Endothelial MMP14 is Required for Endothelial Dependent Growth Support of Human Airway Basal Cells

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Abstract

Human airway basal cells (BC) are the stem/progenitor population of the airway epithelium, and play a central role in anchoring the epithelium to the basement membrane. The anatomic position of BC allows for potential paracrine signaling between BC and the underlying non-epithelial stromal cells. In support of this, we previously demonstrated endothelial cells (EC) support growth of BC during co-culture via vascular endothelial growth factor A (VEGFA)-mediated signaling. Building on these findings, RNA sequencing analysis demonstrated that BC express multiple fibroblast growth factor (FGF) ligands (FGF2, 5, 11 and 13) with only FGF2 and FGF5 capable of functioning in a paracrine manner to activate classical FGF receptor (FGFR) signaling. Antibody mediated blocking of FGFR1 during BC-EC co-culture significantly reduced EC dependent BC growth. Stimulation of EC via BC-derived growth factors resulted in EC expression of matrix metallopeptidase 14 (MMP14) and shRNA mediated knockdown of EC MMP14 significantly reduced EC dependent growth of BC. Overall, these data characterize a novel growth factor mediated reciprocal “cross-talk” between human airway BC and EC that regulates proliferation of BC.
Introduction

The human airway epithelium consists of multiple cell types including basal, secretory and ciliated cells lining a basement membrane (BM) (Knight and Holgate, 2003; Crystal et al., 2008; Tam et al., 2011). Below the BM lie multiple non-epithelial cell types including fibroblasts, smooth muscle and capillary endothelial cells (EC) (Tam et al., 2011). Basal cells (BC) are the stem/progenitor population of the airway epithelium that differentiate into the other specialized epithelial cell types during turnover and repair (Evans et al., 2001; Hajj et al., 2007; Rock et al., 2009; Rock et al., 2010; Hackett et al., 2011; Shaykhiev et al., 2013; Staudt et al., 2014; Hogan et al., 2014). In addition, BC play a central role in anchoring the epithelium to the BM helping to protect the underlying non-epithelial cell types from the external environment (Knight and Holgate, 2003; Tam et al., 2011). The positioning of BC allows for potential paracrine signaling from other non-epithelial cell types to regulate BC function including proliferation and differentiation. Therefore, understanding the cross-talk between BC and other airway cell types is important for understanding the processes that regulate normal airway epithelial architecture.

Previous work from our laboratory demonstrated functional cross-talk between BC and EC, whereby EC in response to BC secreted vascular endothelial factor A (VEGFA) could support growth of BC in co-culture (Curradi et al., 2012). The present study builds on these findings and demonstrates that, in addition to VEGFA, BC express multiple fibroblast growth factor (FGF) ligands, a family of growth factors known to regulate EC activation (Presta et al., 2005; Lieu et al., 2011; Belov and Mohammadi, 2013; Carter et al., 2014). These ligands function in a paracrine manner to activate EC via FGF receptor 1 (FGFR1) dependent signaling. Further, secreted BC derived growth factors induce EC expression of matrix metallopeptidase 14 (MMP14), a mediator required for EC dependent growth support of BC.
Results and Discussion

Expression of FGF Ligands in Human Airway BC

Fibroblast growth factor ligands play an essential role in regulating diverse processes in multiple cell types during embryonic development and in differentiated tissues (Presta et al., 2005; Lieu et al., 2011; Belov and Mohammadi, 2013; Carter et al., 2014). Majority of FGF ligands function by binding to four tyrosine kinase fibroblast growth factor receptors (FGFR1-4) to activate downstream kinase responses including Akt and MAPK signaling. However, some ligands (FGF11-14) do not bind FGFRs and have distinct modes of action (Olsen et al., 2003). Based on our prior work demonstrating cross-talk between EC and BC (Curradi et al., 2012), and the knowledge that FGF ligands play a role in regulating EC function, we assessed expression of FGF ligands in BC. RNA sequencing demonstrated BC express only FGF2, 5, 11 and 13 (Fig. 1A). Of the ligands expressed, only FGF2 and FGF5 mediate signaling via binding to FGFRs and are capable of functioning in both an autocrine and paracrine manner, whereas FGF11 and 13 function independent of FGFRs (Olsen et al., 2003; Presta et al., 2005; Lieu et al., 2011; Belov and Mohammadi, 2013; Carter et al., 2014). Basal cell expression of FGF2 and FGF5 at the protein level was confirmed by immunohistochemistry (Fig. 1B). In addition, secretion of both FGF2 and FGF5 was confirmed by ELISA analysis of supernatants from BC cultures (Fig. 1C, D). To investigate whether FGF2 and FGF5 function in an autocrine manner to activate signaling cascades in BC, growth factor starved BC were stimulated with basal media, basal media supplemented with recombinant FGF2 or FGF5 or growth media as a positive control. Western analysis for activation of Akt and p44/42 MAPK demonstrated robust phosphorylation of both proteins in cells stimulated with growth media (Fig. 1E, lane 6). However, in cells stimulated with basal media or basal media supplemented with FGF2 or FGF5 there were low levels of basal phosphorylation of both proteins with no increased activation in response to each stimuli (Fig. 1E, lanes 1-5). Equal loading of protein in each lane was confirmed by staining for total Akt and p44/p42 MAPK (Fig. 1E, lanes 1-5). Overall, these data suggest that BC expressed FGF2 and FGF5 function in a paracrine manner.

Fibroblast growth factor 2 is a pro-angiogenic factor known to regulate EC function through FGFR1 dependent signaling mechanisms (Presta et al., 2005). The role of FGF5 in regulating EC function is less well characterized, although it is overexpressed in human
glioblastoma and promotes malignant progression via FGFR1-dependent signaling (Allerstorfer et al., 2008). Based on these functions we focused on characterizing the role of FGFR1 signaling in regulating cross-talk between BC and EC.

**Blocking of FGFR1 with Specific Antibody Suppresses EC-dependent Proliferation of BC**

Using our previously developed cytokine- and serum-free co-culture system that utilizes modified primary human umbilical vein endothelial cells (HUVEC) constitutively expressing active Akt (HUVEC-Akt) (Curradi et al., 2012), we assessed the role of FGFR1-mediated signaling in regulating EC dependent proliferation of BC. Co-cultures of BC and EC were untreated, or incubated with control IgG or an antibody against FGFR1 (which blocks FGF ligand dependent signaling through this receptor) in the absence and presence of recombinant FGF2 or FGF5. Immunofluorescence analysis with specific markers for EC (VE-cadherin) and BC (KRT5) following 4 days of co-culture in the presence of IgG or anti-FGFR1 antibody (with or without FGF2 or FGF5) demonstrated little difference in the numbers of VE-cadherin+ EC between conditions. However, we observed a reduction in numbers of KRT5+ BC in cultures treated with anti-FGFR1 even in the presence of recombinant FGF2 or FGF5. Stimulation of co-cultures with exogenous FGF2 or FGF5 in the absence of anti-FGFR1 had little effect on KRT5+ BC numbers, suggesting over-stimulation of FGF signaling has no positive effect on BC growth during co-culture. We next quantified the number of BC and EC at specific times points by flow cytometric analysis (Fig. 2B). Over 4 days, untreated BC proliferated with a 14.8-fold increase in cell numbers compared to day 0 (Fig. 2C). Relative to untreated cells, incubation with IgG had no significant effect (p>0.4) on BC proliferation (13.6-fold; Fig. 2C). However, anti-FGFR1 significantly suppressed BC growth (2.6-fold) compared to untreated (p<0.0005) and IgG controls (p<0.0005).

We next analyzed the effect of FGFR1 blockade on the EC population in the same experiments. Over 4 days, the untreated EC proliferated with a 1.3-fold increase in cell numbers compared to day 0 (Fig. 2D). Treatment with IgG had no significant effect on EC numbers (1.1-fold, p>0.1) relative to untreated cells (Fig. 2D). For EC incubated with anti-FGFR1, there was a small significant (p<0.05) decrease in cells numbers relative to untreated cells (Fig. 2D). However, there was no significant difference (p>0.3) in cell numbers compared to IgG treatment (Fig. 2D).
To confirm reduced BC growth in co-culture was due to specific blockade of FGFR1 in EC, BC were cultured alone in regular growth media in the absence and presence of anti-FGFR1 antibody or control IgG (Fig. 2E). Compared to untreated cells, no significant change in BC growth was observed following 4 days of culture in the presence of IgG (19.2-fold vs 21.9-fold, p>0.6) and anti-FGFR1 (17.1-fold vs 21.9-fold, p>0.4). Further, no significant difference in cell numbers was observed between anti-FGFR1 and IgG treated cells (17.1-fold vs 19.2-fold, p>0.5). Overall, these data demonstrate that EC specific FGFR1 signaling is required for efficient EC dependent proliferation of BC.

**Endothelial Expressed MMP14 is required for EC-dependent Proliferation of BC**

Murine studies have shown that epithelial-endothelial cross-talk plays an important role in promoting alveologenesis following unilateral pneumonectomy (Ding et al., 2011). Pneumonectomy stimulates pulmonary capillary EC via VEGFR2 and FGFR1 signaling dependent mechanisms to produce EC growth factors, specifically MMP14, that induce proliferation of epithelial progenitor cells to support alveologenesis. Therefore, we hypothesized that similar mechanisms may be conserved in humans and that EC expressed MMP14 may play a role in regulating EC dependent growth support of BC. To assess whether MMP14 expression is up-regulated in EC following stimulation with BC derived growth factors, EC were growth factor starved, then stimulated with either basal media, conditioned media (basal media exposed overnight to cultured BC) or basal media containing recombinant FGF2 or FGF5 and then harvested to analyze MMP14 mRNA expression. Relative to EC stimulated with basal media, there was a significant increase of MMP14 expression in EC stimulated with BC conditioned media (1.31-fold, p<0.005) and FGF2 (1.52-fold, p<0.03; Fig. 3A), however, FGF5 had no effect (1.03-fold, p>0.7; Fig. 3A). These data were verified at the protein level by Western analysis for MMP14 (Fig. 3B). Overall, these data demonstrate that BC secreted growth factors (specifically FGF2) can stimulate expression of MMP14 in EC in a paracrine manner.

The functional role of EC expressed MMP14 in regulating EC dependent growth support of BC was assessed by knockdown of MMP14 in EC. Lentivirus infection of EC with MMP14 specific shRNA resulted in a significant knockdown of MMP14 expression at the mRNA level relative to scrambled shRNA (>95% knockdown, p<0.009; Fig. 3C). These data were further validated at the protein level (Fig. 3D). Co-cultures of BC and uninfected EC or EC infected with scrambled or MMP14 shRNA were established and the growth of BC and EC quantified (Fig. 3E). Over 4 days, BC cultured with uninfected EC proliferated with a 14.4-fold increase in cell numbers compared to day 0 (Fig. 3F). Endothelial cells infected
with scrambled shRNA had no significant effect (p>0.9) on BC proliferation over 4 days (14.4-fold) relative to uninfected cells. However, knockdown of EC MMP14 significantly suppressed BC growth (3.5-fold) compared to uninfected (p<0.002) and scrambled shRNA cells (p<0.002).

We next analyzed the effect of MMP14 knockdown on the EC population in the same experiments. Over 4 days, the uninfected EC proliferated in co-culture with BC with a 1.35-fold increase in cell numbers compared to day 0 (Fig. 3G). Relative to uninfected cells, treatment with scrambled or MMP14 specific shRNA had no significant effect (both p>0.4) on EC numbers at day 4 (1.3-fold increase for both; Fig. 3G). Overall these data demonstrate that EC expressed MMP14 is essential for efficient growth support of BC during co-culture.

In summary, we have identified a novel function of airway BC to regulate activation of EC in a paracrine manner via FGFR1 mediated mechanisms. In turn, activated EC upregulate expression of EC-specific factors including MMP14 that support growth of BC in the absence of exogenous growth factors. In vivo studies of smoking dependent airway remodeling demonstrate elevated expression of FGF2 in bronchial epithelial cells of patients with COPD (Kranenburg et al., 2005), enhanced expression of FGF/FGFR1 during vascular remodeling in COPD (Kranenburg et al., 2002) and altered distribution of vessels in the airway of smokers and smokers with COPD compared to healthy nonsmokers (Soltani et al., 2010). Therefore, cross-talk between BC and EC may play a significant role in maintaining normal airway epithelial structure with alterations of this cross-talk contributing towards smoking dependent airway remodeling.
Materials and Methods

Culture of Primary Human Airway Basal Cells

Basal cells were isolated from the large airway epithelium of healthy nonsmokers as described previously (Hackett et al., 2011). The BC were maintained in Bronchial Epithelial Growth Media (BEGM, Lonza, Walkersville, MD, USA) and passaged by seeding at a cell density of 3000 cells/cm². Each culture was passaged one time before study in co-culture with endothelial cells.

RNA Sequencing

RNA sequencing of nonsmoker primary BC (n=10) was assessed as previously described (Ryan et al., 2014). The data are publically available at the Gene Expression Omnibus (GEO) site (http://www.ncbi.nlm.nih.gov/geo/), accession number 64464. FGF ligand expression was characterized as fragments per kilobase of exon per million fragments sequenced (FPKM) ≥0.04 in every sample.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Walters et al., 2013). The following primary antibodies were used for FGF2 (2 µg/ml; Cat# 3196, Cell Signaling Technology, Danvers, MA, USA) and FGF5 (0.2 µg/ml, ab88118, Abcam, Cambridge, MA, USA).

ELISA

The secretion of FGF2 and FGF5 by BC was assessed by ELISA (FGF2, Cat# ab99979, Abcam and FGF5, Cat# ELH-FGF5-1, RayBiotech, Inc., Norcross GA, USA) following incubation of BC overnight in basal media (BEBM) as described previously (Walters et al., 2013).

Western Analysis

Western analysis was performed as described previously (Curradi et al., 2012) using NuPAGE 4 to 12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA, USA). The following primary antibodies were used: phospho-Akt (1/1000, Cat# 4060), Akt (1/1000, Cat# 9272), p44/42 MAPK (1/1000, #9102); phospho-p44/42 MAPK (1/1000, Cat# 9101); β-actin (1/1000; Cat# 4967) (all from Cell Signaling Technology), GAPDH (1/5000, SC-32233, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and MMP14 (1/1000; ab51074, Abcam).
Culture and Maintenance of Endothelial Cells

Human umbilical cord vein endothelial cells (HUVECs) were isolated and cultured as previously described (Kobayashi et al., 2010). HUVEC-Akt cells were generated as previously described (Kobayashi et al., 2010) and maintained in an identical manner to HUVEC cells.

Co-culture Proliferation Assays

Co-culture assays were used to assess the ability of EC (HUVEC-Akt) to support BC proliferation in cytokine- and serum-free conditions as previously described (Curradi et al., 2012). To assess the role of FGFR1 mediated signaling on BC proliferation, human anti-FGFR1 neutralizing antibody (Clone FR1-H7, ImClone, New York, NY, USA) or IgG control was added at a final concentration of 1 µg/ml. In a subset of experiments recombinant FGF2 (Cat# 8910LC, Cell Signaling Technology) or FGF5 (Cat# 237-F5-050, R&D Systems, Minneapolis, MN, USA) were added. Fresh media and antibody with or without growth factors was added every 2 days and at the desired time points, cells were trypsinized and cell numbers measured with a hemocytometer and the viability assessed by counting of trypan blue dye-excluded cells. The EC were quantified as the GFP⁺VE-cadherin⁺ population by flow cytometric analysis and the GFP⁺VE-cadherin⁻ population quantified as expanded BC.

To assess the role of EC expressed MMP14 on BC proliferation in co-culture, EC were infected with lentivirus containing either pooled MMP14 specific shRNA (Cat# TRCN0000050853-56, GE Dharmacon, Lafayette, CO, USA) or scrambled control shRNA with knockdown of MMP14 confirmed by TaqMan quantitative PCR and Western analysis. Co-culture with BC was carried out as described above.

Immunofluorescence

Cells were fixed directly with 4% paraformaldehyde in PBS for 20 min and then permeabilized with 0.1% triton X-100 in PBS, followed by blocking with normal serum. The samples were stained with primary antibodies against KRT5 (1 µg/ml, PA1-37974, Thermo Scientific, Rockford, IL, USA) and VE-cadherin (1 µg/ml, Cat# AF938, R&D Systems). To visualize the antibody binding, Alexa Fluor® 594 Donkey anti-rabbit IgG (2 µg/ml, Cat# 711-585-152) and Alexa Fluor® 488 Donkey anti-goat IgG (2 µg/ml, Cat# 705-546-147) from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA) were used for KRT5 and VE-cadherin respectively.
Basal Cell Proliferation

Basal cells (2 x 10^4) were seeded into each well of a 12-well plate in BEGM growth media. The next day (day 0) fresh growth media was added to the cells with and without anti-FGFR1 antibody or IgG control (final concentration of 1 µg/ml). Fresh media and antibody was added every 2 days and at the desired time points the cells were trypsinized and cell numbers quantified as described above.

Stimulation of Endothelial MMP14 Expression

HUVEC cells were (1.5 x 10^5) seeded into each well of a 12-well plate in the appropriate growth media. The following day, cells were washed twice with PBS and then growth factor starved for 12 hr. Following starvation, the cells were stimulated for 6 hr with either basal media (BEBM), conditioned media (BEBM exposed overnight to cultured BC) or basal media supplemented with recombinant FGF2 or FGF5. Following stimulation, the cells were placed in TRIzol (Invitrogen) for extraction of total RNA or harvested for Western analysis.

TaqMan Quantitative PCR

The expression of MMP14 in EC was assessed using TaqMan quantitative PCR as described previously (Walters et al., 2013). Relative expression levels were determined using the dCt method with 18S ribosomal RNA (TaqMan® Ribosomal RNA Control, VIC, #4308329, Applied Biosystems, Foster City, CA, USA) as the endogenous control. A premade TaqMan Gene Expression Assay for MMP14 (Hs01037009_g1) was obtained from Applied Biosystems.

Statistical Analysis

Statistical comparisons were calculated using an unpaired, 2-tailed Student’s t test with unequal variance. A p value <0.05 was considered significant.
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Competing Interests
The authors declare that they have no competing interests, but notify that S.R. is the founder of and consultant to Angiocrine Bioscience New York, NY.

Author Contributions
BSD conceived of the study, performed research, data analysis and manuscript writing.
KG performed research and data analysis.
SR conceived of the study and performed data analysis.
RGC conceived of the study, performed data analysis and manuscript writing.
MSW conceived of the study, performed research, data analysis and manuscript writing.

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Figures

A. FGF ligands (RNA sequencing)

B. Immunohistochemistry

C. FGF2 ELISA

D. FGF5 ELISA

E. Western analysis
**Fig. 1.** Basal cell expression of FGF family ligands. **A.** RNA sequencing analysis of FGF ligands in BC. Data shown represents the average ± s.d FPKM expression from n=10 independent samples. **B.** Immunohistochemical staining of cytopreps of BC for FGF2 and FGF5 and isotype control. Bar = 20 µm. **C-D.** FGF2 and FGF5 levels assessed by ELISA in media from BC. Secreted FGF2 and FGF5 were normalized to cell number and calculated as pg/cell/ml. Data shown is the average ± s.d of 3 independent samples each performed in triplicate. **E.** Airway BC were growth factor starved for 6 hr and then stimulated for 15 mins with basal media (lane 1), basal media containing FGF2 (10 and 100 ng/ml) (lane 2 and 3) or FGF5 (10 and 100 ng/ml) (lane 4 and 5) or growth factor containing media (lane 6). Activation of Akt and MAPK signaling was evaluated by Western analysis and staining for phosphorylated Akt and p44/42 MAPK. Total Akt and p44/42 MAPK were evaluated as a loading control.
Fig. 2. Blocking of FGFR1 suppresses EC-dependent proliferation of BC. **A-D.** Airway BC were co-cultured with EC (HUVEC-Akt) and incubated with control IgG or anti-FGFR1. For A, FGF2 (10 ng/ml) or FGF5 (10 ng/ml) were also added. **A.** Immunofluorescence of BC and EC in co-culture. BC (KRT5, red) and EC (VE-cadherin, green). Bar = 100 µm. **B.** Representative flow cytometric analysis of BC and EC in co-culture. **C.** Proliferation of BC co-cultured with EC. **D.** Proliferation of EC in co-culture with BC. **E.** Proliferation of BC cultured alone in growth media and incubated with control IgG or anti-FGFR1. For panels C-E shown are untreated (black), IgG (gray), and anti-FGFR1 (white). Data shown in panels C-E is the average ± s.d of 4 independent experiments each performed in triplicate.
A. Endothelial MMP14 mRNA

- Bar graph showing MMP14 expression compared to basal media (fold-change) with p-values: p<0.03 and p<0.005.

B. Endothelial MMP14 protein

- Conditioned media vs. FGF2 vs. FGF5 with protein bands at kDa 65 and 36 for MMP14 and GAPDH.

C. mRNA, MMP14 knockdown

- Graph showing percentage of MMP14 expression relative to scrambled control with p=0.009.

D. Protein, MMP14 knockdown

- Graph showing cell number compared to day 0 (fold-change) with time in culture (days) and p<0.004.

E. Basal cells + endothelial cells

- Scatter plot showing GFP and VE-cadherin with BC and EC groups.

F. Basal cells in co-culture

- Bar graph showing cell number compared to day 0 (fold-change) with time in culture (days) and p<0.004.

G. Endothelial cells in co-culture

- Bar graph showing cell number compared to day 0 (fold-change) with time in culture (days) and p<0.04.
**Fig. 3.** Endothelial cell MMP14 is required for EC-dependent induction of basal cell (BC) proliferation. **A-B.** Basal cell-derived growth factors stimulate expression of EC MMP14. **A.** mRNA, TaqMan analysis of MMP14 expression in growth factor starved EC (HUVEC) stimulated with either basal media, basal media conditioned on BC, or basal media containing FGF2 (10 ng/ml) or FGF5 (10 ng/ml). Data shown is the average ± s.d of at least 3 independent experiments each performed in triplicate. **B.** Protein, representative Western analysis. **C-D.** shRNA mediated knockdown of EC (HUVEC-Akt) MMP14 expression. **C.** mRNA, TaqMan. **D.** Protein, representative Western analysis. **E-G.** Analysis of BC and EC co-culture. **E.** Representative flow cytometric analysis of BC and EC scrambled or MMP14 shRNA cells in co-culture. **F.** Basal cell number in co-culture. **G.** Endothelial cell numbers in co-culture. For panels **F-G** shown is EC (black), EC containing scrambled shRNA (grey) and MMP14 shRNA (white). Data shown in panels **F-G** is the average ± s.d of 3 independent experiments each performed in triplicate.