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Discoidin domain receptor 1 regulates bronchial epithelial repair and matrix metalloproteinase production

M.E. Roberts, L. Magowan, I.P. Hall and S.R. Johnson

ABSTRACT: Discoidin domain receptor (DDR)1 is an extracellular matrix (ECM)-sensing receptor tyrosine kinase, which is activated by collagen and expressed in bronchial epithelium. DDR1 is responsible for maintaining the normal structure of skin and kidney epithelia and we hypothesised that DDR1 plays a regulatory role in bronchial epithelial integrity by transducing signals from the airway ECM.

Effects of DDR1 depletion were studied using RNA interference in primary human bronchial epithelial cells (HBECs) and BEAS-2B cells. The effects of overexpression of DDR1a and DDR1b in BEAS-2B cells were studied using a plasmid vector. We measured the effects on epithelial repair using a scratch wounding model, and levels of matrix metalloproteinases (MMPs) by gelatin zymography (MMP-2 and -9) and ELISA (MMP-7).

We showed that knockdown of DDR1 slowed epithelial repair by 50%, which was associated with a reduction in levels of MMP-7, whilst DDR1 overexpression enhanced epithelial repair. DDR1 knockdown reduced proliferation of HBECs, but had no significant effect on adhesion to collagen I or other matrix substrates.

These data suggest that ECM signalling *via* DDR1 regulates aspects of bronchial epithelial repair, integrity and MMP expression in the airways.

KEYWORDS: Bronchial epithelial repair, discoidin domain receptor, matrix metalloproteinase

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Maintenance of epithelial integrity is essential for normal function of the bronchial epithelium, including host defence and epithelial barrier function. Bronchial epithelial injury and repair are features of respiratory diseases, such as asthma and the response to viral infection. Direct toxic insults, trauma or inflammatory processes cause the loss of epithelial cells, leading to exposure of basal epithelial cells and basement membrane, which then institute a repair process [1, 2]. Epithelial repair is a complex process comprising proliferation, migration and adhesion. Initially, the remaining epithelial cells secrete a provisional matrix, predominantly composed of collagens I and III, and fibronectin. By adhering to this provisional matrix, these cells migrate to the site of injury. Later, cells distant from the wound edge undergo a proliferative response [2, 3]. The process of restitution is also associated with and dependent on the production of secreted factors, such as epidermal growth factor (EGF) and trefoil factors, which promote bronchial epithelial repair by increasing the motility of epithelial cells [3–5]. Matrix metalloproteinases (MMPs) remodel the extracellular matrix (ECM), affect cell–ECM interactions and are known to be essential components of epithelial repair. MMP-7 is expressed constitutively by bronchial epithelial cells and in an MMP-7 knockout mouse model, bronchial epithelial repair is completely abolished following mechanical wounding [6]. Substrates of MMP-7 include the ECM component fibronectin, the cell–cell interaction molecule E-cadherin and proteases, including pro-MMP-2, which is also upregulated during the epithelial repair process [7].

The integrin family of ECM receptors and CD44, a cell surface glycoprotein, interact with ECM components and promote adhesion and migration of epithelial cells [8–16]. Discoidin domain receptors (DDRs) are a family of receptor tyrosine kinases whose cognate ligands are the collagen family, particularly collagens I–V: collagens I and III are significant components of the ECM produced during epithelial repair [17–19]. DDR1 is expressed in the bronchial epithelium and has been shown to be important in the maintenance of the normal structure of epithelia in the skin and kidney [20–22]. DDR1 is also required for arterial wound repair after injury [23]. In whole-lung

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samples from patients with idiopathic pulmonary fibrosis, a disease characterised by persisting epithelial injury [24], DDR1 mRNA together with MMP-7, MMP-2 and MMP-9 were strongly overexpressed when compared with normal tissue [25]. Taken together, these data suggest that DDR1 may play a role in this disease, perhaps by modulating epithelial repair and MMP activity.

We hypothesised that DDR1 plays a role in airway epithelial repair by transduction of signals between the ECM and epithelial cells to regulate epithelial repair and effectors of repair, such as MMPs. In this study, we undertook experiments to confirm the presence of DDR1 in bronchial epithelium and to investigate the influence of DDR1 on epithelial wound healing, proliferation, adhesion and levels of MMP-7, MMP-2 and MMP-9. We also investigated DDR1 expression in epithelium from patients with asthma (a disease characterised by abnormal epithelial integrity and repair) and nonasthma controls to examine if DDR1 is differentially expressed in asthma.

METHODS

Cell culture

BEAS-2B Cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Yeovil, UK) at 37°C in 5% CO_2. Subculture was undertaken using trypsin–EDTA (Sigma, Poole, UK). Human bronchial epithelial cells (HBECs) were obtained from Lonza (Wokingham, UK) or from biopsies obtained at bronchoscopy. Endobronchial biopsies were immediately transferred to the cell culture laboratory in warm PBS (Invitrogen). Biopsies were then dissected to isolate epithelial tissue under a low-power dissection microscope. Explants were transferred to six-well plates with a minimum volume of bronchial epithelial growth medium (bronchial epithelial basal medium (BEBM) supplemented with bronchial epithelial growth medium SingleQuot kit; Lonza) and incubated at 37°C in 5% CO_2 overnight to facilitate adhesion. The volume of medium was then increased and explants were grown in submerged cultures until cell numbers had expanded sufficiently to transfer to flasks. HBECs and cells derived from explants were harvested using trypsin–EDTA. Trypsin was neutralised using soybean trypsin inhibitor (Invitrogen) in PBS.

DDR1 knockdown

Cultured BEAS-2B cells were washed in bronchial epithelial ''wounding'' medium (BEWM) [3], consisting of BEBM containing retinoic acid, triiodothyronine, bovine pituitary extract and recombinant human insulin. They were then exposed to RNA interference (RNAi) transfection complexes composed of 2 $\mu L \cdot mL^{-1}$ Lipofectamine™ 2000 (Invitrogen) pre-incubated with 20 nM small interfering RNA (siRNA) to DDR1 or Control Number 1 (DDR1 sense sequence: GCC AGU GAC ACU AAA ACA Att; DDR1 antisense sequence: UUG UUU UAG UGU CAC UGG Ctt; Applied Biosystems, Warrington, UK). For HBECs, cells were exposed to RNAi for 4 h and then recovered in BEWM for 48 h.

DDR1 overexpression

BEAS-2B cells were transfected with the pIRES2-EGFP vector (Clontech, Saint-Germain-en-Laye, France), either with no insert used as the control, or the vector with active DDR1a, active DDR1b or a kinase-dead (kd) DDR1a variant that has previously been shown to prevent autophosphorylation at all tyrosine residues of DDR1 in response to collagen I activation (K618A, a loss of function substitution in the kinase domain) [26]. All transfections were performed using Fugene (Roche, Burgess Hill, UK). DDR1 constructs were kindly supplied by the late W. Vogel, (University of Toronto, Toronto, Canada; personal communication). For overexpression studies, stable transfections were used with selection for stably transfected pIRES2-EGFP expressing cells achieved by antibiotic selection using Geneticin (Invitrogen) according to standard procedures.

DDR1 expression: Western blotting

Cell lysates were obtained using Cytobuster (Merck Biosciences, Nottingham, UK) with complete protease inhibitors (Roche Diagnostics). Cells were incubated on ice for 30 min, insoluble cell debris was removed by centrifugation at 10,000 $\times g$ for 3 min and samples combined with an equal volume of loading buffer. Proteins were resolved by electrophoresis using 10% polyacrylamide–sodium dodecylsulfate gels, blotted on to polyvinylidene fluoride membranes and probed with an anti-DDR1 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) followed by a horseradish peroxidase-conjugated polyclonal goat anti-rabbit secondary antibody (Sigma). Bands were visualised using enhanced chemiluminescence (Amersham Biosciences, Amersham, UK). Equal protein loading was confirmed by reprobing blots for α-actin (Sigma).

Quantitative PCR

RNA was extracted using the QIAGEN RNeasy kit (QIAGEN, Crawley, UK) and contaminating genomic DNA removed using DNAse I (QIAGEN). cDNA was synthesised using Superscript II (Invitrogen). RT-PCR was performed on a Biometra Thermal Cycler using Invitrogen Recombinant *Taq* DNA polymerase. Procedures were carried out according to manufacturers' instructions. The conditions were 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for 35 cycles. PCR products were resolved on a 2% agarose gel stained with ethidium bromide. For quantitative PCR, the SYBR Green technique was used on a Stratagene Mx3005P real-time PCR system (Agilent Technologies, Stockport, UK), with the following settings for 40 cycles: 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Each reaction contained 12.5 μL Brilliant Green (Agilent Technologies), 6.5 μL water, 5 μL cDNA and 1 μL 5 μM primer mix. Dissociation curves showed a single peak. Primer efficiency was calculated using a dilution curve. Relative expression of transcripts was measured by calculating concentration values relative to a housekeeping gene (β_2-microglobulin) [27]. Primers were obtained from published literature as follows [28–30]. β_2-microglobulin-forward: GAGTGCTGTCTCCATGTTTGATGT; β_2-microglobulin-reverse: AAGTTGCCAGCCCTCCTAGAG; DDR1-forward: ATGGAGCAACCACAGCTTCTC; DDR1-reverse: CTCAGCCGGTCAAACTCAAACT; DDR2-forward: GGAGG TCATGGCATCGAGTT; DDR2-reverse: GAGTGCCATCCCG ACTGTAATT.

ECM coating of tissue culture plastics

Rat's tail collagen I (Sigma) diluted to 10 $\mu g \cdot mL^{-1}$ was applied to tissue culture plastics in a thin layer and allowed to air-dry overnight. After extensive washing, collagen-treated and

control wells were blocked for 1 h with 1% bovine serum albumin (BSA; Sigma) in PBS then washed extensively again.

Wound healing assay

Two epithelial cell types were used. Stable DDR1a-, b- and kd-overexpressing, and control BEAS-2B cells, and BEAS-2B cells exposed to DDR1 or control RNAi were seeded in DMEM/FBS at 1 mL per well at equal density in 24-well plates (Corning, Artington, UK). After 4 h, cells were serum starved by exchange of medium for BEBM for 24 h. HBECs were seeded at 60% confluence and then cultured to confluence in a 24-well plate with 2 mL per well BEGM exchanged every 48 h. HBECs were transfected when confluent and incubated in BEWM for 48 h prior to wounding. Two scratch wounds were created in parallel in each well of a 24-well plate [3]. Medium was exchanged for further BEBM or BEWM and photographs were obtained at a fixed point in each wound using an Insight QE Digital camera and software (Image Solutions, Preston, UK) attached to a Nikon Diaphot 300 microscope (Nikon Instruments Inc., Melville, NY, USA) with a 2.5× NA 0.08 Zeiss lens or 4.0× NA 0.075 Zeiss lens (Carl Zeiss Ltd., Welwyn Garden City, UK). Further images were obtained at 8 h for HBECs or 24 h for BEAS-2B cells. Wound areas were calculated by tracing wounds using Spot software (version 4.6; Image Solutions).

Gelatin zymography

Supernatants from wounding experiments were combined with an equal volume of 2× Novex sample buffer (Invitrogen) and separated by electrophoresis through 10% gelatin gels (Invitrogen) using the Invitrogen Novex system. Gels were exposed to Novex renaturing buffer after electrophoresis and then Novex developing buffer overnight at 37°C. Recombinant MMP-2 was used as a positive control. Gels were stained using Coomassie blue (VWR International, Lutterworth, UK) dissolved in 40% methanol, 10% acetic acid in distilled water, washed and destained using the same solution without Coomassie blue. Images were acquired using SynGene GeneSnap v7.04g attached to a GeneGenius image acquisition system (Synoptics, Cambridge, UK). Calculation of relative sizes of MMP bands was performed using ImageJ 1.39 (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). The largest band on each gel was selected as the reference size for the region of interest. The same size region of interest was then applied to bands consecutively and the average density determined. The validity of this method was confirmed by determining the density of known concentrations of an MMP-2 standard in a gelatin gel and confirming a linear relationship between density and MMP-2 concentration (data not shown).

MMP-7 ELISA

MMP-7 was measured by ELISA (minimum detectable concentration of MMP-7 0.016 $ng \cdot mL^{-1}$) according to the manufacturer's instructions (R&D Systems, Abingdon, UK). Supernatants from wounding experiments were concentrated five-fold prior to analysis using Vivaspin 500 columns (Scientific Laboratory Supplies, Nottingham, UK).

Cell number

Equal numbers of cells were seeded into 12-well plates (Corning). For BEAS-2B cells, these were either collagen I-coated or BSA-coated controls. BEAS-2B cells were exposed to RNAi transfection complex for 48 h and then seeded at equal density. After 4 h, the medium was exchanged for further BEWM and cells were incubated overnight then fixed with 4% paraformaldehyde. HBECs were seeded in equal numbers in each well and transfected at 20% confluence. The medium was exchanged after 4 h. At 48 h, the medium was exchanged again. Cells were fixed after a further 24 h. Nuclear staining was achieved by incubation with 10 $\mu g \cdot mL^{-1}$ 4′,6-diamidino-2-phenylindole (Sigma) in PBS for 30 min at room temperature. Fluorescence images were obtained with a 4× Zeiss lens attached to a Nikon Diaphot 300 microscope using Spot 4.7 software and a Slider RT3 camera (Image Solutions). Nuclei were counted using ImageJ 1.41. The accuracy of this method was confirmed by counting serial dilutions of a cell suspension (data not shown).

Thymidine incorporation

Equal numbers of cells were seeded into 48-well plates (Corning). For BEAS-2B cells, these were either collagen I-coated or BSA-coated controls. BEAS-2B cells overexpressing DDR1 or empty vector control were seeded in DMEM/FBS and then serum starved for 24 h once adherent. In HBECs, cells were seeded at equal density, exposed to transfection complexes for 4 h and then incubated overnight in BEWM. 10 $ng \cdot mL^{-1}$ EGF was then added and after 8 h incubation, tritiated thymidine was added at an activity of 1 μCi (37 kBq) per well. After a further 16 h, cells were exposed to 10% trichloroacetic acid (Sigma) for 30 min at 4°C and DNA was precipitated by incubation with 0.2 M NaOH (Sigma) overnight at 4°C. The number of disintegrations was assessed on a Wallac scintillation counter (PerkinElmer, Waltham, MA, USA) after the addition of 10 mL scintillation fluid per vial.

Adhesion assay

HBECs were incubated for 48 h after exposure to RNAi transfection complexes. Adhesion was assessed using the Chemicon cytomatrix adhesion assay kit comprising pre-coated ECM substrates (vitronectin, fibronectin, laminin, collagen I, collagen IV and albumin control; Fisher Scientific, Loughborough, UK). After treatment, cells were harvested, incubated on various cell matrix factors for 2 h at 37°C in 5% CO_2 and adhesion quantitated according to manufacturer's instructions.

Apoptosis

HBECs were seeded to eight-well chamber slides at equal density. 24 h after seeding, cells were exposed to RNAi transfection complexes and incubated for 48 h. HBECs were then fixed using 3.7% paraformaldehyde, permeabilised with PBS containing 0.1% Triton X-100 and 0.1% sodium citrate (both Sigma), and then incubated with the Fluorescein *In Situ* Cell Death Detection Kit (Roche), according to the manufacturer's instructions. Fluorescence images were obtained as described and fluorescent cells were counted using ImageJ 1.41.

Vital dye exclusion

Cells were seeded and transfected as for the apoptosis assay. Cells were then incubated with 0.02% trypan blue in PBS for

5 min at room temperature. Brightfield images were obtained as above and cells were counted using ImageJ 1.41.

DDR1 expression: immunohistochemistry

Bronchial biopsies were obtained by fibre optic bronchoscopy. Patients with stable asthma were recruited. Control patients without asthma who were undergoing bronchoscopy for other indications, including possible lung cancer and haemoptysis, were also recruited. These patients had normal spirometry and histologically normal airways. The study was approved by the Nottingham Research Ethics Committee (Nottingham, UK) and informed consent was obtained from all patients. Bronchial biopsies were obtained from a first or second subdivision carina. Specimens for staining were fixed in 4% paraformaldehyde, wax embedded, cut and mounted. Slides were dewaxed in histoclear (RA Lamb Ltd, Eastbourne, UK) and rehydrated through an ethanol series. Sections were either stained with 1% eosin (Nustain, Nottingham, UK) and haematoxylin (Sigma) or prepared for immunostaining using a Vectastain ABC kit (Vector Laboratories, Peterborough, UK) according to the manufacturers' instructions. Briefly, sections were boiled in 10 mM citrate buffer for 20 min, peroxidase-activity blocked, incubated overnight with anti-DDR1 C20 antibody (Santa Cruz) and the secondary antibody applied according to the manufacturer's instructions. Diaminobenzidine was then applied using the Vectastain DAB kit (Vector Laboratories). Nuclear counterstaining was achieved using haematoxylin. Slides were then dehydrated through a reverse ethanol series and mounted using Vectamount (Vector laboratories).

Statistical analysis

Observations were compared using unpaired t-tests or one-way ANOVA analysis. Between-group analyses were undertaken using Bonferroni's post-test. Data are presented as mean$\pm$SEM and n $\geqslant$3.

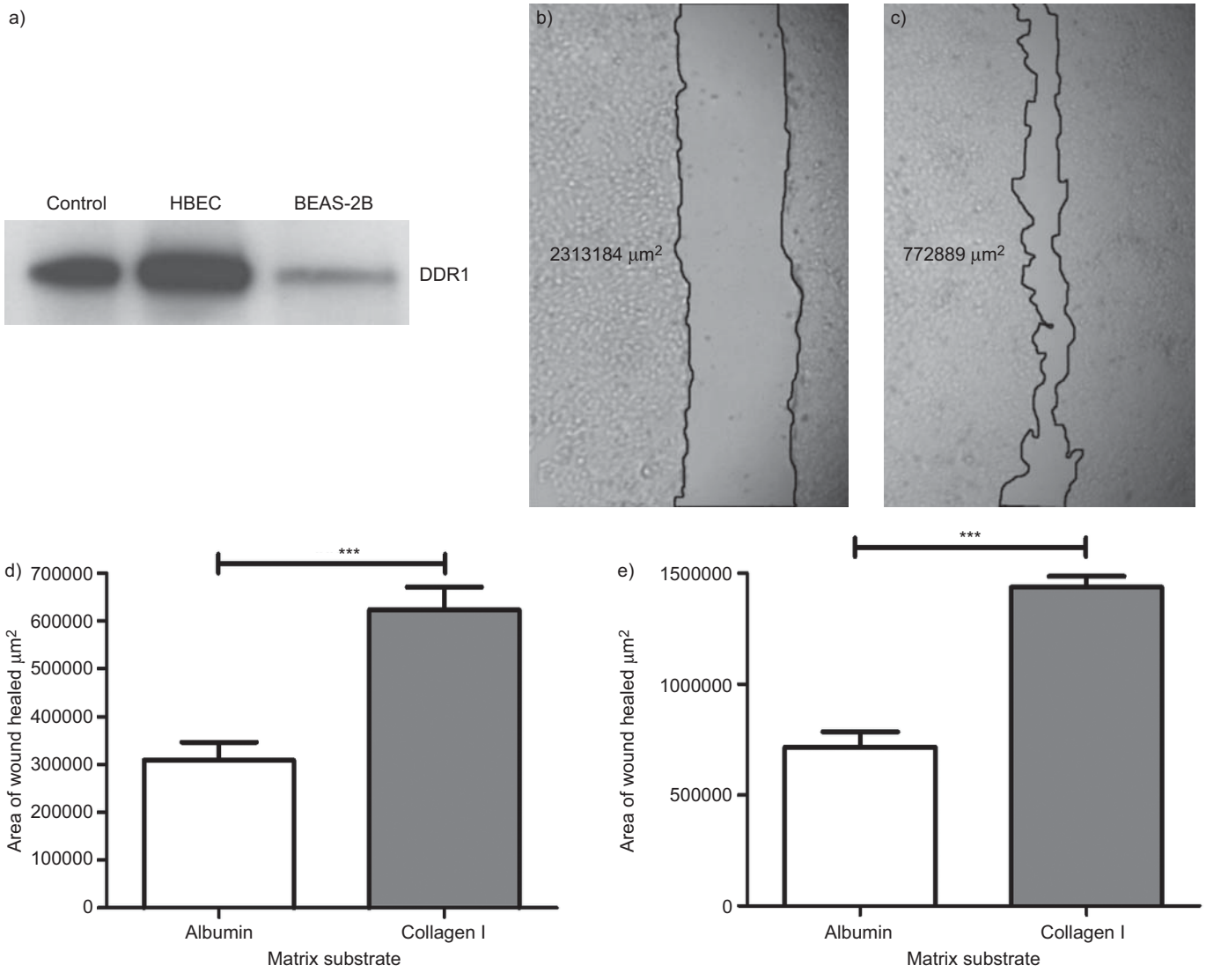

FIGURE 1. a) Western blot showing discoidin domain receptor (DDR)1 expression in human bronchial epithelial cells (HBECs) and BEAS-2B cells. Representative scratch wound in BEAS-2B cells at b) 0 and c) 24 h. Collagen I enhances the area of scratch wound repair in d) a HBEC monolayer at 8 h and e) a BEAS-2B monolayer at 24 h. Data are presented as mean$\pm$SEM. n $\geqslant$3. ***: $p<0.001$.

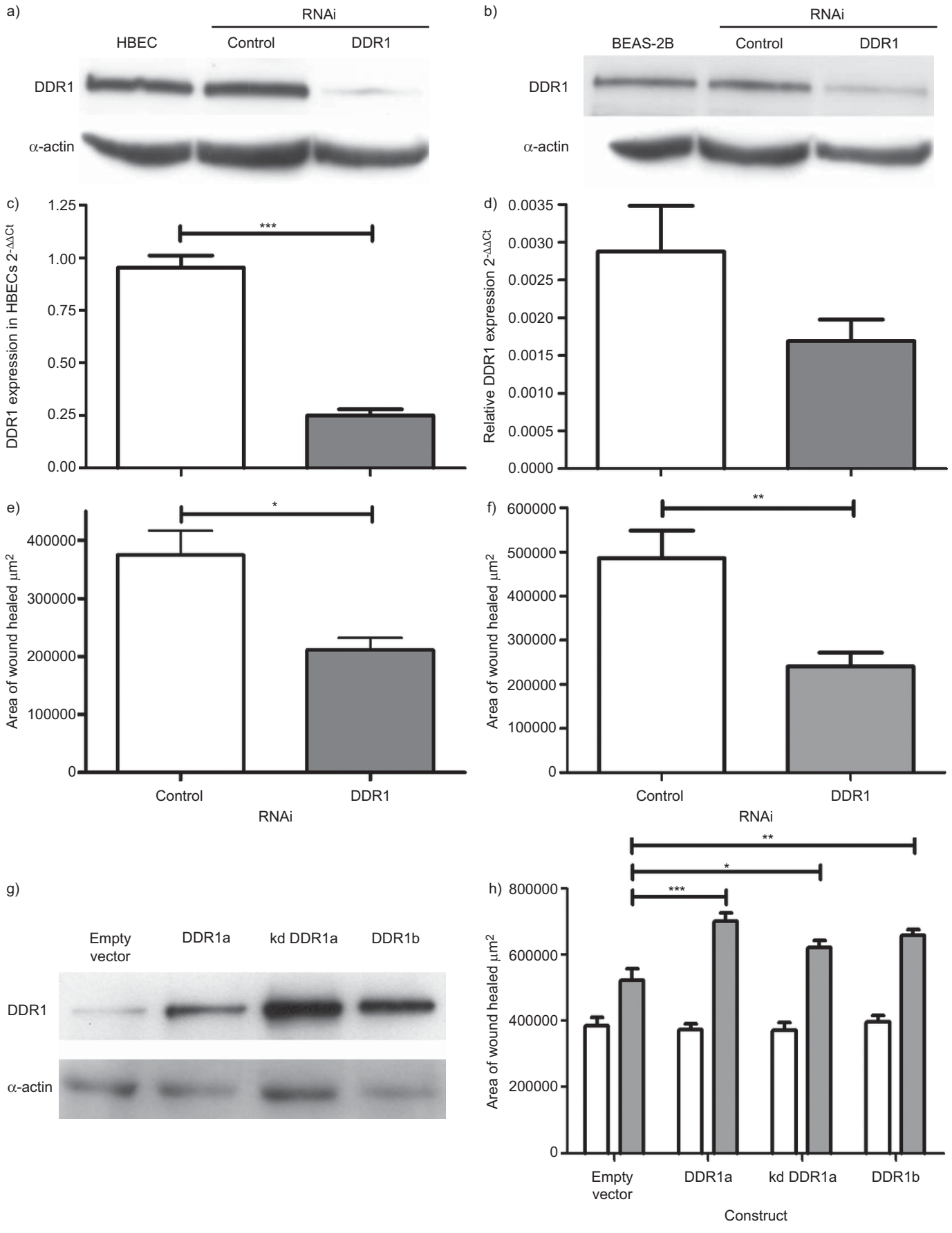
a)
RNAi
HBEC
Control
DDR1
DDR1
α-actin
b)
RNAi
BEAS-2B
Control
DDR1
DDR1
α-actin
c)
DDR1 expression in HBECs 2-ΔΔCt
1.25
1.00
0.75
0.50
0.25
0.00
***
d)
Relative DDR1 expression 2-ΔΔCt
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
e)
Area of wound healed µm2
400000
300000
200000
100000
0
*
Control
DDR1
RNAi
f)
Area of wound healed µm2
600000
500000
400000
300000
200000
100000
0
**
Control
DDR1
RNAi
g)
Empty vector
DDR1a
kd DDR1a
DDR1b
DDR1
α-actin
h)
Area of wound healed µm2
800000
600000
400000
200000
0
**
*
***
Empty vector
DDR1a
kd DDR1a
DDR1b
Construct

FIGURE 2. Discoidin domain receptor (DDR)1 protein knockdown was confirmed by Western blotting in a) human bronchial epithelial cells (HBECs) and b) BEAS-2B cells 48 h after transfection. c) Knockdown of DDR1 transcript in HBECs was also confirmed by quantitative RT-PCR 48 h after transfection. d) There was no significant effect on levels of DDR2 transcript. DDR1 knockdown leads to inhibition of wound repair in both e) a HBEC monolayer and f) a BEAS-2B cell monolayer on a collagen I substrate, compared with control RNA interference (RNAi). g) Overexpression of DDR1 was confirmed by Western blotting. h) Overexpression of all DDR1 isoforms, including kinase-dead (kd) DDR1, leads to enhanced wound repair on a collagen I substrate. Data are presented as mean±SEM. n ⩾3. *: p<0.05; **: p<0.01; ***: p<0.001.

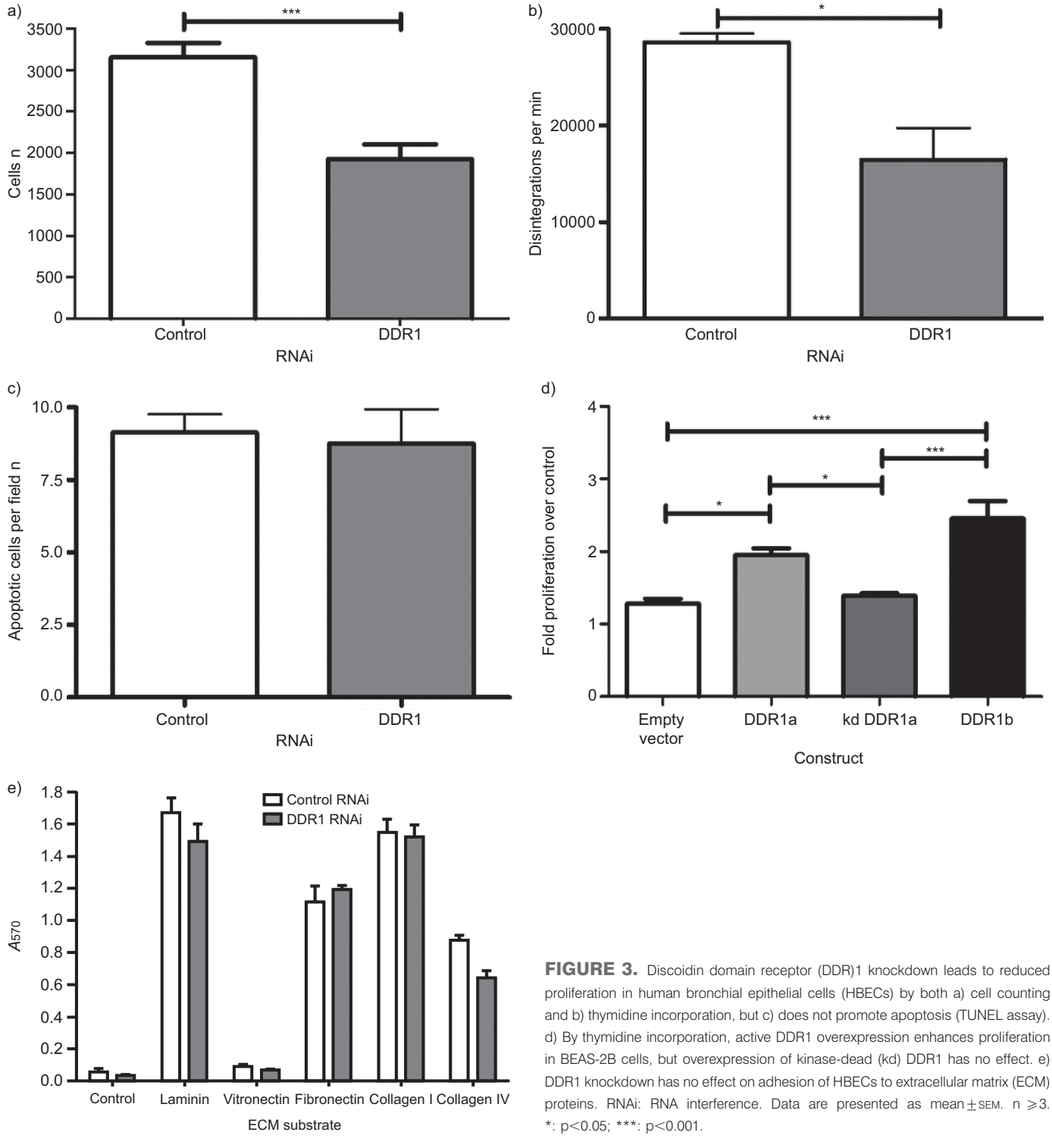

FIGURE 3. Discoidin domain receptor (DDR)1 knockdown leads to reduced proliferation in human bronchial epithelial cells (HBECs) by both a) cell counting and b) thymidine incorporation, but c) does not promote apoptosis (TUNEL assay). d) By thymidine incorporation, active DDR1 overexpression enhances proliferation in BEAS-2B cells, but overexpression of kinase-dead (kd) DDR1 has no effect. e) DDR1 knockdown has no effect on adhesion of HBECs to extracellular matrix (ECM) proteins. RNAi: RNA interference. Data are presented as mean±SEM. n ⩾3. *: p<0.05; ***: p<0.001.

RESULTS

DDR1 is expressed in cultured epithelial cells

To confirm that DDR1 is expressed in both HBECs and BEAS-2B cells, we performed Western blotting of cell lysates of both BEAS-2B cells and HBECs (fig. 1a). DDR1 was strongly expressed in HBECs and present at a lower level in BEAS-2B cells. The positive control is a sample validated against a cell lysate known to contain DDR1 obtained from W. Vogel (University of Toronto; personal communication).

Collagen I enhances epithelial repair

In order to investigate the contribution of collagen I (a major component of provisional matrix) to epithelial repair, we compared epithelial repair in the scratch wounding model on collagen I- *versus* albumin-coated tissue culture plastic [31]. Cells were seeded to near-confluence and then starved of growth factors overnight so that cellular production of other ECM factors could be minimised. In preliminary experiments, we showed that wound healing was significantly faster in HBECs compared with BEAS-2B cells, with ~50% wound

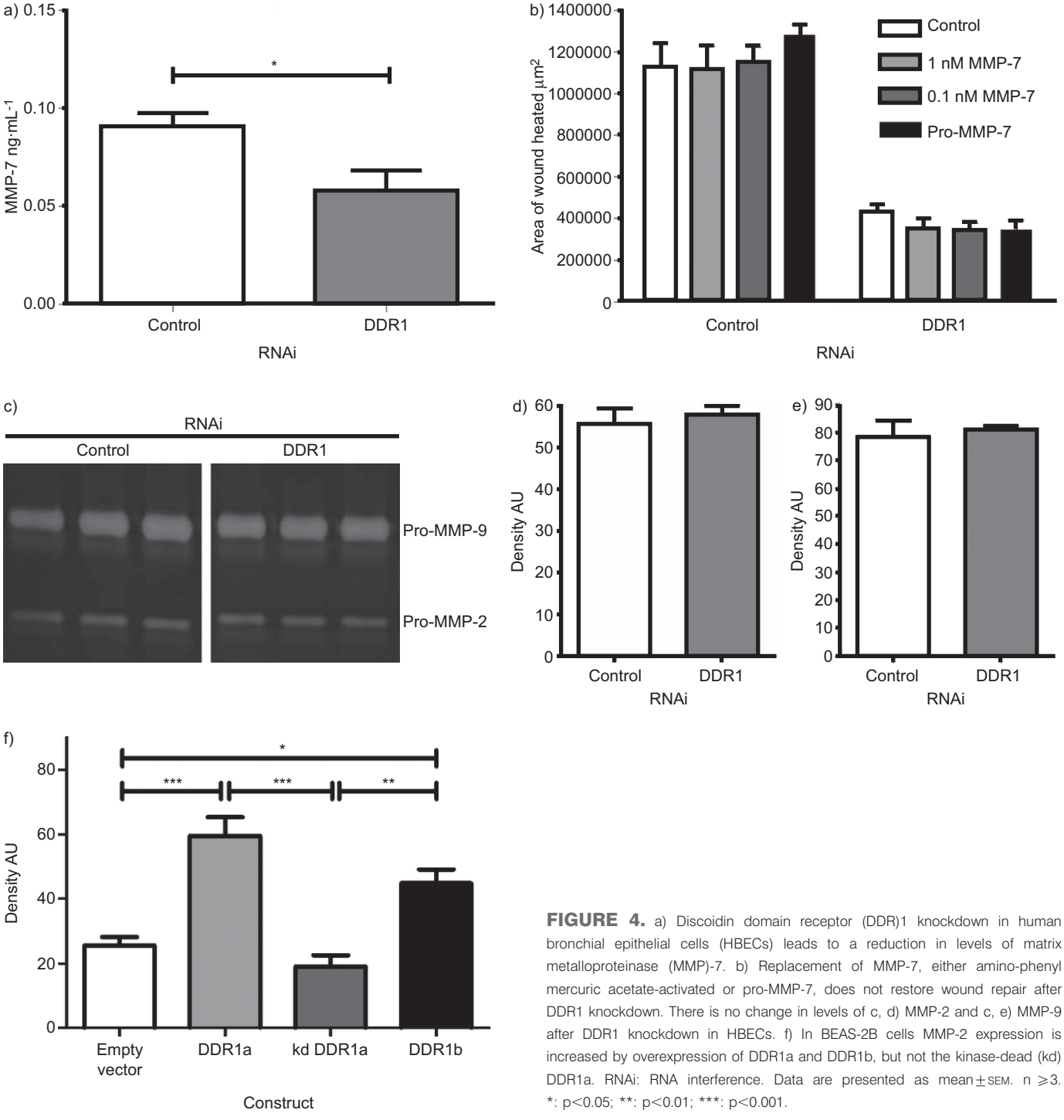

FIGURE 4. a) Discoidin domain receptor (DDR)1 knockdown in human bronchial epithelial cells (HBECs) leads to a reduction in levels of matrix metalloproteinase (MMP)-7. b) Replacement of MMP-7, either amino-phenyl mercuric acetate-activated or pro-MMP-7, does not restore wound repair after DDR1 knockdown. There is no change in levels of c, d) MMP-2 and c, e) MMP-9 after DDR1 knockdown in HBECs. f) In BEAS-2B cells MMP-2 expression is increased by overexpression of DDR1a and DDR1b, but not the kinase-dead (kd) DDR1a. RNAi: RNA interference. Data are presented as mean±SEM. n ⩾3. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

healing after 8 and 24 h, respectively. These time-points were therefore used for further experiments with these cell types (fig. 1b and c). Collagen I increased the area of wound healing over that of the albumin control in a BEAS-2B cell monolayer at 24 h (2.0-fold enhancement, 95% CI 1.58–2.57; $p<0.001$) and in HBECs at 8 h (2.0-fold enhancement, 95% CI 1.41–3.36; $p<0.001$) (fig. 1d and e).

What is the contribution of DDR1 to epithelial repair?

As collagen I is the cognate ligand for DDR1, and $DDR1^{-/-}$ smooth muscle cells from mice have been shown to exhibit impaired attachment to and migration towards collagen I [32], we hypothesised that the observed effect of collagen on epithelial repair was mediated by DDR1. We first studied the effect of DDR1 knockdown on epithelial repair in both HBECs and BEAS-2B cells using RNAi techniques. In initial experiments we showed by Western blotting that at 48 h after transfection, DDR1 siRNA resulted in an 89% reduction in DDR1 protein in HBECs and a 53% reduction in BEAS-2B cells, when compared with Ambion Control Number 1 (Ambion, Warrington, UK) siRNA (fig. 2a and b). In order to examine the selectivity of the siRNA, we also measured the level of DDR transcripts in epithelial cells (fig. 2c and d). In HBECs, DDR1 transcript was reduced by 74% by RNAi, whereas DDR2 mRNA was expressed at a low level (300-fold lower than DDR1) and, although it was reduced slightly by DDR1 siRNA, this was not a significant effect. Compared with control siRNA, DDR1 knockdown led to a 46% reduction in the area of wound healed in BEAS-2B cells at 24 h (95% CI 13–79%; $p=0.01$) and a 44% reduction in the area of wound healed in HBECs at 8 h (95% CI 16–72%; $p=0.006$) (fig. 2e and f).

As epithelial repair is reduced by knockdown of DDR1, we went on to investigate the effect of overexpression of DDR1 on epithelial repair and whether this differs between DDR1 isoforms. Additionally, we assessed the contribution of signalling through DDR1 phosphorylation to epithelial repair. We used BEAS-2B cells overexpressing the two main isoforms of DDR1, DDR1a and DDR1b. A kd version of DDR1a (with a single amino acid substitution, K618A, in the kinase domain) was used to examine whether the repair process was dependent on phosphorylation of DDR1 after activation. Overexpression of all three DDR1 isoforms was confirmed by Western blotting (fig. 2g). In these experiments on an albumin substrate, there was no significant difference in wound healing between empty vector control cells and cells overexpressing DDR1a, kd DDR1a or DDR1b. However, on a collagen I substrate, overexpression of DDR1a, kd DDR1a and DDR1b in BEAS-2B cells were all associated with enhanced wound healing (fig. 2h). For DDR1a, the enhancement over empty vector control was 1.34-fold (95% CI 1.17–1.52; $p<0.001$), for kd DDR1a it was 1.19-fold (95% CI 1.01–1.36; $p<0.05$) and for DDR1b it was 1.26-fold (95% CI 1.09–1.44; $p<0.01$).

DDR1 knockdown was associated with reduced proliferation of HBECs but had no effect on adhesion

In order to determine which aspect of epithelial repair is affected by DDR1, we went on to investigate the effects of DDR1 knockdown on HBEC proliferation and adhesion. To allow time for DDR1 turnover based on our preliminary findings, as in previous experiments, we measured the increase in cell number induced by 48 h of exposure to collagen I in HBECs 48 h after DDR1 knockdown. We

TABLE 1 Characteristics of asthma patients and nonasthma controls

Sex	Age yrs	Lung function % pred		Smoking history	Medication					Diagnosis	Sample use
		FEV_1	FVC		SABA	ICS	LABA	OCS	ACh receptor antagonist		
M	70	59	70	Ex-smoker since 1997						Control	IHC
M	68	86	71	20 pack-yrs						Control	IHC
M	65	87	101	Nonsmoker						Control	IHC, culture
F	76			25 pack-yrs	+					Control	IHC
M	76	76	84	Nonsmoker						Control	IHC, culture
F	69	82	96	25 pack-yrs						Control	Culture
M	29	95	99	Nonsmoker	+	+				Asthma	IHC
F	19	93	113	Nonsmoker	+	+				Asthma	IHC
M	54	72	77	Nonsmoker	+	+	+			Asthma	IHC
M	24	107	104	Nonsmoker	+	+				Asthma	IHC
M	37	81	111	Nonsmoker	+	+				Asthma	IHC
M	26	117	117	Nonsmoker	+					Asthma	IHC
M	39	91	93	Nonsmoker	+	+	+			Asthma	IHC
M	63	74	121	Nonsmoker	+	+	+		+	Asthma	IHC
F	41	70	90	4 pack-yrs		+	+	+	+	Asthma	Culture

% pred: % predicted; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; SABA: short-acting β-agonist; ICS: inhaled corticosteroid; LABA: long-acting β-agonist; OCS: oral corticosteroid; ACh: acetylcholine; M: male; F: female; IHC: immunohistochemistry; +: medication prescribed.

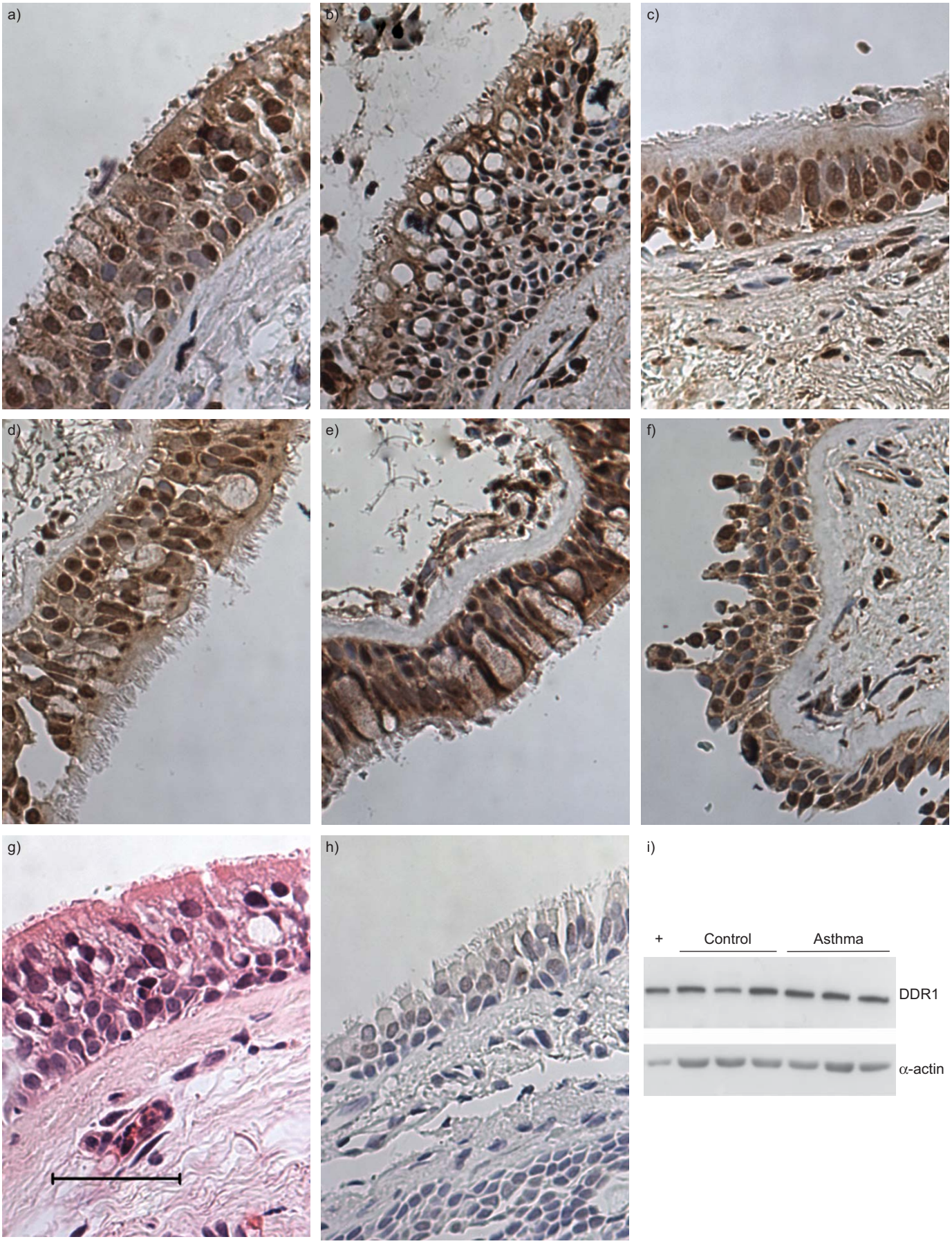
a)
b)
c)
d)
e)
f)
g)
h)
i)
+
Control
Asthma
DDR1
α-actin

FIGURE 5. Discoidin domain receptor (DDR)1 immunostaining of three representative a–c) nonasthma controls and d–f) asthma patients. g) Haematoxylin and eosin, and h) isotype controls are shown for specificity of staining. i) Western blotting of cell lysates confirms DDR1 expression in epithelial cells from patients with asthma and controls. +: positive control. Scale bar=50 μm.

incubated cells in BEWM and demonstrated a 30% reduction in cell number at 48 h compared with control RNAi by cell counting (fig. 3a) and by thymidine incorporation (fig. 3b). DDR1 has previously been shown to influence levels of p53, a vital regulator of apoptosis [33]. In order to confirm that the effects seen were not due to a reduction in cell viability by DDR1 siRNA, in parallel experiments we confirmed that, although DDR1 siRNA reduced cell proliferation, this was not due to cell death, as the number of cells undergoing apoptosis as confirmed by TUNEL (terminal deoxynucleotidyltransferase uridine triphosphate nick end-labelling) assay did not differ between DDR1 siRNA and control siRNA (fig. 3c). Similar findings were observed in parallel experiments using a trypan blue exclusion assay of cell viability (data not shown). In BEAS-2B cells, proliferation was enhanced in the presence of active DDR1, but not kd DDR1, compared with empty vector control (fig. 3d). DDR1a overexpression enhanced proliferation 1.6-fold (95% CI 1.1–2.3 fold; $p<0.05$) and DDR1b overexpression enhanced proliferation 2.2-fold (95% CI 1.6–2.7 fold; $p<0.001$).

To examine the role of DDR1 on cell adhesion, cells were harvested 48 h after DDR1 knockdown and seeded in equal numbers onto strips coated with ECM factors. Adhesion was significantly higher on components of the provisional matrix and basement membrane (laminin, fibronectin, and collagens I and IV) than on vitronectin or control (fig. 3e). Adhesion was unaffected by DDR1 siRNA on any ECM substrate.

MMP expression

MMPs are implicated in the repair of the bronchial epithelium. As MMP-7 is constitutively expressed by HBECs and is a necessary effector of airway epithelial repair [34], we tested the hypothesis that MMP expression is related to DDR1 signalling. We harvested supernatants of DDR1 siRNA and control-transfected HBECs 24 h after wounding on a collagen I substrate, and measured MMP-7 protein by ELISA, and pro- and active-MMP-2 and MMP-9 by gelatin zymography. In HBECs, DDR1 depletion was associated with a 50% reduction in MMP-7 protein, similar to the reduction in wound healing (fig. 4a). To determine whether the effects of DDR1 on epithelial repair are mediated solely through MMP-7, we performed wounding experiments in the presence of DDR1 siRNA and added recombinant MMP-7; pro-MMP-7 was added to 1 nM, or amino-phenyl mercuric acetate-activated MMP-7 was added to 0.1 nM or 1 nM. The addition of MMP-7 did not restore epithelial repair after DDR1 knockdown (fig. 4b). Increasing concentrations of MMP-7 above these levels resulted in epithelial detachment (data not shown). Pro-MMP-2 and pro-MMP-9 levels were unaffected by DDR1 knockdown in HBECs (fig. 4c–e). Next, we examined the effect of DDR1 overexpression on MMP expression in BEAS-2B cells on a collagen I substrate: although BEAS-2B cells do not produce MMP-7 (data not shown) we observed by zymography that overexpression of DDR1a led to a 2.3-fold increase in MMP-2 protein (95% CI 1.6–3.0; $p<0.001$) and overexpression of DDR1b led to a 1.8-fold increase in MMP-2 protein (95% CI 1.1–2.4; $p<0.05$). However, overexpression of kd DDR1 had no effect on MMP-2 protein expression (0.74-fold change, 95% CI 0.054–1.4) (fig. 4f).

DDR1 is expressed in bronchial epithelium

Having demonstrated that DDR1 is expressed in cultured primary and transformed HBECs, and contributes to epithelial repair, we next tested DDR1 expression in both normal bronchial epithelium and in patients with asthma, a disease characterised by epithelial damage and repair. Samples were obtained from nine asthma patients and six controls by fibre optic bronchoscopy. One of the patients with asthma was treated at step 1, four at step 2, two at step 3, and one each at steps 4 and 5 of the British Thoracic Society Asthma guidelines [35]. All control patients were undergoing bronchoscopy for another indication and had normal spirometry. Patient characteristics are shown in table 1. Specimens from eight asthma patients and five controls underwent immunostaining for DDR1. Histologically, most epithelial specimens were of normal appearance. Specimens from five patients with asthma showed characteristic features of basement membrane thickening or epithelial cell shedding. All epithelia from both control- and asthma-derived bronchial biopsies were strongly positive for DDR1 (fig. 5). Staining appeared uniform in nature throughout the epithelium. There was minimal staining of subepithelial structures. Substitution of an immunoglobulin G isotype control for the DDR1 primary antibody abolished this staining. In six patients (three asthma and three control), HBECs were subcultured from biopsies. Protein lysates obtained from these cells underwent Western blotting for DDR1. All patients expressed a single protein band consistent with DDR1.

DISCUSSION

We have shown that DDR1 is expressed in normal bronchial epithelium and is involved in epithelial repair due to a combination of effects on cell proliferation and migration. DDR1 is required for expression of MMP-7 in HBECs, which is also likely to contribute to epithelial repair.

It is probable that the effects we have observed here are relevant to epithelial repair *in vivo*, where epithelial wounding exposes ECM containing collagen I, leading to activation of DDR1. Our work and that of others suggests that DDR1 may contribute to these effects by more than one mechanism. In the NIH3T3 mouse fibroblast cell line, activation of DDR1 by collagen I leads to co-localisation of DDR1 with the cytoskeletal protein nonmuscle myosin heavy chain (NMHC)-IIA in 30–60 min, which is faster than the maximal phosphorylation kinetics of DDR1 [19]. NMHC-IIA subsequently binds to the actin cytoskeleton. DDR1-transfected NIH3T3 cells at a wound edge migrate more rapidly than wild-type (DDR1-null) cells [36]. This correlates well with the known pro-migratory effects of DDR1 in mesangial cells, vascular smooth muscle, glioma cells and leukocytes [23, 37–39]. In bronchial epithelium, our study suggests the mechanism of DDR1-enhanced epithelial migration may be due to facilitation of other ECM receptor

interactions independent of DDR1 autophosphorylation, as all DDR constructs enhanced wound repair, irrespective of phosphorylation. In NIH3T3 cells, collagen I binding to DDR1 dimers leads to dimer aggregation on the cell surface prior to internalisation [40]. Receptor internalisation occurs much more quickly than receptor phosphorylation, suggesting a possible dual mechanism for DDR1 action: interaction with other cell surface receptors independent of phosphorylation at position 618 facilitates migration, while phosphorylation leads to effects on cell synthetic function [26, 40, 41].

Consistent with the observation that the kd variant of DDR1 abolishes phosphorylation and subsequent downstream signalling [26], in our overexpression model, although the kd mutant DDR1 enhanced epithelial repair, unlike wild-type DDR1, it had no effect on MMP-2 production or cell proliferation.

MMPs are known effectors of epithelial repair. MMP-7 is constitutively expressed in HBECs, is upregulated in regenerating lung epithelium [7] and MMP-7 knockout in mice abolishes epithelial repair [6]. Our findings show that MMP-7 is reduced in association with DDR1 knockdown in an *in vitro* wounding model. However, absolute levels of MMP-7 in supernatants were low, which may reflect the relatively small wound area and low number of cells involved in the repair process in this model. MMP-7 replacement at physiological levels did not restore epithelial repair, suggesting that the effect of DDR1 on epithelial repair in this model is likely to work through MMP-dependent and -independent pathways. We propose a complementary role of DDR1 and MMP-7 in epithelial repair, where DDR1 promotes migration, which is facilitated by the effect of MMP-7 on cell detachment. This is supported by observations in A549 cells in which either active or pro-MMP-7 was overexpressed. No change in migration of A549 cells was observed, but when active MMP-7 was overexpressed, more cells detached [42]. This is consistent with our observation that MMP-7 replacement does not restore bronchial epithelial repair that has been inhibited by DDR1 knockdown.

DDR1 has been shown to be essential for the maintenance of normal structure and function in skin and kidney [21, 22], and we propose a similar role in lung epithelium. It has been shown that DDR1 co-localises with E-cadherin and that E-cadherin sequesters DDR1 to cell–cell junctions in an intact epithelium [43]. Epithelial cell loss leads to exposure of DDR1 to ECM components with subsequent DDR1 activation, cell migration and, in our model, upregulation of MMP-7. To facilitate cell detachment, MMP-7 may cause loss of cell surface E-cadherin [44], further promoting the effects of DDR1 until the wound is closed, at which point DDR1 activation is reduced, less MMP-7 is produced and E-cadherin can sequester DDR1 to cell–cell junctions. In our study, epithelial repair was enhanced in the presence of DDR1 overexpression, even where the tyrosine kinase was inactive in BEAS-2B cells. This may be explained by the cell-surface molecule expression profile of BEAS-2B cells, which lack E-cadherin, allowing DDR1 to interact with other cell-surface ECM receptors to facilitate migration. Downstream signalling to MMP-7 would not be required to interrupt cell–cell interactions [45].

Our observation that DDR1 knockdown in HBECs did not significantly affect adhesion to various ECM factors may reflect the abundance of other cell adhesion molecules, such as integrins, which together are likely to have a larger effect on cell adhesion than DDR1. Consistent with this idea, it has previously been shown in NIH3T3 cells that adhesion to collagen I after 10 min is unaffected by DDR1 expression [36].

DDR1 was strongly expressed in the epithelium of the bronchial biopsies from both patients with asthma and nonasthma controls. This suggests that DDR1 expression is not affected by the asthmatic epithelial damage–repair process. However, DDR1 may still play a role in the disease through transduction of signals from the abnormal deposition of subepithelial ECM that is characteristic of the disease. Further studies are required to address this point.

In summary, we have shown that DDR1 regulates bronchial epithelial repair, proliferation and expression of MMPs. These findings warrant further investigation in diseases where epithelium is disrupted, including idiopathic pulmonary fibrosis and acute lung injury, particularly in view of the known upregulation of DDR1 in idiopathic pulmonary fibrosis.

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STATEMENT OF INTEREST

None declared.

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