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The metalloproteinase ADAM-12 regulates bronchial epithelial cell proliferation and apoptosis

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Abstract. *Objectives*: The ADAMs (a disintegrin and metalloproteinase) enzymes compose a family of membrane-bound proteins characterized by their multi-domain structure and ADAM-12 expression is elevated in human non-small cell lung cancers. The aim of this study was to investigate the roles played by ADAM-12 in critical steps of bronchial cell transformation during carcinogenesis. *Materials and methods*: To assess the role of ADAM-12 in tumorigenicity, BEAS-2B cells were transfected with a plasmid encoding human full-length ADAM-12 cDNA, and then the effects of ADAM-12 overexpression on cell behaviour were explored. Treatment of clones with heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) neutralizing antibodies as well as an EGFR inhibitor allowed the dissection of mechanisms regulating cell proliferation and apoptosis. *Results*: Overexpression of ADAM-12 in BEAS-2B cells promoted cell proliferation. ADAM-12 overexpressing clones produced higher quantities of HB-EGF in their culture medium which may rely on membrane-bound HB-EGF shedding by ADAM-12. Targeting HB-EGF activity with a neutralizing antibody abrogated enhanced cell proliferation in the ADAM-12 overexpressing clones. In sharp contrast, targeting of amphiregulin, EGF or transforming growth factor-α failed to influence cell proliferation; moreover, ADAM-12 transfectants were resistant to etoposide-induced apoptosis and the use of a neutralizing antibody against HB-EGF activity restored rates of apoptosis to be similar to controls. *Conclusions*: ADAM-12 contributes to enhancing HB-EGF shedding from plasma membranes leading to increased cell proliferation and reduced apoptosis in this bronchial epithelial cell line.

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INTRODUCTION

Together with snake venom metalloproteinases and ADAMTS (ADAMs with thrombospondin motifs), ADAMs (a disintegrin and metalloproteinase) constitute the adamalysin subfamily of metalloproteinases (Huovila *et al*. 2005; Lu *et al*. 2005). ADAMs are membrane-bound proteins with a multi-domain structure, which participate in many physiological and pathological functions. One important role of ADAMs relates to their metalloproteinase domain, which endows them with the ability to shed membrane-bound growth factors [e.g. tumour necrosis factor-α or transforming growth factor-α (TGF-α); Black *et al*. 1997; Peschon *et al*. 1998] or other cell surface molecules, including ligands and receptors (Asakura *et al*. 2002; Maretzky *et al*. 2005). These events mediate intracellular pathways and signalling. ADAM-12, initially described as meltrin-α (Yagami-Hiromasa *et al*. 1995), comprises an active metalloproteinase domain (Loechel *et al*. 1998) enabling shedding of growth factors, binding proteins (e.g. HB-EGF or IGFBP3; Loechel *et al*. 2000; Shi *et al*. 2000; Asakura *et al*. 2002; Higashiyama & Nanba 2005) and extracellular matrix components (Roy *et al*. 2004). The pro-domain (which remains non-covalently associated with the mature molecule) may rule ADAM-12 activities not only inside the cell, but also in the extracellular environment (Wewer *et al*. 2006). Two isoforms of ADAM-12 arise from alternative splicing: the secreted (short) form (ADAM-12S) which lacks transmembrane and cytoplasmic domains and the membrane-bound (long) form (ADAM-12L) (Gilpin *et al*. 1998). The short secreted form of ADAM-12 is, however, not expressed in lung tissue (Rocks *et al*. 2006).

Having first been described as a homologue of ADAM-1 and -2 displaying similar effects in myoblast fusion (Yagami-Hiromasa *et al*. 1995), ADAM-12 was shown to be implicated in cell–cell and cell–matrix interactions leading to myogenesis (Gilpin *et al*. 1998) or adipogenesis (Kawaguchi *et al*. 2002; Masaki *et al*. 2005). The cystein-rich domain of ADAM-12 supports mesenchymal cell adhesion through syndecans, and further spreading through integrin β1 (Iba *et al*. 2000). Furthermore, it appears that the cystein-rich domain of ADAM-12 is important in supporting tumour cell adhesion using heparin sulfate proteoglycans as receptors or co-receptors (Iba *et al*. 1999).

ADAM-12, produced in high amounts in placental tissue (Gilpin *et al*. 1998) and also present in maternal serum during pregnancy, has been proposed as a good early marker to predict the presence of Down syndrome (Laigaard *et al*. 2006), foetal trisomy 18 (Laigaard *et al*. 2005a) or pre-eclampsia, during the first trimester of pregnancy (Gack *et al*. 2005; Laigaard *et al*. 2005b).

Given its multiple functions, it is conceivable that this proteinase could be linked to various pathologies, such as cancer development and progression. Indeed, ADAM-12 expression is up-regulated in various tumours (Le Pabic *et al*. 2003; Carl-McGrath *et al*. 2005; Kveiborg *et al*. 2005; Lendeckel *et al*. 2005; Rocks *et al*. 2006) and can be detected in urine from breast or bladder cancer patients, where its levels correlate with disease stage (Frohlich *et al*. 2006; Roy *et al*. 2004). In a murine mammary cancer model, ADAM-12 overexpression is linked to enhanced tumour progression (Kveiborg *et al*. 2005). Moreover, ADAM-12 transgenic mice display less apoptosis of tumour cells while stromal cells display increased sensitivity to apoptotic signals (Kveiborg *et al*. 2005). Several studies have described ADAMs, including ADAM-12, as being key enzymes in epidermal growth factor receptor (EGFR) transactivation signalling by shedding EGFR ligands (Yan *et al*. 2002; Lemjabbar *et al*. 2003; Sahin *et al*. 2004; Schafer *et al*. 2004; Itoh *et al*. 2005; Tanaka *et al*. 2005; Liu *et al*. 2006). Indeed, shedding of HB-EGF by ADAM-12 plays an important role in cardiac hypertrophy (Asakura *et al*. 2002). However, to date, mechanisms implicating ADAM-12 in lung cancer progression have not been studied at the molecular level.

We recently described human full-length ADAM-12 overexpression in human non-small cell lung carcinoma (Rocks *et al*. 2006). This prompted us to decipher the exact roles of ADAM-12 in cancer development and progression, by transfection of immortalized bronchial epithelial cells (BEAS-2B cell line) with human full-length ADAM-12. Here, we show that overexpression of ADAM-12 leads to an increase in cell proliferation while etoposide-stimulated apoptosis is reduced in cells transfected with ADAM-12. These effects are mediated through increased pro-HB-EGF shedding in cells overexpressing ADAM-12.

MATERIALS AND METHODS

Cell culture

Human BEAS-2B cells (a bronchial epithelial cell line resulting from immortalization of bronchial epithelial cells by SV40) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Airway Epithelial Cell Basal Medium (Promocell, Belgium) supplemented with Supplement Pack (Promocell, Heidelberg, Germany), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen Corporation, Paisley, UK) at 37 °C in a humidified 5% CO_2 atmosphere.

Stable transfection of BEAS-2B cells with human ADAM-12L cDNA

BEAS-2B cells, which spontaneously express very low amounts of ADAM-12L (Rocks *et al*. 2006), were stably transfected with pcDNA3-*neo* plasmid encoding human full-length ADAM-12L cDNA, kindly provided by Ulla Wewer (Copenhagen, Denmark). As controls, parental BEAS-2B cells were transfected with the empty pcDNA3-*neo* vector containing only the neomycin resistance gene. Transfection was performed by electroporation at 250 V and 960 μF using a gene pulser system (Bio-Rad Laboratories, Hercules, CA, USA). Culture of transfected cells in neomycin-containing medium (G418, 0.5 mg/mL; Life Technologies, Grand Island, NY, USA) allowed selection of clones, and all those obtained were screened by RT-PCR for their ADAM-12 expression. Selected clones were assessed by qRT-PCR and flow cytometry for their ADAM-12 mRNA expression and protein production. Stable transfectants were grown in G418-containing medium (0.1 mg/mL; Invitrogen Corporation).

Semi-quantitative RT-PCR analysis and real-time quantitative PCR

For semi-quantitative RT-PCR, total RNA was extracted from cells by RNA Easy Qiagen kit as recommended by the manufacturer (Qiagen, Germantown, MD, USA). RT-PCR was performed on 10 ng of total RNA using the GeneAmp thermostable RNA RT-PCR Kit (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers were designed as follow: ADAM-9 (forward 5′-AGAAGAGCTGTCTTGCCACAGA-3′, reverse 5′-TTTTCCCGCCACTGCACGAAGT-3′); ADAM-12 (forward 5′-TTGGCTTTGGAGGAAGCACAGA-3′, reverse 5′-TTGAGGGGTCTGCTGATGTCAA-3′); ADAM-17 (forward 5′-TACAAAGGAAGCTGACCTGGTT-3′, reverse 5′-TTCATCCACCCTCGAGTTCCCA-3′). Samples were separated by acrylamide gel electrophoresis and were stained with Gel Star (Cambrex, Verviers, Belgium) and intensity of each band was measured using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). In order to normalize mRNA levels in different samples, value of the band corresponding to each mRNA level was divided by intensity of the corresponding 28S rRNA value.

For qRT-PCR, clones and parental BEAS-2B cells were cultured for 24 h in standard culture medium. Total RNA was obtained using the TRIzol reagent (Invitrogen Corporation) and 100 ng of RNA were used for retro-transcription. Real-time quantitative PCR for ADAM-12 was

performed using SuperScript Platinum SYBR Green Two-Step qRT-PCR with ROX (Invitrogen Corporation). In order to obtain a normalized target value, actin gene was used as control. Results were calculated as mean ± SEM of relative gene expression expressed as folds $2^{-(\Delta\Delta Ct)}$ compared to parental BEAS-2B cells.

Flow cytometry analysis

Clones and parental BEAS-2B cells were cultured for 24 h in standard culture medium. The epithelial layer was dissociated with phosphate-buffered saline–ethylenediaminetetraacetic acid (5 mM) solution, and cells were detached by scraping. Epithelial cells were centrifuged and re-suspended in phosphate-buffered saline with 2% foetal calf serum. Cells were incubated with rabbit immunoglobulin G (IgG) anti-ADAM-12 (N-terminal domain) or normal rabbit IgG (2 μg/mL; Abcam, Cambridge, MA, USA) with or without permeabilization with the Perm-Wash kit (BD Biosciences, San Diego, CA, USA). After washing, antibody binding was detected with phycoerythrin-conjugated goat anti-rabbit IgG (Invitrogen Corporation). Cells were then washed, fixed in paraformaldehyde and 5000 events were analysed on a FACScalibur flow cytometer with CellQuest software (Becton Dickinson, Erebodegem, Belgium). Results are expressed as the difference between median fluorescence intensity (MFI) with specific antibody minus the isotype control MFI (ΔMFI).

Preparation of conditioned media and Western blot analysis

For conditioned media, 10^6 cells were seeded on 100 mm diameter dishes, in standard culture medium for 24 h and then were cultured for 48 h in 6 mL of serum-free medium. Conditioned media were collected, clarified by centrifugation and stored at –20 °C. For Western blotting, samples were separated on 12% polyacrylamide gels and were transferred on polyvinylidene difluoride membranes. Samples were blocked for 2 h and then were incubated with anti-human HB-EGF goat polyclonal antibody (1/1000; R&D Systems, Minneapolis, MN, USA) overnight at 4 °C. Signals were detected by secondary horse radish peroxidase-coupled rabbit anti-goat antibody (DAKO, Glostrup, Denmark) diluted 1/1000 by using an enhanced chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA, USA).

Proliferation assay

The cells were cultured in triplicate in 24-well plates at a density of 10^4 cells per well and were maintained in standard culture conditions. Cells were harvested each day for 1 week until confluence was reached. Fluorimetric DNA titration was then performed as described previously (Labarca & Paigen 1980) and used as an indicator of cell density. Anchorage-independent growth was determined by soft agar analysis (Laboisse *et al*. 1981) and cells were plated into 24-well plates at a density of 2×10^4 cells per well, in growth medium containing 0.3% agar, on the surface of a 0.6% agar gel. After 9 days of culture, cells were stained with crystal violet for 1 h and were photographed. Colonies were counted under a microscopic field at 2× magnification. Each assay was performed in triplicate on two independent assays.

To investigate proliferation of cells treated with antibodies neutralizing the effects of EGFR ligands (amphiregulin, EGF, HB-EGF or TGF-α), or with an EGFR inhibitor, clones were seeded in triplicate into 96-well plates at a density of 1250 cells per well. After adhesion, cells were cultured in standard culture medium or were treated either with anti-human HB-EGF (5 μg/mL; R&D Systems), anti-human amphiregulin (10 μg/mL; R&D Systems), anti-human EGF (8 μg/mL; R&D Systems), anti-human TGF-α (0.5 μg/mL; R&D Systems) neutralizing antibodies or with an EGFR inhibitor [4-(3-chloroanilino)-6,7-dimethoxyquinazoline] (AG1478; 5 μg/mL; Calbiochem, San Diego, CA, USA). Quantification of cell proliferation was performed at days 0, 1, 2, 4 and

6 by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis, following the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Results are expressed as time-dependent BrdU incorporation measured by chemiluminescence (absorbance A450 nm–A690 nm).

Invasion or migration capacities of cells using Boyden chamber assays

In vitro migration properties of transfected cells were assessed using a modified Boyden chamber assay. Cells (10^4 cells per well) were suspended in serum-free medium supplemented with 0.1% bovine serum albumin and were placed in quadruplicate in the upper compartment of a 48-well invasion chamber (Nucleopore, Pleasanton, CA, USA). The lower compartment of the chamber was filled with 30 μL Airway Epithelial Cell Basal Medium containing 10% foetal bovine serum and 1% bovine serum albumin. For investigation of invasive properties, cells were allowed to migrate through filters with 8 μm pores (Nucleopore) pre-coated with 25 μg of Matrigel. After 24 h incubation at 37 °C in a humid atmosphere, filters were fixed with methanol at –20 °C and were stained with Giemsa solution (Fluka Chemie AG, Buchs, Switzerland) for 20 min. Non-migrating cells were removed by gentle wiping. Cells recovered from the lower surface of the filters were counted using a microscope, at 400× magnification (30 fields). Experiments were performed twice in quadruplicate. Data are expressed as mean ± SEM.

Apoptosis measured by TUNEL assay

BEAS-2B cells and transfected clones were seeded at 5×10^4 cells per well, in triplicate into 24-well plates. After adhesion, cells were either grown in standard culture medium or were stimulated with etoposide (20 μM; Calbiochem) for 24 h. They were then harvested for TUNEL staining. The proportion of cells showing DNA fragmentation was measured by incorporation of fluorescein isothiocyanate (FITC)-12-dUTP into DNA using terminal deoxynucleotidyltransferase (*in situ* Cell Death Detection, Roche Diagnostics GmbH) as described by the manufacturer. In order to determine total cell number, cell nuclei were labelled with bisbenzimide. Total number of cells and TUNEL-labelled cells were counted in six random fields at 400× magnification. Quantitative analysis of apoptosis is represented as the ratio of TUNEL-labelled cells to total number of cells. Experiments were performed at least twice in triplicate.

To investigate the effects of HB-EGF neutralization on apoptosis, cells were seeded at 5×10^4 per well, in quadruplicate, into 24-well plates. After adhesion, they were either grown in standard culture medium or were treated with etoposide (20 μM) alone, in combination with a general caspase inhibitor (10 μM; Z-VAD-FMK, R&D Systems) or with an anti-HB-EGF neutralizing antibody (5 μg/mL), for 24 h. Cells were then harvested for TUNEL staining and apoptotic cells were counted. Experiments were performed twice in quadruplicate.

Subcutaneous injection of clones into immunodeficient mice

Matrigel was prepared as described previously (Noel *et al*. 1991). Transfected BEAS-2B clone suspensions (10^6 cells) were prepared in standard medium and mixed with an equal volume of cold Matrigel, just before subcutaneous injection into 6- to 8-week-old male SCID mice (Harlan, Indianapolis, IN, USA). A final volume of 400 μL of mixed Matrigel and medium-containing cells was injected into each flank of the mouse.

Statistical analysis

Data are expressed as mean ± SEM. Differences between experimental groups were assessed using the Mann–Whitney test. $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered as statistically significant.

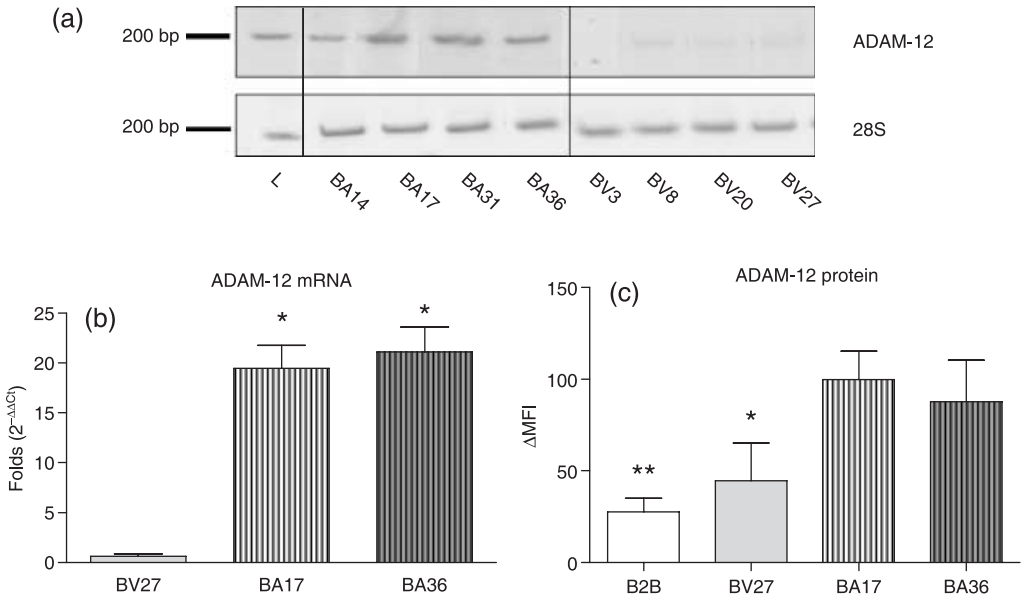

Figure 1. Characterization and selection of transfectants. (a) Semi-quantitative PCR analysis of transfected clones. ADAM-12 mRNA expression is higher in cells transfected with ADAM-12 cDNA-containing pcDNA3 plasmid (BA) than in cells transfected with the empty vector used as control (BV). L: 200 bp molecular weight marker (Smart Ladder, Eurogentec, Belgium). (b) Real-time quantitative PCR measuring ADAM-12 expression in clones. ADAM-12 expression is higher in ADAM-12 transfected clones than in control clones. Results are the mean ± SEM of relative gene expression expressed in folds ($2^{-\Delta\Delta Ct}$) compared with BEAS-2B gene expression ($n = 3$). *$P < 0.05$, Mann–Whitney test. (c) ADAM-12 protein production was determined by flow cytometry. ADAM-12 protein production is higher in ADAM-12 transfected clones than in control clones. Results are expressed as mean ± SEM of ΔMFI (difference between mean fluorescence intensity versus control isotype) ($n = 3$). **$P < 0.01$, BEAS-2B versus BA clones; *$P < 0.05$, BV27 versus BA17, Mann–Whitney test.

RESULTS

Transfection of BEAS-2B cells with full-length ADAM-12 and clone characterization

As ADAM-12 mRNA was weakly expressed in the basal state of BEAS-2B cells (Rocks *et al*. 2006), we stably transfected this cell line with pcDNA3-*neo* vector containing full-length ADAM-12 cDNA (BA clones) or with the pcDNA3-*neo* plasmid containing only the neomycin resistance gene, serving as control (BV clones). Clones obtained by G418 selection were initially screened by RT-PCR. PCR reactions for the neomycin gene confirmed the presence of the pcDNA3-*neo* plasmid in transfected clones (data not shown). For further *in vitro* studies, four clones overexpressing ADAM-12 (referred to as BA clones) and four control clones (referred to as BV clones) were selected (Fig. 1a). Both real-time quantitative PCR and flow cytometry studies revealed higher ADAM-12 mRNA expression (*$P < 0.05$ versus BV27) and protein production in BA clones than in BV control clones (*$P < 0.05$ versus BA17) or BEAS-2B cells (**$P < 0.01$ versus BA clones) (Fig. 1b,c).

ADAM-12 overexpression increased cell proliferation *in vitro*

Cell proliferation of clones and parental cells was first measured by determining DNA concentration as an indicator of cell density. Interestingly, ADAM-12 overexpressing clones displayed

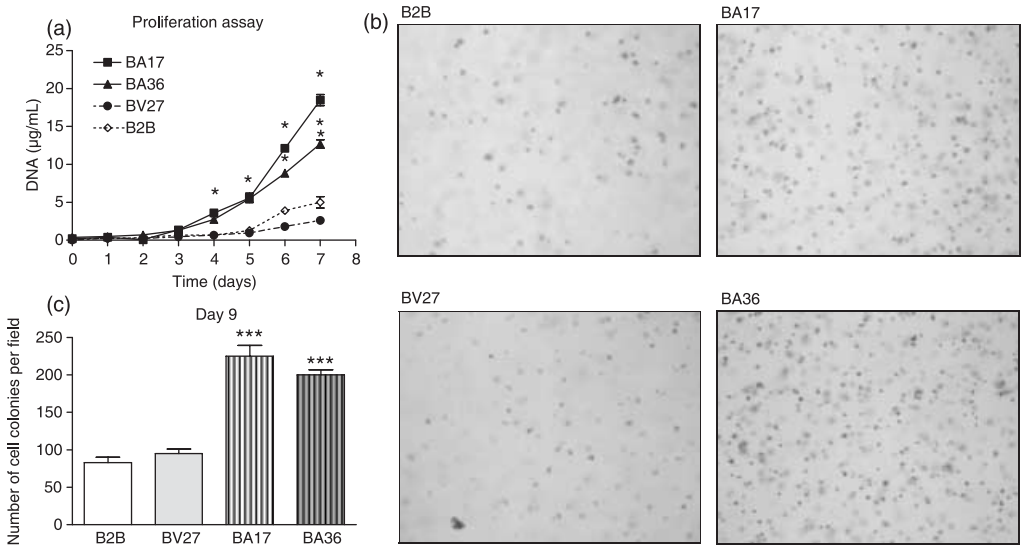

Figure 2. ***In vitro*** **proliferation of BEAS-2B clones**. (a) Cells cultured in complete culture medium were incubated (in triplicate) in 24-well plates (10^4 cells/well) and cell number was determined by measuring DNA concentration at 1-day interval ($n = 3$); *$P < 0.05$, BA17 and BA36 versus BV27 and parental BEAS-2B (B2B) cells, Mann–Whitney test. Results shown are those of a representative assay out of three different experiments. (b and c) Clones were grown in 24-well dishes in soft agar and cell growth was determined by counting number of colonies developed after 9 days of incubation. ADAM-12 overexpression significantly enhanced growth abilities of cells without any anchorage support (***$P < 0.001$ versus BV27 and BEAS-2B cells). Results are expressed as mean number of colonies counted per field at 2× magnification, Mann–Whitney test (results are representative of three independent experiments).

higher *in vitro* cell population growth rates than controls (Fig. 2a). It is worth noting that this difference in cell population growth rates was clearly apparent after 4 days of culture (*$P < 0.05$ versus BV27 and parental BEAS-2B cells). In addition, ADAM-12 overexpression also significantly enhanced anchorage-independent cell colony formation after 9 days of culture in soft agar (***$P < 0.001$ versus controls) (Fig. 2b,c).

ADAM-12-mediated enhanced proliferation is dependent on exaggerated production of mature HB-EGF

As HB-EGF is a key player in cell proliferation regulatory mechanisms and appears to be matured by ADAM-dependent shedding, we sought a possible direct effect for ADAM-12 overexpression on HB-EGF maturation in clones. Protein levels of mature HB-EGF were higher in conditioned media from clones overexpressing membrane-bound ADAM-12 (BA clones), as compared to control clones (Fig. 3a). These differences were dependent on ADAM-12 overexpression and could not be ascribed to shedding of pro-HB-EGF by ADAM-9 or ADAM-17. Indeed, ADAM-9 and ADAM-17 mRNA levels were similar in parental cells, ADAM-12 overexpressing clones or control clones as assessed by RT-PCR (data not shown).

We further addressed the effects of HB-EGF or EGF receptor tyrosine kinase inhibition on proliferation of ADAM-12 overexpressing clones and corresponding controls. When cells were cultured in standard media, in the presence of an anti-EGFR compound (AG1478) or an anti-HB-EGF neutralizing antibody, exaggerated proliferation found in ADAM-12 overexpressing clones was suppressed (**$P < 0.01$, ***$P < 0.001$, standard conditions versus inhibitors; °$P < 0.05$, °°$P < 0.01$ versus BV27) (Fig. 3b,c). This inhibitory effect in cell proliferation was not observed

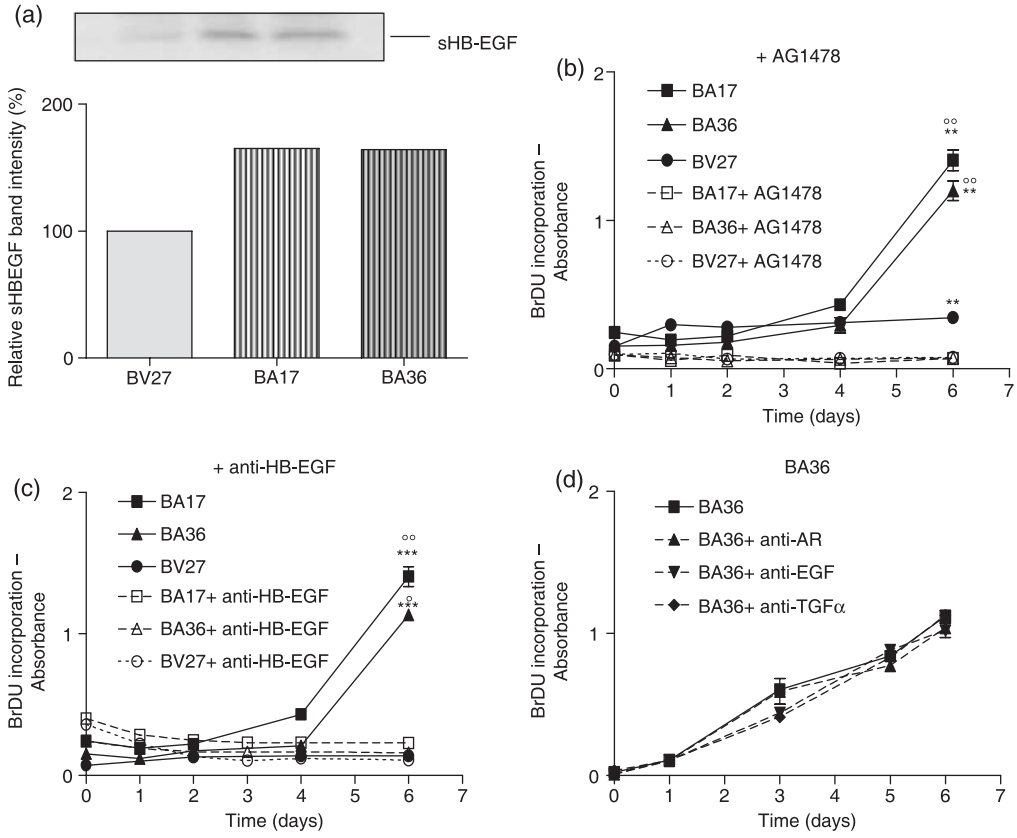

Figure 3. Mature HB-EGF expression in clones overexpressing ADAM-12 and effects of HB-EGF inhibition on cell proliferation. (a) Mature (soluble) HB-EGF protein (sHB-EGF) production measured by Western blot analysis in conditioned media of transfected cells (top panel). Data expressed as relative sHB-EGF band intensities, considering BV27 intensity as 100% (lower panel). (b–d) Cells were grown either in standard media or in media supplemented with a specific inhibitor of EGF receptor tyrosine kinase (AG1478) (b) or an anti-HB-EGF neutralizing antibody (c), or neutralizing antibodies against amphiregulin (AR), EGF, TGF-α (d). Results are representative of three independent experiments. In (d), results are shown for BA36 clone, as a representative example. $^{**}P < 0.01$, $^{***}P < 0.001$ versus stimulation with inhibitors. $^{\circ}P < 0.05$ versus BV27; $^{\circ\circ}P < 0.01$ versus BV27, Mann–Whitney test.

with neutralizing antibodies targeting amphiregulin, EGF or TGF-α (Fig. 3d). Taken together, these results showed that increase in ADAM-12-dependent cell proliferation was related to activation of the HB-EGF pathway.

ADAM-12 induces HB-EGF-dependent resistance to apoptosis

To determine etoposide-induced apoptosis in BEAS-2B clones, an *in vitro* experiment was conducted using a TUNEL assay. Baseline apoptotic rate was determined in the cells cultured in standard conditions without any etoposide treatment. As shown in Fig. 4a, stimulation of BEAS-2B cells with etoposide significantly enhanced apoptosis when compared to baseline levels ($^{\circ\circ}P < 0.01$). In addition, when treated with etoposide, cells overexpressing ADAM-12 displayed significantly lower apoptosis than those transfected with the empty vector or in parental BEAS-2B cells ($^{**}P < 0.01$) (Fig. 4a). As a control, cells were treated with the broad caspase inhibitor Z-VAD-FMK, which drastically diminished apoptosis, demonstrating a caspase-dependent

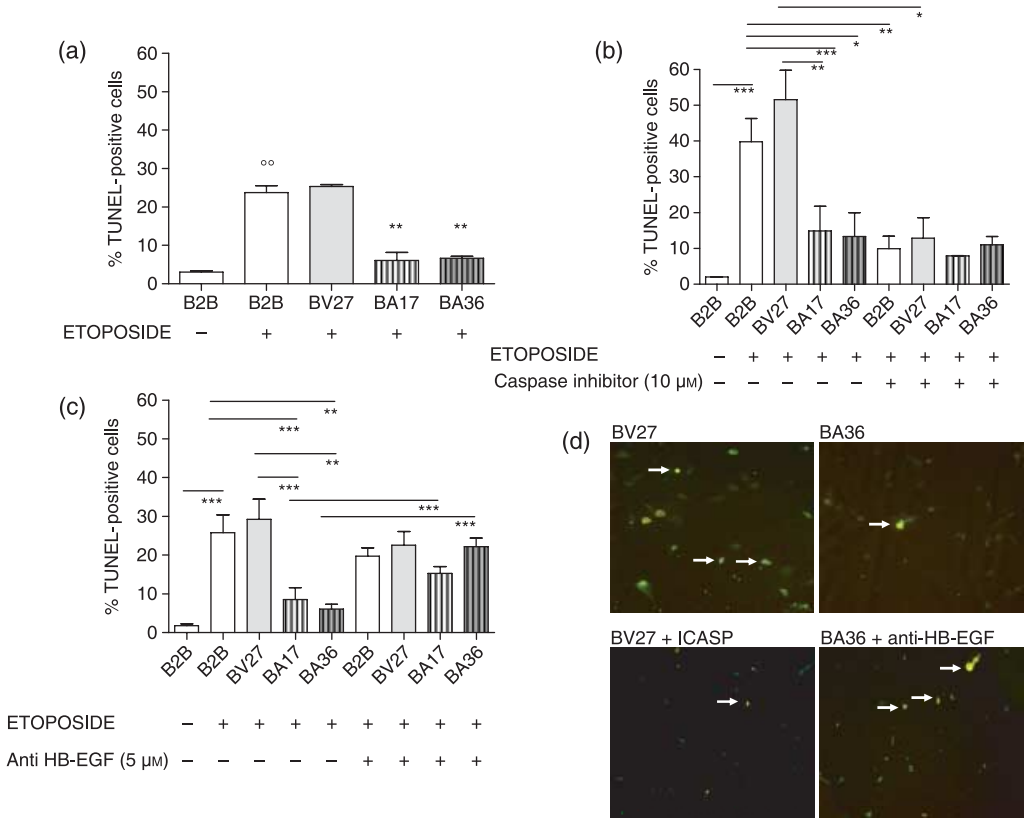

Figure 4. Representative results of TUNEL assay, measuring role of ADAM-12, in cell resistance to etoposide-induced apoptosis, effects of general caspase inhibition and HB-EGF neutralization. (a) Cells were immunostained with FITC-12-dUTP to investigate baseline or etoposide-induced apoptosis (presented data are representative of two independent experiments). Results are expressed as percentage of mean number of TUNEL-positive cells compared to total cell number for each group ± SEM. $^{**}P < 0.01$, compared to parental or control transfected cells; $^{\circ\circ}P < 0.01$, compared to baseline apoptosis in parental BEAS-2B cells, Mann–Whitney test. (b) Cells cultured in presence of a general caspase inhibitor and subjected to apoptosis inducing conditions showed reduced apoptotic levels when compared to standard apoptosis inducing experiments ($^{*}P < 0.05$, $^{***}P < 0.001$). (c) Inhibition of HB-EGF by a neutralizing antibody enhanced apoptotic levels of ADAM-12 overexpressing cells. Apoptotic levels in parental and control cells were not different when cells were cultured with etoposide alone or with etoposide and anti-HB-EGF neutralizing antibody. (d) Representative microphotographs of TUNEL staining in ADAM-12 overexpressing clones and controls. White arrows indicate examples of cells positive for TUNEL staining.

mechanism for apoptosis in our model (Fig. 4b). Neutralizing HB-EGF antibody abrogated protection against apoptosis observed in ADAM-12 overexpressing cells ($^{***}P < 0.001$, etoposide versus etoposide and HB-EGF inhibitor) (Fig. 4c). In sharp contrast, addition of anti-HB-EGF neutralizing antibody did not affect the apoptotic level of parental ($P = 0.94$) or control BV27 ($P = 0.78$) cells treated with etoposide. This result clearly underlines a role for ADAM-12 overexpression in protection of cells against apoptosis, through an HB-EGF-mediated mechanism.

Role of ADAM-12 in *in vitro* cell migration and invasion

In vitro cell migration or invasion capacities were compared between transfected cells using a modified Boyden chamber assay. Parental cells and clones were evaluated for their potential to

migrate through filters or invade a Matrigel layer, for 24 h. In this assay, ADAM-12 overexpression did not affect cell migration or invasion ($P > 0.05$) (data not shown).

Subcutaneous injection of clones into immunodeficient mice

To further evaluate the *in vivo* tumorigenic phenotype of ADAM-12 transfectants, clones were subcutaneously injected into immunodeficient *SCID* mice. However, neither the ADAM-12 transfected cells nor the control cells developed detectable tumours when injected into *SCID* mice (data not shown). These data suggest that ADAM-12 was not *per se* sufficient to initiate cancer development.

DISCUSSION

Over the last number of years, matrix metalloproteinases have been studied extensively, and increased activity of these proteolytic enzymes has been associated with the malignant phenotype (Egeblad & Werb 2002; Rocks *et al*. 2008). ADAM-12 is a proteinase of the ADAM family which is highly expressed in rapidly growing tissues, such as placenta and malignant tumours (Iba *et al*. 1999; Le Pabic *et al*. 2003). Based on our previous studies showing overexpression of full-length ADAM-12 mRNA and proteins, in human non-small cell lung cancer samples (Rocks *et al*. 2006), we investigated the effects of full-length ADAM-12 overexpression on the phenotype of human bronchial epithelial non-transformed BEAS-2B cells. Here, we focused our attention on membrane-bound ADAM-12, because the short secreted form of ADAM-12 is expressed neither in lung tissue nor in lung tumours (Rocks *et al*. 2006).

Both *in vitro* and *in vivo* studies were performed in order to characterize ADAM-12 transfected clones. Proliferation assays measuring cell density by DNA titration and BrdU incorporation revealed higher proliferation rates in ADAM-12 overexpressing clones as compared to control clones.

Normally, numerous proteins are secreted as soluble molecules but some proteins playing key roles in cell behaviour, such as EGFR ligands, are first synthesized as membrane-bound precursors. These molecules require proteolytic cleavage through zinc-dependent proteinases for activation. ADAM proteinases, such as ADAM-9, -12 and -17, are important regulators of cleavage for EGFR ligands (Gee & Knowlden 2003; Ohtsu *et al*. 2006). In order to provide further insight into the exaggerated cell proliferation levels found in ADAM-12 overexpressing clones, we sought potential factors whose activation could be regulated by ADAM-12. Of interest was our finding of higher levels of soluble active HB-EGF in media conditioned by ADAM-12 over-expressing cells, while production of other ligands of EGFR was not affected. Moreover, incubation of cells with an anti-HB-EGF blocking antibody or a specific inhibitor of EGFR reversed the enhanced cell proliferation in ADAM-12 overexpressing clones, confirming a role for this factor in control of proliferation of these cells. A specific effect on the HB-EGF/EGFR pathway is supported by failure of other neutralizing antibodies targeting amphiregulin, EGF or TGF-α, to control cell proliferation. These data are reinforced by previous studies showing the predominant role of HB-EGF shedding in glioblastoma or in HB-EGF-dependent cardiac hypertrophy. These studies suggested that processing of HB-EGF by ADAM-12 could play a central role in many pathological processes implicating cell proliferation (Asakura *et al*. 2002; Kodama *et al*. 2004). ADAM-9 and ADAM-17, two potentially important regulators of pro-HB-EGF shedding, are, however, not involved in the amplified HB-EGF shedding observed in ADAM-12 transfectants. Indeed, no difference regarding their expression was found among the clones.

A role for ADAM-12 in mechanisms leading to apoptosis has been suggested by previous authors, who reported lower apoptosis levels in tumour cells from ADAM-12 overexpressing mice (Kveiborg *et al*. 2005). In the present study, control clones were found to be more sensitive to etoposide-induced apoptosis than ADAM-12 overexpressing clones. To challenge the hypothesis that higher, active HB-EGF production could help protect cells from apoptosis in our model, neutralizing antibodies of HB-EGF were used. Interestingly, neutralization of HB-EGF led to increased apoptosis. This is the first description of an anti-apoptotic effect of ADAM-12 through HB-EGF shedding in bronchial cells. Our study, therefore, clearly identifies ADAM-12 as a potential therapeutic target in cancer. It is interesting to note that etoposide is a chemotherapeutic drug used as a treatment option in some types of cancer, including lung cancer (Rigas & Kelly 2007). Data presented here argue for a possible role of ADAM-12 in chemoresistance against etoposide *in vivo* and imply a role for this enzyme in resistance of some tumours to chemotherapy regimens. These facts should be handled carefully and further experiments are still needed to demonstrate that ADAM-12 inhibition, for example, by SiRNAs or neutralizing antibodies, can actually reverse this phenotype characteristic.

ADAM-12 has been proposed as a reliable biomarker for bladder and breast cancer, because it can be detected in urine from breast cancer or bladder cancer patients, confirming the biological relevance of this mediator. Our data also suggest that HB-EGF is another potential target in bronchial cell lines and indicate that further research with a neutralizing antibody to HB-EGF or with an EGFR inhibitor should be conducted *in vitro* and *in vivo*. Because *in vitro* results converge towards a modification of the BEAS-2B cell phenotype, it seemed essential to analyse *in vivo* behaviour of cells. At the first attempt, a soft agar assay was applied to evaluate anchorage-independent growth capacity of the cells, a major hallmark of transformed cells. Indeed, the ability of cells to proliferate without firm attachment is one of the *in vitro* indicators of tumorigenicity (Freedman & Shin 1974). Most cancer cells overcome anchorage growth dependency and become capable of non-anchored proliferation. In our work, ADAM-12 overexpression in BEAS-2B cells led to enhancement of their ability to form colonies during soft agar assay. These data pinpoint the impact of ADAM-12 overexpression on modulation of cell phenotype. *In vivo* tumorigenicity was then investigated by subcutaneously inoculating cell transfectants into immunodeficient SCID mice. Neither ADAM-12 transfected cells nor the control cells developed detectable tumours. Therefore, ADAM-12 expression was not *per se* sufficient to induce development of tumours *in vivo*. It is now well accepted that tumour progression is not only dependent on acquisition of new properties by tumour cells themselves, but is also influenced by the tumour microenvironment composed of extracellular matrix and host cells, including stromal cells (Kalluri & Zeisberg 2006; Noel *et al*. 2008). Despite inoculation of cells with Matrigel, known as a tumour promoter (Noel *et al*. 1993), results of the tumour implantation were unsuccessful. In addition, inoculation of cells into the lung by intratracheal instillation also failed to promote tumour development (data not shown). These data demonstrate that although ADAM-12 is able to control cell proliferation, it cannot convert immortalized non-transformed cells, such as BEAS-2B cells into tumorigenic cells. Moreover, ADAM-12 overexpressing clones did not display any invasive phenotype *in vitro* in modified Boyden chambers, suggesting again that ADAM-12 is not sufficient *per se* to acquire invasive properties. Interestingly, Kveiborg *et al*. described a higher frequency of metastasis in polyomavirus middle T oncoprotein transgenic mice expressing ADAM-12 (Kveiborg *et al*. 2005), suggesting that ADAM-12 confered increased malignancy in this mouse model, whose oncogenesis was induced by expression of the polyomavirus middle T oncoprotein. Hence, ADAM-12 might act as an enhancer of malignancy in cells which already have acquired a transformed phenotype, while it contributes to enhance the proliferation in non-transformed cells.

In conclusion, we show for the first time that ADAM-12 overexpression in lung epithelial cells contributes to increased cell proliferation and reduced apoptosis. These effects appeared to be mediated through ADAM-12-dependent pro-HB-EGF shedding and further EGFR transactivation. ADAM-12 should, therefore, be considered as one of the key factors participating in the complex interplay between growth factors and proteinases. ADAM-12 contributes to enhance cell proliferation levels and to reduce apoptosis thereupon endowing normal epithelial cells with tumour cell features.

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