

Apremilast Induces Apoptosis of Human Colorectal Cancer Cells with Mutant *KRAS*

KENSUKE NISHI^{1,2*}, HAO LUO^{1*}, SHUHEI ISHIKURA^{1,3}, KEIKO DOI^{1,3},
YURI IWAIHARA¹, LAUREN WILLS⁴, GEORGE S. BAILLIE⁴, TOSHIFUMI SAKATA²,
SENJI SHIRASAWA^{1,3} and TOSHIYUKI TSUNODA^{1,3}

Departments of ¹Cell Biology, and ²Otorhinolaryngology, Faculty of Medicine, and
³Central Research Institute for Advanced Molecular Medicine, Fukuoka University, Fukuoka, Japan;
⁴Institute of Cardiovascular and Medical Science, University of Glasgow, Glasgow, Scotland, U.K.

Abstract. *Background/Aim:* We previously reported the crucial roles of oncogenic Kirsten rat sarcoma viral oncogene homologue (*KRAS*) in inhibiting apoptosis and disrupting cell polarity via the regulation of phosphodiesterase type 4B2 (*PDE4B2*) expression in human colorectal cancer (CRC) HCT116 cells in a three-dimensional culture (3DC). Here, we evaluated the effects of apremilast, a selective *PDE4* inhibitor, on luminal apoptosis in 3DC and nude mice assay using HKe3 human CRC cells stably expressing wild-type (wt)*PDE4B2* (HKe3-wt*PDE4B2*), mutant (mt)*PDE4B2* (kinase dead) (HKe3-wt*KRAS*), wt*KRAS* (HKe3-wt*KRAS*) and mt*KRAS* (HKe3-mt*KRAS*). *Materials and Methods:* Apoptosis was detected by immunofluorescence using confocal laser scanning microscopy or western blot in HKe3-wt*PDE4B2*, HKe3-mt*PDE4B2*, HKe3-wt*KRAS* and mt*KRAS* cells treated with or without apremilast in 3DC. Tumourigenicity was assessed in nude mice assay using these cells. *Results:* Apremilast did not inhibit the proliferation of HKe3-wt*PDE4B2* cells or HKe3-mt*KRAS* in two-dimensional cultures, whereas the number of apoptotic HKe3-wt*PDE4B2* cells and HKe3-mt*KRAS* cells increased after apremilast treatment in 3DC, leading to formation of a luminal cavity. Tumour growth in nude mice was dramatically reduced by intraperitoneal injection of apremilast. Notably, a decreased level of caspase-1 expression was observed in HKe3-wt*PDE4B2* and HKe3-mt*KRAS* cells.

Conclusion: Apremilast induces tumour regression in nude mice, possibly by inducing caspase-1 expression.

The impairment of cyclic AMP (cAMP) generation by overexpression of phosphodiesterase 4 (*PDE4*) isoforms, including *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D* (1, 2), has been described in various cancer pathologies (3). Indeed, many recent studies have indicated that *PDE4* inhibitors are effective against different types of cancer cells (4-9). In our recent studies, we found that *PDE4B* is specifically up-regulated by mutated Kirsten rat sarcoma viral oncogene homologue (*KRAS*) in three-dimensional (3D) culture, and *PDE4* inhibitors, including rolipram and resveratrol, were found to induce luminal apoptosis of human colorectal cancer cells with mutated *KRAS* in 3D culture (10, 11), suggesting that *PDE4* is a novel effector protein downstream of mutated *KRAS* signaling within the *in vivo* microenvironment. Currently, there are no effective drugs that directly target effectors of *KRAS* signaling or mutated *KRAS* in clinical use. Therefore, *PDE4* inhibitors may provide a novel strategy for targeting different types of cancer cells with mutated *KRAS*, including colorectal and pancreatic cancers.

Apremilast, an oral small molecule inhibitor of *PDE4*, is approved by the United States Food and Drug Administration for treating patients with chronic inflammatory disorders, such as psoriasis and psoriatic arthritis (12, 13). Notably, apremilast shows favourable adverse reaction profiles compared to other *PDE* inhibitors, which are milder than emesis with cilomilast (14) and central nervous system effects with rolipram (15), suggesting its appropriateness for cancer treatment and chemoprevention. Our study intended to determine the effects of apremilast in cancer with mutated *KRAS* (*in vivo* and *in vitro*). For this reason, we performed 3D floating culture and nude mice assays using the HKe3 cells overexpressing wild type (wt) *PDE4B2*, mutated (mt) *PDE4B2* (kinase dead) mt*KRAS* and wt*KRAS*. Our results

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*These Authors contributed equally to this study.

Correspondence to: Toshiyuki Tsunoda, MD, Ph.D., Department of Cell Biology, Faculty of Medicine Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011, Fax: +81 928643865, e-mail: tsunoda@fukuoka-u.ac.jp

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suggest that apremilast may be an effective treatment for human colorectal cancer.

Materials and Methods

Antibodies and reagents. Antibody to cleaved caspase-3 (5A1), cleaved caspase-7 (D6H1), cleaved poly (ADP-ribose) polymerase (PARP) (Asp214; D64E10) and caspase-1 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody to actin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Apremilast was a kind gift from Celgene Corp, NJ, USA.

Retroviral production and generation of stable cell line. Dasher Green Fluorescent Protein (DGFP) cDNA from DNA2.0's Cas9 vectors (pD1401-AD) was subcloned into a pMSCVpuro vector (Clontech, Palo Alto, CA, USA) at a multi-cloning site to generate retrovirus vector to produce a pMSCV-DGFP vector. cDNAs for Hemagglutinin (HA)-tagged human wtPDE4B2 (wtPDE4B2-HA) and HA-tagged human mtPDE4B2 (H234S, mt PDE4B2-HA) were inserted into the pMSCV-DGFP vector at multi-cloning sites to generate the mammalian expression plasmids pMSCV-wtPDE4B2-HA-DGFP and pMSCV-mtPDE4B2-HA-DGFP, which were produced by the transfection of the retrovirus vectors together with the pAmpho vector (Clontech) into GP2-293 packaging cells (Clontech) by standard calcium phosphate transfection in the presence of 25 μ M chloroquine (Sigma-Aldrich). After 48 hours since transfection, the viral supernatants were collected and supplemented with 8 μ g/ml polybrene (Sigma-Aldrich). The HKe3 cells in six-well plates were infected with the viruses by being spun for 2 h at 32°C and 1,000 \times g. At 48 h after infection, the cells were treated with 2 μ g/ml puromycin (Sigma-Aldrich) for 1 week to establish HKe3-derived cells stably expressing wtPDE4B2 or mtPDE4B2. The cells were further maintained in the medium containing 2 μ g/ml puromycin.

Cell culture. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in the presence or absence of apremilast (100 μ M) for 6 days in two-dimensional (2D) or 3D culture as previously described (10, 16-23).

Cell growth assay in 2D culture. Herein, 4×10^4 cells for HKe3-wtPDE4B2, HKe3-mtPDE4B2 and HKe3-wtKRAS, and 1×10^4 cells for HKe3-mtKRAS cells, which were established earlier (23), were seeded in a 96-well microplate (product number CLS3596; Corning Inc., Corning, NY, USA) at 37°C for six days, and the absorbance of formazan solutions was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay-based Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the supplier's instructions. The relative growth rate was calculated from a comparison of the absorbance obtained from cells treated with dimethyl sulfoxide (DMSO) alone as a vehicle control.

3D floating cell culture. Cells were seeded in a 96-well plate with an ultra-low attachment surface and a round bottom (product number 7007; Corning Inc., Corning, NY, USA). Cells were cultured for 6 days with or without apremilast in a CO₂ incubator, as previously described (23). Photomicrographs of cells were taken and analysed using an IN Cell Analyzer 1000 (GE Healthcare, Little Chalfont, UK) and IN Cell Developer Toolbox (GE Healthcare).

The relative growth rate was calculated from a comparison of the area of control spheroids at day three.

Quantification of apoptotic cells in spheroids. Apoptotic cells were stained with Magic Red Caspase 3&7 Assay Kit (ImmunoChemistry Technologies, LLC, Bloomington, MN, USA) on day 6, according to the supplier's instructions, and imaged using CS-SP5 laser scanning confocal microscopy (Leica, Wetzlar, Germany). The number of apoptotic cells in spheroids was counted as previously described (10, 18).

Assay for tumorigenicity. Four-week-old female SHO mice (Crl: HA-Prkdc^{scid}Hr^{hr}) were purchased from Charles River Laboratories (Yokohama, Japan). Cells for implantation were trypsinised and resuspended in a 1:1 mix of phosphate-buffered saline and Matrigel (BD Bioscience, Bedford, MA, USA). 100 μ l containing either 1.5×10^6 HKe3-mtKRAS or 1×10^7 of HKe3-wtPDE4B2, HKe3-mtPDE4B2 or HKe3-wtKRAS cells was subcutaneously injected into the flank of mice. At the beginning of apremilast treatment, the average diameter of these tumours was approximately 5 mm. Apremilast was intraperitoneally administered from day 0 to day 7 daily. Tumour volumes were calculated using the formula: $(L \times W^2) \times 0.5$, where L was the tumour length and W the tumour width. The tumour volume at day n was expressed as the relative tumour volume (RTV) and calculated according to the following formula: $RTV = TV_n / TV_0$, where TV_n was the tumour volume at day n and TV_0 the tumour volume at day 0. Animal studies were conducted in accordance with our institutional guidelines, and the experimental procedures were approved by the Fukuoka University Animal Care Committee (approval number 1611991).

Immunoblotting. Cells treated with or without apremilast were lysed in a RIPA buffer [(50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and protease inhibitor cocktail) (Roche, Basel, Switzerland)] and were subjected to immunoblotting as previously described (10, 24). Quantitative analysis of the immunoblotting was performed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistical analyses. Data are presented as means \pm standard deviations. Statistical analyses were performed using unpaired two-tailed Student's t -test. p -Values of less than 0.05 were considered statistically significant.

Results

Induction of luminal apoptosis by apremilast in HKe3-wtPDE4B2 and HKe3-mtKRAS cells grown in 3D culture. To address the effects of apremilast on cell proliferation in 3D culture, cells were treated with apremilast or DMSO alone. No significant differences were observed in the growth rates between HKe3-wtPDE4B2 cells, HKe3-mtPDE4B2 cells or HKe3-wtKRAS cells treated with apremilast and those treated with DMSO (Figure 1A). Interestingly, however, the relative growth rate of HKe3-mtKRAS cells treated with apremilast was lower than in those treated with DMSO alone (Figure 1A), suggesting that apremilast, *via* its inhibitory action on PDE4, regulates the signalling pathway altered by mutated KRAS in 3D culture.

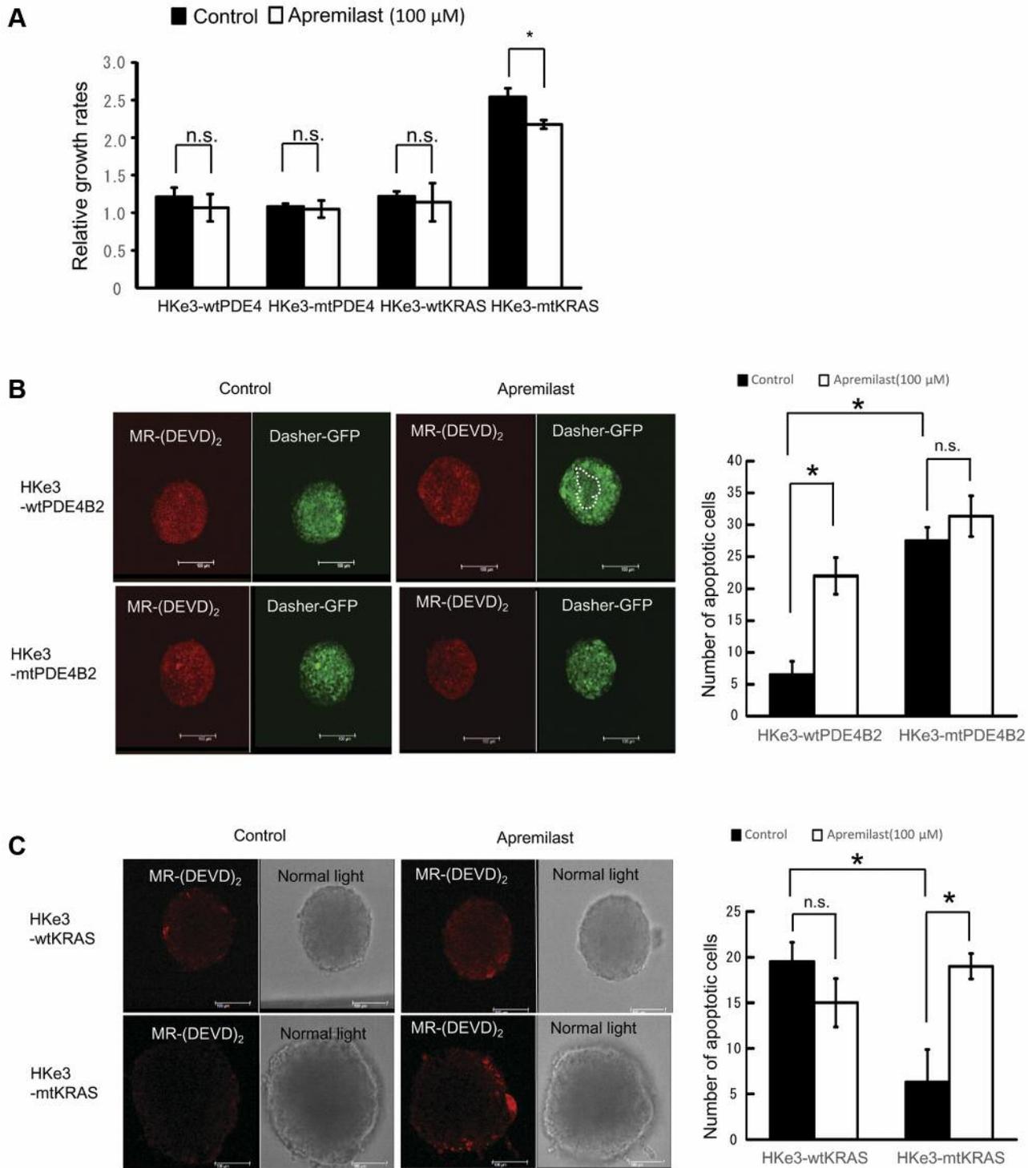


Figure 1. Luminal apoptosis was induced by apremilast in human colorectal cancer HKe3 cells stably expressing wild-type phosphodiesterase 4B2 (PDE4B2) (HKe3-wtPDE4B2) and mutant Kirsten rat sarcoma viral oncogene homologue (KRAS) (HKe3-mtKRAS) cells grown in 3D culture. A: Relative growth rates of HKe3-wtPDE4B2, HKe3-mutant (mt) PDE4B2, HKe3-wtKRAS and HKe3-mtKRAS with or without apremilast treatment in 3D culture at day 6. B, C: Left panels: The signals for cleaved caspase-3/7 in HKe3-wtPDE4B2 and HKe3-mtPDE4B2 cells (B) and HKe3-wtKRAS and HKe3-mtKRAS (C) treated with dimethyl sulfoxide alone (control) or apremilast at day 6 in 3D culture. Cleaved caspase-3/7, red. Dasher GFP, green. Dots represent luminal cavities. Scale bar=100 μm. Right panels: Number of apoptotic cells in single spheroids. *Significantly different at $p < 0.05$; n.s., not significant.

To address whether the influence of apremilast extended to luminal apoptosis in 3D culture, we evaluated the apoptotic activity in these cells grown in 3D culture for 6 days by evaluating cleaved caspase-3/7 using confocal microscopy. In HKe3-wtPDE4B2 control cells, the number of apoptotic cells was 4.23-fold lower compared to HKe3-mtPDE4B2 control cells (Figure 1B; $p < 0.05$), suggesting that the overexpression of wtPDE4B2 was able to inhibit luminal apoptosis, whereas mtPDE4B2 (kinase dead) was not. In HKe3-wtPDE4B2 cells treated with apremilast, the number of apoptotic cells increased 3.38-fold in comparison to the DMSO control (Figure 1B; $p < 0.05$). In addition to increased apoptosis, a luminal cavity was observed in cells treated with apremilast, suggesting that apremilast directly inhibits the effect of wtPDE4B2. In HKe3-mtPDE4B2 cells, no significant difference was observed between DMSO control and apremilast-treated cells (Figure 1B), suggesting that apremilast acts in this system by inhibiting the PDE activity of PDE4B2. The signals for dasher GFP representing the localisation of wtPDE4B2 or mtPDE4B2 were similar, suggesting that PDE4B2 localisation was not affected by PDE4B2 mutation (Figure 1B). These results suggest that apremilast induces luminal apoptosis in colon cancer cells by inhibiting the activity of PDE4B2.

In HKe3-mtKRAS control cells, the number of apoptotic cells decreased 3.08-fold in comparison with HKe3-wtKRAS control cells (Figure 1C; $p < 0.05$), suggesting that the overexpression of mtKRAS was able to inhibit luminal apoptosis, whereas that of wtKRAS was not. In HKe3-mtKRAS cells treated with apremilast, the number of apoptotic cells increased 3-fold in comparison with DMSO alone (Figure 1C; $p < 0.05$), suggesting that apremilast directly counteracts the oncogenic effects of mtKRAS. In HKe3-wtKRAS cells, no significant difference was observed between DMSO control and apremilast (Figure 1C), suggesting that apremilast targets signaling downstream of mtKRAS, possibly affecting cAMP dynamics orchestrated by PDE4B2. The data presented here suggests that apremilast may represent a new therapeutic avenue for colon cancer as the PDE4 inhibitor induces luminal apoptosis by inhibiting the activity of PDE4B2.

Apremilast inhibits growth of tumours expressing wtPDE4B2 and mtKRAS in nude mice. For HKe3-wtPDE4B2 cells, tumour volume experienced a 2.65-fold increase in comparison to those in HKe3-mtPDE4B2 from day 0 to day 8 (Figure 2A and B), suggesting that wtPDE4B2 promotes tumour growth *in vivo*. Tumour volume was dramatically reduced in HKe3-wtPDE4B2 tumours in mice treated with apremilast in comparison to those administered DMSO alone (Figure 2A). In contrast, no difference was observed between HKe3-mtPDE4B2 cell tumours treated with apremilast and DMSO controls (Figure 2B), suggesting that apremilast acts

via PDE4 inhibition activity *in vivo*. In HKe3-mtKRAS cells, tumour volume increased 9.04-fold in comparison with that in HKe3-wtKRAS from day 0 to day 8 (Figure 2C and D). Tumour volume of HKe3-mtKRAS tumours treated with apremilast was dramatically reduced than in those treated with DMSO controls (Figure 2D); conversely, no difference was observed between apremilast-treated mice with HKe3-wtKRAS tumours and DMSO-treated controls (Figure 2C), suggesting that apremilast induces apoptosis by inhibiting the activity of PDE4B2 regulated by mtKRAS *in vivo*.

mtKRAS and PDE4B2 prevent apoptosis through down-regulation of caspase-1. In order to understand the underlying signaling events regulated by PDE4B2 in a 3D environment, caspases were examined by immunoblotting in cells from 2D and 3D culture (Figure 3). In 3D culture, the amount of cleaved caspase-3, caspase-7 and PARP were detected robustly in HKe3-wtKRAS and HKe3-mtPDE4B2 cells and were decreased in HKe3-mtKRAS or HKe3-wtPDE4B2 cells, suggesting that mtKRAS and PDE4B2 are involved in the inhibition of luminal apoptosis in 3D culture. Surprisingly, the expression of full-length caspase-1 was strongly increased by wtKRAS and mtPDE4B2 and was reduced by mtKRAS and wtPDE4B2 in 3D culture, suggesting that mtKRAS and PDE4B2 down-regulate the protein expression of caspase-1.

Discussion

In this study, we demonstrated for the first time that the recently approved PDE4 inhibitor apremilast significantly promoted apoptosis of HKe3-mtKRAS and HKe3-wtPDE4B2 cells *via* a signalling route that includes PDE4B2 and caspase-1. Our previous work showed that mtKRAS prevents luminal apoptosis *via* the induction of PDE4B2 in HCT116 cells (18), and PDE4B2 inhibitors, including rolipram and resveratrol, induce luminal apoptosis (10, 11) by a previously undefined signalling route. Apremilast is known to be highly specific for PDE4 and mediates its effects in monocytes and T-cells *via* protein kinase A (PKA) and nuclear factor kappa B pathways (13). PDE4 is the predominant cAMP-degrading enzyme expressed in inflammatory cells in diseases, such as psoriasis (25). The most common downstream effector of cAMP is PKA (26), and PKA action can result in up-regulation of caspase-1 expression (27). Hence, it is logical to suggest that regulation of cAMP dynamics by PDE4B2 could appropriately regulate local PKA activity to affect caspase-1 expression. Our results showed that PDE4B2 and mtKRAS down-regulate the expression of caspase-1 and promote tumour growth (Figures 2 and 3). In agreement with this result, recent work has shown that caspase-1-deficient mice demonstrate increased colonic epithelial cell

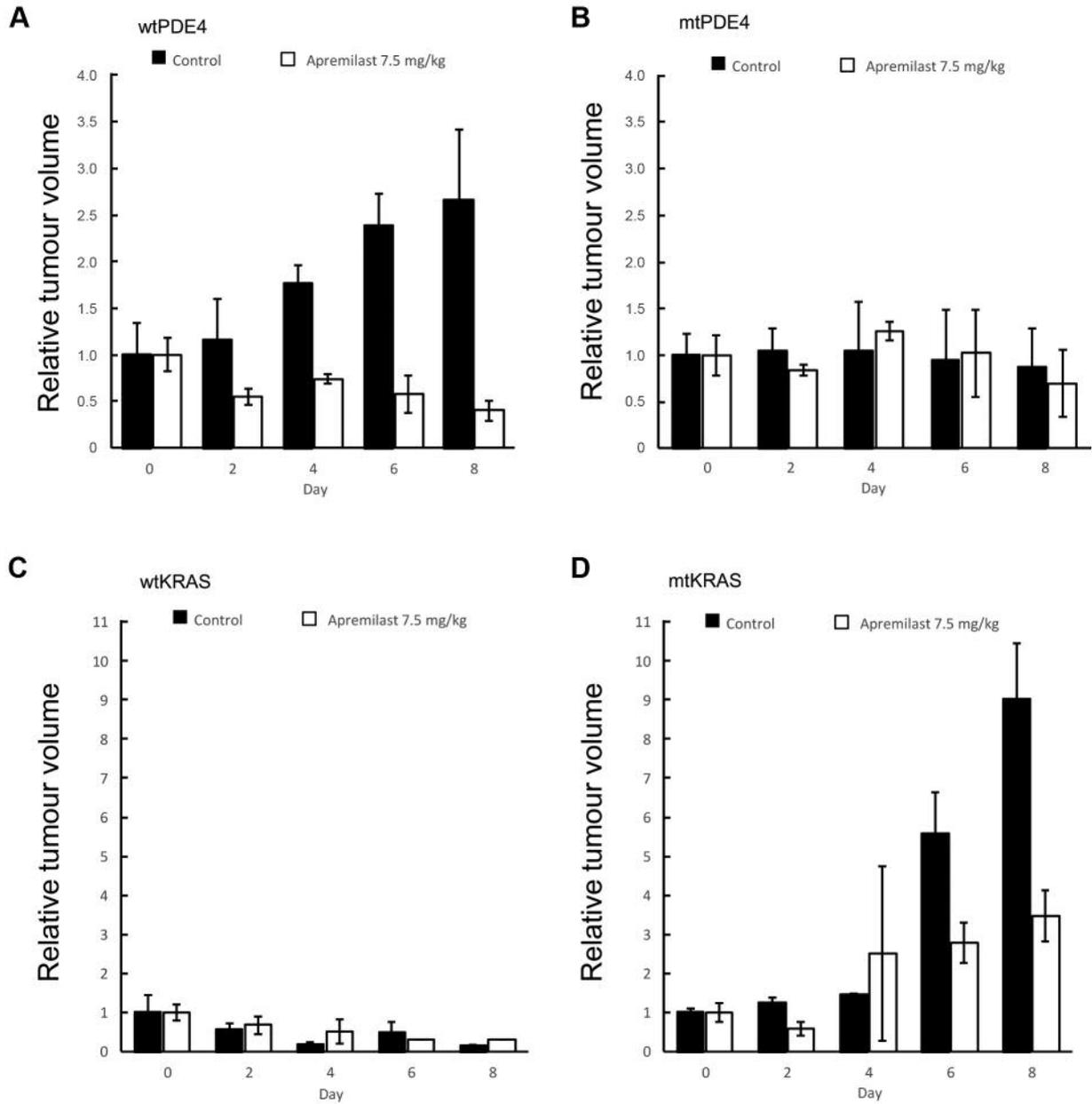


Figure 2. Assays of *in vivo* tumorigenicity for human colorectal cancer HKe3 cells. HKe3 cells stably expressing wild-type phosphodiesterase 4B2 (PDE4B2) (HKe3-wtPDE4B2) (A), mutant PDE4B2 (HKe3-mtPDE4B2) (B), wild-type Kirsten rat sarcoma viral oncogene homologue (KRAS) (HKe3-wtKRAS) (C) and mutant KRAS (HKe3-mtKRAS) (D) were injected subcutaneously into the flanks of nude mice. Relative tumour volumes are shown for mice treated with and without apremilast.

proliferation in early stages of injury-induced tumour formation and reduced apoptosis in advanced tumours (28). Furthermore, recent reports also suggest that caspase-7 and not caspase-3 is activated by caspase-1 *in vitro* and *in vivo* (29, 30), and caspase-7 is a substrate of caspase-1 that cleaves PARP (31). In our study, the expression changes of caspase-7 by mtKRAS or PDE4B were larger than those of

caspase-3 (Figure 3). These results together suggest that mtKRAS and PDE4B are protective against apoptosis by blocking the caspase-1–caspase-7 axis. We also propose that caspase-1 signalling may offer novel downstream targets for apremilast which underpin the mechanism driving apoptosis of colon cancer cells. Further elucidation of the molecular mechanisms triggered by novel PDE4

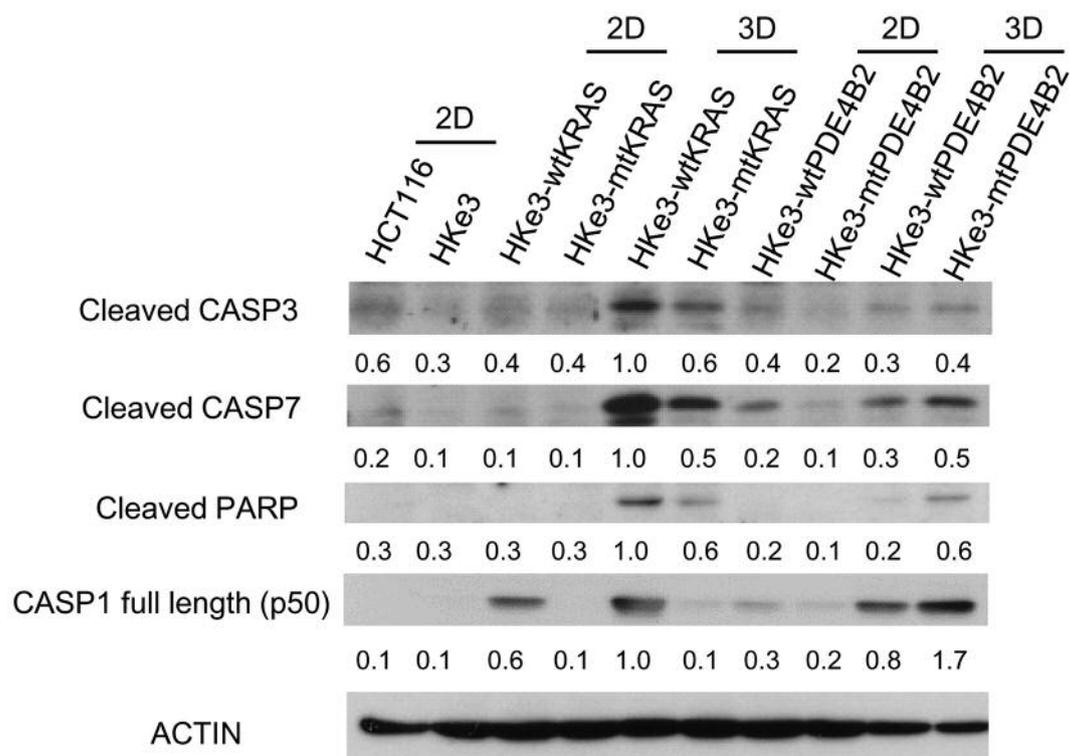


Figure 3. Mutant (mt) Kirsten rat sarcoma viral oncogene homologue (KRAS) and wild-type (wt) phosphodiesterase 4B2 (PDE4B2) prevent apoptosis through down-regulation of caspase-1. Human colorectal cancer HKe3 cells stably expressing wtPDE4B2 (HKe3-wtPDE4B2), mtPDE4B2 (HKe3-mtPDE4B2), wtKRAS (HKe3-wtKRAS) and mtKRAS (HKe3-mtKRAS) cells grown in 2D or 3D culture were lysed. Expression of the indicated proteins in the lysates was examined by immunoblotting using specific antibodies. Actin was used as a loading control. Levels of proteins were quantified by densitometry and normalised to actin levels. The values represent the relative ratio to levels in HKe3-wtKRAS grown in 3D culture. Data are representative of three independent experiments.

inhibitors (32) such as apremilast, which drives apoptosis or pyroptosis is required to bolster the notion that PDE4 inhibition is a future therapeutic avenue for the treatment of cancer.

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