Int J Clin Exp Pathol 2018;11(5):2356-2363
www.ijcep.com /ISSN:1936-2625/IJCEP0073141

Original Article

$CD44^{high}/ESA^{low}$ squamous cell carcinoma cell-derived prostaglandin E_2 confers resistance to 5-fluorouracil-induced apoptosis in $CD44^{high}/ESA^{high}$ cells

Hideo Shigeishi[1], Miho Hashikata[2], Sho Yokoyama[2], Miyuki Sakuma[2], Hiroshi Murozumi[2], Hiroki Kato[2], Mohammad Zeshaan Rahman[2,3], Sayaka Seino[2], Yasuki Ishioka[2], Kouji Ohta[2], Masaaki Takechi[2], Masaru Sugiyama[1]

[1]Department of Public Oral Health, Program of Oral Health Sciences, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan; [2]Department of Oral and Maxillofacial Surgery, Program of Dentistry, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan; [3]Department of Oral and Maxillofacial Surgery, Pioneer Dental College & Hospital, Ka-40/1, Lichu Bagan Road, Joar Sahara, Baridhara, Dhaka-1229, Bangladesh

Received January 20, 2018; Accepted February 22, 2018; Epub May 1, 2018; Published May 15, 2018

Abstract: We previously found that $CD44^{high}/ESA^{low}$ head and neck squamous cell carcinoma (HNSCC) cells harboring high dihydropyrimidine dehydrogenase (DPD) expression exhibited potent resistance to 5-fluorouracil (5-FU)-induced apoptosis. In addition, susceptibility of HNSCC cells to 5-FU was compromised in the presence of cyclooxygenase 2 (COX2)-derived prostaglandin E_2 (PGE_2). In this study, we examined 5-FU-induced apoptosis in sorted cell populations (i.e., $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ cells from the HNSCC cell line A-253) to clarify the anti-apoptotic effect of PGE_2 on $CD44^{high}$ cells. Notably, $CD44^{high}/ESA^{low}$ cells upregulated PGE_2, compared with other populations. To investigate the effect of $CD44^{high}/ESA^{low}$ cell-derived PGE_2 on $CD44^{high}/ESA^{high}$ cells, direct and indirect co-culture assays were performed. The percentage of apoptotic cells in a culture of $CD44^{high}/ESA^{high}$ cells was significantly reduced when they were directly and indirectly co-cultured with $CD44^{high}/ESA^{low}$ cells. Furthermore, 5-FU-induced apoptosis of $CD44^{high}/ESA^{high}$ cells was significantly increased in the presence of an inhibitor of the PGE_2 receptors (EP1/EP2) when $CD44^{high}/ESA^{high}$ cells were co-cultured with $CD44^{high}/ESA^{low}$ cells. These results suggest that $CD44^{high}/ESA^{low}$ cell-derived PGE_2 may contribute to the inhibition of 5-FU-induced apoptosis in $CD44^{high}/ESA^{high}$ cells. Additionally, NR4A2 knockdown enhances 5-FU-induced apoptosis in $CD44^{high}/ESA^{high}$ cells, suggesting that PGE_2 attenuates 5-FU-induced apoptosis in an NR4A2-dependent manner in $CD44^{high}/ESA^{high}$ cells. In conclusion, $CD44^{high}/ESA^{low}$ cells contribute to induction of resistance to 5-FU in $CD44^{high}/ESA^{high}$ cells through provision of PGE_2. $CD44^{high}/ESA^{low}$ cell-targeted therapy may be effective in treatment of HNSCC.

Keywords: Head and neck squamous cell carcinoma (HNSCC), apoptosis, prostaglandin E_2 (PGE_2), CD44, NR4A2

Introduction

While the mortality rate of head and neck squamous cell carcinoma (HNSCC) has improved recently, survival rates remain lower than those of other malignant tumours, such as colorectal and breast cancers [1]. In particular, patients with advanced-stage HNSCC exhibit high rates of recurrence and metastasis [2, 3]. This worsened prognosis may be associated with the behaviour of a small population of cancer stem cells that may be less sensitive to conventional cancer therapy [4]. In HNSCC, populations of $CD44^{high}$ cells exhibit cancer stem cell (CSC) properties, as well as phenotypic plasticity, such as mesenchymal (i.e., $CD44^{high}/ESA^{low}$ cells) to epithelial transition (i.e., $CD44^{high}/ESA^{high}$ cells) [5]. In addition, $CD44^{high}/ESA^{low}$ cells exhibited strong resistance to 5-fluorouracil (5-FU)-induced apoptosis, along with high dihydropyrimidine dehydrogenase (DPD) expression [6]. Therefore, we hypothesized that surviving $CD44^{high}/ESA^{low}$ cells may play an important role in the poor prognosis associated with HNSCC. Additionally, we have reported that susceptibility of HNSCC cells to 5-FU might be compromised following cyclooxygenase 2 (COX2)-derived prostaglandin E_2 (PGE_2) delivery to the cancer microenvironment [7]. We suspect that PGE_2 might be involved in enhancement of the

anti-apoptotic effect in HNSCC cells. However, it remains unknown whether PGE_2 can block apoptosis in $CD44^{high}$ cells (i.e., HNSCC cells with CSC properties). In this study, we examined the effect of exogenous PGE_2 on 5-FU induced apoptosis in $CD44^{high}$ subpopulations in HNSCC cells. In addition, we examined 5-FU-induced apoptosis of $CD44^{high}/ESA^{high}$ cells that were co-cultured directly or indirectly with $CD44^{high}/ESA^{low}$ cells.

Materials and methods

Cell culture and treatment

A HNSCC cell line, A-253 (American Type Culture Collection (ATCC), Manassas, VA, USA), was used in this study. Cells were cultured in a highly supplemented epithelial growth medium (Dulbecco's Modified Eagle's Medium: DMEM), with 10% FBS, under 5% CO_2 in air at 37°C [8]. For re-plating and for assays, cells were released into suspension using Accutase (Nakalai Tesque, Kyoto, Japan). To investigate the effect of the $CD44^{high}/ESA^{low}$ cells on $CD44^{high}/ESA^{high}$ cells, direct co-culture assays were performed by culturing the two cellular populations together. An indirect co-culture system, using Transwell cell culture inserts with microporous membranes (pore size: 1.0 μm) (BD Falcon, Milian, Italy) was also employed to prevent direct cell-to-cell communication. PGE_2 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO). DMSO was used as control for PGE_2. PGE_2 was added after cells had been cultured without FBS for 48 h. SC-51089 (Cayman Chemical, Ann Arbor, MI, USA) and AH-6809 (Cayman) were used as EP1 receptor and EP1/EP2 receptor antagonist, respectively. H-89 (Enzo Life Sciences, Farmingdale, NY, USA) was used as a cAMP-dependent protein kinase (PKA) inhibitor.

Fluorescence-activated cell sorting

Anti-CD44-PE-conjugated antibody (BD Pharmingen, San Diego, CA, USA) and anti-ESA-APC-conjugated antibody (BD Pharmingen) were used for fluorescence-activated cell sorting (FACS). 7-AAD (BD Pharmingen) was used to exclude dead cells during FACS analysis. According to our previously published methodology [9], samples were assayed on a Becton Dickenson FACSCalibur™ (BD Biosciences, San Jose, CA, USA); $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ cells were sorted using Becton Dickenson FACSAria equipment.

Apoptosis analyses

We examined apoptosis induced by 5-FU (Wako, Osaka, Japan) in A-253 cells. 1.0×10^4 cells were seeded in 6-well plates and grown for 24 h, then exposed to 25 μg/mL 5-FU for 48 h. Cells were collected and stained with 7-AAD and AnnexinV-Cy5 (BD Pharmingen), then AnnexinV-positive apoptotic fractions were analysed by FACS. Results are expressed as the mean ± SD for three independent experiments.

Introduction of green fluorescent protein (GFP) through viral infection

Introduction of GFP into the cells was performed according to our previously published method [10]. Briefly, virus host cells (HEK293FT cells) were cotransfected with a viral vector plasmid encoding EGFP (pLenti 6.3) (Invitrogen) and packaging plasmid Virapower mix (Invitrogen), using the EGFP X-treme GENE HP reagent (Roche, Basel, Switzerland). After 48 h, the viral supernatant was mixed with 8 μg/mL polybrene (Sigma-Aldrich), and then used to infect the target cells by spinoculation.

Measurement of PGE_2 concentration

Enzyme-linked immunosorbent assay (ELISA) was performed to measure PGE_2 concentrations via the Prostaglandin E_2 Monoclonal Kit (Oxford Biomedical Research, Oxford, UK), according to the manufacturer's instructions. Briefly, after 24 h incubation of the cells in culture medium with or without FBS, culture media were harvested to determine the PGE_2 concentration. The absorbance values were measured using a microplate reader with a 450-nm filter. Results are expressed as the mean ± SD for three independent experiments.

Quantitative RT-PCR analysis

The RNAeasy Micro Kit (Qiagen, Hilden, Germany) was used for RNA extraction and the resulting total RNA was subjected to reverse transcription using the ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan). Quantification of mRNA levels was performed using the CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and SYBR Green PCR

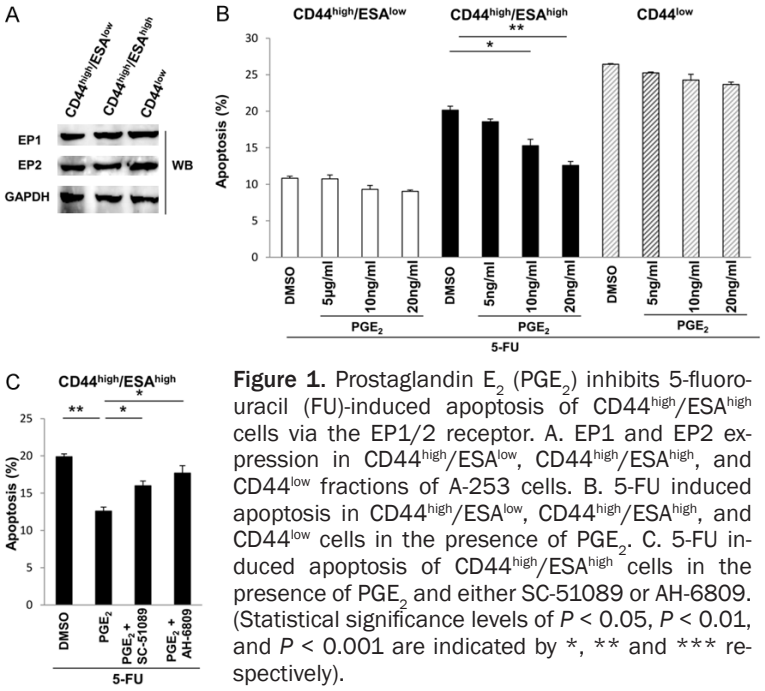

Figure 1. Prostaglandin E_2 (PGE_2) inhibits 5-fluorouracil (FU)-induced apoptosis of $CD44^{high}/ESA^{high}$ cells via the EP1/2 receptor. A. EP1 and EP2 expression in $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ fractions of A-253 cells. B. 5-FU induced apoptosis in $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ cells in the presence of PGE_2. C. 5-FU induced apoptosis of $CD44^{high}/ESA^{high}$ cells in the presence of PGE_2 and either SC-51089 or AH-6809. (Statistical significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, ** and *** respectively).

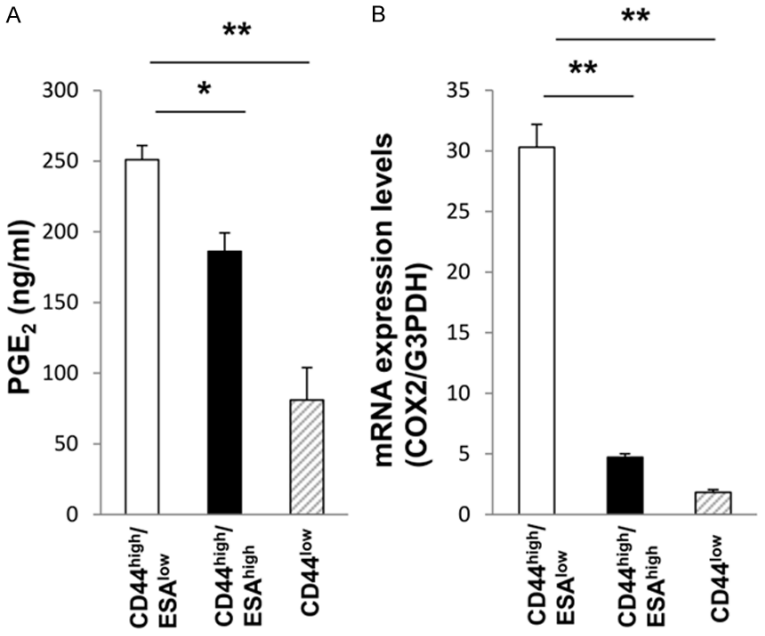

Figure 2. Prostaglandin E_2 (PGE_2) concentration and cyclooxygenase 2 (COX2) mRNA expression of $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$ and $CD44^{low}$ cells. A. PGE_2 concentration of $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ cells. B. COX2 mRNA expression levels of $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ cells. (Statistical significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, ** and *** respectively).

Master Mix (TOYOBO). The reaction mixture contained 1.0 μg of cDNA, 10.0 μL of SYBR Green Mix, and 10 μmol of each pair of oligonucleotide primers. GAPDH was used as a reference control. The primer sequences were as follows: Cox2, 5'-TTGCTGGAACATGGAATTACC-3' (sense), 5'-TGCCTGCTCTGGTCAATG-3' (antisense); NR4A2, 5'-GTCTCAGCTGCTCGACACG-3' (sense), 5'-TTTTGCACT-GTGCGCTTAAA-3' (antisense); Bcl-2, 5'-CCCTGTGGATGACTGAGTAC-3' (sense), 5'-GCATGTTGACTTCACTTGTG-3' (antisense); Bax, 5'-GGCCCACCAGCTCTGAGCAGA-3' (sense), 5'-GCCACGTGGGCGTCCCAAAGT-3' (antisense); and G3PDH, 5'-GTGAACCATGAGAAGTATGACAAC-3' (sense), 5'-ATGAGTCCTTCCACGATACC-3' (antisense). The PCR program was as follows: initial melting at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, 57°C for 30 sec and 72°C for 40 sec. Results are expressed as the mean ± SD for three independent experiments.

Western blotting

Protein samples were solubilized in sample buffer by boiling, then separated in a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Western blot bands were detected using an enhanced chemiluminescence Western blotting reagent (GE Healthcare, Piscataway, NJ, USA). Antibodies (all diluted at 1:1000) consisted of an anti-human EP-1 mouse monoclonal anti-body (Abcam, Cambridge, UK), an anti-human EP-2 mouse monoclonal antibody (Abcam), and an anti-human GAPDH mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

siRNA knockdown

Stealth™ siRNAs were used for NR4A2 knockdown (Life Technologies, Gaithersburg, MD, USA) and a Stealth siRNA negative control (Life Technologies) served as the control for knockdown. Cells were transfected with siRNA using

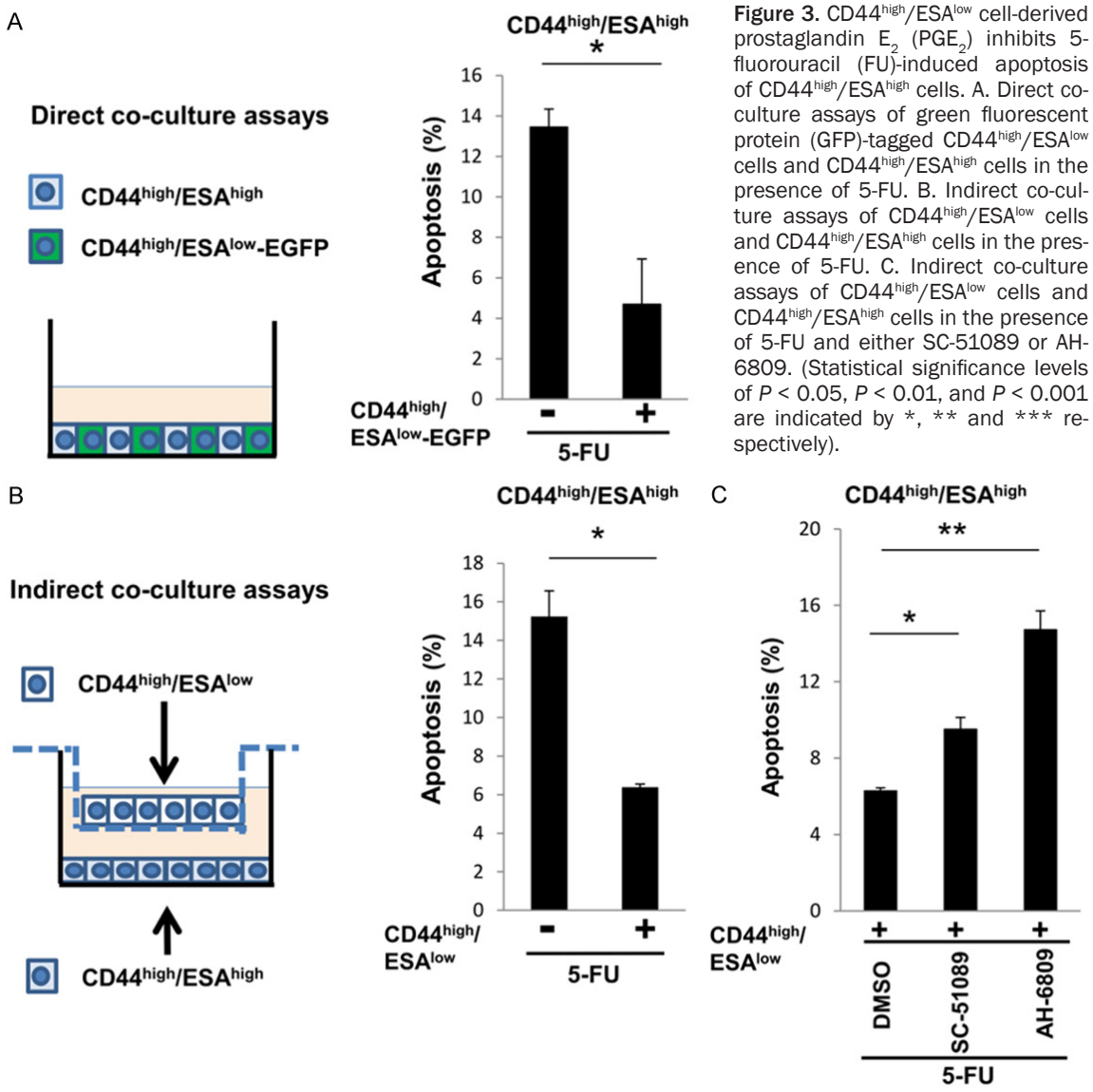

Figure 3. $CD44^{high}/ESA^{low}$ cell-derived prostaglandin E_2 (PGE_2) inhibits 5-fluorouracil (FU)-induced apoptosis of $CD44^{high}/ESA^{high}$ cells. A. Direct co-culture assays of green fluorescent protein (GFP)-tagged $CD44^{high}/ESA^{low}$ cells and $CD44^{high}/ESA^{high}$ cells in the presence of 5-FU. B. Indirect co-culture assays of $CD44^{high}/ESA^{low}$ cells and $CD44^{high}/ESA^{high}$ cells in the presence of 5-FU. C. Indirect co-culture assays of $CD44^{high}/ESA^{low}$ cells and $CD44^{high}/ESA^{high}$ cells in the presence of 5-FU and either SC-51089 or AH-6809. (Statistical significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, ** and *** respectively).

HiPerFect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's recommendations.

Statistical methods

Statistical analysis was performed using Student's t-test. P values < 0.05 were regarded as statistically significant.

Results

PGE_2 inhibit 5-FU-induced apoptosis of $CD44^{high}/ESA^{high}$ cells via EP1/2 receptor

All cell fractions expressed PGE_2 receptor, EP1, and EP2 proteins, as shown by Western blotting (**Figure 1A**). Thus, we analysed the effect of exogenous PGE_2 on 5-FU-induced apoptosis in $CD44^{high}/ESA^{low}$, $CD44^{high}/ESA^{high}$, and $CD44^{low}$ cells. $CD44^{high}/ESA^{low}$ cells exhibited the most potent resistance to 5-FU-induced apoptosis. Notably, PGE_2 significantly reduced the percentage of 5-FU-induced apoptotic cells in $CD44^{high}/ESA^{high}$ cells at concentrations of 10 and 20 ng/mL (**Figure 1B**). However, PGE_2 did not significantly reduce 5-FU-induced apoptosis in $CD44^{high}/ESA^{low}$ and $CD44^{low}$ cells (**Figure 1B**). 5-FU-induced apoptosis was examined in the presence of PGE_2 and either EP1 or EP1/EP2 receptor inhibitor. PGE_2-inhibited apoptosis was significantly increased after the addition of either EP1 or EP1/EP2 receptor antagonist (**Figure 1C**).

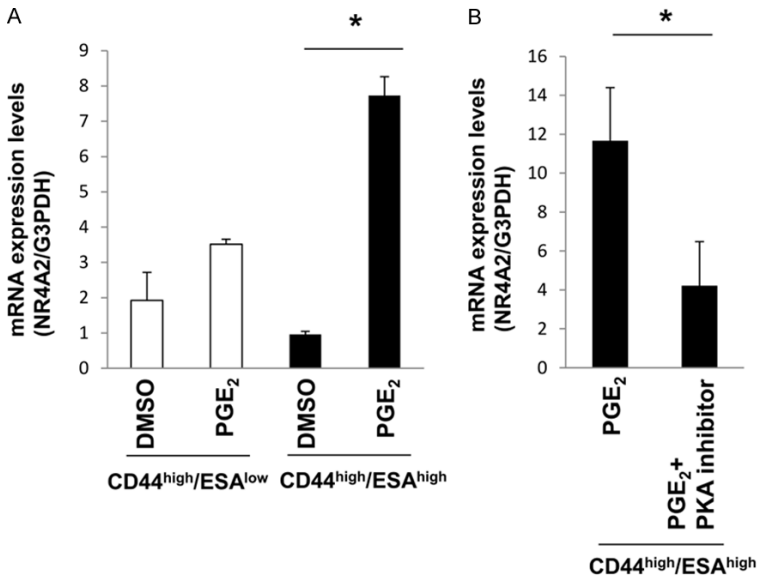

Figure 4. Prostaglandin E_2 (PGE_2)-induced NR4A2 expression was reduced in the presence of protein kinase A (PKA) inhibitor. A. NR4A2 mRNA expression in the presence of 20 ng/mL PGE_2. B. NR4A2 mRNA expression in the presence of PGE_2 and PKA inhibitor in $CD44^{high}/ESA^{high}$ cells. (Statistical significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, ** and *** respectively).

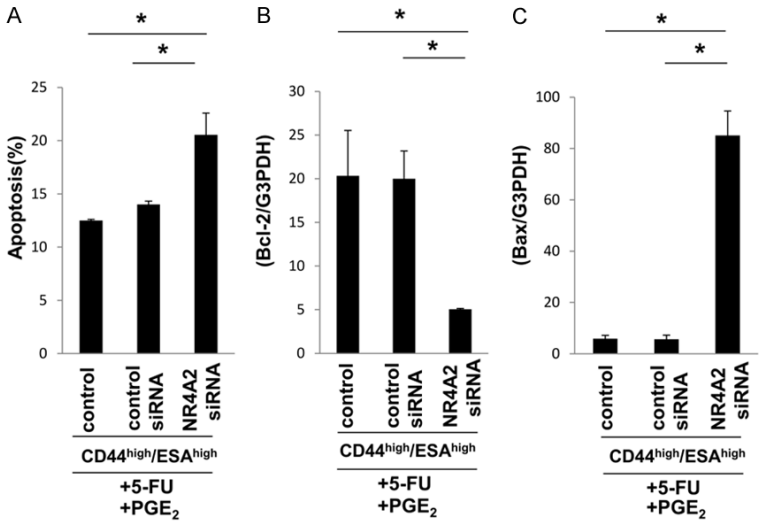

Figure 5. NR4A2-knockdown enhances 5-fluorouracil (FU)-induced apoptosis of $CD44^{high}/ESA^{high}$ cells. A. The percentage of apoptotic cells after siRNA knockdown of NR4A2. B. Bcl2 mRNA expression following NR4A2 siRNA knockdown in $CD44^{high}/ESA^{high}$ cells. C. Bax mRNA expression following NR4A2 siRNA knockdown in $CD44^{high}/ESA^{high}$ cells. (Statistical significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, ** and *** respectively).

$CD44^{high}/ESA^{low}$ cells produce an increased amount of PGE_2 compared than $CD44^{high}/ESA^{high}$ cells

Next, we examined the PGE_2 production ability of sorted cells. After 24 h incubation of the cells in culture medium without FBS, culture media were collected to determine the PGE_2 concentration. $CD44^{high}/ESA^{low}$ cells exhibited significantly higher PGE_2 concentration than $CD44^{high}/ESA^{high}$ cells or $CD44^{low}$ cells (**Figure 2A**). In addition, COX2 mRNA expression was significantly elevated in $CD44^{high}/ESA^{low}$ cells, compared with $CD44^{high}/ESA^{high}$ cells or $CD44^{low}$ cells (**Figure 2B**).

Endogenous PGE_2 inhibits 5-FU-induced apoptosis of $CD44^{high}/ESA^{high}$ cells

To investigate the effect of $CD44^{high}/ESA^{low}$ cell-derived PGE_2 on $CD44^{high}/ESA^{high}$ cells, direct co-culture assays were performed by culturing the two cellular populations together (**Figure 3A**). GFP-tagged $CD44^{high}/ESA^{low}$ cells and $CD44^{high}/ESA^{high}$ cells were co-cultured in the presence of 5-FU, followed by determination of the percentage of apoptotic cells within the GFP-negative $CD44^{high}/ESA^{high}$ population by FACS. The percentage of apoptotic cells within a culture of $CD44^{high}/ESA^{high}$ cells significantly decreased in the presence of $CD44^{high}/ESA^{low}$ cells, relative to $CD44^{high}/ESA^{high}$ cultures grown in the absence of $CD44^{high}/ESA^{low}$ cells (**Figure 3A**). Next, indirect co-culture of $CD44^{high}/ESA^{low}$ and $CD44^{high}/ESA^{high}$ cells was performed using Transwell cell culture inserts, in the presence of 5-FU (**Figure 3B**). The percentage of apoptotic cells within a culture of $CD44^{high}/ESA^{high}$ cells was significantly reduced when $CD44^{high}/ESA^{high}$ cells were indirectly co-cultured with $CD44^{high}/ESA^{low}$ cells (**Figure 3B**). Furthermore, the percentage of apoptotic cells within a culture of $CD44^{high}/ESA^{high}$ cells was significantly increased by the addition of

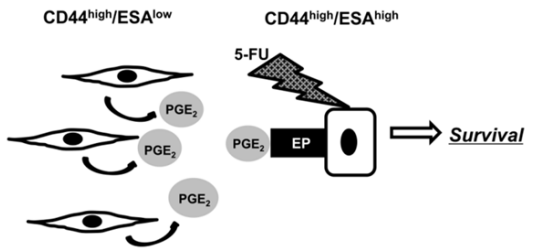

Figure 6. $CD44^{high}/ESA^{low}$ cell-derived prostaglandin E_2 (PGE_2) inhibits 5-fluorouracil (FU)-induced apoptosis in $CD44^{high}/ESA^{high}$ cells. PGE_2 release from $CD44^{high}/ESA^{low}$ cells may contribute to enhanced resistance to 5-FU-induced apoptosis in $CD44^{high}/ESA^{high}$ cells. (Statistical significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, ** and *** respectively).

SC-51089 and AH-6809 in the presence of 5-FU, when $CD44^{high}/ESA^{high}$ cells were indirectly co-cultured with $CD44^{high}/ESA^{low}$ cells (**Figure 3C**). These results suggest that $CD44^{high}/ESA^{low}$ cell-derived PGE_2 may contribute to the inhibition of 5-FU-induced apoptosis in $CD44^{high}/ESA^{high}$ cells.

PGE_2 induces NR4A2 expression via PKA dependent manner in $CD44^{high}/ESA^{high}$ cells

We previously revealed that exogenous PGE_2 induces NR4A2 expression in a PKA-dependent manner in oral SCC cells [7]. Therefore, we aimed to examine the effect of PGE_2 on NR4A2 expression in $CD44^{high}/ESA^{low}$ cells. $CD44^{high}/ESA^{low}$ cells exhibited a slight increase in NR4A2 mRNA expression in the presence of PGE_2 (**Figure 4A**). However, NR4A2 mRNA expression was significantly upregulated in the presence of PGE_2 in $CD44^{high}/ESA^{high}$ cells. Moreover, PGE_2-induced NR4A2 expression was inhibited by the addition of PKA inhibitor in $CD44^{high}/ESA^{high}$ cells (**Figure 4B**).

NR4A2-knockdown enhances 5-FU-induced apoptosis in $CD44^{high}/ESA^{high}$ cells

Finally, we examined the effect of NR4A2 siRNA knockdown on 5-FU-induced apoptosis in $CD44^{high}/ESA^{high}$ cells. Following siRNA knockdown of NR4A2, the percentage of apoptotic cells was significantly increased (**Figure 5A**). In addition, we examined expression of apoptosis-related genes, such as anti-apoptotic Bcl2 and pro-apoptotic Bax, during NR4A2 siRNA knockdown in $CD44^{high}/ESA^{high}$ cells. Bcl2 mRNA expression was significantly downregulated after siRNA knockdown of NR4A2 (**Figure 5B**). In contrast, Bax expression was significantly upregulated after siRNA knockdown of NR4A2 (**Figure 5C**).

Discussion

Several types of cancer can produce pro-inflammatory cytokines such as PGE_2, thereby inducing localized infiltration of inflammatory cells [11-13]. PGE_2 is suspected to contribute to modulation of the cancer microenvironment (i.e., cell proliferation, anti-apoptosis, and angiogenesis, as well as migration and invasion of tumor cells) [14]. There is mounting evidence to support the oncogenic role of PGE_2 within the context of the COX2-PGE_2-EP1/2-dependent signalling pathway [15, 16]. Several studies have reported a significant relationship between COX2 expression and CSC properties [17-20]. For example, there is greater sphere-forming ability within breast cancer cells that upregulate expression of COX-2 and its receptor, EP4. Notably, sphere forming ability is suspected to be a significant feature of CSCs [18]. COX-2 stimulates self-renewal of cancer stem-like cells of glioma via PGE_2 [19]. In addition, CSC repopulation has been blocked by the inhibition of the COX2-PGE_2 signalling pathway in bladder cancers [20]. These results indicate that PGE_2 may play a significant role in the maintenance of CSCs in different types of cancers. In this study, PGE_2 was involved in the inhibition of 5-FU-induced apoptosis of $CD44^{high}/ESA^{high}$ cells, indicating that PGE_2 may contribute to resistance to chemotherapeutics in HNSCC CSCs with epithelial character.

It remains unclear which type of cells (i.e., cancer cells, cancer-associated stromal cells, blood cells, or other normal cells) are the main source of PGE_2 in HNSCC. This study revealed that $CD44^{high}/ESA^{low}$ cells exhibit a greater capacity to produce PGE_2, compared with other populations of cells. $CD44^{high}/ESA^{low}$ cells with both CSC and mesenchymal properties may contribute to PGE_2 production in HNSCC. In addition, we found that 5-FU-induced apoptosis was significantly blocked in $CD44^{high}/ESA^{high}$ cells when they were co-cultured with $CD44^{high}/ESA^{low}$ cells. Thus, PGE_2 release from $CD44^{high}/ESA^{low}$ cells may confer anti-apoptotic capacity to $CD44^{high}/ESA^{high}$ cells, likely via EP1/EP2 receptor activation. Previously, we found that

$CD44^{high}/ESA^{low}$ cells exhibit strong resistance to other chemotherapeutics (e.g., cisplatin and docetaxel) [9]. Therefore, $CD44^{high}/ESA^{low}$ cells may be involved in the strong resistance to the conventional chemotherapy that is observed in HNSCC. $CD44^{high}$ subpopulations with potent resistance to chemotherapeutics (e.g., $CD44^{high}/ESA^{low}$ cells) may not only survive but also aid in the survival of other $CD44^{high}$ subpopulations (e.g., $CD44^{high}/ESA^{high}$ cells) in the presence of chemotherapeutics such as 5-FU.

NR4A2 is a member of the orphan nuclear hormone receptor subfamily and is implicated in a wide variety of biological processes [21, 22]. NR4As may be capable of inhibiting tumour suppressor signalling [23]. In this study, PGE_2-induced NR4A2 expression was inhibited by a PKA inhibitor, suggesting that PGE_2 induces NR4A2 expression in a PKA dependent manner within $CD44^{high}/ESA^{high}$ cells. The combination of Bcl2 upregulation and Bax downregulation, following NR4A2 siRNA knockdown, strongly supports the notion that NR4A2 contributes to anti-apoptosis signalling in $CD44^{high}/ESA^{high}$ cells.

Collectively, our results indicate that $CD44^{high}/ESA^{low}$ cells play a significant role in the induction of resistance to 5-FU within $CD44^{high}/ESA^{high}$ cells, largely by providing PGE_2 (**Figure 6**). Our results suggest that cancer-associated PGE_2 may regulate chemotherapeutic resistance in HNSCC. Thus, $CD44^{high}/ESA^{low}$ cell-targeted chemotherapies (or other potential therapies) may be effective in treatment of HNSCC.

Acknowledgements

This work was supported by a Grant-in-aid for Scientific Research (C) (No. 26463005) from the Japanese Ministry of Education, Culture, Sports, and Technology.

Disclosure of conflict of interest

None.

Address correspondence to: Hideo Shigeishi, Department of Public Oral Health, Program of Oral Health Sciences, Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. Tel: +81-82-257-5954; Fax: +81-82-257-5954; E-mail: shige@hiroshima-u.ac.jp

References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[2] Bernier J, Domenge C, Ozsahin M, Matuszewska K, Lefèbvre JL, Greiner RH, Giralt J, Maingon P, Rolland F, Bolla M, Cognetti F, Bourhis J, Kirkpatrick A, van Glabbeke M; European Organization for Research and Treatment of Cancer Trial 22931. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. N Engl J Med 2004; 350: 1945-1952.

[3] Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, Kish JA, Kim HE, Cmelak AJ, Rotman M, Machtay M, Ensley JF, Chao KS, Schultz CJ, Lee N, Fu KK; Radiation Therapy Oncology Group 9501/Intergroup. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med 2004; 350: 1937-1944.

[4] Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF and Ailles LE. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A 2007; 104: 973-978.

[5] Biddle A, Liang X, Gammon L, Fazil B, Harper LJ, Emich H, Costea DE and Mackenzie IC. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res 2011; 71: 5317-5326.

[6] Shigeishi H, Biddle A, Gammon L, Rodini CO, Yamasaki M, Seino S, Sugiyama M, Takechi M and Mackenzie IC. Elevation in 5-FU-induced apoptosis in head and neck cancer stem cells by a combination of CDHP and GSK3β inhibitors. J Oral Pathol Med 2015; 44: 201-207.

[7] Shigeishi H, Higashikawa K, Hatano H, Okui G, Tanaka F, Tran TT, Rizqiawan A, Ono S, Tobiume K and Kamata N. PGE_2 targets squamous cell carcinoma cell with the activated epidermal growth factor receptor family for survival against 5-fluorouracil through NR4A2 induction. Cancer Lett 2011; 307: 227-236.

[8] Harper LJ, Piper K, Common J, Fortune F and Mackenzie IC. Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. J Oral Pathol Med 2007; 36: 594-603.

[9] Hashikata M, Shigeishi H, Okui G, Yamamoto K, Tobiume K, Seino S, Uetsuki R, Kato H, Ishioka Y, Ono S, Ohta K, Higashikawa K, Sugiyama M and Takechi M. Int J Clin Exp Pathol 2016; 9: 7908-7918.

[10] Okui G, Tobiume K, Rizqiawan A, Yamamoto K, Shigeishi H, Ono S, Higashikawa K and Kama-

ta N. AKT primes snail-induced EMT concomitantly with the collective migration of squamous cell carcinoma cells. J Cell Biochem 2013; 114: 2039-2049.
[11] DuBois RN. Cyclooxygenase-2 and colorectal cancer. Prog Exp Tumor Res 2003; 37: 124-137.
[12] Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslow RA, Koki AT, Woerner BM, Masferrer JL and Dannenberg AJ. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 1999; 59: 991-994.
[13] Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H and Isola J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 2002; 62: 632-635.
[14] Wang MT, Honn KV and Nie D. Cyclooxygenases, prostanoids, and tumor progression. Cancer Metastasis Rev 2007; 26: 525-534.
[15] Cha YI and DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. Annu Rev Med 2007; 58: 239-252.
[16] Brouxhon S, Konger RL, VanBuskirk J, Sheu TJ, Ryan J, Erdle B, Almudevar A, Breyer RM, Scott G and Pentland AP. Deletion of prostaglandin E_2 EP2 receptor protects against ultraviolet-induced carcinogenesis, but increases tumor aggressiveness. J Invest Dermatol 2007; 127: 439-446.
[17] Pang LY, Hurst EA and Argyle DJ. Cyclooxygenase-2: a role in cancer stem cell survival and repopulation of cancer cells during therapy. Stem Cells Int 2016; 2016: 2048731.
[18] Kundu N, Ma X, Kochel T, Goloubeva O, Staats P, Thompson K, Martin S, Reader J, Take Y, Collin P and Fulton A. Prostaglandin E receptor EP4 is a therapeutic target in breast cancer cells with stem-like properties. Breast Cancer Res Treat 2014; 143: 19-31.
[19] Wu M, Guan J, Li C, Gunter S, Nusrat L, Ng S, Dhand K, Morshead C, Kim A and Das S. Aberrantly activated Cox-2 and Wnt signaling interact to maintain cancer stem cells in glioblastoma. Oncotarget 2017; 8: 82217-8223.
[20] Liu Q, Yuan W, Tong D, Liu G, Lan W, Zhang D, Xiao H, Zhang Y, Huang Z, Yang J, Zhang J and Jiang J. Metformin represses bladder cancer progression by inhibiting stem cell repopulation via COX2/PGE_2/STAT3 axis. Oncotarget 2016; 7: 28235-28246.
[21] Wang Z, Benoit G, Liu J, Prasad S, Aarnisalo P, Liu X, Xu H, Walker NP and Perlmann T. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 2003; 423: 555-560.
[22] Law SW, Conneely OM, DeMayo FJ and O'Malley BW. Identification of a new brain-specific transcription factor, NURR1. Mol Endocrinol 1992; 6: 2129-2135.
[23] Beard JA, Tenga A and Chen T. The interplay of NR4A receptors and the oncogene-tumor suppressor networks in cancer. Cell Signal 2015; 27: 257-266.