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Regulation of *EP* receptors in non-small cell lung cancer by epigenetic modifications

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ABSTRACT

Background: Cyclooxygenase (COX)-2 is frequently overexpressed in non-small cell lung cancer (NSCLC) and results in increased levels of prostaglandin E2 (PGE_2), an important signalling molecule implicated in tumourigenesis. PGE_2 exerts its effects through the E prostanoid (*EP*) receptors (*EPs1–4*).

Methods: The expression and epigenetic regulation of the *EPs* were evaluated in a series of resected fresh frozen NSCLC tumours and cell lines.

Results: EP expression was dysregulated in NSCLC being up and downregulated compared to matched control samples. For *EPs1*, 3 and 4 no discernible pattern emerged. *EP2* mRNA however was frequently downregulated, with low levels being observed in 13/20 samples as compared to upregulation in 5/20 samples examined. In NSCLC cell lines DNA CpG methylation was found to be important for the regulation of *EP3* expression, the demethylating agent decitabine upregulating expression. Histone acetylation was also found to be a critical regulator of *EP* expression, with the histone deacteylase inhibitors trichostatin A, phenylbutyrate and suberoylanilide hydroxamic acid inducing increased expression of *EPs2–4*. Direct chromatin remodelling was demonstrated at the promoters for *EPs2–4*.

Conclusions: These results indicate that *EP* expression is variably altered from tumour to tumour in NSCLC. *EP2* expression appears to be predominantly downregulated and may have an important role in the pathogenesis of the disease. Epigenetic regulation of the *EPs* may be central to the precise role COX-2 may play in the evolution of individual tumours.

1. Introduction

The molecular and cellular basis of inflammation has become a topic of great interest in the pathogenesis of malignant disease. One of the inflammatory mediators that may play a critical role in cancer is cyclooxygenase (COX)-2. This enzyme is a rate-limiting step in the prostanoid synthesis pathway, which converts arachidonic acid into prostaglandin H2, a substrate for specific prostaglandin synthases. Overexpression of COX-2 has been observed in various malignancies including NSCLC.[1,2] One of the metabolites generated by COX-2 activity is prostaglandin E2 (PGE_2). Elevated levels of this product have

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been shown to play important roles in tumourigenesis by inducing immunosuppression, cell proliferation, differentiation, angiogenesis, metastasis and inhibition of apoptosis.[3–5] PGE_2 exerts its effects through binding to specific receptors.[6] There are at least four subtypes of PGE_2 receptor, designated as *EPs1–4*, according to their pharmacological profiles and signal transduction pathways. *EP1* activation stimulates the release of intracellular calcium via a mechanism involving G proteins. *EP2* and *EP4* activate adenylate cyclase via stimulatory G proteins, but differ in the response induced by certain ligands. *EP3* induces Ca2+ mobilisation or inhibits adenylate cyclase via inhibitory G-binding proteins. PGE_2 acts on *EP* receptors to trigger intracellular signal transduction cascades,[5,6] impacting on cell growth and survival through the upregulation of bcl-2 family members.[7] Perturbations in *EP* receptor synthesis seem to be present in most cancer types,[5] but the precise role of these receptors in malignant behaviour has yet to be determined.[5]

Molecular genetic studies of lung cancer frequently reveal multiple genetic and epigenetic changes including DNA sequence alterations, copy number changes and aberrant promoter methylation.[8] NSCLC is no exception and both DNA CpG methylation and aberrant histone post-translational modifications are recognised as having predictive and prognostic significance in this disease.[9–11] A direct link to DNA CpG methylation in the regulation of *EP3* has been demonstrated in both colon cancer and oesophageal cancer where treatment of cells with a DNA demethylating agent restored expression of *EP3* in various cell lines.[12,13]

There had been one report identifying aberrant *EP* receptor expression in NSCLC,[14] but the number of primary tumour samples examined was low ($n = 5$). Therefore we sought to examine the expression of *EPs1–4* in a larger series of primary NSCLC tumour samples and an additional panel of NSCLC cell lines, and to directly examine whether epigenetics plays a role in the regulation of *EP* receptor expression in this disease. Our results indicate that *EP* receptor expression is frequently dysregulated in NSCLC, being regulated via epigenetic mechanisms (DNA CpG methylation and histone post-translational modifications), and may be a good candidate target for epigenetic therapy in the treatment of this cancer.

2. Materials and methods

2.1. Cell lines and primary tumour samples

Beas-2B transformed normal human bronchoepithelial, and the H460 (large cell), H647 (adenosquamous carcinoma), A549 (adenocarcinoma) and SK-MES-1 (squamous cell carcinoma) NSCLC cell lines were purchased from the ATCC (LGC Promochem) and were maintained at a constant temperature of 37 °C in a humidified atmosphere of 5% CO_2 in the appropriate cell culture media as defined by the ATCC. Twenty tumour specimens (10 adenocarcinomas and 10 squamous cell carcinomas) from patients presenting with early stage NSCLC (Stages I and II) with the corresponding matched normal tissue from the same individual were collected at surgery at St James's Hospital, Ireland. Of these twenty samples, six had undergone prior chemotherapy (four squamous cell carcinomas and two adenocarcinomas).

2.2. Treatments with trichostatin A, phenylbutyrate, suberoylanilide hydroxamic acid or 5-Aza-2-deoxycytidine

Trichostatin A [TSA] was purchased from Invivogen and dissolved in DMSO at a concentration of 250 mg/ml. Cell cultures were treated for 24 h with trichostatin A, at a final concentration of 250 ng/ml.

Phenylbutyrate [PB] (Tributyrate™) was a generous gift from Triple Crown America. Cell cultures were treated with PB at a final concentration of 10 mM for 24 h.

Suberoylanilide hydroxamic acid [SAHA] (Zolinza/Vorinostat®) was purchased from Cayman Chemicals and dissolved in methanol. Cell cultures were treated for 24 h at a final concentration of 5 μM.

5-Aza-2-deoxycytidine (Decitabine®) [DAC] was purchased from Merck, and dissolved in methanol. Cell cultures were treated for 48 h at a final concentration of 5 μM, with the media and drug replaced every 24 h.

2.3. Cellular proliferation assays

Cellular proliferation was measured using BrdU incorporation according to the manufacturer's instructions (Roche). Briefly, 5000 cells/well (A549/SK-MES-1) were seeded into each well of a 96-well plate, and allowed to recover for 24 h, at which point they were treated with 5-Aza-2-deoxycytidine (5 μM) for 48 h, with the media and drug replaced every 24 h. For treatments with SAHA, the cells were allowed to grow untreated for a further 24 h, following which they were subsequently treated with drug (5 μM) for 24 h. Following treatments cellular proliferation was assayed.

2.4. Nucleic acid isolation

Total RNA was isolated using tri-reagent (MRC Gene) according to the manufacturer's instructions.

2.5. Generation of cDNA and analysis of gene expression

First strand cDNA was prepared from total RNA using Superscript II according to the manufacturer's instructions (Invitrogen). Expression of *EPs1–4* and *Beta-Actin* in A549 and SK-MES-1 cells was examined by RT-PCR using the primers and annealing conditions outlined in Table 1.

PCR cycling conditions were as follows:

Ninety five degree celsius for 5 min followed by 35 cycles of (1 min at 94 °C, 1 min at the indicated annealing temperature as outlined in Table 1, 1 min at 72 °C) with a final extension at 72 °C for 10 min.

Ten microlitres of the *EP* RT-PCR product and 2 μl of the *Beta-Actin* RT-PCR product were loaded onto a 1.2% agarose gel. Quantification of the RT-PCR results was obtained by scanning the gel images and importing the data into TINA 2.09c (Raytest, Isotopenmeßgeräte GmbH, Germany) with *Beta-Actin* levels utilised as the internal control in each case as appropriate. The values for the gene under scrutiny were then normalised to the internal control.

Table 1 – Primers and cycling conditions for RT-PCRs used in this study.

	Annealing temperature (°C)	Number of cycles
EP1 Fwd: 5′-GGTATCATGGTGGTGTCGTG-3′ EP1 Rev: 5′-GGCCTCTGGTTGTGCTTAGA-3′	57	40
EP2 Fwd: 5′-GCCACGATGCTCATCCTCTTCGCC-3′ EP2 Rev: 5′-CTTGTGTTCTTAATGAAATCCGAC-3′	58	35
EP3 Fwd: 5′-GCATAACTGGGGCAACCTTTTCTTCGCC-3′ EP3 Rev: 5′-CTTAACAGCAGGTAAACCCAAGGATCC-3′	62	35
EP4 Fwd: 5′-TGGTATGTGGGCTGGCTG-3′ EP4 Rev: 5′-GAGGACGGTGGCGAGAAT-3′	62	35
Beta-Actin Fwd: 5′-TGTTTGAGACCTTCAACACCC-3′ Beta-Actin Rev: 5′-AGCACTGTGTTGGCGTACAG-3′	56	30

2.6. *Chromatin immunoprecipitation (X-ChIP)*

Chromatin immunoprecipitation was performed as follows: following treatments, cells were fixed with formaldehyde (final concentration 1%), suspended in SDS lysis buffer (Millipore) and sonicated until DNA was fragmented into lengths of between 200 and 1000. Aliquots of this sheared DNA were subsequently immunoprecipitated using the OneDay ChIP Kit™ (Diagenode) according to the manufacturer's instructions. The antibodies used for immunoprecipitations were as follows: pan acetyl-histone H3 (Millipore Cat#06-599), pan acetyl-histone H4 (Millipore Cat# 06-598), acetyl-histone H3 (K9/14ac) (Diagenode Cat# pAb-ACHBHS-044), acetyl-histone H3 (K9ac) (Diagenode Cat# pAb-ACHAHS-044). A no-antibody control was included to test for non-specific carriage of DNA with histones.

PCR primers for studying *EPs2–4* by ChIP either were designed from published data for *EP2*,[15] or were manually designed from the published *EP3* promoter.[16] The *EP4* promoter sequence was generated using two sources. The available *EP4* promoter sequence obtained from the Transcriptional Regulatory Element Database (TRED)[17] was compared and merged with the *EP4* promoter sequence provided by Lee and colleagues.[18] This sequence was compared to the UCSC genome for veracity by BLAT alignment,[19] and ChIP primers were manually designed from it. The PCR primers are presented along with the cycling and annealing parameters in Table 2.

3. Results

3.1. *Expression of EPs1–4 in primary lung cancer tumour specimens*

We examined the expression of *EPs1–4* in a panel of primary NSCLC tumour surgical specimens, comparing expression in the tumour versus matched normal lung tissue obtained from the same individuals. We found that the expression of *EPs1–4* was frequently altered compared to normal tissue with both upregulation and downregulation in the tumours of patients observed (Fig. 1), and the results are summarised in Tables 3 and 4.

3.2. *Expression of EPs1–4 in non-small cell lung cancer cell lines*

Using RT-PCR *EPs1–4* expression was also examined in a panel of NSCLC cell lines (Fig. 2). For the most part, expression of all 4 receptors was observed for all the cells with the following exception. *EP3* expression could not be detected in BEAS-2B or A549 cells.

3.3. *Methylation is involved with regulating EP expression in NSCLC cell lines*

EPs1, 3 and 4 all showed an increase in their mRNA levels in response to treatment with the demethylating agent decitabine (Fig. 3). However, only *EP3* showed a statistically significant increase in both cell lines. These results indicate that epigenetic mechanisms such as DNA CpG methylation are involved with the regulation of expression of the *EP* receptors in NSCLC.

3.4. *Histone acetylation is involved with regulating expression of EPs1–4*

Inhibition of histone deacetylases by the histone deacetylase inhibitor trichostatin A (TSA) led to the induction of *EP2–4* while the expression of *EP1* was reduced (Fig. 4). Subsequently we examined the response of the *EPs* to TSA with other histone deacetylase inhibitors, including phenylbutyrate (PB) (Fig. 5), and the FDA approved HDAC inhibitor

Table 2 – Primers and cycling conditions for EP ChIP-PCRs used in this study.

	Annealing temperature (°C)	Number of cycles
EP2 ChIPFwd: 5′-GATCTTCTGTGTATTCTGCG-3′ EP2 ChIPRev: 5′-TTGCCGGTGTACTGAAGCTG-3′	56	35
EP3 ChIPFwd: 5′-GGGCTCCCGGTCCGGCCAGG-3′ EP3 ChIPRev: 5′-CGCGCGGAGGTGCCGAGTCC-3′	58	35
EP4 ChIPFwd: 5′-GAGAAGTTCCCAGGAGGAAG-3′ EP4 ChIPRev: 5′-TCAGTTGGCAGTGCCCAGAC-3′	56	35

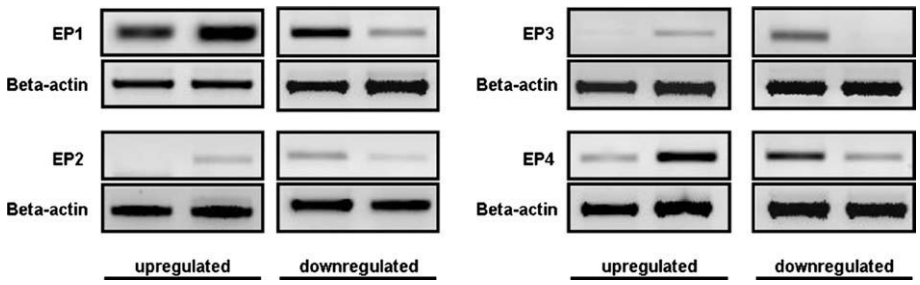

Fig. 1 – Expression of *EPs1–4* mRNA in primary NSCLC tumours. RT-PCR for *EPs1–4* was carried out on a panel of 10 adenocarcinomas and 10 squamous cell non-small cell lung carcinomas with matched normal lung from the same individual. Levels were normalised to *Beta-Actin*. A representative figure showing the altered expression patterns observable in the samples is provided, showing both upregulation and downregulation of the *EP* receptors in the NSCLC samples. The full results for this analysis are summarised in Tables 3 and 4.

Table 3 – Heat map showing alterations in the expression of EP receptors in a panel of matched tumour/normal pairs of NSCLC.

Sample	*EP1*	*EP2*	*EP3*	*EP4*
1 (Adeno)	↑	↓	Unchanged	↓
2 (Adeno)	↑	↓	↑	↑
3 (Adeno)	↑	↓	↑	↓
4 (Adeno)	↓	↓	↓	↓
5 (Adeno)	↓	↑	↑	↑
6 (Adeno)	↓	↑	↓	↓
7 (Adeno)	↓	↓	↑	↓
8 (Adeno)	↑	↑	↑	↑
9 (Adeno)	↓	↓	↓	↓
10 (Adeno)	↑	n.d.	Unchanged	↓
11 (Squam)	↑	↓	↓	↑
12 (Squam)	↑	↓	↓	↑
13 (Squam)	↑	↓	↓	Unchanged
14 (Squam)	↓	↓	↑	Unchanged
15 (Squam)	↓	↑	↑	↑
16 (Squam)	↓	Unchanged?	↑	↑
17 (Squam)	↓	↓	↓	↑
18 (Squam)	↑	↑	Unchanged	Unchanged
19 (Squam)	↓	↓	↓	↓
20 (Squam)	↑	↓	↑	↓

Table 4 – Summary of changes observed overall in 20 NSCLC samples.

	Down (%)	Up (%)	Unchanged (%)	Not detected (%)
EP1	10 (50%)	10 (50%)	–	–
EP2	13 (68%)	5 (26%)	1 (6%)	1
EP3	8 (40%)	9 (45%)	3 (15%)	–
EP4	9 (45%)	8 (40%)	3 (15%)	–

SAHA (Vorinostat®) (Fig. 6). We confirmed the initial observation that *EP2–4* expression can be induced or increased following the inhibition of histone deacetylases (Figs. 5 and 6). Though not statistically significant, we also confirmed the downregulation of *EP1* by treatment with SAHA (Fig. 6).

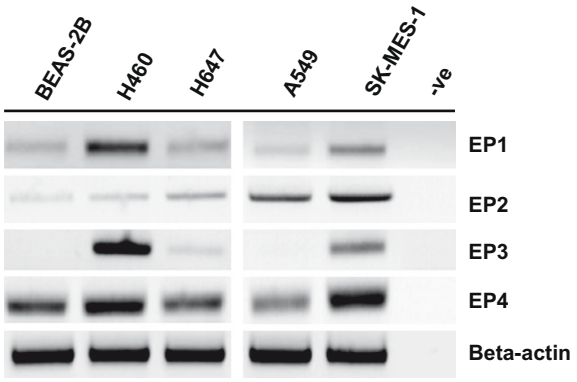

Fig. 2 – Expression of *EPs1–4* in cells derived from lung. RT-PCR analysis of *EPs1–4* expression in a panel of transformed lung and non-small cell lung cancer cell lines: Beas-2B (transformed normal human bronchoepithelial), H460 (large cell lung cancer), H647 (adenosquamous carcinoma), H1299 (adenocarcinoma), A549 (adenocarcinoma) and SK-MES-1 (squamous cell carcinoma). RT-PCR expression for *Beta-Actin* is included as a loading control. Expression of all *EP* isoforms could be observed in most cell lines.

3.5. *Regulation of EPs2–4 occurs through direct chromatin remodelling*

To confirm that the observed effects for HDACi were due to increased histone hyperacetylation at the promoters of the *EP* genes we carried out chromatin immunoprecipitation analysis of the individual *EP* promoters from A549 cells treated with TSA. As can be seen in Fig. 7, treatment with TSA results in an increase in the amount of PCR product for particular *EPs* indicating an increase in histone hyperacetylation around the promoters for the *EP* receptors. Using specific antibodies we show that lysine 9 and lysine 14 are hyperacetylated in this region following treatment with TSA, and clearly demonstrate that chromatin remodelling is directly involved with the activation of *EP2* (Fig. 6A), *EP3* (Fig. 6B) and *EP4* (Fig. 6C) gene expression. In addition, we also observed an increase in the histone H3 lysine 4 tri-methylation (H3K4me3) at the promoters of these genes (Fig. 7). This modification has also

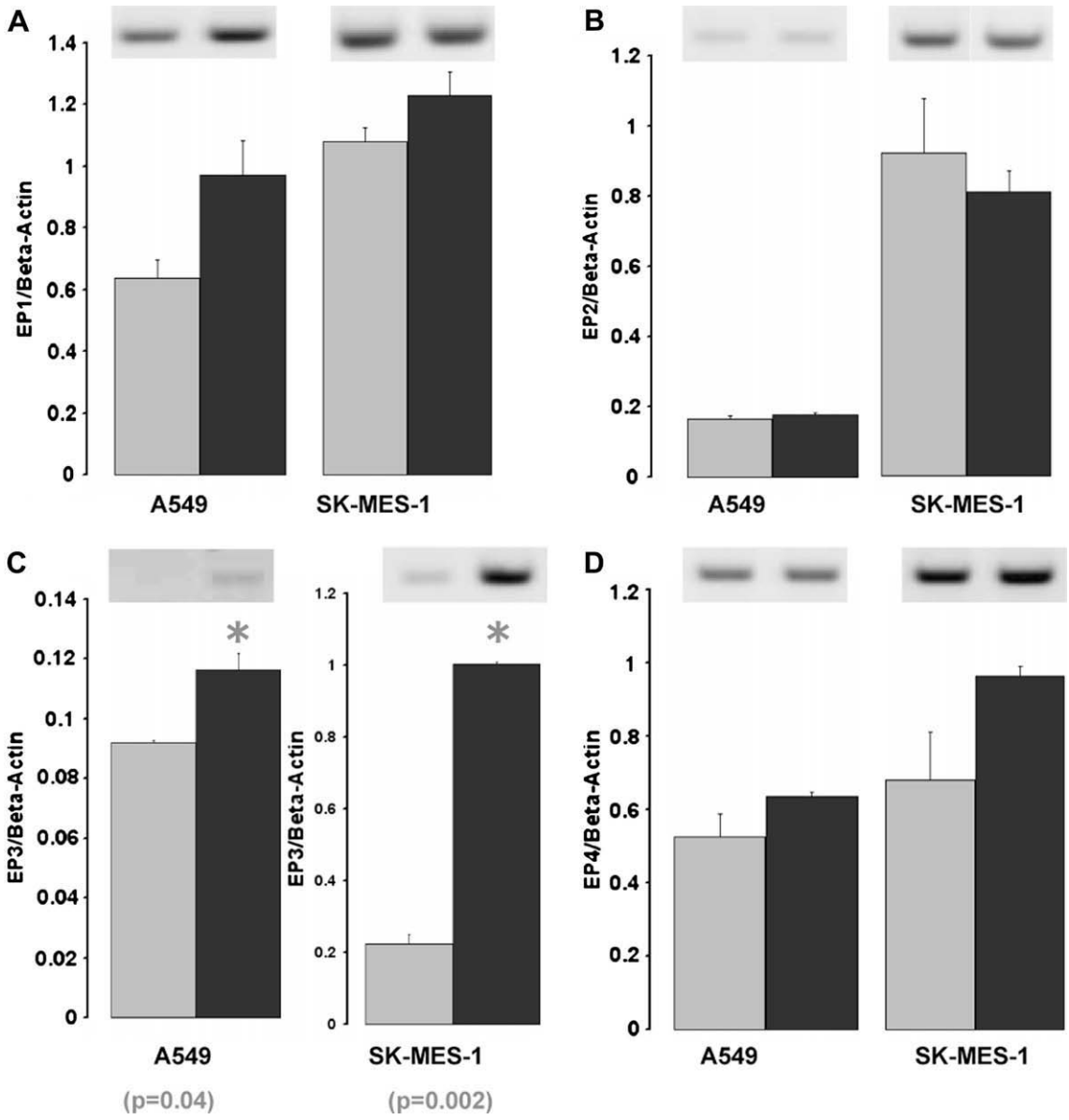

Fig. 3 – Epigenetic regulation of *EP* expression by DNA CpG methylation A549 cells were treated for 48 h in the presence or absence of 5 μM 5-Aza-2-deoxycytidine, a DNA methyltransferase inhibitor. Expression changes to *EP* mRNA were measured using RT-PCR. *Beta-Actin* is used as an internal control for quantification purposes. A representative figure showing the altered expression patterns observable in the samples is provided as follows: (A) *EP1*, (B) *EP2*, (C) *EP3* and (D) *EP4*. Analysis of expression changes in treated versus untreated cells was generated as arbitrary numbers when normalised to *Beta-Actin*. The results are shown as the mean ± standard error of the mean for A549 ($n = 3$), and SK-MES-1 ($n = 3$). Significant upregulation of *EP3* expression was observed in both cell lines. *p*-values were obtained using a Students *t*-tail test.

been associated with the activation of transcription,[20] and adds additional strength to the evidence that *EP* receptor expression is under dynamic regulation by chromatin remodelling.

3.6. *Cellular proliferation in NSCLC is affected by epigenetic therapies*

Using BrdU incorporation we assayed the effects of two of these epigenetic therapeutic agents SAHA and 5-Aza-2-deoxycytidine on cellular proliferation. Both drugs affected cellular proliferation in the squamous cell lung subtype SK-MES-1, while only SAHA affected the proliferation of the adenocarcinoma subtype A549 (Fig. 8).

4. Discussion

Much of our current understanding of *EP* receptors and cancer has come from mouse models of colon cancer. All four *EP* receptors have been implicated in playing roles in colon carcinogenesis, but variable results have been observed depending on the particular model used. A similar picture is emerging from the models of skin carcinogenesis.[5]

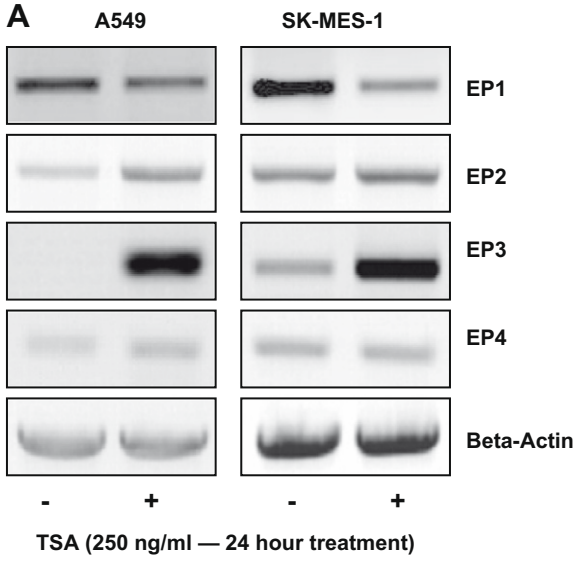

Fig. 4 – Screening for responses to histone deacetylase inhibition on *EP* expression. (A) A549 and SK-MES-1 cells were treated for 24 h in the presence or absence of trichostatin A (TSA), a histone deacetylase inhibitor at a final concentration of 250 ng/ml. Expression changes to *EP* mRNA were measured using RT-PCR. *Beta-Actin* is used as an internal loading control. A representative figure showing the altered expression patterns observable in the samples is shown.

We have summarised the overall effects of epigenetic targeting on *EP* receptors in NSCLC cell lines in Table 5. DNA methyltransferase inhibitors and histone deacetylase inhibitors were found to upregulate *EPs3 and 4*. While the effect of HDACi on *EPs2 and 4* would appear to be specific to A549 (adenocarcinoma), this may simply reflect the fact that SK-MES-1 (squamous cell carcinoma) has high endogenous levels of *EPs2 and 4*, and any effect on transcriptional activity is minor. If we consider the effects of the different therapies on cellular proliferation (Fig. 8), it is interesting to note that only the HDAC inhibitors affect adenocarcinoma proliferation while the squamous subtype is affected by both HDACi and DNA methyltransferase inhibitors at the concentrations used.

Strikingly, DNA methyltransferase inhibition resulted in an elevation of *EP1*, while HDAC inhibitors were shown to reduce *EP1*. This may have important implications for therapy as in *EP1* knockout models long-term melanoma tumour growth was found to be attenuated,[21] and colon cancer development is inhibited.[22,23] Selective antagonists of *EP1* have been shown to inhibit or protect against tumour formation in both colon and skin models.[5] In colon cancer, PGE_2 has recently been shown to increase the Fas ligand (FasL), a major inhibitor of the anti-tumour immune response, via *EP1*.[24] As we show that *EP1* is downregulated by histone deacetylase inhibitors in NSCLC cell lines (Figs. 4 and 6), this represents a means to target NSCLC by restoring the potential for anti-tumour immune responses and reducing pro-angiogenic activity. Indeed within the setting of lung adenocarcinoma cyclooxygenase-2 has been shown to affect lymphangiogenesis through the induction of *EP1*- and HER-2/Neu-dependent vascular endothelial growth factor-C upregulation.[25] In NSCLC *EP1* has been implicated as the receptor involved in PGE2-mediated activation of MAPK/ERK-induced cellular proliferation.[26] In this regard, HDACi may represent a better therapeutic option for targeting *EP1*. To functionally determine whether the downregulation of *EP1* may be of therapeutic importance, it will be necessary to target this receptor via siRNA or a selective antagonist such as ONO-8711 or ONO-8713 (Ono Pharmaceutical Group) or SC-19220 (Cayman chemicals) in a panel of NSCLC cell lines and assay cellular growth responses.

The initial observation that the epigenetic downregulation of *EP2* is important in tumourigenesis came from studies on neuroblastoma, where downregulation of this gene by DNA CpG methylation was observed to correlate with the progression of this disease.[15] The literature in this area is controversial as the presence of *EP2* has been implicated in both colon cancer and skin cancer, while its overexpression has been observed in breast and cervical cancer.[5] *EP2* –/– mice challenged with a Lewis lung carcinoma cell line had significantly attenuated tumour growth, lower tumour burden and longer survival.[3,27] Loss of *EP2* in human keratinocytes has also been shown to result in increased cellular invasiveness,[28] while in human gastric carcinoma activation of *EP2* and *EP4* induces cancer growth inhibition.[29] Ligands for PPARgamma such as rosaglitazone suppress *EP2* in human NSCLC cell lines resulting in the inhibition of cell growth.[30] The data from our analysis of patient samples found that *EP2* was downregulated in approximately 70% of tumours examined (Table 4), suggesting that the suppression of *EP2* expression is associated with NSCLC. In addition we find that the inhibitors of DNA CpG methylation do not reactivate or induce expression of *EP2* (Fig. 3), but find that the inhibitors of histone deacetylase can upregulate expression of this gene (Figs. 4–6), and remodel its promoter (Fig. 7), indicating that aberrant chromatin remodelling may be a factor in the downregulation of this gene in primary tumours. As such re-expressing *EP2* via epigenetic therapies may reduce both the growth and metastatic capacity of NSCLC. In lung cancer cells fibronectin has been shown to stimulate lung carcinoma cell proliferation via the induction of COX-2 expression with subsequent PGE(2) protein biosynthesis. PPARgamma ligands have been shown to inhibit this proliferative response via *EP2* (PMID: 14751245). As such if *EP2* can be reactivated in NSCLC via epigenetic therapy, it may be possible to utilise compounds such as rosiglitazone to enhance therapeutic benefit. If reactivation/re-expression of *EP2* is an important target for re-expression strategies in NSCLC as suggested above, it will be essential to generate NSCLC cell lines which stably overexpress *EP2*. From this one can test (a) whether the expression of *EP2* will reduce NSCLC cell line invasiveness as assayed by invasion assays and (b) whether or not it affects cellular proliferation (e.g. by MTT or BrdU proliferation assays). Furthermore, as PPARgamma ligands have been shown to inhibit proliferative responses via *EP2*, it may be possible to determine whether there may be synergy between PPARgamma ligands and *EP2* agonists such as butaprost or CP-533,536 (Pfizer) on NSCLC cell line cellular responses.

Loss of *EP3* expression has been associated with colon[5,12] and skin carcinogenesis.[5,31] Absent expression of *EP3* has also

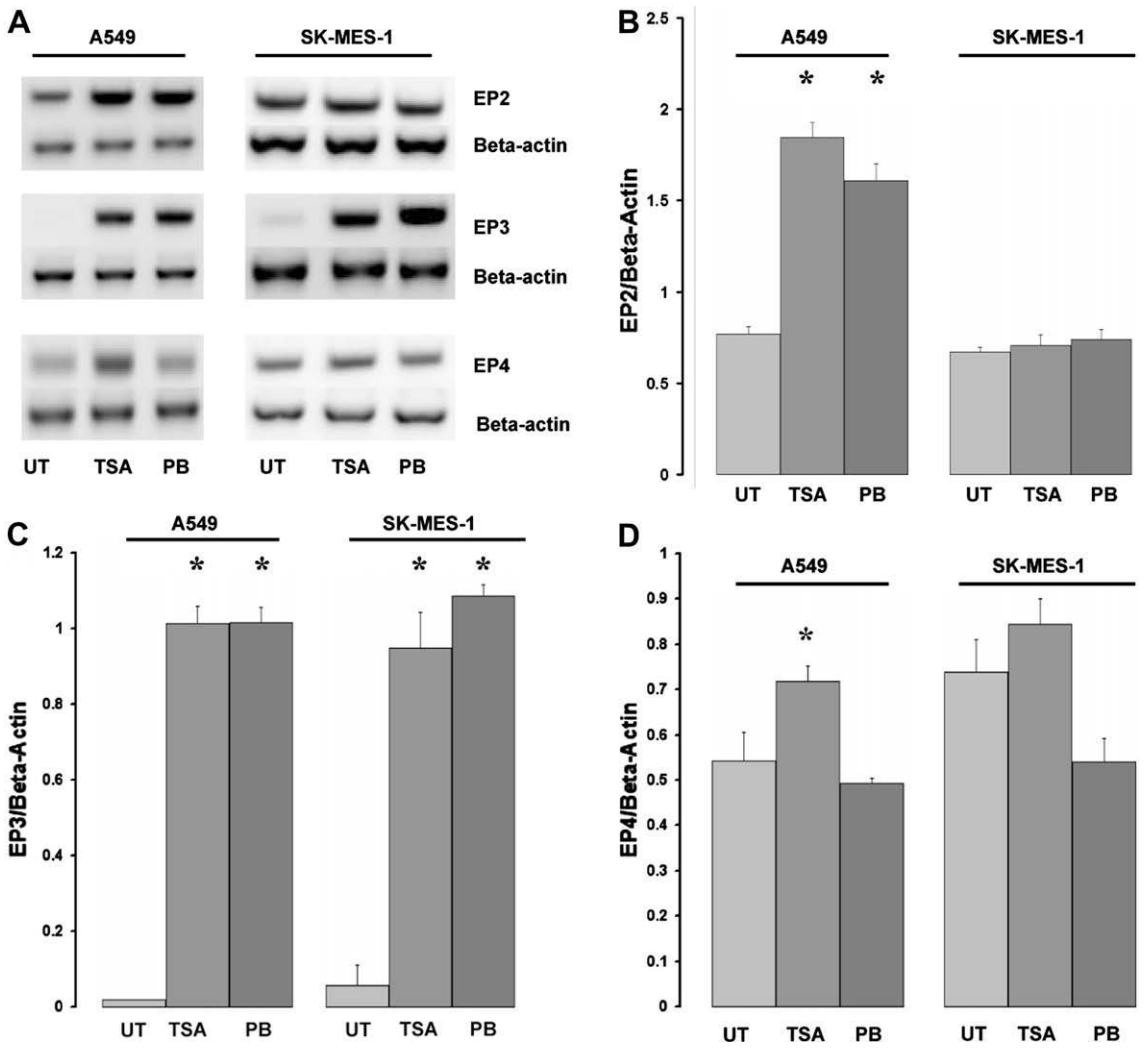

Fig. 5 – Effect of histone deacetylase inhibitors TSA and PB on *EP* expression. A549 and SK-MES-1 cells were treated with TSA (250 ng/ml) or PB (10 mM) for 24 h. Expression changes to *EP2–4* mRNA were measured using RT-PCR with *Beta-Actin* used as the internal control for quantification purposes. The figure shows the following: (A) representative RT-PCR products, and densitometric analysis of the responses to (B) *EP2*, (C) *EP3* and (D) *EP4* expression by histone deacetylase inhibition. The results are shown as the mean ± standard error of the mean ($n = 3$), and statistical significance was evaluated using a Student's *t*-tail test.

been observed in a Barrett's-derived oesophageal cell line,[13] while knockout *EP3* (–/–) mice have been shown to promote early onset of tumour growth.[21] Conversely, overexpression of *EP3* has been shown to reduce tumour cell proliferation and tumourigenesis *in vivo*, by inducing enhanced cell-cell contact and reducing cell proliferation *in vitro* in a Rho-dependent manner.[32] This makes *EP3* a good target for therapy in NSCLC. We have shown that *EP3* mRNA can be significantly induced following treatment with DNA methyltransferase inhibitors (Fig. 3c) and with histone deacetylase inhibitors (Figs. 4–6), including two FDA approved drugs phenylbutyrate (Fig. 5) and SAHA Fig. 6). One issue concerning the data regarding *EP3* expression is that this gene can generate 10 mRNA transcript variants that encode for eight protein isoforms, and which have different internalisation patterns.[33] As such individual isoforms may have differential roles in PGE_2-mediated responses. The primer set used by us for the mRNA analysis in this manuscript were designed to amplify from a region conserved across all *EP* mRNA variants. Therefore a more careful characterisation of which isoforms is expressed in NSCLC tumours and cell lines will be required to further delineate both the effects of epigenetic therapies targeting *EP3* expression and the molecular pathways which may subsequently be affected. As overexpression of *EP3* has been shown to reduce tumourigenic potential in mice, NSCLC cell lines overexpressing this gene should be produced and tested for (a) reduced proliferative and invasive capacity and (b) their ability to form tumours via clonogenic or soft agar assays.

In breast, colorectal and Lewis lung cancer models, selective antagonism or targeting of *EP4* has been shown to have a profound reduction in tumour metastasis and migration,[4,5,34,35] while fibronectin, a matrix glycoprotein highly expressed in tobacco-related lung disease has been shown to

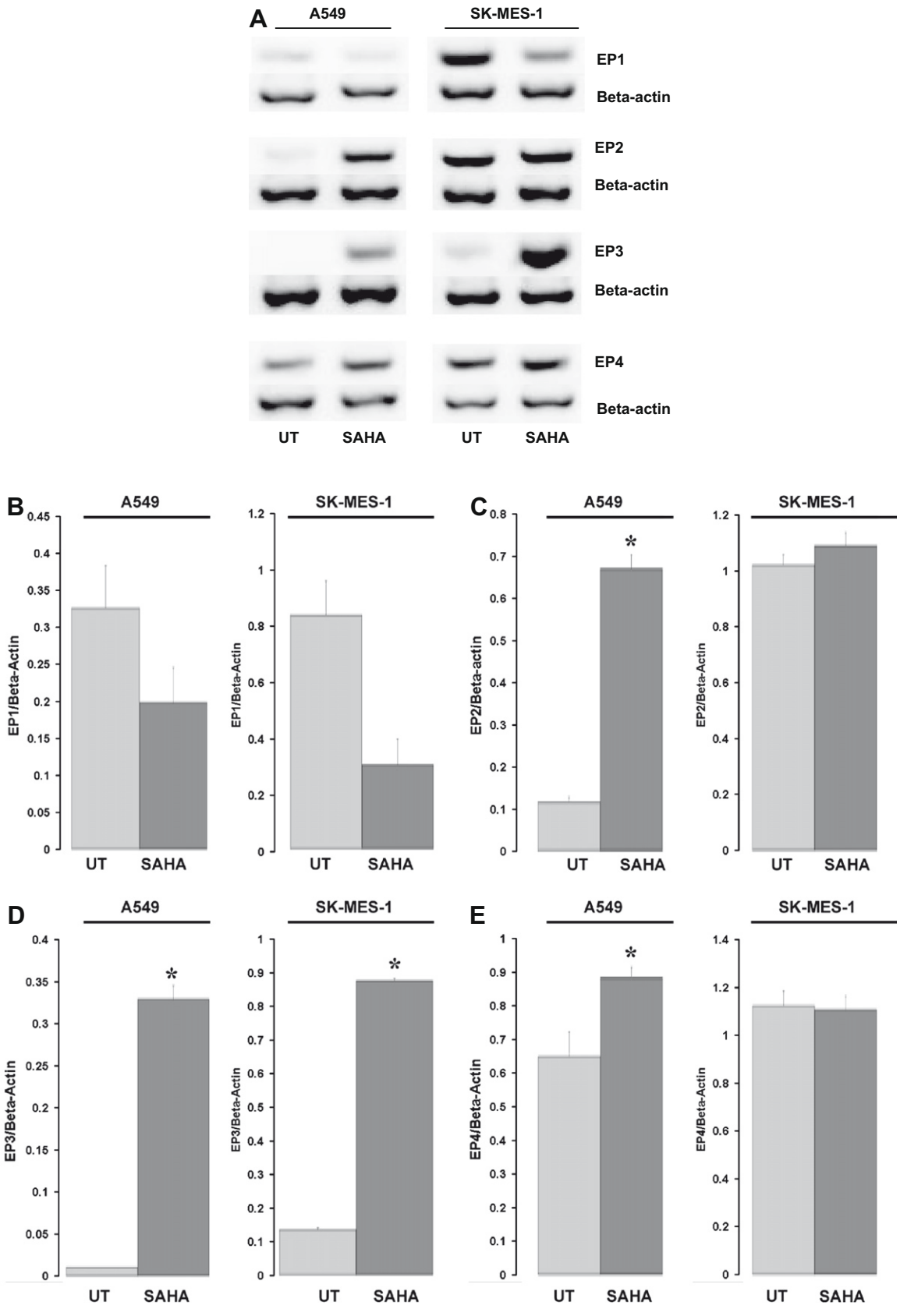

Fig. 6 – Effect of SAHA on *EP* expression. A549 and SK-MES-1 cells were treated with SAHA (5 μm) for 24 h. Expression changes to *EP1–4* mRNA were measured using RT-PCR with *Beta-Actin* used as the internal control for quantification purposes. The figure shows the following: (A) representative RT-PCR products, and densitometric analysis of the responses to (B) *EP1*, (C) *EP2*, (D) *EP3* and (E) *EP4* expression by histone deacetylase inhibition with Vorinostat. The results are shown as the mean ± standard error of the mean ($n = 3$), and statistical significance was evaluated using a one-tailed Student's *t*-tail test ($p < 0.05$).

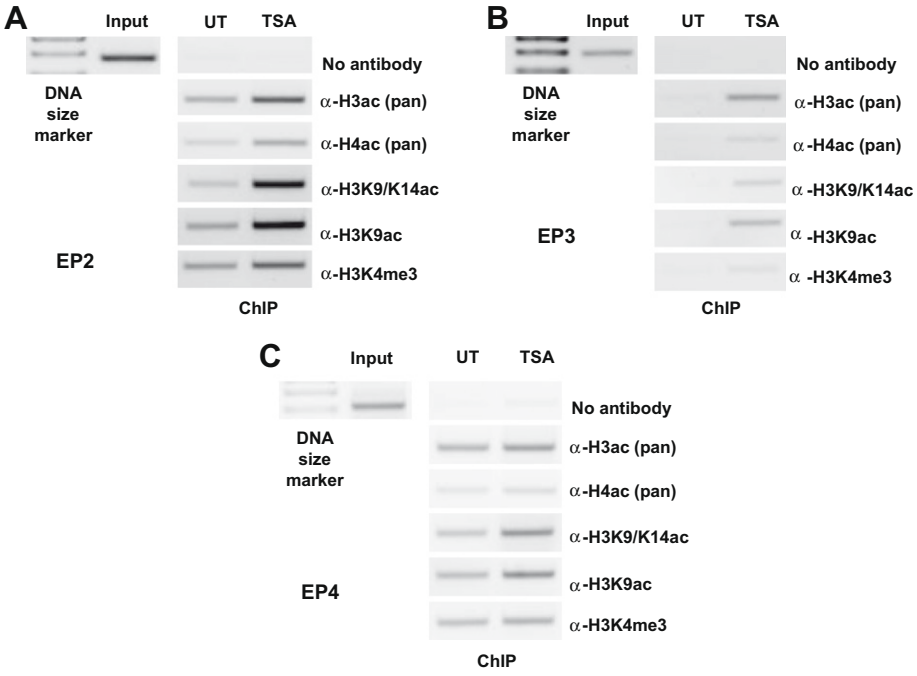

Fig. 7 – Histone acetylation occurs directly at the *EP2–4* promoters. The chromatin immunoprecipitation assay demonstrates increased histone H3 and H4 acetylation locally at the *EP* promoters as a consequence of TSA treatment. A549 cells were cultured in the presence or absence of 250 ng/ml nM TSA for 24 h. Subsequently, Chromatin Immunoprecipitation (ChIP) was performed with antibodies to (A) pan-acetylated Histone H3 (AcH3), (B) pan-acetylated Histone H4 (AcH4), (C) Histone H3 acetylated at lysines 9 and 14 (H3K9/K14ac) and (D) Histone H3 acetylated at lysine 9 (H3K9ac) and the status of chromatin assembled on the (A) *EP2*, (B) *EP3* and (C) *EP4* promoters were assayed by PCR as described under 'Section 2'. Input represents a positive control consisting of 1/10th of the original fixed chromatin prior to immunoprecipitation as recommended by the manufacturer (Diagenode). A no-antibody control was included to test for non-specific carriage of DNA with histones.

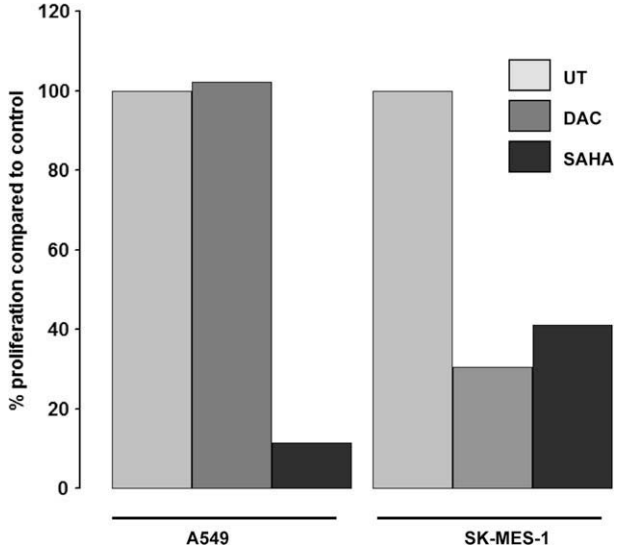

Fig. 8 – Effects of epigenetic therapies on cell line proliferation. The effects of 5-Aza-2-deoxycytidine (DAC – 5 μM, 48 h treatment) and Suberoylanilide hydroxamic acid (SAHA – 5 μM, 24 h treatment) on cellular proliferation were assayed by BrdU incorporation in A549 and SK-MES-1 cells (*n* = 2).

stimulate lung carcinoma growth, via *EP4*-mediated signalling.[36] Our results demonstrate that *EP4* can be affected by epigenetic therapies including DNA methyltransferase inhibitors (Fig. 3), and histone deacetylase inhibitors (Figs. 5 and 6). In addition, NSCLC cell lines expressing *EP4* should be treated with selective *EP4* inhibitors such as MF498 (Merck Frosst), CJ-023,423 (Pfizer) or AH23848 (Cayman chemicals) to see if they have effects on NSCLC cellular proliferation and invasiveness similar to those observed in breast.

Our results clearly demonstrate for the first time that the expression of the *EP* receptor family is commonly disrupted in NSCLC. Furthermore we demonstrate that these receptors are functionally regulated epigenetically, and may represent good targets for epigenetic therapies in the treatment of NSCLC. Taken together, the therapeutic benefit of epigenetic therapies targeting *EPs* in NSCLC may be multifactorial. Further experimental work will be necessary to

Table 5 – Summary of response to epigenetic therapy in NSCLC cell lines.

	DAC	HDi
EP1	↑	↓
EP2	–	↑[a]
EP3	↑	↑
EP4	↑	↑[a]
Effect on proliferation	None	Cell cycle arrest

[a] Effect observed in adenocarcinoma only.

delineate these possibilities. In addition, studies will be required to define which isoforms of *EP3* are expressed or altered in NSCLC, and studies will be required to determine if aberrant *EP* receptor expression correlates with prognosis in NSCLC.

Conflict of interest statement

None declared.

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