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IL-20 is epigenetically regulated in NSCLC and down regulates the expression of *VEGF*

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ABSTRACT

Background: IL-20 is a pleiotrophic member of the IL-10 family and plays a role in skin biology and the development of haematopoietic cells. Recently, IL-20 has been demonstrated to have potential anti-angiogenic effects in non-small cell lung cancer (NSCLC) by down regulating COX-2.

Methods: The expression of *IL-20* and its cognate receptors (*IL-20RA/B* and *IL-22R1*) was examined in a series of resected fresh frozen NSCLC tumours. Additionally, the expression and epigenetic regulation of this family was examined in normal bronchial epithelial and NSCLC cell lines. Furthermore, the effect of IL-20 on VEGF family members was examined.

Results: The expression of *IL-20* and its receptors are frequently dysregulated in NSCLC. *IL-20RB* mRNA was significantly elevated in NSCLC tumours ($p < 0.01$). Protein levels of the receptors, IL-20RB and IL-22R1, were significantly increased ($p < 0.01$) in the tumours of NSCLC patients. IL-20 and its receptors were found to be epigenetically regulated through histone post-translational modifications and DNA CpG residue methylation. In addition, treatment with recombinant IL-20 resulted in decreased expression of the VEGF family members at the mRNA level.

Conclusions: This family of genes are dysregulated in NSCLC and are subject to epigenetic regulation. Whilst the anti-angiogenic properties of IL-20 require further clarification, targeting this family via epigenetic means may be a viable therapeutic option in lung cancer treatment.

1. Introduction

IL-20 was initially identified in a human keratinocyte library.[1] It is a pleiotrophic member of the extended IL-10 cytokine family, including IL-19, 22, 24, 26, 28 and 29, all of which have well defined immune functions.[2,3] IL-20 is a pro-inflammatory cytokine, playing key roles in skin diseases, particularly psoriasis,[4,5] and has been implicated in arthritis, atherosclerosis and lupus nephritis,[6–9] and treatment with an anti-IL-20 antibody produced a therapeutic benefit in arthritis.[10] Lipopolysaccharide (LPS) induced IL-20 expression in glial cells suggests that IL-20 may also play a role in inflammatory conditions in the brain.[11]

IL-20 is expressed in a wide variety of cell types, particularly in lung and skin,[4,7] as well as bronchial epithelial cells.[7,12] In addition, expression of IL-20 has been found in breast[13] and occurs in cells of the immune system such as monocytes, T cells and maturing dendritic cells.[14,15]

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IL-20 signals through a hetero-dimeric receptor complex, which consists of IL-20RB, combined with either IL-20RA (Type 1 receptor complex), or IL-22R1 (Type 2 receptor complex).[7,16,17] The expression pattern of the IL-20 receptors indicate that cells in the skin, lung and reproductive organs are targets of IL-20 and other members of the extended IL-10 family.[1,7] IL-20 signalling has been shown to induce cellular proliferation and activate STAT3 through both receptor complexes.[1,16,17]

IL-20 may also have either pro- or anti- angiogenic properties, but this would appear somewhat cell line and *in vivo*/*in vitro* dependant.[18,19] However, it has recently been shown to demonstrate anti-angiogenic properties in NSCLC (non-small cell lung cancer).[18]

Inflammation plays a role in lung carcinogenesis.[20,21] As IL-20 has such a significant role in inflammatory skin biology, this suggests that IL-20 may be important in lung disease. Evidence has emerged linking decreased expression of IL-20RA via DNA CpG hypermethylation[22] with poorer disease free survival in NSCLC.[23] This suggests that the IL-20 signalling pathway may be important to NSCLC pathogenesis and could potentially be amenable to epigenetic targeting therapies.

We examined the expression of IL-20 and its receptors in a series of primary NSCLC tumour samples and an additional panel of normal and NSCLC cell lines, and tested whether epigenetic mechanisms play a role in their regulation. Our results demonstrate that the expression of IL-20 and its receptors are; frequently dysregulated in NSCLC; regulated via epigenetic mechanisms (DNA CpG methylation and histone post-translational modifications); and may represent a candidate anti-angiogenic therapeutic approach in treating NSCLC as IL-20 treatment results in the down regulation of VEGF family members.

2. Methods

2.1. Cell lines

The A549 (adenocarcinoma), SK-MES-1 (squamous cell carcinoma), H460, H647 and H1299 (large cell carcinoma) and BEAS2B (transformed normal bronchoepithelial) cell lines were purchased from the ATCC (LGC Promochem, Teddington, United Kingdom). HBEC cell lines[24] were a gift from Prof. John D Minna (Hamon Centre for Therapeutic Oncology Research, UTSoutwestern, Dallas, TX, United States of America). All cell culture reagents were purchased from Lonza (Walkersville, MD, USA) unless stated otherwise. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the following media; A549 – F-12 (Ham) medium supplemented with 10% (v/v) FBS, penicillin streptomycin (500 U/mL) and 2 mM L-glutamine. BEAS2B were also maintained in F-12 (Ham) medium without the addition of FBS. SK-MES-1 – EMEM with the addition of 10% (v/v) FBS, penicillin streptomycin (500 U/mL), 2 mM L-glutamine and 0.1 M non-essential amino acids. All large cell carcinoma lines were maintained in RPMI with 10% FBS and penicillin streptomycin (500 U/mL). HBEC lines were maintained in Keratinocyte serum-free media (SFM), with L-glutamine (GIBCO Invitrogen, Paisley, Scotland) and supplemented with 2.5 μg human recombinant epidermal growth factor (rEGF), and 25 μg bovine pituitary extract (GIBCO Invitrogen).

2.2. Primary tumour samples

Eighteen tumour specimens (8 adenocarcinoma, 10 squamous cell carcinoma) were taken from surgical resections of chemotherapy naïve patients presenting with Stages I and II NSCLC at St. James's Hospital, Dublin. Matched normal tissue was taken in parallel for each patient and samples were evaluated by a pathologist immediately following dissection. Informed consent was obtained from each patient, and the study was conducted after formal approval from the SJH/AMNCH Hospital Ethics Committee.

2.3. Reagents

Trichostatin A (TSA) was purchased from Calbiochem (San Diego, CA, USA) and dissolved in DMSO to a concentration of 250 mg/mL. Cell cultures were treated for a period of 16 h, at a final concentration of 250 ng/mL.

Phenylbutyrate (PB) (Tributyrate™) was a gift from Triple Crown America, Perkasie, PA, USA. Cell cultures were treated at a final concentration of 10 mM for 16 h.

5-Aza-2′-deoxycytidine (DAC) was purchased from Merck (Darmstadt, Germany) and dissolved in methanol. Cell cultures were treated with DAC (final concentration – 1 μM) for 48 h with DAC and media replaced every 24 h.

Recombinant human IL-20, IL-19 and IL-24 were purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel) and reconstituted in sterile distilled water.

2.4. RNA isolation and RT-PCR amplification

Total RNA was extracted using TRI reagent® (Molecular Research Center, Montgomery Road, OH, USA) according to manufacturer's instructions. Prior to first strand cDNA synthesis, 10 μg of total RNA was pre-treated by digestion with RQ1 DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. cDNA was generated using Superscript III (Invitrogen Corp., Carlsbad, CA, USA) and Oligo dT(20) primers (Eurofins MWG Operon, Ebersberg, Germany) according to the manufacturer's instructions.

Cell lines were examined for the expression of *IL-20*, *IL-20RA/B*, *IL-22R1* and *beta-actin* by RT-PCR, using primers, annealing temperatures and cycling conditions outlined in Table 1.

Experiments were carried out in triplicate and products electrophoresed on a 1% agarose gel. Product quantification was performed using TINA 2.09c (Raytest, Isotopenmeßgeräte GmbH, Straubenhardt, Germany) densitometry software. The mRNA expression was normalised to Beta-actin controls, and was expressed as a ratio of target mRNA expression: *beta-actin* expression.

2.5. Western blot analysis

Protein lysates were extracted from cell cultures using RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA (ethylene diamine tetra-acetic acid), 1% (v/v) Triton-X 100,

Table 1 – Primers and annealing temperatures for RT-PCR.

Primer name	Sequence	Annealing temp (°C)
IL-20 (526 bp)	F: 5′ ATGAAAGCCTCTAGTCTTGC 3′ R: 5′ CTGTCTCCTCCATCCATTGC 3′	58
lL-20RA (392 bp)	F: 5′ TCAAACAGAACGTGGTCCCAGTG 3′ R: 5′ TCCGAGATATTGAGGGTGATAAAG 3′	64
IL-20RB (406 bp)	F: 5′ GCTGGTGCTCACTCACTGAAGGT 3′ R: 5′ TCTGTCTGGCTGAAGGCGCTGTA 3′	64
IL-22R1 (354 bp)	F: 5′ CTCCACAGCGGCATAGCCT 3′ R: 5′ ACATGCAGCTTCCAGCTGG 3′	61
Beta-actin (510 bp)	F: 5′ TGTTTGAGACCTTCAACACCC 3′ R: 5′ AGCACTGTGTTGGCGTACAG 3′	55

PCR cycling conditions consisted of: IL-20: 95 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at target annealing temperature and 1 min at 72 °C with a final extension at 72 °C for 10 min.
IL-20RA/B: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 64 °C for 1 min, 72 °C for 2 min, with a final extension at 72 °C for 5 min.
IL-22R1: 35 cycles of 95 °C for 45 s, 61 °C of 45 s and 72 °C for 45 s.

0.1% (w/v) SDS), supplemented with 10 μL phenylmethylsulfonyl fluoride (87 mg/mL in 96% EtOH), and 100 μL protease inhibitor cocktail (2 mM AEBSF, 1 mM EDTA, 130 μM Bestatin, 14 μM E-64, 1 μM Leupepin, 0.3 μM Aprotinin). Primary tissue protein lysates were also extracted from the 18 NSCLC specimens with corresponding matched normal lung tissue from the same individual using TRI reagent® according to the manufacturer's instructions. Lysates were separated by SDS/PAGE and subsequently transferred onto a pre-activated polyvinylidene fluoride nitrocellulose membrane (PVDF). Membranes were blocked for 1 h at RT in TBST (10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 0.1% (v/v) Tween 20) containing 5% non-fat dry milk powder. Membranes were immunoblotted over night at 4 °C in TBST with 5% non-fat dry milk powder with IL-20 (2 μg/mL), IL-20RA (2 μg/mL), IL-20RB (0.4 μg/mL), or IL-22R1 (2 μg/mL) (R&D systems, Minneapolis, MN, USA) as appropriate. All secondary antibodies were HRP labelled and bound antibody complexes were detected by the Supersignal West Pico Chemiluminescent kit (Pierce, Rockford, IL, USA). Protein expression was normalised to Beta-actin (Merck Biosciences, Nottingham UK), and was expressed as a ratio of target protein expression: Beta-actin expression. Analysis was performed using TINA 2.09c software.

2.6. ELISA (enzyme-linked immunosorbent assay)

Levels of IL-20 in conditioned media were measured using an ELISA (Strathmann Biotech, Hamburg, Germany), according to the manufacturer's instructions.

2.7. 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, Billerica, MA, USA) and sonicated until DNA was fragmented into lengths of between 200 and 1000 bp. Aliquots of this sheared DNA were subsequently immunoprecipitated using the OneDay ChIP Kit™ (Diagenode, Liege, Belgium) according to the manufacturer's instructions.

The antibodies used for immunoprecipitation were as follows: pan acetyl-histone H3 (AcH3) (Millipore Cat#06-599), pan acetyl histone H4 (AcH4) (Millipore Cat# 06-598), acetyl-histone H3 (K9/14ac) (Diagenode Cat# pAb-ACHBHS-044), acetyl-histone H3 (K9ac) (Diagenode Cat# pAb-ACHAHS-044), tri acetyl-histone H3 (H3K9Me3) (Diagenode Cat# pAb-003-024), acetyl phospho-histone H3 (K9pS10) (Sigma Cat# H0788), di methyl-histone H3 (K9Me2) (Sigma Cat# D5567), di methyl histone H3 (K4Me2) (Sigma Cat# D5692) and methyl-histone H3(K4Me) (Sigma Cat# M4819). A no antibody control was included to test for non-specific binding.

Primers used to study the promoter region of *IL-20* by ChIP were designed to incorporate the known 5′ UTR contained within the nucleotide sequence NM_018724.3 aligned with the human genome. Primers and annealing temperatures for ChIP are presented in Table 2.

2.8. Cellular proliferation assays

Cell proliferation was measured using a Cell Proliferation BrdU ELISA (Roche Diagnostics Ltd., Sussex, UK). Briefly, cells were seeded at 5×10^3/well in a 96-well plate and adhered overnight. Subsequently the complete media were removed and the cells washed with 100 μL PBS. Serum depleted media (0.5% FBS) were added to the lung cancer cells only (HBEC media does not contain serum), as this mimics more closely physiological conditions. Following overnight incubation,

Table 2 – Primers and annealing temperatures for ChIP.

Primer name	Sequence	Annealing temp (°C)
IL-20	F: 5′ CCTCACCCCGT GGACACTTGG 3′ R: 5′ CAGTCCAGTGG AAGGAGTCC 3′	60

PCR cycling conditions consisted of 95 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C with a final extension at 72 °C for 10 min.

cells were treated for 24 h with either human recombinant IL-20 (200 ng/mL), IL-19 (100 ng/mL) or IL-24 (100 ng/mL). Absorbance was measured on a plate reader at 450 nm with a reference wavelength set to 690 nm and untreated wells were used for normalisation purposes.

2.9. Statistical analysis

The data are expressed as mean ± SEM. Statistical analysis was performed with InStat (Graphpad software, La Jolla, CA, USA) using a paired one tailed Student's *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Gene expression analysis of IL-20, IL-20RA/B and IL-22R1 in primary lung cancer tumour specimens

RT-PCR was performed to assess the expression of IL-20 family members in a panel of normal/tumour matched chemonaïve patient samples (Fig. 1A). Densitometric analysis of the gels revealed a significant increase in the expression of IL-20RB in NSCLC tumour samples compared with normal ($p < 0.01$) (Fig. 1B). Overall, increased gene expression was observed for *IL-20* (10/18, 55.5%), *IL-20RB* (14/18, 77.7% – $p < 0.01$) and *IL-22R1* (13/18, 72.2%), whilst the *IL-20RA* receptor had reduced expression in the tumour compared to normal (11/18, 61.1%) as summarised in Table 3.

Table 3 – Summary of mRNA expression in 18 NSCLC samples.

Gene	Down (%)	Up (%)	ND (%)
IL-20	5 (27.7)	10 (55.5)	3 (16.6)
IL-20RA	11 (61.1)	7 (38.8)	–
IL-20RB	3 (16.6)	14 (77.7)	1 (5.5)
IL-22R1	5 (27.7)	13 (72.2)	–

ND – not detected.

3.2. Protein expression of IL-20, IL-20RA/B and IL-22R1 in primary lung cancer tumour specimens

The protein levels of the IL-20 family members were determined in the same patient samples by Western Blot analysis. Representative images are shown in Fig. 2A. Densitometric analysis revealed an increase in the expression of IL-20RB ($p < 0.01$) and IL-22R1 ($p < 0.01$) in NSCLC tumour samples compared with normal (Fig. 2B). The average expression values of IL-20RA differed from that observed at the mRNA level, with an increase observed at the protein level (tumour versus normal). IL-20 was not detected in either normal or tumour samples, however, a positive control using recombinant IL-20 demonstrated the efficacy of the IL-20 antibody (data not shown). The overall results are summarised in Table 4.

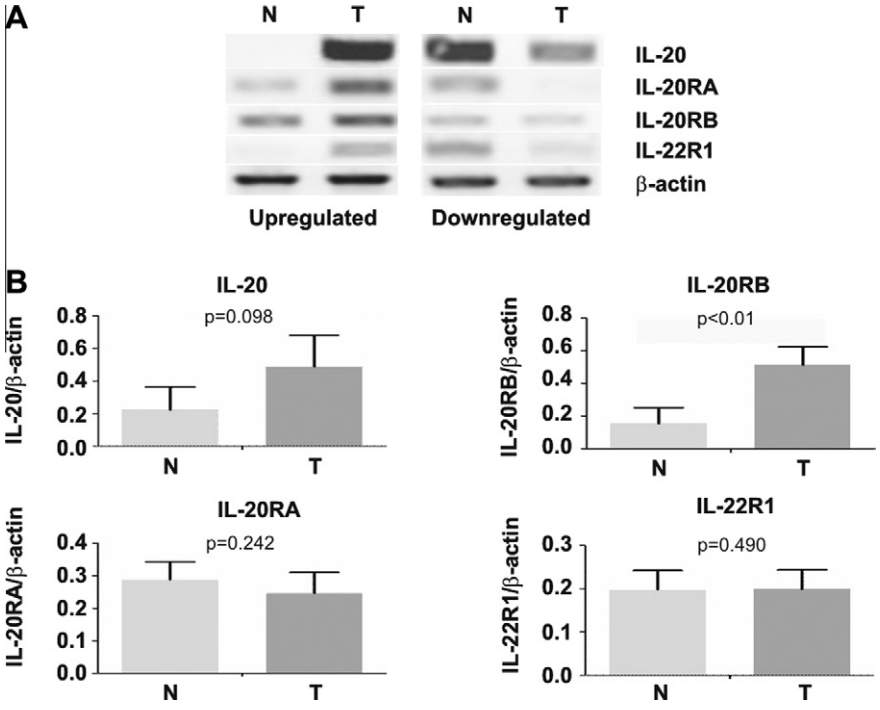

Fig. 1 – IL-20 and its cognate receptor mRNA expression in normal/tumour NSCLC matched pairs (A) Levels of *IL-20*, *IL-20RA/B* and *IL-22R1* were examined by RT-PCR on a panel of 18 NSCLC (adenocarcinoma ($n = 8$) and squamous cell carcinoma ($n = 10$)) patient samples. Representative images of up and down regulated samples are shown. (B) Overall densitometry analyses of tumour/normal matched patient NSCLC samples. *Beta-actin* levels were used for normalisation purposes. Data are expressed as mean ± SEM ($n = 18$). Statistical analysis was performed using a paired one tail Student's t test (N – normal, T – tumour).

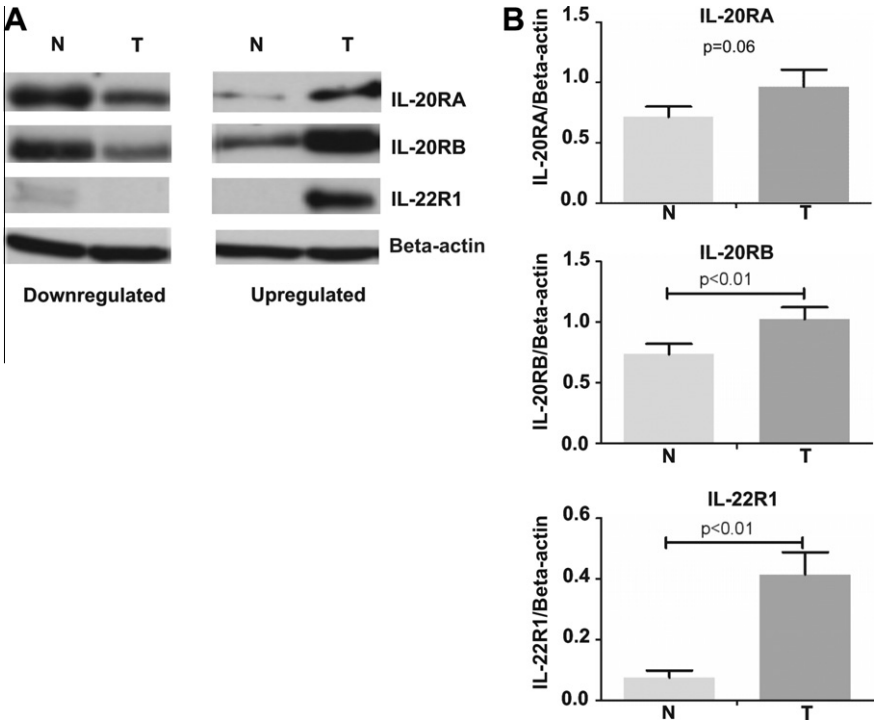

Fig. 2 – Protein expression of IL-20 and its receptors in normal/tumour NSCLC matched pairs. (A) Levels of IL-20, IL-20RA/B and IL-22R1 were examined by Western Blot on a panel of adenocarcinoma ($n = 8$) and squamous cell carcinoma ($n = 10$) patient samples. Representative images of samples *demonstrating* up- or down- regulation of IL-20 and its cognate receptors expression in normal/tumour NSCLC matched pairs are shown. There was no IL-20 detected in any patient sample and there were no down regulated IL-22R1 squamous cell carcinoma samples. (B) Densitometric analysis of IL-20RA/B and IL-22R1 on a panel of adenocarcinoma and squamous cell carcinoma patient samples. Beta actin levels were used for normalisation purposes. Data are expressed as mean ± SEM. Statistical analysis was performed using a paired one tail Student's *t* test. ($n = 18$).

Table 4 – Summary of protein expression for IL-20 family members in NSCLC patients.

Protein	Down (%)	Up (%)	ND (%)
IL-20	–	–	18 (100)
IL-20RA	6 (33.3)	12 (66.6)	–
IL-20RB	3 (16.6)	15 (83.3)	–
IL-22R1	1 (5.5)	15 (83.3)	2 (11.1)

ND – Not detected.

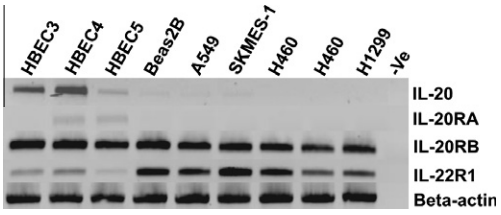

Fig. 3 – Expression of the *IL-20* family in a panel of normal and lung cancer cell lines. The panel examined included A549 (adenocarcinoma), SK-MES-1 (squamous cell carcinoma), H460, H647 and H1299 (large neuro-endocrine), BEAS-2B (SV40 transformed normal bronchoepithelial) and HBEC cell lines (normal bronchial epithelial cell lines immortalised in the absence of viral oncoproteins).

3.3. *Expression of IL-20, IL-20RA/B and IL-22R1 in a panel of normal and lung cancer cell lines*

Utilising RT-PCR, the IL-20 family members were examined in a panel of normal and NSCLC cell lines (Fig. 3). All cell lines tested expressed high levels of *IL-20RB*. Levels of *IL-22R1* were decreased in the normal (HBEC) cell lines compared with the NSCLC cell lines, conversely *IL-20RA* expression was only observed in HBEC4/5. Additionally, IL-20 could only be robustly detected in the HBEC cell lines, however, a small basal level of mRNA could be observed in some of the NSCLC cell lines, namely A549 and SK-MES-1 (Fig. 3).

3.4. *Histone acetylation is involved in the regulation of IL-20 family member expression*

Using the histone deacetylase inhibitor (HDACi), Trichostatin A (TSA), an induction of *IL-20* was observed in both the normal and lung cancer cell lines (Fig. 4A) and was significant across all cell lines (HBEC4/SK-MES-1 – $p < 0.05$, A549 – $p < 0.01$) (Fig. 4B). TSA was unable to reactivate *IL-20RA*

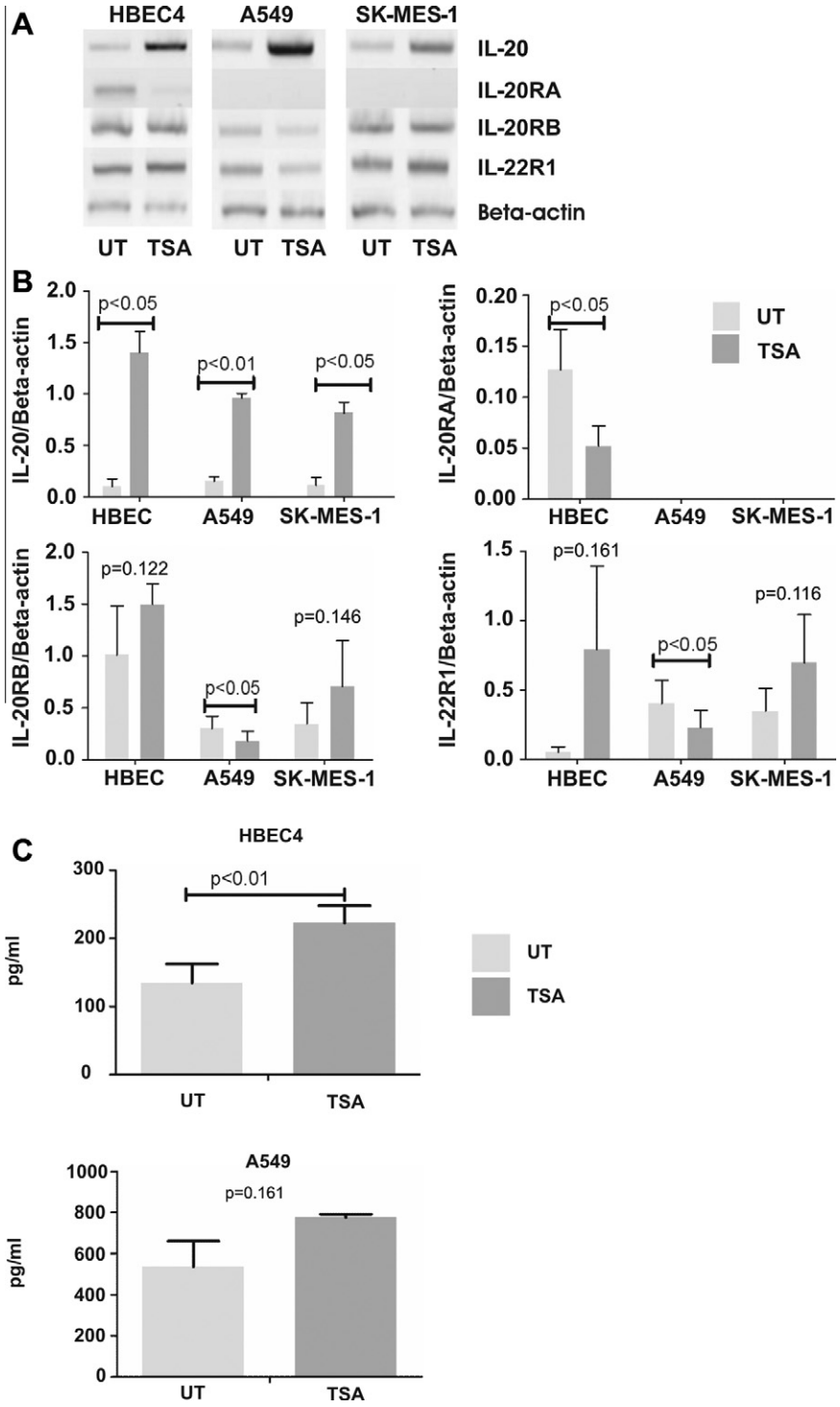

Fig. 4 – Cell line response to histone deacetylase inhibition. (A) The effect of TSA treatment (250 ng/mL for 16 h) on the expression of *IL-20*, *IL-20RA/B* and *IL-22R1*. (B) Densitometry analysis of expression in treated versus untreated samples when normalised to *Beta-actin*. Data are graphed as mean ± SEM ($n = 3$). (C) Treatment with TSA also effects the production of IL-20 at protein level in HBEC4 and A549 cells. IL-20 was quantified in conditioned media removed from culture after exposure to TSA (250 ng/mL for 16 h). Data are graphed as mean ± SEM ($n = 3$) (UT – untreated, TSA – trichostatin A).

expression in the NSCLC lines, but significantly reduced *IL-20RA* expression in HBEC4 ($p < 0.05$) (Fig. 4B). Overall, expression of *IL-20RB*, or *IL-22R1* was not affected by TSA in HBEC4 and SK-MES-1 (Fig. 4B), however, both receptors were significantly down regulated by TSA in the adenocarcinoma cell line (A549 – $p < 0.05$; Fig. 4B). The induction of *IL-20* expression indicates that this gene is epigenetically regulated at the level of histone acetylation, and treatment of cell lines with an

additional histone deacetylase inhibitor, phenylbutyrate (PB), confirmed that the induction of *IL-20* was an HDACi specific effect (data not shown). Levels of IL-20 in the media were subsequently measured by ELISA, and increased IL-20 protein was observed in the supernatant of cells treated with TSA in HBEC4 ($p < 0.01$), and A549 (Fig. 4C). Although IL-20 was detected in the untreated SK-MES-1 cell line, there was little difference observed in levels of the protein post TSA treatment (data not shown).

3.5. *Regulation of IL-20 occurs through direct chromatin remodelling*

To confirm that the observed effects for HDACi were due to increased histone hyperacetylation at the promoter of the *IL-20* gene, we carried out chromatin immunoprecipitation (ChIP) analysis of the promoter from A549 cells treated with TSA. As shown in Fig. 5, treatment with TSA results in an increase in the amount of PCR product for *IL-20* indicating an increase in histone hyperacetylation around the promoter region of this gene. We show that lysines in both histone H3 and histone H4 become hyperacetylated in this region following treatment with TSA (Fig. 5), and demonstrate that chromatin remodelling is directly involved with the activation of *IL-20* gene expression. In addition, we also observed an increase in histone H3 lysine 4 tri- (H3K4Me3) and di- methylation (H3K4me3) markers (Fig. 5). These modifications have been associated with the activation of transcription, and adds additional strength to the evidence that *IL-20* is dynamically regulated by histone post-translational modifications. Other post-translational modifications of histones which occur in the IL-20 promoter following HDACi treatment include decreased levels of histone H3 lysine 4 monomethylation (H3K4me) and histone H3 lysine 9 di-methylation (H3K9me2) (Fig. 5).

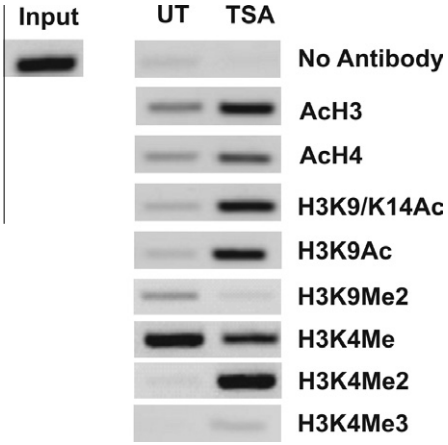

Fig. 5 – Chromatin remodelling within the promoter region of *IL-20* in response to HDACi. The ChIP assay demonstrates that HDACi treatment results in changes to the acetylation and methylation status of histones H3 and H4. A549 cells were cultured in the presence or absence of TSA (250 ng/mL) for a period of 16 h, and a ChIP assay performed using the following antibodies; pan acetyl-histone H3, pan acetyl histone H4, acetyl-histone H3 (K9/14ac), acetyl-histone H3 (K9ac), tri methyl-histone H3 (H3K9Me3), acetyl phospho-histone H3 (K9pS10) (Sigma Cat# H0788), di methyl-histone H3 (K9Me2), di methyl histone H3 (K4Me2) and methyl-histone H3(K4Me). Input DNA serves as a positive control as recommended by the manufacturer (Diagenode).

3.6. *Methylation is also involved in the regulation of the IL-20 family*

As the IL-20RA receptor has been shown to be downregulated in NSCLC by DNA CpG methylation,[22] cell lines were treated with 5-aza-2′-deoxycytidine (DAC) and its effects on IL-20 and its cognate receptors gene expression were examined using RT-PCR (Fig. 6A). DAC treatment significantly reduced the expression of; *IL-20RB* ($p < 0.05$) in HBEC4, *IL-20RB* ($p < 0.05$) and *IL-22R1* ($p < 0.01$) in SK-MES-1 (Fig. 6B) and was able to reactivate *IL-20RA* expression in A549 cells ($p < 0.01$). In all cell lines, DAC treatment increased the expression of IL-20 (Fig. 6B). *IL-20RA* has previously been demonstrated to be down regulated in NSCLC by DNA CpG methylation[22] and our study supports this finding as DAC reactivated IL-20RA expression in the adenocarcinoma cell line ($p < 0.01$).

3.7. *Cellular proliferation of SKMES-1 is increased in the presence of recombinant IL-20, whilst HBEC4 is unchanged*

There is extensive receptor sharing within the newer IL-10 family members.[16] IL-24 can signal through both IL-20 receptor complexes (IL-20RA/B and IL-20RB/IL-22R1) and IL-19 through IL-20RA/IL-20RB.[16] We examined which receptor sub types are utilised in our panel of cell lines and if treatment with recombinant protein could promote cellular proliferation; IL-20 (200 ng/mL), IL-19 (100 ng/mL) and IL-24 (100 ng/mL) for a period of 24 h and proliferation measured with a BrdU ELISA (Fig. 7). None of the three cytokines significantly changed the proliferative capacity of HBEC4. Although, IL-19 increased proliferation in the A549 cell line (IL-19 versus UT – 105.32 ± 0.73% versus 100 ± 0%, $p < 0.05$) it is possibly not biologically relevant. All three cytokines resulted in enhanced proliferation in the SK-MES-1 cell line (IL-19 – $p < 0.01$, IL-24/IL-20 – $p < 0.05$). These results would indicate cytokine effects are cell line dependant and may not drive cellular proliferation in certain subtypes.

3.8. *IL-20 reduces mRNA levels of VEGF, Neuropilin-1 and KDR in normal and NSCLC cell lines*

IL-20 has previously been implicated in reducing angiogenesis in NSCLC through the down regulation of COX-2.[18] COX-2 contributes to increased VEGF expression in NSCLC[25] and levels of VEGF have also been linked to poor prognosis in patients with this disease.[26] We, therefore, examined the effect of recombinant IL-20 on the gene expression of members of the VEGF signalling family. Three cell lines (A549, SK-MES-1 and HBEC4) were treated with recombinant IL-20 (200 ng/mL) for 24 h and levels of VEGF, Flt-1 (VEGFR1), KDR (VEGFR2), NP-1 were examined at the mRNA level (Fig. 8A). Densitometry analysis revealed (Fig. 8B) that IL-20 treatment resulted in a decrease in the expression of VEGF in the normal ($p < 0.05$) and lung cancer cell lines (A549 – $p < 0.05$). Overall, there was a decrease in receptor expression observed in all

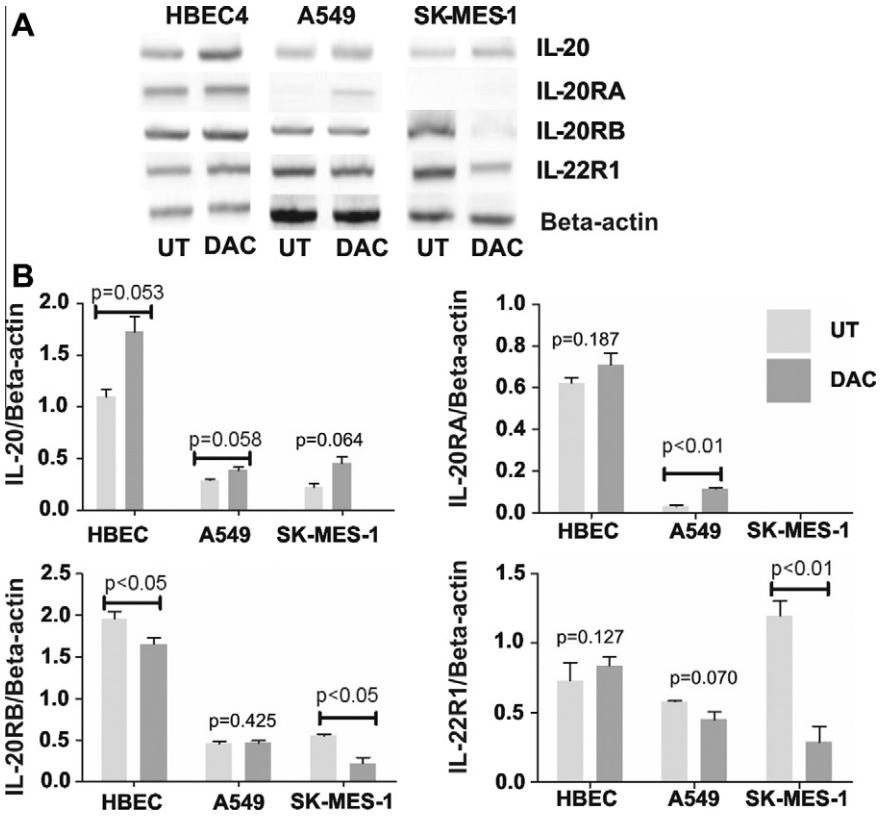

Fig. 6 – Cell line response to a DNA methyltransferase inhibitor (DNMTi). (A) The effect of 5-aza-2'deoxycitidine (DAC) treatment on the expression of *IL-20*, *IL-20RA/B* and *IL-22R1*. Cells were cultured in 1 μM DAC for 48 h with media and drug replaced every 24 h. A PCR was also carried out for *beta-actin* to determine loading efficiency and for normalisation purposes. (B) Densitometry analysis of expression in treated versus untreated samples when normalised to *beta-actin*. Data are graphed as mean ± SEM. Statistical analysis was performed using a paired one tail Student's *t* test ($n = 3$).

cell lines following IL-20 treatment adding strength to the argument that IL-20 is anti-angiogenic in NSCLC.

4. Discussion

The IL-20 cytokine is a member of the IL-10 family and has a number of key roles in pro-inflammatory joint and skin conditions but has yet to be studied to any great extent in the lung.

In a series of tumour/normal matched chemo-naïve patient samples, the mRNA expression of IL-20 and its receptors were examined. In general, increased mRNA was observed in the tumours compared to their matched normal counterparts (Fig. 1B). In particular, significantly elevated levels of *IL-20RB* were observed across the NSCLC samples ($p < 0.01$, 14/18 patients or 77.7%). The exception was *IL-20RA*, where a slight decrease in the mRNA level was observed. In contrast, we observed elevated amounts of IL-20RA protein in the tumours (Fig. 2B). In many of the patients examined there appears to be an inverse correlation between decreased IL-20RA mRNA and elevated IL-20RA protein in patient samples and vice versa (highlighted in Table 5).

Despite expressing significant amounts of the IL-20 receptors at the protein level, we were only able to detect IL-20 at the mRNA level in the NSCLC samples. However, IL-20 was detected in the supernatants of the normal and lung cancer cell lines (Fig. 4C) with A549 having increased basal levels compared to the HBEC4 cell line (535.70 ± 126.317 pg/mL versus 134.50 ± 27.84 pg/mL). These different levels of expression of IL-20 are most likely due the secreted nature of the ligand and the receptors being membrane bound. Other studies have observed IL-20 in serum and synioval fluids by ELISA[6,27] and tissues by immunohistochemistry[4,9] but not by western blot to the best of our knowledge.

Additionally, we observed significantly elevated protein levels of IL-20RB ($p < 0.01$) and IL-22R1 ($p < 0.01$) in NSCLC tumours compared with matched normal tissue (Fig. 2B). The significant increase of IL-20RB at both mRNA and protein level, may be related to its role as the common receptor subunit in both IL-20 receptor complexes. The high levels of IL-22R1 may signify that the IL-20RB/IL-22R1 complex is the main receptor complex used NSCLC.

Our results confirm those of Parrish-Novak et al.[17], which found that all three receptor subunits are expressed in the normal lung (Fig. 3). However, the co-expression of receptor subunits would seem to vary between normal and cancerous cells as their study also found that *IL-22R1* and *IL-20RB* mRNA were only observed in tissues that also expressed *IL-20RA*, however, this was not the case in our NSCLC cell line panel (Fig. 3). IL-20 mRNA expression was more robustly expressed in the HBECs compared with NSCLC cell lines (Fig. 3) reflecting results observed previously.[18]

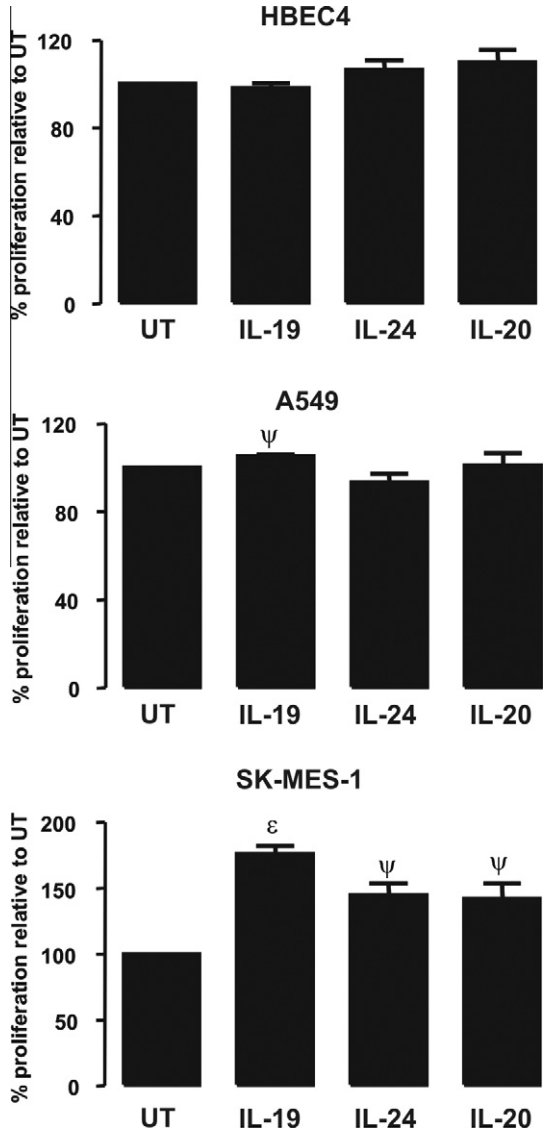

Fig. 7 – IL-20 receptor complexes are functional in NSCLC cell lines. Cell proliferation was examined by BrdU ELISA following 24 h of stimulation with either human recombinant IL-20 (200 ng/mL), IL-19 (100 ng/mL) or IL-24 (100 ng/mL) in normal and NSCLC cell lines. IL-20, IL-19 and IL-24 caused proliferative changes in the NSCLC cell lines. (ε $p < 0.01$ – IL-19 treatment versus UT, ψ $p < 0.05$ – IL-24, IL-20 treatment versus UT) Data are represented as a percentage of the untreated control (UT), which was set to 100% and is expressed as mean ± SEM. Statistical analysis was performed using a paired one tail Student's t test ($n = 3$).

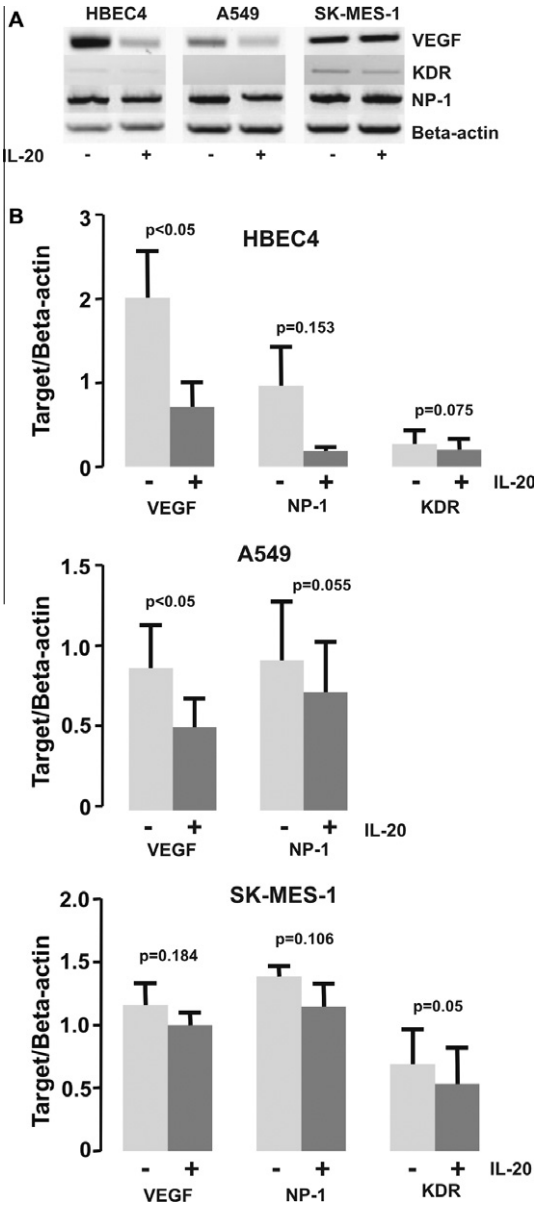

Fig. 8 – IL-20 affects expression of VEGF signalling pathway members. (A) HBEC4, A549 and SK-MES-1 cells were stimulated with recombinant IL-20 (200 ng/mL) for 24 h. Following treatment the expression levels of various members of the VEGF signalling pathway were examined by RT-PCR. (B) Densitometry analysis of expression in treated versus untreated samples when normalised to *beta-actin*. Data are graphed as mean ± SEM. Statistical analysis was performed using a paired one tail Student's t test ($n = 3$).

We, therefore, sought to determine if epigenetic therapies such as HDACi or demethylating agents could reactive the expression of this cytokine. In normal and lung cancer cell lines treated with HDACi there was an increase in *IL-20* ($p < 0.05$, HBEC4/SK-MES-1, $p < 0.01$ A549) (Fig. 4B), this increase was also observed at the protein level (Fig. 4C). The effect of HDACi on receptor expression varied between cell lines with decreases in *IL-20RB/IL-22R1* observed in A549 ($p < 0.05$) and *IL-20RA* in HBEC4 ($p < 0.05$). The increase in expression of *IL-20* in all cell lines would indicate that they are under epigenetic regulation at the level of histone modification. The general dogma associated with HDACi is that they function to induce gene expression. However, in this study, TSA caused both an up- and down- regulation of the IL-20 receptors, an effect seen for other genes in other studies. For example, HDACi have been shown to down-regulate Wilms tumour gene 1 (Wt1)[28] and EGFR,[29] either through limited availability of specific transcription factors, or by destabilising mRNA species.

Table 5 – Summary of mRNA/protein expression for IL-20 family members in NSCLC patients.

		IL20 mRNA	IL20 protein	IL-20RA mRNA	IL-20RA protein	IL-20RB mRNA	IL-20RB protein	IL-22R mRNA	IL-22R protein
1	Adeno	↑	nd	↓	↑	↑	↓	↑	↑
2	Adeno	↑	nd	↑	↑	↑	↑	↓	↑
3	Adeno	↑	nd	↑	↑	↑	↑	↑	↑
4	Adeno	↑	nd	↓	↓	↑	↑	↑	↑
5	Adeno	↑	nd	↑	↓	nd	↑	↑	↑
6	Adeno	↓	nd	↓	↑	↓	↑	↑	↓
7	Adeno	↓	nd	↓	↑	↑	↑	↓	↑
8	Adeno	↑	nd	↓	↑	↑	↓	↑	↑
9	Squam	↑	nd	↓	↑	↑	↑	↓	↑
10	Squam	↓	nd	↓	↓	↑	↑	↓	↑
11	Squam	↑	nd	↓	↑	↑	↑	↑	↑
12	Squam	↑	nd	↓	↑	↓	↑	↑	nd
13	Squam	↓	nd	↓	↑	↑	↑	↓	nd
14	Squam	nd	nd	↑	↓	↑	↓	↑	↑
15	Squam	nd	nd	↑	↓	↑	↑	↑	↑
16	Squam	↓	nd	↓	↑	↑	↑	↑	↑
17	Squam	nd	nd	↑	↑	↑	↑	↑	↑
18	Squam	↑	nd	↑	↓	↑	↑	↑	↑

ChIP results indicate that TSA acts by directly remodelling the promoter region of the *IL-20* gene (Fig. 5). Increased levels of acetylated histones H3 and H4 were found at the promoter region in response to HDACi. Furthermore we observed concomitant alterations to various histone H3 methylation patterns following HDACi treatment. These included increased levels of histone H3 lysine 4 di and tri-methylation (H3K4me2/3) and decreases in histone H3 lysine 4 monomethylation (H3K4me) and histone H3 lysine 9 di-methylation (H3K9me2) (Fig. 5). These results verify that HDACi treatments cause alterations to histone post-translational modifications around the promoter region, and that chromatin remodelling is an active element in the regulation of this gene.

Epigenetic loss of gene expression by aberrant DNA CpG methylation is an important event in lung cancer.[30] To determine if IL-20 family members were epigenetically regulated via DNA CpG methylation, cell lines were treated with DAC. Increased expression of *IL-20* mRNA occurred suggesting that its promoter region may be partially hypermethylated (Fig. 6A). *IL-20RA* is located on the lung cancer susceptibility locus 6q23-25.[31] In a study by Tessema et al.[22] 26% of primary adenocarcinoma cases show loss of *IL-20RA* expression, and this was determined to be due to dense DNA CpG methylation of the promoter region of this gene. Our study confirms the observations of Tessema and colleagues[19] as DAC resulted in the reactivation of *IL-20RA* in the adenocarcinoma cell line ($p < 0.01$) (Fig. 6B). In addition, our adenocarcinoma cohort had reduced expression of *IL-20RA* (5/8–62.5%) and IL-20RA (2/8–25%) in the tumour compared with normal. In support of the notion that loss of *IL-20RA* expression is an important aspect of NSCLC pathogenesis we show that normal bronchial epithelial cells express *IL-20RA* mRNA, NSCLC cell lines do not (Fig. 3).

As we were unable to detect IL-20 at the protein level in our NSCLC cohort, we, therefore, sought to determine if the IL-20 receptors were functional in our cell line panel. Previously, IL-20 has been suggested to be pro-proliferative in certain cell types,[1,16,17] however, as there is extensive receptor sharing within the extended IL-10 family we also treated cells with IL-19 and IL-24 in addition to IL-20. Although there was a trend to suggest an increase in proliferation with cytokine treatment in the normal cell line, this was not significant (Fig. 7), however, a significant increase was observed in the SK-MES-1 cell line with all treatments (IL-20, IL-24 – $p < 0.05$, IL-19 – $p < 0.01$) and IL-19 in the A549 cell line ($p < 0.05$) (Fig. 7). These results would indicate that all three receptor units, which form IL-20 receptor Type 1 and 2 complexes, promote proliferation in the squamous sub type and agree with prior results in relation to IL-24 treatment in the A549 cell line.[32]

To further examine the anti-angiogenic potential of IL-20, we examined its effect on the VEGF family members. A decrease was observed in *VEGF* and its receptors in all three cell lines, with *VEGF* significantly reduced in HBEC4 and A549 cell lines ($p < 0.05$) and *KDR* (*VEGFR2*) was down regulated in SK-MES-1 ($p < 0.05$) (Fig. 8). This data would support the initial findings of Heuze-Vourc'h et al. of IL-20's anti-angiogenic properties in NSCLC.[18] It has also been demonstrated within our laboratory that HDACi themselves, can reduce VEGF expression in NSCLC (Dr. Martin Barr, manuscript submitted).

We have shown than IL-20 possesses anti-angiogenic properties within the NSCLC setting and is significantly up regulated by HDACi. In addition, DAC treatment reactivates the expression of the *IL-20RA* receptor which is found on the 6q23-25 lung cancer susceptibility locus.[31] In conclusion, the potential clinical significance of epigenetic therapies in targeting this family in NSCLC may be of therapeutic benefit. However, supplementary studies are warranted to further dissect the anti-angiogenic potential of IL-20 in NSCLC and to determine levels of IL-20 in either serum or tissue of NSCLC patients.

Cell lines

The HBEC cell lines were a gift from Prof. John D. Minna (Hamon Centre for Therapeutic Oncology Research, UTSouthwestern, Dallas, TX, USA).

Conflict of interest statement

None declared.

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