Expression of prostaglandin E$_2$ receptors in oral squamous cell carcinomas and growth inhibitory effects of an EP3 selective antagonist, ONO-AE3-240

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Abstract. Prostaglandin E$_2$ (PGE$_2$) can stimulate tumor progression by both direct and indirect mechanisms. However, its influence on cell proliferation is still unclear. The present study characterized expression of subtypes of PGE$_2$ receptors in oral squamous cell carcinomas, while also investigating the effects of EP3 and EP4 selective antagonists on oral carcinoma cell lines. EP1, EP2, EP3 and EP4 receptor mRNAs were detected in 4, 5, 10 and 10 of 11 surgical specimens respectively. Application of an EP3 antagonist (ONO-AE3-240) strongly inhibited cell growth in COX-2 and PGE$_2$ high expression cells (Ca9-22) but not in COX-2 and PGE$_2$ low expression cells (HSC4). The antagonist also reduced the production of endogenous PGE$_2$ and induced G0/G1 phase cell arrest. Addition of exogenous PGE$_2$ only partly abrogated the growth inhibition, indicating that the anti-proliferative effect via EP3 receptor signaling was not only due to PGE$_2$-dependent but also PGE$_2$-independent mechanisms. In contrast, an EP4 antagonist (ONO-AE3-208) did not inhibit growth in either of the cancer cell lines. In summary, PGE$_2$ receptor EP3 signaling probably contributes to development of oral carcinomas and use of EP3 antagonist may be a new therapeutic strategy for head and neck cancer.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the enzyme cyclooxygenase (COX) and suppress prostaglandin (PG) synthesis have been widely used as anti-inflammatory, anti-pyretic and analgesic agents. Epidemiological studies have recently shown that rheumatoid arthritis patients taking aspirin long-term have a lower incidence of colorectal cancer (1,2).

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Two COX isoforms have been identified: COX-1, constitutively expressed in various tissues and COX-2 induced by mitogens, cytokines and tumor promoters. Disruption of the COX-2 gene in mice was found to reduce the number and size of intestinal polyps generated by a mutation in the adenomatous polyposis (APC) gene, thus verifying the role of COX-2 in generation of colon tumors (3). NSAIDs inhibit COX and thereby suppress synthesis of prostaglandin E$_2$ (PGE$_2$), which can stimulate cell proliferation, angiogenesis and also inhibit apoptosis and immune surveillance.

PGE$_2$ induces its effects mainly through G protein-coupled PGE receptors designated EP1, EP2, EP3 and EP4 (4). These PGE$_2$ receptors are coupled to different intracellular signal-transcription systems and affect cellular functions in different ways (5-7). The EP1 receptor is associated with increases in intracellular Ca^{2+} while the EP2 and EP4 receptors are known to be coupled to Gs protein and stimulate cAMP production by activation of adenylate cyclase. In contrast, the major signal pathway for the EP3 receptor leads to inhibition of adenylate cyclase via Gi. Different specific patterns of PGE$_2$ receptor expression has been reported in numerous tumor cell lines and tumor tissues (8-12). Therefore inconsistent results of research published previously concerning PGE$_2$-induced cellular proliferation may have been due to differences in PGE$_2$ receptor expression among different cell types.

Regarding head and neck cancers, up-regulated expression of COX-2 mRNA, protein and PGE$_2$ has been reported (13-16). These observations suggest that COX-2-derived PGE$_2$ may play an important role in carcinogenesis and COX-2 inhibitor is a promising target molecule as chemopreventive agents (17-19). However, there have been few studies regarding the expression and functional significance of PGE$_2$ receptors. The present study was therefore performed to assess variation in expression of PGE$_2$ receptors in oral squamous cell carcinomas. In addition, the effect of EP3 and EP4 selective antagonists were compared using human oral cancer cell lines.

Materials and methods

Reagents. NS-398, a selective COX-2 inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI, USA). EP receptor selective antagonists, ONO-AE3-240 (EP3 antagonist) and ONO-AE3-208 (EP4 antagonist), were kindly supplied by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). These agents were

dissolved in dimethyl sulfoxide (DMSO) and then diluted to working concentrations with culture medium. The final concentration of DMSO, used as the solvent, was <0.5%.

Tumor samples and cell lines. Surgical specimens of oral cancer tissues were obtained from patients at Kagawa University Hospital after written informed consent in all cases. Samples were immediately placed in RNA*later* RNA stabilization regent (Qiagen Benelux B.V., The Netherlands). Two human squamous cell carcinoma cell lines (Ca9-22 and HSC4) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in EMEM (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FBS (Gibco BRL, Gaithersburg, MD, USA) and 1% antibiotics in an atmosphere of 95% air and 5% CO_2 at 37˚C.

Cell growth assay. Cells ($2.5x10^3$/well) were plated on 96-well plates with medium including 10% FBS. After 24 h incubation, various concentrations of NS398 or/and EP receptor selective antagonists were added to six wells each and the cells were further cultured for 72 h. The net number of viable cells was then determined using a water-soluble tetrazolium [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; Nacalai Tesque] colorimetric assay. The cell surviving fraction was determined as the ratio of absorbance at 72 h to that at 0 h. The percent change was calculated as follows:
(72 h absorbance - 0 h absorbance) x 100 / (72 h absorbance - 0 h absorbance) control
The cell growth assays were performed three times.

RT-PCR. Transcripts encoding COX-2, EP1, EP2, EP3, EP4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by RT-PCR analysis. Total cellular RNA was isolated using an RNeasy mini kit (Qiagen Benelux B.V.,) and cDNA was synthesized from 1 μg of total RNA with the use of an oligo-p (dT) 15 primer and AMV reverse transcriptase. PCR was performed in a Takara PCR thermal cycler MP (Takara Biomedicals, Tokyo, Japan). The cycle parameters were as follows: 1 cycle at 95˚C for 1 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. The following primers were used: 5'-GCG GGA ACA CAA CAG AGT ATG C-3' (sense) and 5'-CAG CAT AAA GCG TTT GCG GTA CT-3' (antisense) for COX-2, 5'-GGT ATC ATG GTG GTG TCG TG-3' (sense) and 5'-GGC CTC TGG TTG TGC TTA GA-3' (antisense) for EP1, 5'-GCC ACG ATG CTC ATG CTC TTC GCC-3' (sense) and 5'-CTT GTG TTC TTA ATG AAA TCC GAC-3' (antisense) for EP2, 5'-CGT GTC GCG CAG CTA CCG GCG-3' (sense) and 5'-CGG GCC ACT GGA CGG TGT ACT-3' (antisense) for EP3, 5'-CCT CCT GAG AAA GAC AGT GCT-3' (sense) and 5'-AAG ACA CTC TCT GAG TCC T-3' for EP4, and 5'-AAG GTC GGA GTC AAC GGA TTT GG-3' (sense) and 5'-ACG AAC ATG GGG GCA TCA GC-3' (antisense) for GAPDH.

Real-time RT-PCR. Quantitative RT-PCR was carried out using Taq Man universal PCR master mix (Perkin Elmer, Foster City, CA) with the following Taq Man assay-on-demand gene expression (Applied Biosystems) predesigned
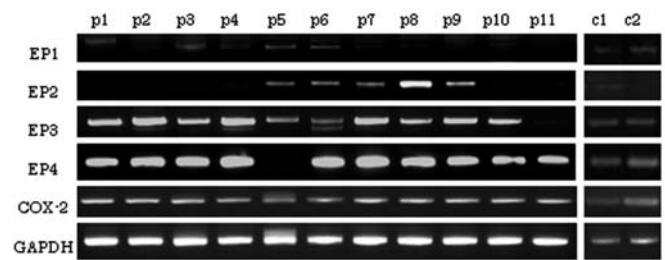

Figure 1. RT-PCR detection of the four subtypes of PGE_2 receptor in surgical specimens and cancer cell lines. p1-11, tumor samples of the patients. c1, HSC4 cell line and c2, Ca9-22 cell line.
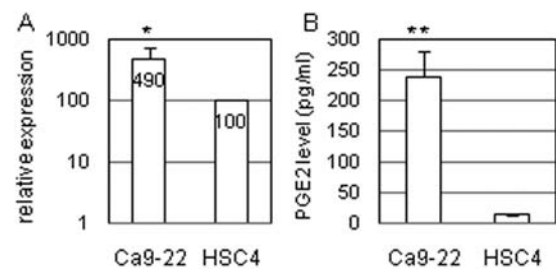

Figure 2. (A) Relative expression of COX-2 mRNA in cancer cell lines as determined by real-time quantitative PCR. (B) PGE_2 production in cancer cell lines. The cells were plated at $2x10^5$ cells/well in 1.5 ml volume in 6-well plate and cultured for 24 h. PGE_2 levels were determined using PGE_2 EIA kits. $^*P<0.05$. $^{**}P<0.01$.

primers and probes: CDK2, Hs00608082_m1; CDK4, Hs00262861_m1; cyclin D1, Hs00277039_m1; p53, Hs00153349_ml and p21, Hs00355782_ml.

Thermal cycling was initiated with incubation at 95˚C for 10 min. After this initial step, 40 cycles of PCR were performed, each consisting of heating at 95˚C for 15 sec for melting and 60˚C for 1 min for annealing and extension. The ABI7700 model software program was employed to construct amplification plots with the fluorescent emission data collected during PCR amplification. Standard curves were constructed by plotting CT values against the input quantities for both GAPDH and the target molecules in every PCR assay. The relative concentrations of GAPDH and the target molecules of unknown samples were then calculated to give the relative concentrations of target molecules to those of GAPDH (target molecule/GAPDH).

Prostaglandin E_2 immunoassay. Cells were plated at $2x10^5$ cells/well in 1.5 ml volume in 6-well plate and grown for 24 h at 37˚C. The indicated concentrations of NS398 and ONO-AE3-240 (EP3 antagonist) were added to the wells followed by culture for 24 h. The supernatant was removed and 1.5 ml of new medium was added to each well and then conditioned medium was collected after 24 h. PGE_2 assays were performed using a prostaglandin E_2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA).

Cell cycle analysis. Flow cytometry was conducted using a FACSEpics XL flow cytometer (Beckman Coulter, Fullerton, CA). Cells were washed twice with phosphate-buffered saline
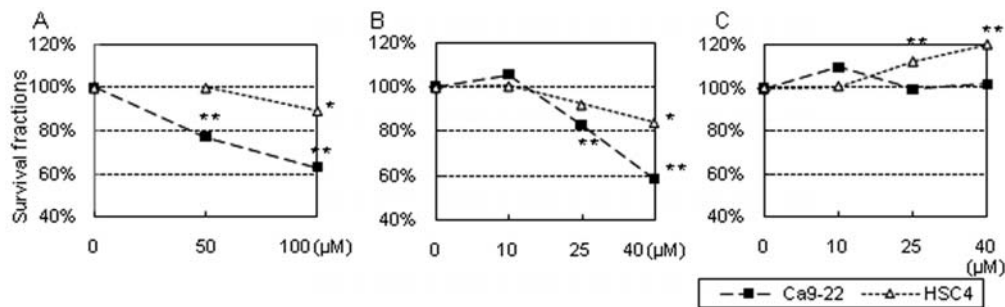
Figure 3. Effect of NS398 (A), ONO-AE3-240 (EP3 antagonist) (B) and ONO-AE3-208 (EP4 antagonist) (C) treatment on cell growth of carcinoma cell lines. Cells were seeded onto 96-well plates. After 24 h, drugs were added and then cells were cultured for 72 h. Cell numbers were measured using the MTT assay. *P<0.05 and **P<0.01.
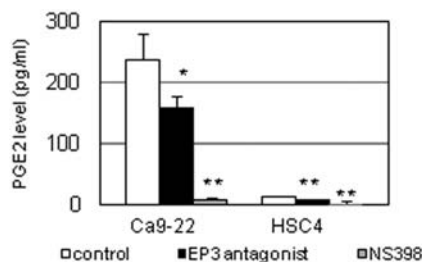

Figure 4. PGE$_2$ production in cancer cell lines. The cells were treated with 100 μM of NS398 or 40 μM of ONO-AE3-240 for 24 h. PGE$_2$ levels were determined using PGE$_2$ EIA kits. *P<0.05 and **P<0.01.

(PBS), fixed in 1 ml of 70% ethanol for 2 h at 4˚C, treated with 200 μg RNase A and stained with 50 μg propidium iodide. The cell distribution in the cell cycle was analyzed using the System II software program (Beckman Coulter).

Measurement of apoptosis. Cells were plated at 5x10^3 cells/ well in a 96-well plate and grown for 24 h at 37˚C. After a further 48 h treatments with 100 μM of NS398 and 40 μM of ONO-AE3-240, apoptosis was measured with a single-stranded DNA (ssDNA) apoptosis ELISA kit (Chemicon International, Inc., Temecula, CA, USA).

Statistical analysis. Data are expressed as mean ± SD. Comparisons between two groups were performed with the Student's t-test. A difference of P<0.05 was considered to be significant.

Results

Expression pattern of PGE$_2$ receptors in oral cancer biopsy specimens and cell lines. EP1, EP2, EP3 and EP4 mRNAs were detected in 4, 5, 10 and 10 surgical specimens respectively (Fig. 1). In the cancer cell lines, EP3 and EP4 were both detected but the expression of EP1 and EP2 was only very weak. COX-2 was detected in all surgical specimens and both cell lines.

Different expression levels of COX-2 and PGE$_2$ in oral cancer cell lines. To compare characteristics of the two cancer cell lines, the mRNA expression level of COX-2 was examined by real-time-PCR and PGE$_2$ production was measured by

ELISA. The mRNA expression level of COX-2 in the Ca9-22 cell line was 4.9 times that of the HSC4 cell line (Fig. 2A). The production of PGE$_2$ was also higher in the Ca9-22 case (238±42 vs. 14±1 pg/ml) (Fig. 2B).

Effects of COX-2 inhibitor and the PGE$_2$ receptor antagonists on growth of head and neck cancer cell lines. With Ca9-22 cells, growth was significantly suppressed by NS398 in a dose-dependent manner (p<0.01). However, 100 micromoles of NS398 only weakly inhibited growth of HSC4 cells (p<0.05). As shown in Fig. 3, cell numbers were decreased in both cell lines by ONO-AE3-240. On day 3, the number of Ca9-22 cells was significantly decreased in a dose-dependent manner by 17.2 and 41.6% (p<0.01 and p<0.01) in the presence of 25 and 40 μM ONO-AE3-240, respectively. The decrease in the HSC4 cell number was more limited, amounting to 7.8 and 16.1% (p<0.05). On the other hand, treatment with ONO-AE3-208 did not inhibit cell growth of either of the cell lines. The numbers of HSC4 cells were significantly increased by 12.1 and 20% (p<0.01 and p<0.01), respectively, in the presence of 25 and 40 μM ONO-AE3-208 on day 3.

Inhibition of PGE$_2$ production by NS398 and ONO-AE3-240. When cells were treated with 100 μM of NS398 or 40 μM of ONO-AE3-240 for 24 h and medium was changed with fresh reagents and samples were collected after 24 h, production of PGE$_2$ was found to be strongly inhibited by NS398 in both Ca9-22 and HSC4 cases. Although ONO-AE3-240 also reduced the production of PGE$_2$, the effect was weak in comparison to that of NS398 (Fig. 4). To investigate whether the anti-proliferative effect of NS398 and ONO-AE3-240 was due to suppression of PGE$_2$ production, Ca9-22 cells were treated with NS398 or ONO-AE3-240 for 24 h and then the supernatants were changed to medium including 500 pg/ml of PGE$_2$. As a result, growth inhibition with NS398 and ONO-AE3-240 was partially abrogated by the added PGE$_2$ (Fig. 5). However, no significant differences were found between PGE2 (-) and PGE2 (+) groups.

In addition, the anti-proliferative effects of the EP3 antagonist were not affected by pretreatment with 10 μM of NS398 (Fig. 6).

Cell cycle arrest and apoptosis induction by ONO-AE3-240. ONO-AE3-240 (40 μM) significantly induced G0/G1 phase
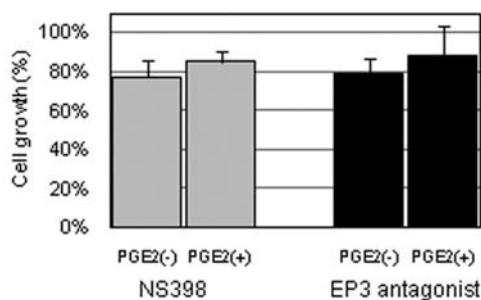
Figure 5. Effect of PGE2 on drug-induced growth inhibition in the Ca9-22 cell line. The cells were treated with NS398 (50 μM) or ONO-AE3-240 (25 μM) for 24 h and then incubated with or without 500 pg/ml of PGE2 for 48 h. Cell growth was assessed by MTT assay.
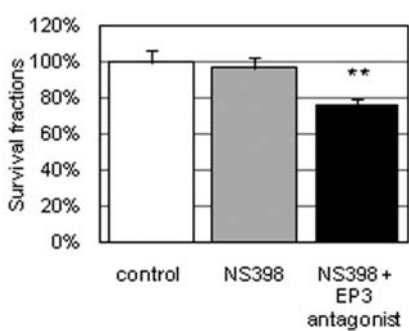

Figure 6. Effect of the EP3 antagonist after the PGE2 inhibition. The Ca9-22 cells were treated with NS398 (10 μM) for 24 h and then incubated with 25 μM of ONO-AE-240 for 72 h. Cell growth was assessed by MTT assay. **$P < 0.01$.

block in the two cell lines ($p < 0.05$) (Fig. 7). Cyclin-dependent kinases (CDK2 and CDK4) and cyclin D1 mRNA expression of the two cell lines were also decreased in comparison to those of the control group. Furthermore, p21 mRNA expression was significantly increased in the presence of ONO-AE3-240 in HSC4 cells (Fig. 8).

However, no significant difference was found between the apoptosis indices for the control and NS398 and ONO-AE3-240 treatments (data not shown).

Discussion

In the present study, EP3 and EP4 receptors were detected in 10 of 11 surgical specimens and the EP3 receptor antagonist

ONO-AE3-240 but not the EP4 receptor antagonist ONO-AE3-208 was found to have anti-proliferative effect *in vitro*. The EP3 antagonist thus inhibited the oral carcinoma cell growth in a dose-dependent manner. There are conflicting studies regarding involvement of EP3 receptor for carcinogenesis and cell proliferation. The receptor signaling with EP3 receptor suppresses colon carcinogenesis (20), but it enhances chemically-induced skin carcinogenesis, where most tumors induced are of squamous cell origin (21). Another recent study indicated that PGE2-EP3 signaling plays a critical role in tumor-associated angiogenesis, tumor progression and tumor growth in a mouse tumor implantation model (22). Yamaki *et al* (12) described PGE2-dependent activation of Src
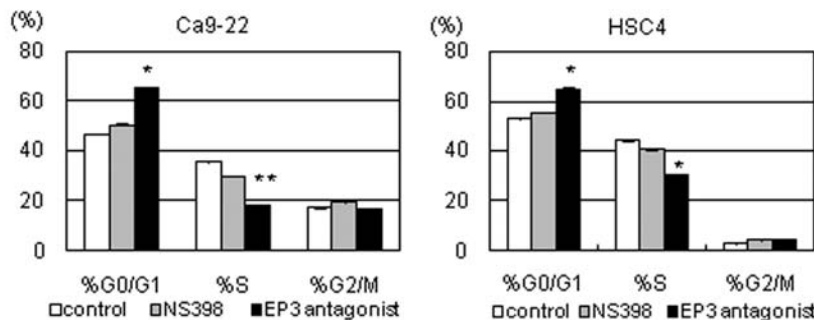

Figure 7. A cell cycle analysis by flow cytometry. The cells were treated with 100 μM of NS398 or 40 μM of ONO-AE3-240 for 48 h. *$P < 0.05$ and **$P < 0.01$.
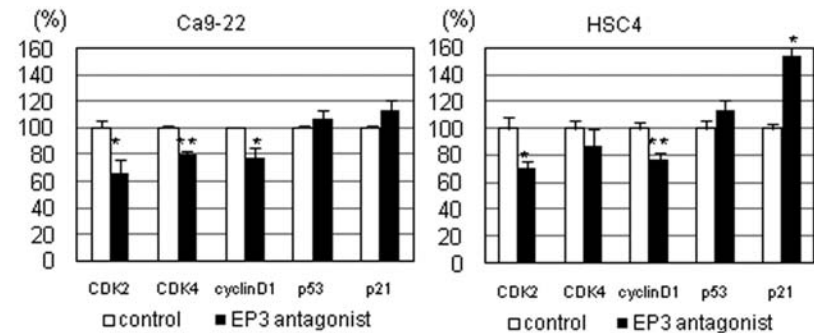

Figure 8. Alterations in the mRNA levels of CDK2, CDK4, p53 and p21 in the cell lines after treatment with ONO-AE3-240 for 24 h. The data are presented as the mean ± SD of the percent change in comparison with 3 PBS-treated control samples. *$P < 0.05$ and **$P < 0.01$.

signaling via EP3 to play an important role in growth of lung adenocarcinoma cells.

In our present study, The EP3 antagonist had more effects on the PGE$_2$-high production cell line (Ca9-22) but also weakly suppressed growth of the PGE$_2$-low production cell line (HSC4). Interestingly, it reduced the production of PGE$_2$ in both cell lines, although this influence was relatively weak in comparison to that of NS398. The mechanism is unclear but the inhibition of PGE$_2$-EP3 receptor signaling may act to down-regulate endogenous PGE$_2$ production.

Such as the COX-2 selective inhibitor, the EP3 antagonist could partially inhibit the cell growth in the presence of a large amount of exogenous PGE$_2$. Furthermore, the pretreatment with 10 μM of NS398 which inhibited the PGE$_2$ production had no effect on cell growth inhibition by the EP3 antagonist. Our data indicate that the anti-proliferative effects via EP3 receptor signaling are not only due to PGE$_2$-dependent but also PGE$_2$-independent mechanisms.

In our experiment, there was no evidence of NS398-induced apoptosis or cell cycle arrest. On the other hand, the EP3 antagonist reduced cell growth with cell cycle arrest at the G0/G1 phase. This was thought attributable to decreased expression of cyclin D1 and cyclin-dependent kinases (CDK2 and CDK4). It is possible that cell cycle arrest is one of the primary mechanisms responsible for the anti-proliferative effects of EP3 antagonists.

Generally, EP2 and EP4 receptors are known to be coupled to Gs protein and stimulate cAMP production by activation of adenylate cyclase. Several studies have provided evidence that an increase of intracellular cAMP through EP2 or EP4 receptors plays an important role in carcinogenesis (23-25). In some human gastric cancer cells, PGE$_2$ has been suggested to have an anti-proliferative effect via increased cAMP production, so that the activation of selective EP2 and EP4 receptors is linked to decreased cell proliferation (26). In the present study, the EP4 antagonist had no effect on Ca9-22 cells while increasing growth of the HSC4 cell line. These results suggest that the signal pathway through EP4 receptor impacts on cell proliferation in oral squamous cell carcinomas. Further studies are needed to investigate whether intracellular cAMP in oral cancer cells is decreased by EP4 antagonists.

Makita *et al* (27) earlier reported that an EP1-selective antagonist suppressed 4-NQO induced rat tongue carcinogenesis and speculated that EP1 antagonists can decrease the inflow of Ca^{2+} to cells, slow the speed at which cells mature and lower the cell proliferation activity. In the current study, the EP1 mRNA expression by real-time PCR was much lower than that of other EP receptors (data not shown) and the detection rate by RT-PCR in the surgical specimens were also low. Its potential role in carcinogenesis remains unclear. However, Tober *et al* (28) reported that UVB-induced changes in EP3 receptor localization were prevented by the EP1 antagonist ONO-8713, so that the EP1 and EP3 receptor may thus interact during events that occur following both acute and chronic UVB irradiation. It is therefore important to investigate possible cross-regulation of PGE$_2$ receptors.

In summary, expression of PGE$_2$ receptors in the majority of oral squamous cell carcinomas was here identified and the EP3 receptor antagonist ONO-AE3-240 was also found to have an anti-proliferative effect *in vitro*, reducing PGE$_2$ production and leading to cell cycle arrest at the G0/G1 phase. Further studies are now needed to elucidate the underlying molecular mechanisms and assess tumor suppressive effects *in vivo*.

Acknowledgements

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