YS) had been established from surgically resected tumors (Saito-Ohara *et al.*, 2003). The DLD-1 colon-cancer cell line was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). Cells were maintained in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 µg/ml streptomycin. NB cells were treated with or without 1 or 5 µM of 5-aza-dCyd for 5 days, and/or 100 ng/ml of TSA for the last 12 h.

Primary tumor samples were obtained at surgery from 49 patients with NB and two with ganglioneuroma who underwent tumor resection at University Hospital, Kyoto Prefectural University of Medicine from 1986 to 2003, with written consent from the parents of each patient in the formal style and after approval by the local ethics committees. Staging of each NB case was determined according to the criteria of the International Neuroblastoma Staging System (Brodeur *et al.*, 1993). Of the 49 NB patients, 41 (83.7%) were infants less than 18 months of age at diagnosis, 36 (73.5%) were detected by a mass-screening program, 12 were classified as stage 1, 11 as stage 2, nine as stage 3, 12 as stage 4 and five as stage 4S; *MYCN* amplification was detected in the tumors of nine (18.4%) patients. Genomic DNA was available for analysis from all 49 NB and two ganglioneuroma samples; total RNA was available from 39 NB samples.

Reagents and plasmids

PGE2, butaprost and 8-Br-cAMP were obtained respectively from Calbiochem (San Diego, CA, USA), Cayman Chemicals (Ann Arbor, MI, USA) and Sigma-Aldrich (St Louis, MO, USA). Anti-PTGER2 polyclonal antibody was from Cayman Chemicals. Anti-acetylated histone H3 (anti-AcH3), anti-acetylated histone H4 (anti-AcH4), anti-dimethylated histone H3 lysine 9 (anti-2Me-H3K9) and anti-trimethylated histone H3 lysine 9 (anti-3Me-H3K9) antibodies were from Upstate (Lake Placid, NY, USA); anti-Myc-tag and anti-p44/42 antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Plasmids expressing PTGER2 alone (pcDNA3.1-PTGER2) C-terminally Myc-tagged PTGER2 (pcDNA3.1-PTGER2-Myc) were prepared by cloning the RT-PCR product of the full coding sequence of PTGER2 alone, or in-frame along with

the Myc-epitope in the C terminus, respectively, into the vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). The empty vector pcDNA3.1 or plasmid expressing Myc-tagged GFP (pcDNA3.1-GFP-Myc) was used as a control.

RT-PCR and real-time quantitative RT-PCR

Single-stranded cDNAs generated from total RNAs were amplified with primers specific for each gene (Supplementary Table S1). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was amplified at the same time to allow estimation of the efficiency of cDNA synthesis. RT-PCR products were electrophoresed, and quantified with LAS-3000 (Fujifilm, Tokyo, Japan). Levels of mRNA expression in primary tumors were measured using a quantitative real-time fluorescence detection method (PRISM 7900HT, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The expression of PTGER2 mRNA in each sample was normalized on the basis of the respective *GAPDH* content and recorded as a relative expression level. PCR amplification was performed in duplicate for each sample.

Methylation analysis

Genomic DNAs were treated with sodium bisulfite, and subjected to PCR using primer sets designed to amplify regions of interest (Supplementary Table S1). For COBRA, PCR products were digested with *TaqI*, and electrophoresed (Xiong and Laird, 1997). For bisulfite sequencing, the PCR products were subcloned and then sequenced.

For MSP, sodium bisulfite-treated DNA was amplified using primers specific to the methylated and unmethylated forms of DNA sequences (Supplementary Table S1). DNAs from cell lines recognized as unmethylated or highly methylated by bisulfite sequencing were used as controls.

Promoter assay

DNA fragments around the CpG-island predicted by the CpGPLOT program (<http://www.ebi.ac.uk/emboss/cpgplot/>) were ligated into the vector pGL3-Basic (Promega, Madison, WI, USA). Promoter assay using each construct or control vector with an internal control vector (pRL-hTK, Promega) was performed as described elsewhere (Misawa *et al.*, 2005).

ChIP assay

ChIP assays were performed as described previously (Sonoda *et al.*, 2004). Chromatin was immunoprecipitated with anti-AcH3, anti-AcH4, anti-2Me-H3K9, anti-3Me-H3K9, or no antibody, after which a quantitative real-time PCR was performed with 1/30 of the immunoprecipitated DNA, using primers designed to amplify regions of interest (Supplementary Table S1); 1/600 of the solution before adding antibody was amplified as an internal control for the amount of DNA.

Immunohistochemistry

Formalin-fixed, paraffin-embedded surgical specimens were sliced into 5 µm-thick sections, deparaffinized, then immersed for 30 min in methanol containing 0.3% hydrogen peroxide. After retrieval of epitope by boiling, the sections were incubated with anti-PTGER2 antibody (1:500 dilution) and then with a biotinylated secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA). After staining with Vectastain ABC reagent (Vector Laboratories), the sections were immersed in 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% hydrogen peroxide, and counterstained with hematoxylin.

Transient transfection and colony-formation assays

pcDNA3.1-*PTGER2*, pcDNA3.1-*PTGER2*-Myc or the empty-vector (pcDNA3.1-mock) were transiently transfected into SJ-N-CG and GOTO cells using FuGENE6 (Roche Diagnostics, Tokyo, Japan) for colony-formation assays. Expression of *PTGER2* protein in transfected cells was confirmed by western blotting, using anti-Myc-Tag or anti-*PTGER2* antibody as described elsewhere (Misawa *et al.*, 2005). After 2–3 weeks of incubation with G418 (600 and 250 $\mu\text{g}/\text{ml}$ for SJ-N-CG and GOTO, respectively), cells were stained with crystal violet.

Immunofluorescent staining

Transiently or stably transfected cells seeded into slide chambers were fixed in cold methanol for 3 min. The cells were covered with blocking solution (1% skim milk in phosphate-buffered saline) for 30 min, and incubated overnight at 4°C with anti-Myc-tag antibody (1:200 dilution) in blocking solution. There followed 1 h of incubation with Alexa 594-conjugated goat anti-mouse IgG (1:500 dilution; Molecular Probes, Eugene, OR, USA). The cells were counterstained with 4',6-diamidino-2-phenylindole, and viewed with an ECLIPSE E800 fluorescence microscope (Nikon, Tokyo, Japan).

Establishment of stable transfectants and cell-growth assay

Stable transfectants of *PTGER2* and mock vector were obtained by transfecting pcDNA3.1-*PTGER2*-Myc, and pcDNA3.1-mock, respectively, into SJ-N-CG and GOTO cells, and selected by G418. For measurements of cell growth, 2×10^3 cells were seeded in 96-well plates. To determine the effects of PGE2, butaprost, or 8-Br-cAMP on growth of NB cells, wild type cell lines or stable transfectants of SJ-N-CG cells were treated with various concentrations of each reagent for 72 h, after 24-h preculture in media containing 1% FBS (for PGE2 and butaprost) or 10% FBS (for 8-Br-cAMP). The numbers of viable cells were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay as described elsewhere (Misawa *et al.*, 2005). The DLD-1 cell line was served as a

positive control for the PGE2 and butaprost experiments (Castellone *et al.*, 2005).

Enzyme immunoassay

After 24-h pre-culture in medium containing 1% FBS, stable transfectants of SJ-N-CG cells were treated with 0 or 5 μM of butaprost for 20 min. Intracellular cAMP was measured by means of a cAMP Enzyme immunoassay (EIA) system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's protocol.

Flow cytometry

Stable transfectants of SJ-N-CG cells were treated with butaprost (5 μM) or 8-Br-cAMP (0.1 or 1 mM) for 72 h. For FACS analysis, harvested cells were fixed in 70% cold ethanol before treatment with RNaseA and propidium iodide. Samples were analysed on a FACSCalibur HG (Becton-Dickinson, San Jose, CA, USA). Data were analysed using BD CellQuest Pro (Becton-Dickinson).

TUNEL stain

Wild-type or stable transfectants of SJ-N-CG cells were treated with or without butaprost or 8-Br-cAMP, and apoptosis was detected by enzymatic labeling of DNA strand-breaks using a TUNEL staining kit (MEBSTAIN Apoptosis Kit Direct; MBL, Aichi, Japan) according to the manufacturer's protocol.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan; by a Grant-in-Aid from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation (JST); and by a 21st Century Center of Excellence (COE) Program for Molecular Destruction and Reconstitution of Tooth and Bone.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).