Published in final edited form as:

J Allergy Clin Immunol. 2019 October ; 144(4): 1116–1118.e4. doi:10.1016/j.jaci.2019.06.006.

Elevated extracellular maspin after mechanical compression *in vitro* or allergen challenges *in vivo*

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Capsule summary

Maspin secretion from bronchial epithelial cells is substantially elevated by mechanical compression and in BAL fluid of patients with asthma following an allergen challenge, suggesting a potential role of maspin in asthma pathophysiology.

Keywords

maspin; asthma; bronchial epithelium; mechanical compression; bronchoconstriction; mechanotransduction

To the editor

During asthma exacerbations, bronchoconstriction causes buckling of the airway, which imposes compressive mechanical stress on airway epithelial cells.[1] In our previous studies

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Conflict of Interest

Dr. Israel reports personal fees from AstraZeneca, Merck, Philips Respironics, Regeneron Pharmaceuticals, Campbell, Campbell, Edwards & Conroy, Ficksman & Conley, Fox Rothschild, LLP, Ryan Ryan Deluca LLP, other from Novartis, UpToDate, grants from Genentech, other support from Research in Real Life (RiRL), TEVA Specialty Pharmaceuticals, and non-financial support from Boehringer Ingelheim, GlaxoSmithKline, Merck, Sunovion, TEVA. None of the listed support influenced the submitted work. All other authors have no conflicts of interest to report.

Disclosures: No conflicts of interest, financial or otherwise are declared by the author(s).

using *in vitro* mechanical compression that mimics the mechanical environment of buckled epithelium, we have shown that mechanical compression of well-differentiated human bronchial epithelial (HBE) cells leads to the recapitulation of key features of airway remodeling, including collagen deposition, goblet cell hyperplasia, and contraction and proliferation of airway smooth muscle cells, even in the absence of inflammatory cells.[2, 3] Some of these *in vitro* findings were validated in studies performed in animal models and in humans.[4, 5] These studies suggest that compressed bronchial epithelial cells initiate and propagate airway remodeling during asthma exacerbations. However, the impact of asthma exacerbations on the production of pathophysiologic mediators from bronchial epithelial cells and how these mediators could contribute to airway remodeling remains to be elucidated.

Maspin, a non-inhibitory member of the mammary serine protease inhibitor (serpin) superfamily, was originally identified as a tumor suppressor protein in human mammary epithelial cells.[6] Maspin is suggested to contribute to tissue remodeling processes through the modulation of cell-cell interactions and extracellular matrix (ECM) by inhibition of collagen degradation and promotion of epithelial adhesion to ECM.[7, 8] Here, using both mechanically compressed well-differentiated primary HBE cells and bronchoalveolar lavage (BAL) collected from a mouse model of allergic asthma and patients with mild asthma challenged with an allergen, we identified secreted maspin as a potential mediator of airway remodeling in asthma (see the Methods section in this article's Repository at www.jacionline.org for detailed methodology).

Culture of primary HBE cells seeded in transwells was maintained in submerged condition until confluence and switched to air-liquid interface (ALI). In submerged condition, which is comprised solely of basal stem cells, intracellular maspin was detectable, suggesting that maspin is expressed in basal stem cells (Fig 1, A). During differentiation in ALI condition, the level of intracellular maspin progressively increased and plateaued on ALI day 7 (Fig 1, A), suggesting that maspin may also be also abundantly expressed in differentiated cells. In well-differentiated HBE cells, secreted maspin was barely detectable in the basolateral media from the control condition. After compression, the level of secreted maspin was noticeably increased as early as 3 hours and continuously increased up to 24 hours (Fig 1, B). Mechanical compression with 5 or 10 cm H_2O pressure did not induce the secretion of maspin above baseline, whereas compression with 20 or 30 cm H2O markedly induced the secretion of maspin (Fig 1, C). Maspin mRNA expression was modestly and transiently increased up to 1.5-fold at 8 hours following the initiation of compression (Fig E1 in this article's Repository at www.jacionline.org). Blocking of EGFR activity using AG1478 did not inhibit compression-induced maspin secretion (data not shown). However, blocking of PKC activity using bisindolylmaleimide-1 (BIS-1) significantly attenuated compression-induced maspin secretion by 53% (Fig 1, D and E). Though mechanical compression activates both EGFR and PKC pathways,[3] the secretion of maspin was independent of EGFR but dependent on partially PKC pathway.

To determine if maspin may be implicated in asthma pathogenesis, we examined the level of maspin in the BAL fluid collected from a mouse model of asthma using house dust mite (HDM). Compared to that from control mice, the amount of maspin was significantly higher

in the BAL fluid collected from sensitized and challenged mice (Fig 2, A and B). Lastly, to determine if extracellular maspin was elevated during asthma exacerbation induced by allergen challenge, we measured the level of maspin in the BAL fluid collected from patients with mild asthma before and at 48 hours after exposure to an allergen challenge (Table E1 and Fig E1, F) in this article's Repository at www.jacionline.org). Compared to BAL collected prior to allergen challenge, maspin protein levels were significantly increased in BAL collected after the allergen challenge (Fig 2, C and D).

Recent evidence suggests that maspin possesses multiple functions through its interaction with adhesion molecules and ECM proteins.[8] Maspin is detected in multiple organs, but its expression is primarily detected in epithelial cells in the breast, skin, prostate, and airway tissues.[9] The most-well recognized function of maspin is as a tumor suppressor that acts by increasing tumor cell apoptosis or by inhibiting angiogenesis and tumor cell invasion and metastasis.[8] In tumor metastasis, the inhibitory role of maspin has been strongly supported by studies showing that extracellular maspin inhibits cancer cell metastasis by regulation of cell migration, cell-cell adhesion, and ECM degradation.[8] Although functions of extracellular maspin have been primarily linked to cancer metastasis, data from previous studies suggest a potential role for maspin in tissue remodeling.[7, 8] For example, extracellular maspin binds to and thereby inhibits degradation of type I and III collagens.[7] Interestingly, type III collagen is a major ECM component whose expression is increased by mechanical compression and is also increased in the subepithelium of the remodeled airway in asthma.[5] Increased deposition of type III collagen in patients with asthma could be the result of increased maspin, which acted to inhibit collagen degradation. Together, these data indicate that extracellular maspin can affect functions of multiple cell types in multiple contexts and support our hypothesis that airway epithelial-derived maspin may play a role during airway remodeling and asthma pathogenesis.

This is the first study to recognize the potential implication of maspin in the pathophysiology of asthma. Here we identify that human bronchial epithelial cells abundantly express maspin and that secretion of maspin is substantially increased by mechanical compression mimicking bronchoconstriction during asthma exacerbations. The level of maspin secretion depended on the elapsed time following mechanical compression and on the magnitude of pressure applied. Mechanical compression-induced maspin secretion partially depended on PKC signaling. Furthermore, both in a mouse model of allergic asthma and in patients with asthma, the level of maspin detected in BAL fluid was significantly elevated after allergen challenges. We do not yet know the function of extracellular maspin in airway remodeling in asthma. However, given the role of maspin in previous studies, we speculate that maspin secreted from airway epithelial cells may promote airway remodeling by inhibition of collagen degradation or by enhancement of epithelial adhesion to ECM. In this study, we used an in vitro compression model in order to identify the modulation of airway epithelial cells by mechanical force, as exerted during asthma exacerbations, without the confounding effects of the many immunologic mediators that are associated with asthma. Thus, remaining questions should include how/which immunologic or inflammatory factors modulate the impact of bronchoconstriction-induced mechanical compression on the production of maspin in the airway. A limitation of our study was the utilization of materials and data collected from a small number of patients

challenged with an allergen. The impact of our findings would therefore be amplified by immediate follow-up studies, including detection of extracellular maspin in BAL or serum collected from a large cohort with patients with various endotypes, to determine if maspin could be a potential biomarker for asthma. In conclusion, we found that bronchial epithelial cells are a source of maspin, which is increased in the airway after allergen exposures or during bronchoconstriction, suggesting that extracellular maspin might contribute to pathophysiology of asthma.

Methods

Culture of primary normal human bronchial epithelial Cells

Primary normal human bronchial epithelial (NHBE) cells at passage 2 were plated on 12-well transwell plates (Corning Inc, Corning NY) coated with 50 ng/ml of type 1 rat tail collagen (BD Biosciences, San Jose, CA). Cells were grown for 4–6 days in submerged conditions, and after reaching confluence were exposed to air by removing media from the apical surface of cells. NHBE cells were fed with a 1:1 mixture of bronchial epithelial basal medium (BEBM) and high glucose (4.5g/liter) Dulbecco's modified Eagle's medium (DMEM) supplemented. After 14 to 16 days in air-liquid interface (ALI) culture, cells were well-differentiated, as confirmed by staining for MUC5AC and β-tubulin IV, then used for the application of mechanical compression.

Exposure of NHBE cells to mechanical compression

Compressive mechanical stress was applied with a custom pressure setup which allowed for the application of compressed air balanced with 5% CO_2 to the apical side of cells in ALI culture. Unless noted otherwise, in all experiments well-differentiated normal human bronchial epithelial (NHBE) cells were exposed to 30 cm H_2O pressure. This is comparable to that generated during maximum bronchoconstriction and is an order of magnitude higher than that experienced by the airway epithelium during normal breathing or non-asthmatic coughing. NHBE cells were exposed to pressure for 3 hours and then cells and conditioned media (CM) were collected immediately or at indicated time points at 3, 8, or 24 hours following the initiation of pressure. Control cells were connected with a sham setup identical to the compressed cells, except that they were not connected to the pressurized air. In some experiments, to determine the role of transforming growth factor TGF-β2, NHBE cells were treated with vehicle control or TGF- β2 at 10 ng/ml for 3 days and then subsequently exposed to compression.

Inhibitor experiments

To identify intracellular signaling pathways involved in compression-induced maspin secretion, a selective inhibitor for epidermal growth factor receptor (EGFR) (AG1478; 0.5uM) or PKC (bisindolylmaleimide-1; 1uM) was used. Inhibitors or vehicle were spiked into the basal media 30 minutes prior to the application of compression. All inhibitors were initially dissolved in dimethyl sulfoxide (DMSO) as stock solutions and then dissolved to a final concentration in minimal media depleting bovine pituitary extract (BPE) and epidermal growth factor (EGF) from HBE-cell culture media. DMSO was used as a vehicle control.

Western Blot Analysis

Protein lysates were prepared from the cell lysates to detect intracellular protein and from basolateral conditioned media to detect secreted protein by Western blot analysis. Primary antibodies against maspin (1:1000, Cell signaling technology), E-cadherin (1:1000, BD Biosciences, Billerica, MA), GAPDH (1:5000, Cell Signaling Technology, Danvers, MA), and transferrin (1:10000, Thermo Fisher Scientific, Waltham, MA) were used for Western blot analyses. We used GAPDH for intracellular maspin and transferrin for secreted maspin as loading controls. From western blot images, maspin bands were quantified using ImageJ software (NIH, Bethesda, MD). Using recombinant human maspin protein (Sigma, St. Louis, MO), we semi-quantitatively determined the amount of secreted maspin using linear interpolation.

Quantitative RT-PCR

An RNeasy Mini Kit (Qiagen, Germantown, MD) was used to isolate total RNA. MultiScribe reverse transcriptase (Applied Biosystems, Forster City, CA) was used to synthesize cDNA using lug of RNA. 20ng of cDNA was used for quantitative real time PCR using 2X SYBR Green master mix (Life technologies, Grand Island, NY). Primers for maspin and GAPDH were generated by Primer blast or by Primer Express 3.0 software (Table E2). The fold-change of maspin mRNA expression was calculated using the delta-delta Ct method.

Allergen-sensitized and challenged mice

All experiments were performed under protocols approved by the Harvard Medical Area Standing Committee on Animals (Harvard T. H. Chan School of Public Health, Boston, MA). For all experiments, we used both male and female C57BL/6J mice at 4–5 weeks of old age purchased from Jackson Laboratory. House dust mite (HDM) extracts (product number XPB70D3A2.5, lot numbers 343206, 2875 endotoxin units/2.5ml sized-vial) were purchased from Greer Laboratories (Lenoir, NC) and dissolved in PBS. As previously described, mice were exposed to 50ug of HDM extracts dissolved in 30ul of PBS, intranasally. The level of endotoxin contained in the installed HDM was lower than 10.8 endotoxin units/mg of protein. Control mice received 30ul of PBS, intranasally. At 48 hours after the last HDM challenge, BAL fluid was collected. In the BAL, proteins were precipitated with 20% trichloroacetic acid and were used for western blot analysis.

Collection of BAL fluid from patients with asthma

BAL was collected from patients who had been previously diagnosed with mild asthma. The Partners Human Research Committee at Brigham and Women’s Hospital approved the protocol, which was in accordance with ethical standards (IRB #2012P001029). Written informed consent was obtained by a physician from each patient. All patients were not under corticosteroid treatment. For each patient, BAL was collected immediately prior to an allergen challenge, and also at 48 hours following the challenge. As clinical parameters, % eosinophils and FEV_1 were measured.

Statistical analysis

Data are presented as the mean ± SEM or mean ± SD. Statistical analysis was performed using a Student's *t*-test. A Spearman's correlation test was performed between the extent of increased maspin secretion and clinical parameters, FEV_1 (% of baseline) and % of eosinophils. A *P*-value of less than 0.05 was considered as significant.

Acknowledgments

The authors thank Kyle Nelson, Kristen Mclntire, and Angeles Cinelli at Brigham and Women's Hospital for help obtaining human bronchoalveolar lavage samples, and the staff of the Tissue Procurement and Cell Culture Core at the University of North Carolina at Chapel Hill for providing primary human bronchial epithelial cells.

Grant: Parker B. Francis Fellowship (J-A Park), AHA Scientist Development Grant (13SDG 14320004), T32HL007118, P01HL120839, P30 DK065988.

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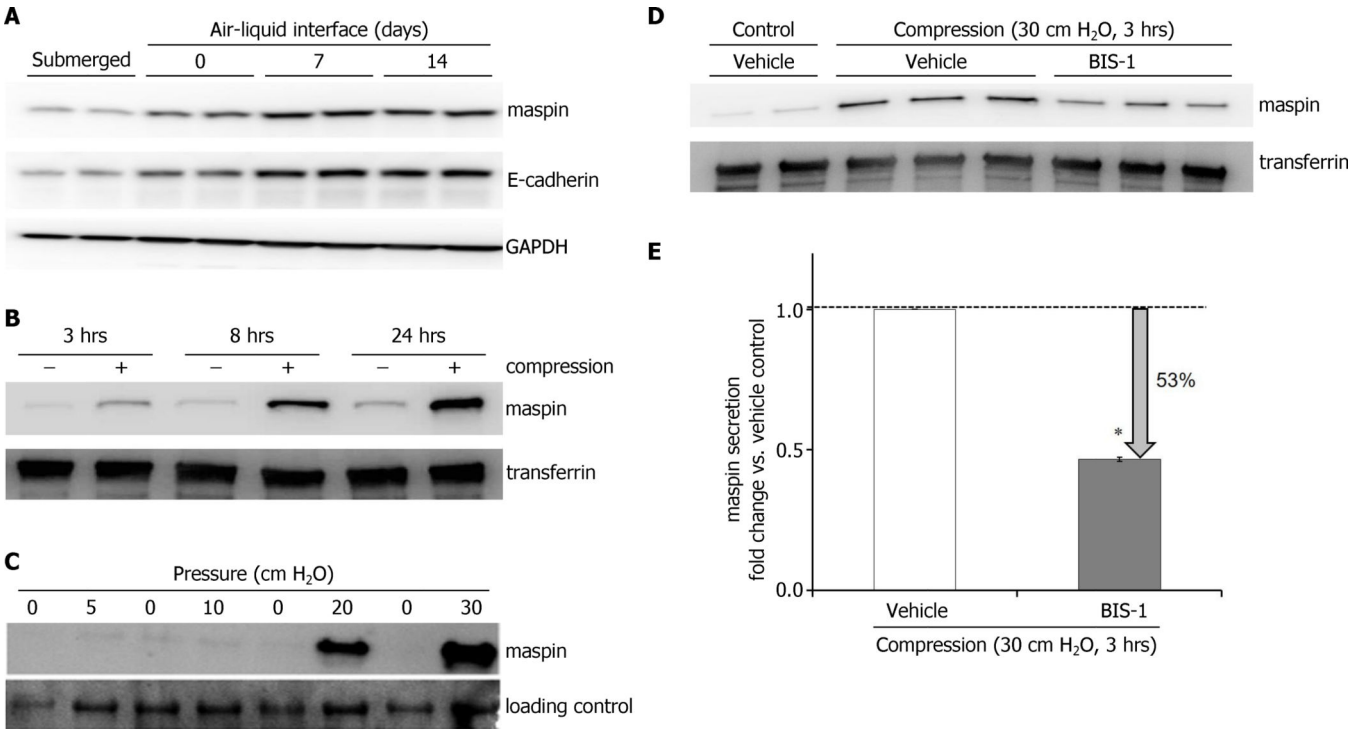

Figure 1. Mechanical compression induces maspin secretion from human bronchial epithelial (HBE) cells.

(A) Representative western blot of maspin in the protein lysates collected from the cells in submerged culture and over ALI days from 3 independent experiments. E-cadherin and GAPDH were loading controls. (B) Time-course of maspin secretion after mechanical compression (30 cm H_2O for 3 hours), representative from 3 independent experiments. (C) Secreted maspin detected in the conditioned media after mechanical compression at various magnitudes of pressure. (D) Western blot of secreted maspin detected in the conditioned media following compression of HBE cells pretreated with vehicle or a selective PKC inhibitor, bisindolylmaleimid-1 (BIS-1). (E) Quantitative analysis of compression-induced maspin secretion shows inhibition by pretreatment with BIS-1, mean ± SEM from 3 independent experiments. *P<0.01 (Student's t-test)

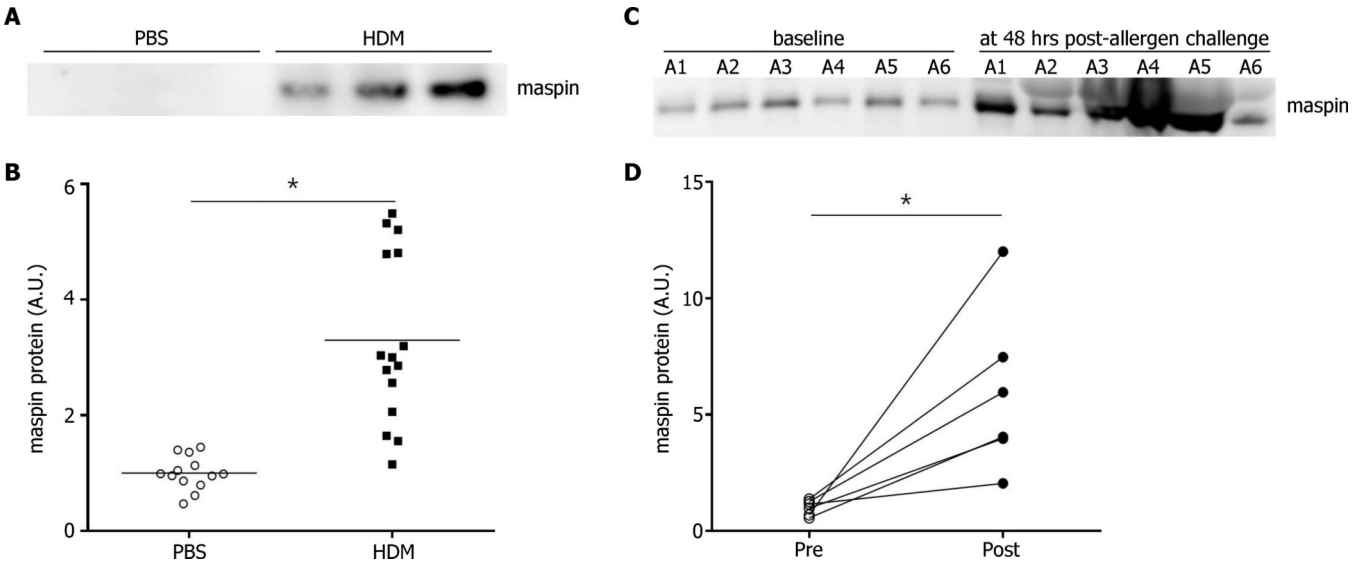

Figure 2. Maspin is elevated in BAL fluid from a mouse model of house dust mite-sensitized allergic asthma and in BAL fluid after an allergen challenge in patients with asthma.

(A) Western blot of maspin in BAL fluid collected from control and HDM exposed mice. (B) Quantification of maspin protein in the BAL fluid from mice. A.U., arbitrary units. **P*<0.01 (Student's *t*-test). (C) Western blot analysis of maspin in BAL fluid collected from the patients with asthma pre- and post-allergen challenge (n=6 patients). (D) Quantification of maspin protein in the BAL fluid. A.U., arbitrary units. **P*<0.05 (paired *t*-test).

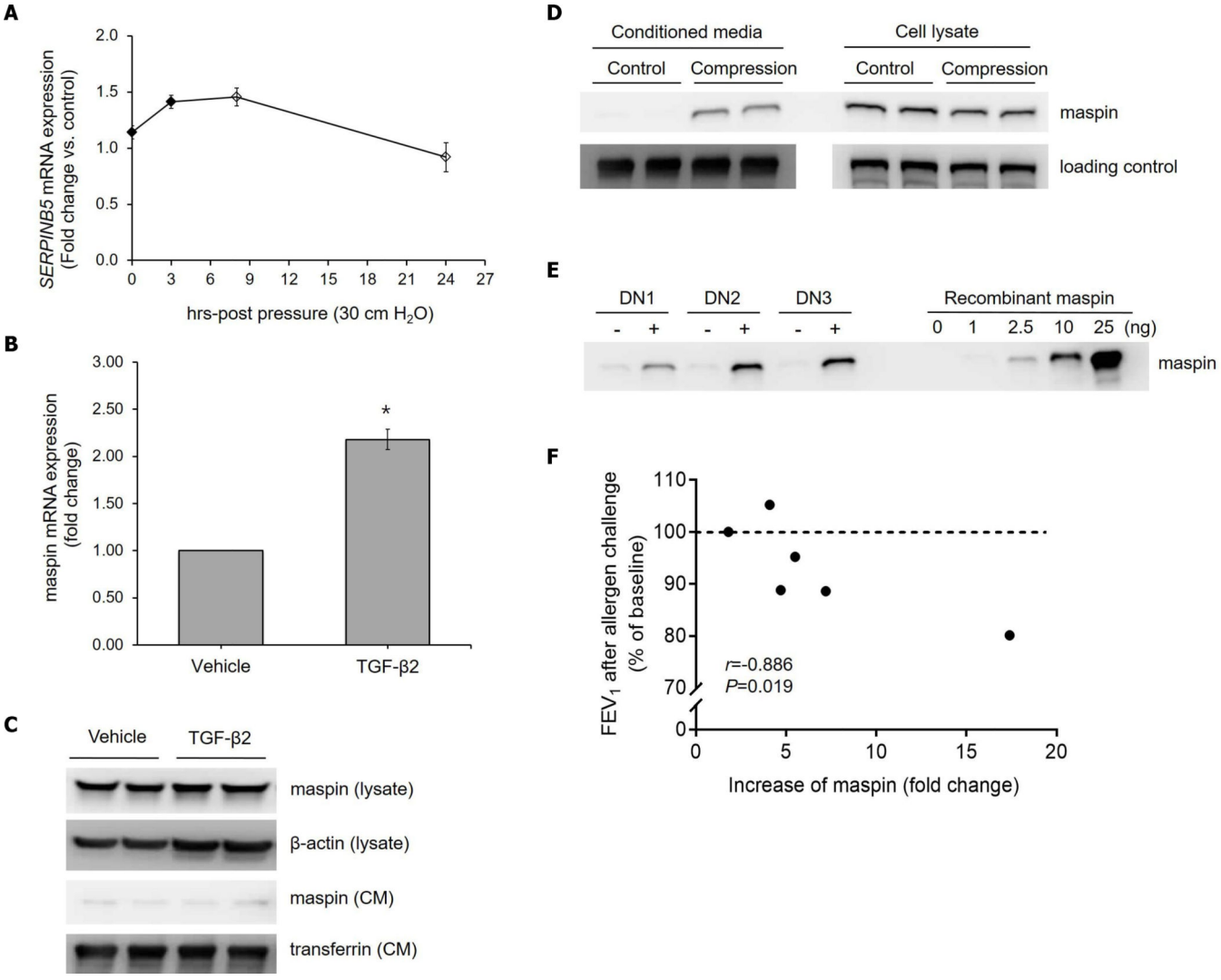

Figure E1.

(A) Time course of maspin mRNA expression after initiation of mechanical compression (30 cm H_2O for 3 hrs), mean ± SD (n=3 independent wells). Filled squares indicate the time points for cells were under compression and unfilled squares indicate the time points for cells were released from compression. (B) Effect of TGF-β2 on maspin mRNA expression in NHBE cells. Following incubation with TGF- β2 for 3 days, maspin mRNA expression was increased, mean ± SEM from 3 independent experiments, **P*<0.01. (C) Effect of TGF-β2 on intracelluar and secreted maspin in NHBE cells. Following incubation with TGF-β2 for 3 days, intracellular maspin in the cell lysates and secreted mapin in the conditioned media were detected. β-actin was used for cell lysates and transferrin was used for conditioned media as loading controls. (D) Maspin detected in conditioned media and cell lysates after mechanical compression. (E) Comparison of secreted maspin from NHBE cells of three different donors (DN) after mechanical compression. Recombinant human maspin was used as a positive control and for quantification based on linear interpolation. (F) Correlation

between the degree of FEV_1 reduction and the degree of increased maspin secretion in BAL fluid after an allergen challenge in patients with asthma.).

Table E1.

Patient information for materials collected at 48hours after an allergen challenge

Donor #	Age	Sex	Ethnicity	% Eosinophils*	FEV_1 (% of baseline)	Fold change of maspin detected in BAL
A1	29	F	Caucasian	5	88.6	7.2
A2	21	F	Hispanic	76.1	105.2	4.1
A3	28	M	Caucasian	84.9	88.8	4.7
A4	19	F	Caucasian	88.5	80.1	17.4
A5	24	F	Caucasian	86.2	95.2	5.5
A6	28	M	Caucasian	82.1	100	1.8

*For all patients, <0.5% of total cells were eosinophils before allergen challenge; number of Eosinophils and FEV1 values were determined at 48 hours post-allergen challenge and compared with the values determined prior to allergen challenge.

Table E2.

Information of primers

Gene	Forward (5’ → 3’)	Reverse (5’ → 3’)	Reference
SERPINB5	AGACAGACACCAAACCAGTGCAGA	TCCCATACAGAACGTGGCCTCCA	Primer blast
GAPDH	TGGGCTACACTGAGCACCAG	GGGTGTCGCTGTTGAAGTCA	Primer express 3,