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Role of Phosphodiesterase2A in Proliferation and Migration of Human Osteosarcoma Cells

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Abstract. *Background/Aim: The prognosis of patients with osteosarcoma is poor; therefore, new treatment strategies are urgently needed. Phosphodiesterase 2 (PDE2) is one of the 11 families (PDE1–PDE11) of the phosphodiesterase superfamily that regulates the intracellular concentrations and effects of cAMP and cGMP. This in vitro study was performed to investigate the role of PDE2 in human oral osteosarcoma HOSM-1 cells. Materials and Methods: PDE2 expression was measured by a cAMP-PDE assay and real-time-PCR. The effects of the PDE2-specific inhibitors, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), 8-bromo-cAMP, and 8-bromo-cGMP on cell proliferation and migration were assessed. Results: PDE2 activity and PDE2A mRNA expression were detected in HOSM-1 cells. Cell proliferation was inhibited by EHNA and 8-bromo-cAMP but not by 8-bromo-cGMP. Cell migration was stimulated by EHNA and 8-bromo-cGMP, but it was inhibited by 8-bromo-cAMP. Conclusion: Cell proliferation is regulated by PDE2-cAMP signaling and cell migration is regulated by PDE2-cGMP signaling in HOSM-1 cells.*

Strategies for managing osteosarcoma including diagnosis, surgery, chemotherapy, radiotherapy, recurrence treatment, palliative care, and survivorship quality are inadequate. Overall outcomes have only been slightly improved and thus new treatments are needed (1).

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cyclic AMP (cAMP) and/or cyclic GMP (cGMP) to regulate their intracellular concentrations. These second messengers are important components of intracellular signaling pathways that regulate many critical physiological processes, including cell proliferation, cell cycle, and cell migration. The PDE superfamily includes 11 gene families (PDE1–PDE11), which differ in their biochemical properties, regulation, and sensitivity to pharmacological agents. PDE inhibitors affect many pathological conditions, but their use as anticancer agents has not been widely examined (2-5).

PDE2 is a member of the PDE superfamily and is encoded by a single gene, *PDE2A*. Although PDE2 hydrolyzes both cGMP and cAMP, PDE2 remarkedly increases cAMP hydrolysis in the presence of cGMP. Therefore, PDE2 is named as cGMP-stimulated cAMP PDE and can provide crosstalk between the cAMP and cGMP signaling pathways (2-5). PDE2 is widely expressed in human tissues, including the adrenal gland, heart, lung, liver, platelets, and endothelial cells (4, 5). In some tumor cells, PDE2 activity was also detected in malignant tumor cells including human mammary carcinoma MCF-7 cells (6). PDE2A mRNA expression has been confirmed in human osteosarcoma MG-63 cells (7). Furthermore, we found that PDE2 regulated cell proliferation, but not migration, of human malignant melanoma PMP cells (8). However, the role of PDE2 in human osteosarcoma cells remains unclear. Thus, this study was conducted to investigate the role of PDE2 in the proliferation and migration of human oral osteosarcoma HOSM-1 cells.

Materials and Methods

Cells and reagents. Human oral osteosarcoma HOSM-1 cells (9) were established in our laboratory. The cells were cultured and maintained at 37˚C and 5% CO_2 in a humidified atmosphere in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Paris, France). Total RNA from human testes was purchased from Agilent Technologies (Santa Clara, CA, USA) and used as a PDE2A-positive control. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was purchased from Cayman Chemical (Ann Arbor, MI, USA). 8-Bromoadenosine 3',5'-cyclic

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Key Words: Phosphodiesterase, phosphodiesterase2A, proliferation, migration, osteosarcoma.

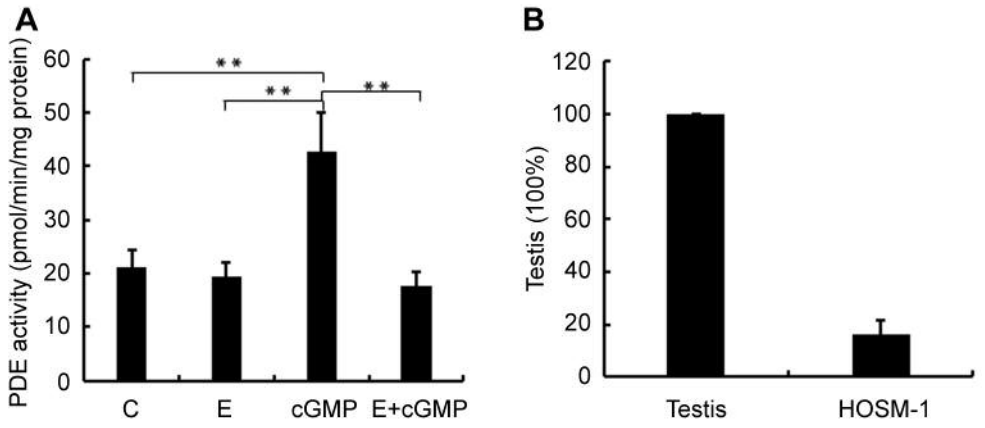

Figure 1. *Identification of phosphodiesterase 2 (PDE2) in HOSM-1 cells. (A) PDE activities were analyzed by cAMP PDE activity assay using the PDE2-specific inhibitor EHNA and/or PDE2 stimulator cGMP. The concentrations of EHNA and cGMP were 20 μM and 10 μM. Experiments were performed independently three times. **p<0.01 indicates a significant difference between the two groups. C: Control; E: EHNA. (B) Expression of PDE2A mRNAs in HOSM-1 cells. Real-time PCR analysis of PDE2A mRNAs was performed. Human testes were used as a positive control (PC) for PDE2A mRNAs. Experiments were performed independently three times.*

monophosphate sodium salt (8-bromo-cAMP) and 8-bromoguanosine 3',5'-cyclic monophosphate sodium salt (8-bromo-cGMP) were from Merck Millipore (Billerica, MA, USA).

cAMP PDE activity assays. HOSM-1 cells were seeded at 1×10^6 cells/25-cm^2 flask. After 3 days, the cells were washed twice with ice-cold PBS, harvested with a rubber policeman, and homogenized in 1 mL of ice-cold homogenization buffer [100 mM TES (pH 7.4), 10 μg/ml each of pepstatin, leupeptin, and aprotinin, 1 mM benzamidine, 0.5 mM pefabloc, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgSO_4$, 10% glycerol]. cAMP PDE activity was evaluated as previously described with some modifications (3). Samples were incubated at 30˚C for 10 min in a total volume of 0.3 ml containing 50 mM Hepes (pH 7.4), 0.1 mM EGTA, 8.3 mM $MgCl_2$, and 0.1 μM (3H) cAMP (18,000 cpm) with or without the specific PDE2 inhibitor EHNA and/or cGMP (which stimulates PDE2). All experiments were performed independently three times.

Quantitative real-time PCR. HOSM-1 cells were seeded at 1×10^5 cells/6-cm plate. After 3 days, the cells were washed twice with PBS, and total RNA was isolated from the cells using the QuickGene RNA cultured cell kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). cDNA synthesis was performed using 2 μg of total RNA with the High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The expression of PDE2A mRNA was quantified by real-time quantitative PCR (7300 real-time PCR system, Thermo Fisher Scientific). Experiments were performed using TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and TaqMan® Gene Expression Assays (Thermo Fisher Scientific). The expression of 18S rRNA was used as an internal control. All experiments were performed independently three times.

Cell proliferation assays. Cell proliferation was assessed using the TACS® MTT Cell Proliferation Assay kit (Trevigen, Gaithersburg, MD, USA). Briefly, the cells were plated at 1,000 cells per well in 96-well plates in RPMI 1640 medium containing 10% FBS. After 24 h of plating, the medium was replaced with the same medium containing drugs. After 72 h, the cell number was determined using the assay kit according to the manufacturer's instructions. The absorbance of each sample was measured at 570 nm using a microplate reader (Promega Corporation, Madison, WI, USA), which was then used to determine the relative cell count in each well. All experiments were performed independently three times.

Cytotoxicity assay. Cytotoxicity was assessed using the CellTox™ Green Cytotoxicity Assay (Promega Corporation). Briefly, the cells were seeded into 96-well black plates (Thermo Fisher Scientific) at a density of 1000 cells/well in RPMI medium containing 10% FBS; 24 h after plating, the medium was replaced with the same medium containing each reagent: EHNA; 8-bromo-cAMP; or 8-bromo-cGMP. Following 72 h incubation, dead cells were detected using an assay kit according to the manufacturer's instructions. The fluorescence of each sample was measured at an excitation wavelength of 485 nm and emission of 538 nm using a absorption spectrophotometer (Thermo Fisher Scientific). All experiments were performed independently three times.

Cell cycle analysis by flow cytometry. Cell cycle progression was assessed using the CycleTEST™ Plus DNA Reagent kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, the cells were plated at 1.2×10^5 cells /10-cm plate (Corning Incorporated, Oneonta, NY, USA) in RPMI 1640 medium containing 10% FBS; 24 h after plating, the medium was replaced with the same medium containing EHNA. After 72 h, the cells were pretreated as described by the manufacturer, and the cells were analyzed by flow cytometry. All experiments were performed independently three times.

In vitro migration assays. HOSM-1 cells (2×10^4 cells) in RPMI 1640 medium containing 0.1% FBS were transferred onto 8-μm pore inserts (BD Biosciences). The inserts were placed in companion wells containing RPMI 1640 medium supplemented

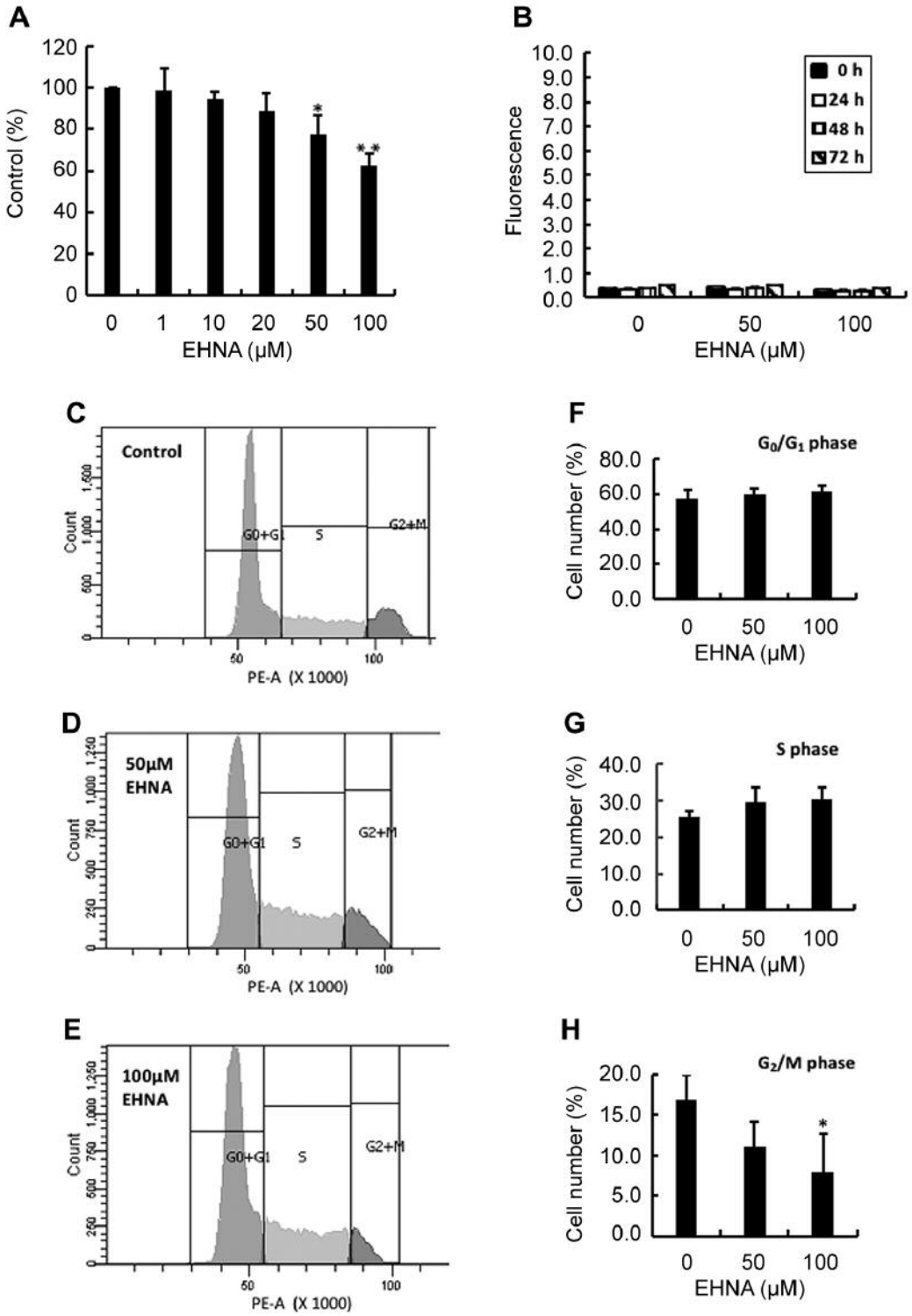

Figure 2. *Effect of phosphodiesterase 2 (PDE2)-specific inhibitor EHNA on proliferation, cytotoxicity, and cycle of HOSM-1 cells. (A) Effect of EHNA on proliferation of HOSM-1 cells. The cells were exposed to increasing concentrations of EHNA for 72 h, and the effects on cell proliferation were measured by MTT assay. (B) The toxic effect of EHNA on HOSM-1 cells. The cells were exposed to EHNA and fluorescence was measured every 24 h. (C-E) The effect of EHNA on cell cycle progression. Cell cycle progression was detected by flow cytometry as described in Materials and Methods. Histograms of the cell count after 3 days of EHNA treatment. (F-H) Data are expressed as the percentage of cells in each phase. *p<0.05 and **p<0.01 indicated a significant difference compared to the control. All experiments were performed independently three times.*

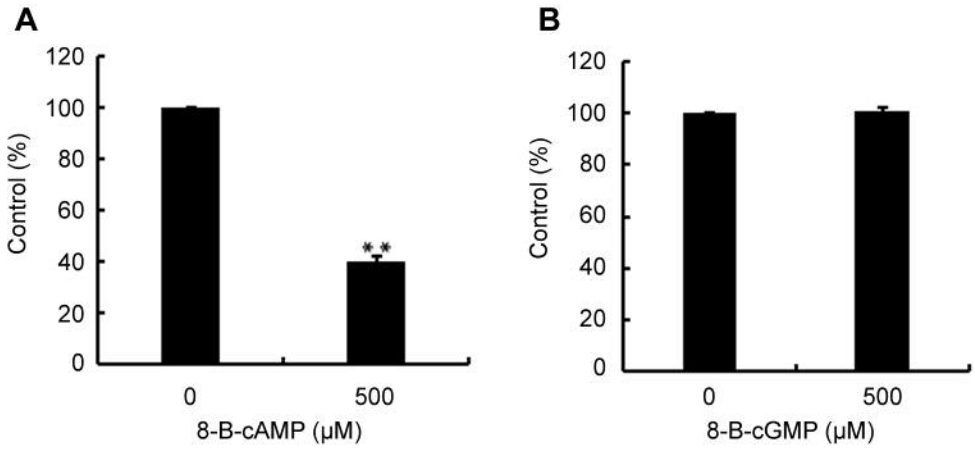

Figure 3. *Effect of 8-bromo-cAMP and 8-bromo-cGMP on proliferation of HOSM-1 cells. (A) Effect of 8-bromo-cAMP on proliferation of HOSM-1 cells. The cells were exposed to 8-bromo-cAMP for 72 h, and the effects on cell proliferation were measured by the MTT assay. **p<0.01. (B) Effect of 8-bromo-cGMP on the proliferation of HOSM-1 cells. The cells were exposed to 8-bromo-cGMP for 72 h, and the effects on cell proliferation were measured by the MTT assay. All experiments were performed independently three times.*

with 10% FBS as a chemoattractant. Following 20 h incubation, the inserts were removed, and nonmigrating cells on the upper surface were removed with a cotton swab. Cells on the lower surface of the membrane were fixed and stained with Diff-Quik™ (Sysmex, Kobe, Japan) and counted under a microscope. All experiments were performed independently three times.

Statistical analysis. All experiments were performed independently three times. Statistical analysis was performed using student's unpaired *t*-test or one-way analysis of variance followed by Tukey's multiple comparison test. Statistical significance was defined by a *p*-value of less than 0.05.

Results

Identification of PDE2 in HOSM-1 cells. Because it is unclear whether PDE2 is expressed in HOSM-1 cells, we examined PDE2 activity and PDE2 mRNA expression. Total cAMP PDE activity in HOSM-1 cell homogenates was inhibited by approximately 10% by the PDE2-specific inhibitor EHNA and stimulated approximately two-fold by cGMP. This cGMP-stimulated PDE activity was completely suppressed by EHNA, indicating the presence of PDE2 activity (Figure 1A). Furthermore, real-time PCR was performed to evaluate the expression of PDE2A (Figure 1B).

Effect of EHNA on cell proliferation. Because the effect of PDE2 on cell proliferation in human osteosarcoma was unclear, the effects of different concentrations of EHNA on cell proliferation were assessed by the MTT assay. EHNA (50 μM) significantly inhibited cell proliferation (Figure 2A). *Effect of EHNA on cell toxicity*. Incubation of cells with EHNA reduced the number of cells; however, it could not be determined whether the cells were dead or whether their proliferative activity was suppressed. Therefore, cell toxicity was evaluated. Following 72 h incubation, the number of dead cells did not change (Figure 2B).

Effect of EHNA on the cell cycle. Next, we predicted that the effect of EHNA on the cell number was due to the inhibition of their proliferation. Thus, we examined the effect of EHNA on the cell cycle. EHNA treatment for 3 days did not alter the proportion of HOSM-1 cells in the G_0/G_1 phase and S phase, but decreased the number of cells in G_2/M phase (Figure 2C–H).

Effects of 8-bromo-cAMP and 8-bromo-cGMP on cell proliferation. Because PDE2 degrades both cAMP and cGMP, we next examined the effect of their derivatives 8-bromo-cAMP and 8-bromo-cGMP on cell proliferation. 8-Bromo-cAMP suppressed cell proliferation (Figure 3A). However, 8-bromo-cGMP did not significantly affect cell proliferation (Figure 3B). These results suggest that PDE2 regulates the cell cycle through PDE2-cAMP signaling.

Effects of EHNA, 8-bromo-cAMP, and 8-bromo-cGMP on cell migration. Because the role of PDE2 in cell migration of human osteosarcoma was unclear, the effects of different concentrations of EHNA on cell migration were assessed in a migration assay. EHNA stimulated migration in dose-dependent manner (Figure 4A). Because PDE2 degrades both cAMP and cGMP, we examined the effect of their derivatives 8-bromo-cAMP and 8-bromo-cGMP on cell migration. 8-

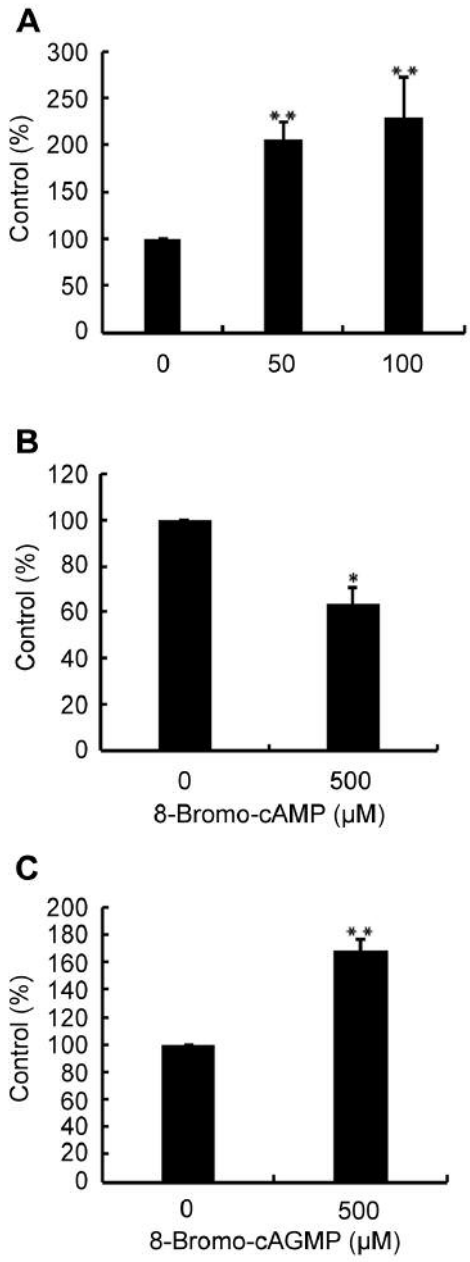

Figure 4. *Effect of EHNA, 8-bromo-cAMP, and 8-bromo-cGMP on migration of HOSM-1 cells. The cells were transferred to inserts for migration assays after treatment with each reagent. The cells were stained and counted as described in the Materials and Methods. (A) Effect of EHNA on cell migration. (B) Effect of 8-bromo-cAMP on cell migration. (C) Effect of 8-bromo-cGMP on cell migration. All experiments were performed independently three times. *p<0.05 and **p<0.01 indicated a significant difference compared to the control.*

Bromo-cAMP suppressed cell migration (Figure 4B) and 8-bromo-cGMP significantly increased cell migration (Figure 4C). These results suggest that PDE2 regulates cell migration through PDE2-cGMP signaling.

Discussion

Both cell proliferation and migration play important roles in the progression of osteosarcoma, and new anticancer therapies that block these processes are required. As we have previously reported that PDE2 regulated cell proliferation, but not migration of human malignant melanoma PMP cells (8), we first investigated whether human oral osteosarcoma HOSM-1 cells have PDE2 activity and express PDE2A mRNA. The results revealed PDE2 activity and PDE2A mRNA expression in HOSM-1 cells. PDE2 activity has also been detected in other malignant tumor cells including human mammary carcinoma MCF-7 cells (6). In addition, PDE2A mRNA expression was also observed in human MG-63 osteosarcoma cells by RT-PCR (7).

PDEs regulate cell proliferation and apoptosis of tumor cells (10, 11). Previously, we have demonstrated the involvement of PDE2 in the regulation cell proliferation (8). In this study, a specific PDE2 inhibitor EHNA was found to inhibit proliferation of HOSM-1 cells in a dose-dependent manner. Although EHNA reduced the number of cells compared to control, was not clear whether EHNA induced cell death or inhibited cell cycle progression. EHNA did not induce the death of HOSM-1 cells or PMP cells. This suggested that PDE2 inhibited cell cycle progression. In PMP cells, EHNA increased the proportion of cells in the G_2/M phase (8). In contrast, EHNA decreased the proportion of HOSM-1 cells in G_2/M phase. PDE2 degrades both cAMP and cGMP, thus affecting cAMP signaling and cGMP signaling. In PMP (8) and HOSM-1 cells, cell proliferation was inhibited by 8-bromo-cAMP but not 8-bromo-cGMP. These data suggested that cell proliferation is regulated by PDE2-cAMP signaling. However, these differences in cell cycle require further analysis.

Cell migration is an important factor in metastasis. EHNA suppressed the migration of PMP cells (8) but stimulated the migration of HOSM-1 cells. The reason for this discrepancy is unclear. In HOSM-1 cells, 8-bromo-cAMP inhibited cell migration, whereas 8-bromo-cGMP stimulated cell migration. EHNA inhibited PDE2 in HOSM-1 cells and as a result the concentration of cAMP and cGMP increased. These results suggested that PDE2-cGMP signaling regulates cell migration. No previous studies have investigated the relationship between PDE2-cGMP signaling and migration. Arozarena et al. have reported that PDE5A downregulation leads to an increase in cGMP, which induces an increase in cytosolic Ca^{2+}, stimulating contractility of melanoma cells (12). In HOSM-1 cells, a similar process is stimulated by PDE2A, and cell migration may be increased.

In conclusion, our results suggest that in HOSM-1 cells, PDE2-cAMP signaling regulates cell proliferation, whereas PDE2-cGMP signaling regulates cell migration. Further studies of these mechanisms are needed.

Conflicts of Interest

The Authors have no conflicts of interest to declare with regard to this study.

Authors' Contributions

All Authors were involved in the preparation of this manuscript. Taku Murata, Kasumi Shimizu and Naoya Arai designed the experiments. Taku Murata and Kasumi Shimizu performed the experiments. Kazuto Kurohara, Akira Tomeoku, Gaku Koizumi and Naoya Arai revised the article. All Authors approved the final version of the manuscript.

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