Reactive oxidant and p42/44 MAP kinase signaling is necessary for mechanical strain-induced proliferation in pulmonary epithelial cells

By

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ABSTRACT

Mechanical strain is necessary for normal lung growth and development. Individuals with respiratory failure are supported with mechanical ventilation, leading to altered lung growth and injury. Understanding signaling pathways initiated by mechanical strain in lung epithelial cells will help guide development of strategies aimed at optimizing strain-induced lung growth while mitigating ventilator-induced lung injury. To study strain-induced proliferative signaling, focusing on the role of reactive oxidant species (ROS) and p42/44 MAP kinase, human pulmonary epithelial H441 and MLE15 cells were exposed to equibiaxial cyclic mechanical strain. ROS were increased within 15 minutes of strain. N-acetylcysteine blocked strain-induced ROS production, p42/44 MAP kinase phosphorylation, and strain-induced proliferation. PD98059 and UO126, p42/44 MAP kinase inhibitors, blocked strain-induced proliferation. To verify the specificity of p42/44 MAP kinase inhibition, cells were transfected with dominant negative MEK1 plasmid DNA. Transfected cells did not proliferate in response to mechanical strain. To determine if strain-induced tyrosine kinase activity is necessary for strain-induced ROS- p42/44 MAP kinase signaling, genistein, a tyrosine kinase inhibitor, was used. Genistein did not block strain-induced ROS production or p42/44 MAP kinase phosphorylation. Gadolinium, a stretch-activated ion channel inhibitor, blocked strain-induced ROS production and p42/44 MAP kinase phosphorylation, but not strain-induced tyrosine phosphorylation. This data support ROS production and p42/44 MAP kinase phosphorylation being involved in a common strain-induced signaling pathway, necessary for strain-induced proliferation in pulmonary epithelial cells, with a parallel strain-induced tyrosine kinase pathway.

Key words: mechanical strain, pulmonary epithelium, ROS, p42/22 MAP kinase
INTRODUCTION

Mechanical ventilation is frequently used as a life-saving intervention in respiratory failure. Infants born prematurely often receive prolonged pulmonary support resulting in potentially fatal pulmonary lung injury [1]. Adults with trauma, aspiration or pneumonia resulting in respiratory failure often progress to Acute Respiratory Distress Syndrome (ARDS), with a mortality of 40-70% [2]. The interventions initiated to support patients with respiratory failure in ARDS, including mechanical ventilation, have been implicated in initiating a systemic response contributing to this mortality [3-6]. Understanding the signaling cascades initiated by mechanical strain could help optimize beneficial strain responses while mitigating detrimental effects of this therapy.

Mechanical strain is known to induce proliferation in lung cells [7,8]. The signaling molecule p42/44 MAP kinase, activated by mechanical strain in lung epithelial cells[7], is a key effector in signaling proliferative responses in other cell types [9,10]. Strain has also been found to initiate an inflammatory response in isolated mouse lungs [11]. Reactive oxygen species (ROS) production is known to be a signaling molecule involved in proliferation in a variety of cell types [12-16]. ROS also plays a role in signaling of inflammatory cytokines [17], and has been linked to p42/44 MAP kinase signaling [18]. We have demonstrated a strain-induced oxidant response in pulmonary epithelial cells [19].

The current work investigates the hypothesis that enhanced ROS production is involved in strain-induced signaling in lung epithelial cells, and that this ROS production mediates strain-induced p42/44 MAP kinase activation and ultimately strain-induced proliferation. While cell-cell
interactions can be studied more effectively in whole animal models, \textit{in vitro} studies allow investigation of specific cell types in complex signal transduction cascades. Experiments in this paper focus on \textit{in vitro} studies that address pulmonary epithelial cell mechanical strain-induced proliferative signaling.
MATERIALS AND METHODS

Cell culture: Human pulmonary adenocarcinoma H441 cells and MLE15 cells were obtained from ATCC (Rockville, Maryland). Cells were plated in RPMI 1640 or DMEM/F12 respectively (Sigma, St Louis MO) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) /0.1% 10mg/ml Gentamicin (Gibco BRL, Grand Island, NY), at 2x10^5 cells/well on flexible bottom, type I collagen coated, silicone elastomer 6-well Bioflex culture plates (Flexcell Corp, McKeesport, PA) for 24h, serum starved for 24 hours, then treated as described. For epidermal growth factor (EGF) experiments, cells were incubated with 10 ng/ml EGF (Upstate Biotechnology, Waltham, Mass). Cell number was determined after cells were removed from the wells by trypsin digestion followed by hemocytometer cell count.

Inhibitor studies: Inhibitor concentrations were determined based on literature review, toxicity as determined by trypan blue dye exclusion assessment, and activity determination. For NAC experiments, cells were pretreated with 20µM NAC (Sigma-Aldrich, St. Louis, MO) while serum starving for 24 hours prior to strain. For other inhibitor experiments, cells were pretreated for 2 hours prior to strain. The following concentrations were used: 10 µM PD98059 (Calbiotech-Novabiochem, LaJolla, CA), 10 µM UO126 (Calbiotech-Novabiochem, LaJolla, CA), 10 µM cyclohexamide (Sigma-Aldrich, St. Louis, MO), 100 µM gadolinium chloride (Sigma-Aldrich, St. Louis, MO), 10 µM genistein (Calbiotech-Novabiochem, LaJolla, CA), and 500 µM allopurinol (Sigma-Aldrich, St. Louis, MO). The stretch-activated ion channel blocker GsMtx-4 isolated from tarantula toxin was added at a concentration of 5 µM [20] just prior to initiating strain.
**Strain application:** Equal biaxial strain was applied using a computer driven Flexercell strain unit with bioflex posts (Flexcell Corp, McKeeseport, PA). Cultured cells were strained at 14 kPa (20% elongation) at 60 cycles per minute to mimic ventilation conditions frequently seen in critically ill patients, for the specified time. Visual inspection confirmed the membrane fully relaxed during each cycle. Control cells were also plated on Bioflex plates to avoid variations based on tissue culture plates.

**ROS activity:** After treatment for time specified, wells were rinsed twice with serum-free medium without phenol red. Cells were incubated with 3 μm 6-carboxy 2’, 7’-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester) C2938 dye (Molecular Probes, Eugene, OR)(DCFDA) and 2 μM Hoechst 33342 (Sigma, St. Louis, MO) in media without phenol red for 15 minutes at 37°C/ 5% CO₂ in darkness. Cells were rinsed 5x with media without phenol red in subdued light. Cells were covered with media without phenol to maintain cell viability, then with #1 cover slips (24x50mm)(Electron Microscopy Sciences, Fort Washington, PA). Samples were illuminated by a 100-watt mercury lamp and viewed with a FITC followed by a DAPI filter on a Nikon E800 microscope (Nikon, Melville, NY) to view DCFDA and Hoechst fluorescence respectively. Fields were viewed at 20x at a constant exposure time within each experiment to minimize variation due to photobleaching. Images were captured with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Relative fluorescence per unit area, and number of fluorescing cells per unit area were determined utilizing Metamorph software, using Hoechst staining of nuclei to normalize to total number of cells per unit area.

**Western Blot Analysis:** Cells were cultured as previously described, grown to confluence, and
changed to serum free media for 24 hours. After treatment, cells were collected in Laemmli sample buffer with 100 µM Na vanadate and 30 mM Na pyrophosphate, and boiled for five minutes. Protein quantification was performed using bicinchoninic acid analysis (Pierce Chemical Co., Rockford, IL). Twenty µg of protein was loaded per well on an SDS polyacrylamide (National Diagnostics, Manville, NJ) gel. After electrophoresis, proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Nonspecific binding sites were blocked using 5% casein in tris buffered saline (TBS) with 1 ml Tween 20 and 10 µl Antifoam A (Sigma, St Louis, MO) per liter for 1 hour at room temperature. For antiphosphotyrosine westerns, the blots were then incubated overnight at 4°C in 1 µg/ml monoclonal mouse antiphosphotyrosine antibody (Upstate Biotechnology Inc (UBI), Lake Placid, NY). Blots were washed with blocking solution for 2x5 minutes, then 1x15 minutes, then incubated for 1 hour at room temperature in 1 µg/ml horse radish peroxidase (HRP) conjugated goat anti-mouse IgG (UBI, Lake Placid, NY). The blots were washed in blocking solution as before, then washed in TBS 2x5 minutes, then 1x15 minutes. The blots were incubated in enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, England) for 1 minute and then exposed to x-ray film (Kodak, Rochester, NY) for 5-15 minutes. For p42/44 MAP kinase western blotting, similar incubation and wash conditions were utilized. The primary antibody was 1 µg/ml polyclonal rabbit phosphospecific p42/p44 MAP kinase antibody or polyclonal rabbit total p42/44 MAP kinase (New England Biolabs, Beverly, MA), secondary antibody was 1 µg/ml horse radish peroxidase conjugated goat anti-rabbit (New England Biolabs), and detection was via enhanced chemiluminescence. Blots were stripped and reprobed with primary actin antibody, 1:5000 dilution (Sigma-Aldrich, St. Louis, MO), goat anti-rabbit IgG-HRP conjugate secondary antibody, 1:5000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) to confirm equal protein loading. Western blot figures are representative of 3 experiments.
**MEK1 dominant negative transfection:** To inhibit signaling of p42/44 MAP kinase, cells were transfected with a dominant negative form of the immediate upstream kinase MEK1. Cells were plated to 50% confluence, incubated with *Mirus* ® liposomal transfection reagent (Panvera, Madison, WI) and 10 µg dominant negative MEK1 DNA (pCMV-HA-MEK1(ala) generously supplied by Dr Roger Davis) for 48h. Inhibition of p42/44 MAP kinase signaling in transfected cells was assessed by assaying serum-induced p42/44 MAP kinase phosphorylation in nontransfected and transfected cells.

**Statistical Analysis:** ANOVA with Scheffe’s post-hoc analysis was used for analysis. A value of the null hypothesis (p)< 0.05 was considered statistically significant. Data are shown as Mean ± Standard Error of the Mean (SEM).
RESULTS

Pulmonary epithelial H441 cells mechanically strained showed an increase in ROS as measured by DCFDA fluorescence, which peaked by 30 minutes (Figure 1). Pretreatment with GsMtx-4 (Figure 1) or gadolinium, mechanosensitive ion channel blockers, or PD98059 or UO126, p42/44 MAP kinase inhibitors, blocked strain-induced increase in DCFDA fluorescence (Figure 2). The xanthine oxidase inhibitor allopurinol, the tyrosine kinase inhibitor genistein or pretreatment with cyclohexamide to inhibit new protein synthesis did not inhibit strain-induced increase in DCFDA fluorescence, suggesting these pathways are not involved in strain-induced ROS production (Figure 2). To determine if strain-induced increase in ROS production is necessary for strain-induced proliferation, cells were pretreated for 24h in NAC, then exposed to unstrained or strained conditions for 24h. NAC pretreatment blocked strain-induced increase in cell number, suggesting ROS production is necessary for strain-induced proliferation in pulmonary epithelial cells (Figure 3).

To determine if ROS production is necessary for strain-induced p42/44 MAP kinase signaling, H441 cells were pretreated with the antioxidant NAC prior to strain. Western blotting demonstrated that NAC pretreatment inhibited strain-induced MAP kinase phosphorylation (Figure 4). This, together with the previous results that demonstrated inhibition of p42/44 MAP kinase signaling inhibits strain-induced ROS production, suggests a feedback signaling mechanism between ROS production and p42/44 MAP kinase signaling. Gadolinium also inhibited strain-induced p42/44 MAP kinase phosphorylation (Figure 4). Pretreatment with genistein did not inhibit strain-induced p42/44 MAP kinase phosphorylation (data not shown). Initial experiments were performed using
H441 cells, due to their relatively long doubling time and more robust increase in cell number in response to strain compared to MLE15 cells (data not shown). H441 cells could not be effectively transfected with the MEK1 dominant negative DNA, so MLE15 cells were used for subsequent studies investigating strain-induced p42/44 MAP kinase signaling. MLE15 cells were strained for 5 min in the presence and absence of PD98059 or UO126, p42/44 MAP kinase inhibitors, followed by western blotting for phospho p42/44 MAP kinase. PD98059 or UO126 at 10 µM concentration blocked strain-induced p42/44 MAP kinase phosphorylation (Figure 5). PD98059 or UO126 also blocked strain-induced increase in cell number (data not shown). Since pharmacologic inhibitors can have unknown unrelated effects, transiently transfected MEK1 dominant negative MLE15 cells were studied. Fetal bovine serum (FBS) is known to cause phosphorylation of p42/44 MAP kinase, so this stimulation was used as a positive control to assess p42/44 MAP kinase signaling. Non-transfected MLE15 cells demonstrated phosphorylation of p42/44 MAP kinase after 5 min exposure to FBS. MEK1 dominant negative MLE15 cells exposed to FBS for 5 min did not demonstrate FBS-induced phosphorylation of p42/44 MAP kinase, demonstrating inhibition of the p42/44 MAP kinase signaling pathway (Figure 6). To determine if p42/44 MAP kinase signaling is necessary for strain-induced proliferation, MEK1 dominant negative MLE15 cells were exposed to unstrained or strained conditions for 24h. MEK1 dominant negative MLE15 cells did not demonstrate strain-induced increase in cell number, providing further evidence to suggest p42/44 MAP kinase signaling is necessary for strain-induced proliferation in pulmonary epithelial cells (Figure 7).

EGF signals a proliferative response through increased ROS production and p42/44 MAP kinase phosphorylation [21,22]. To determine if strain-induced ROS production and EGF stimulation are synergistic, MLE15 cells were strained in the presence and absence of EGF. Strain
and EGF did not have a synergistic response, supporting a common ROS-p42/44 MAP kinase signaling pathway (Figure 8).

A high molecular weight protein, approximately 135 kDa (strain protein 135 or SP135), determined by immunoprecipitation to not be FAK 125 or pp130, has been found to be tyrosine phosphorylated in response to mechanical strain [7]. To determine if ion channel activation or ROS production are necessary for strain-induced SP135 tyrosine phosphorylation, H441 cells were strained in the presence and absence of NAC or gadolinium, Neither NAC nor gadolinium pretreatment blocked SP135 phosphorylation (Figures 9,10). There was no increase in cell death as measured by trypan blue dye exclusion under any of the above conditions (data not shown). Table 1 summarizes the signaling pathways elucidated with the current work, demonstrating the common features of ROS/ p42/44 MAP kinase signaling. It also outlines the divergent pathway of strain-induced tyrosine kinase signaling. The current work provides evidence to support the critical role of ROS production and p42/44 MAP kinase activation in strain-induced proliferation in pulmonary epithelial cells.
DISCUSSION

The lungs are constantly exposed to a variety of environmental stimuli. Normal physiologic lung development requires fetal breathing movements. Without this stretching potentially fatal pulmonary hypoplasia develops, as seen in neurologic anomalies which inhibit fetal breathing, prolonged premature rupture of membranes or congenital diaphragmatic hernia, where herniation of abdominal contents displace the lung, blocking lung distension [23]. While mechanical strain is critical for normal lung growth and development, abnormal strain as seen in mechanical ventilation can lead to lung damage and death [24]. Understanding the mechanisms regulating strain-induced growth and injury can help to optimize beneficial strain effects and leads to the potential for pharmacologic modification of strain-induced changes in the lung, while mitigating damaging sequelae.

We have recently demonstrated that pulmonary epithelial cells exhibit a strain-induced oxidant response [19], but the role of ROS in p42/44 MAP kinase signaling and strain-induced proliferation in the lung has not been elucidated. ROS signaling in response to mechanical strain has been studied in non-pulmonary cell types, most extensively in endothelial cells. Strain is thought to play a major role in atherosclerotic disease, with redox signaling in endothelial cells leading to injury. Strain induces $\text{H}_2\text{O}_2$ production in porcine aortic endothelial cells [25]. Endothelial cell NAD(P)H oxidase activity is enhanced by cyclic strain, as is mobilization of the transcription factor NFκB, an effect that is blocked by a pharmacological inhibitor of NAD(P)H [26]. Pretreatment of cardiac myocytes with NAC inhibits strain-induced activation of p38 MAP kinase [27]. Antioxidants inhibit mechanical strain-induced tenascin-C up regulation in cardiac myocytes [28].
Endothelial cells pretreated with NAC abolish strain-induced ROS generation as well as strain-induced plasminogen activator inhibitor-1 release [29]. Strain-induced increase in MCP-1 mRNA levels in endothelial cells could be inhibited with catalase or NAC [30]. Stretch-induced increases of phosphatidylcholine biosynthesis in astrocytes was significantly reduced by pretreating cells with superoxide dismutase or catalase [31]. Our findings that strain induces ROS production in pulmonary epithelial cells demonstrate a similar strain-induced ROS signaling response compared to these other cell types.

In addition to initiating injury, ROS production signals proliferation. Proliferation of airway smooth muscle cells in culture is inhibited by catalase and NAC [12]. Treatment of cultured hamster lung fibroblasts with oxidants or after partial inhibition of superoxide dismutase (SOD) or glutathione peroxidase increased cell proliferation by approximately 50%. The up regulation of cell proliferation was suppressed by pretreatment with hydroxyl radical scavengers and iron chelating agents [15]. The current study further demonstrates that ROS signaling is necessary for strain-induced proliferation in pulmonary epithelial cells.

The role of p42/44 MAP kinase as a downstream effector of ROS production has been described in other systems. Antioxidant treatment inhibits strain-induced ROS production and p42/44 MAP kinase activation in vascular endothelial cells [32]. Strain-induced p42/44 MAP kinase activation in cardiac myocytes is ROS dependent [33]. Isolated rat extensor digitorum longus muscle exposed to concentric contractions induced a 5-fold increase in p42/44 MAPK phosphorylation, which was blocked by application of NAC or dithiothreitol phosphorylation [34]. Pulsatile flow strain in vascular endothelial cells induced phosphorylation of p42/44 MAP kinase, which was
blocked by NAC [35]. Polyethylene glycol-superoxide dismutase abolished p42/44 MAP kinase activation by pulsatility in isolated rabbit aortas [36]. Our data, which demonstrate that inhibition of ROS signaling by NAC pretreatment blocks strain-induced p42/44 MAP kinase phosphorylation, supports a similar signaling pathway with strain-induced increase in ROS as an early upstream signaling event leading to p42/44 MAP kinase phosphorylation in pulmonary epithelial cells. Most literature supports ROS production as an early mediator of cell signaling, with p42/44 MAP kinase being a downstream effector. Our data demonstrating inhibition of p42/44 MAP kinase phosphorylation blocking strain-induced ROS production suggests a feedback mechanism between the two. This role of MAP kinase signaling as a moderator of ROS signaling has also been found in lung endothelial cells, where hyperoxia-induced p42/44 MAP kinase activation was necessary for hyperoxia-induced NADPH oxidase activation [37].

ROS signaling acts via intracellular calcium mobilization [14]. H$_2$O$_2$ induces an increase in intracellular calcium, which results from release of mitochondrial and sarcoplasmic reticulum calcium stores as well as influx of extracellular calcium [38]. Stimulated human lymphocytes incubated with calcium and ROS sensitive dyes demonstrate that trans-plasma membrane calcium mobilization is necessary for PHA-induced generation of ROS [39]. Human cervical cancer cells exposed to hypotonic swelling activate a trans-plasma membrane calcium influx-dependent p42/44 MAP kinase signaling pathway [40]. Hepatocytes subjected to mechanical strain resulted in increased intracellular calcium [41]. The data presented demonstrates that mechanosensitive ion channel signaling plays a key role in strain-induced ROS and p42/44MAP kinase signaling in pulmonary epithelial cells.
EGF signals via ROS and p42/44 MAP kinase signaling in other cell types [21, 42]. Rat alveolar epithelial cells demonstrate strain-induced p42/44 MAP kinase phosphorylation that is not inhibited by gadolinium, but is blocked by EGF receptor inhibitors [43]. Fetal type II cells demonstrate EGF receptor phosphorylation in response to strain [44]. To determine if strain and EGF signal via common or divergent pathways, cells were strained in the presence or absence of EGF. The lack of synergy in increasing cell number with strain and EGF give further support to a common signaling pathway between strain and EGF in these cells.

In summary, the current work reveals that mechanical strain in pulmonary epithelial cells initiates induction of ROS production, which requires mechanosensitive ion channel activation, and that this ROS induction regulates strain-induced p42/44 MAP kinase activity, both of which are necessary for strain-induced proliferation in lung epithelial cells.
Acknowledgements

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REFERENCES

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Table 1: Summary of modifier/ inhibitor effects on signaling pathways.

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<th>PD98059/UO126</th>
<th>gadolinium</th>
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↓ = inhibition of signaling pathway, * data not shown, ** data in ref [7]
FIGURE LEGENDS

Figure 1: Strain-induced increase in DCFDA fluorescence is partially inhibited by GsMtx-4. H441 cells were exposed to equal biaxial strain, 20% elongation, 60 cycles/min. A: Cells exposed to strain demonstrated increased ROS production measured by relative fluorescence intensity of the REDOX sensitive dye 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate di(acetomethyl ester), which was partially inhibited by the mechanosensitive ion channel blocking peptide GsMtx-4. Arrows demonstrate positive green fluorescence of DCFDA. Blue fluorescence represents Hoechst stained nuclei. B: Quantification of relative fluorescent intensity. Strain increased relative fluorescent intensity by 11.8 fold compared to unstrained control (*p<0.05). GsMtx-4 significantly decreased relative fluorescent intensity after strain compared to untreated strained cells (#p<0.05). Data are Mean ± SEM for N=3.

Figure 2: Gadolinium or p42/44 MAP kinase inhibitor pretreatment blocks the strain-induced increase in DCFDA fluorescence, whereas allopurinol, genistein or cyclohexamide do not. Pretreatment of H441 cells for two hours with the ion channel blocker gadolinium, or p42/44 MAP kinase inhibitors PD98059 or UO126 blocked strain-induced increase in DCFDA fluorescing cells. Pretreatment with the xanthine oxidase inhibitor allopurinol, the tyrosine kinase inhibitor genistein or the inhibitor of protein synthesis cyclohexamide did not block strain-induced increase in DCFDA fluorescing cells. *p<0.05 compared to untreated control. Data are Mean ± SEM for N=4.
Figure 3: NAC pretreatment inhibits strain-induced increase in cell number. H441 cells were incubated for 24h in the presence or absence of 20 μM NAC followed by 24h unstrained or strained conditions. Hemocytometer cell counting demonstrated an increased number of cells after 24h strain, which was blocked by NAC pretreatment. Strain increased cell number by 2.7 fold compared to unstrained control (*p<0.05). Data are Mean ± SEM for N=3.

Figure 4: Gadolinium or NAC pretreatment block strain-induced p42/44 MAP kinase phosphorylation. H441 cells were strained for 0-30 minutes then subjected to SDS-PAGE. Western blotting revealed strain-induced phosphorylation of p42/44 MAP kinase was inhibited by 2h pretreatment with gadolinium or 24h pretreatment with NAC. Actin confirmed equal protein loading. Representative western blot of N=3.

Figure 5: PD98059 or UO126 pretreatment blocks strain-induced p42/44 MAP kinase phosphorylation. MLE15 cells were plated and strained as described (letters in parentheses depict lane labels): unstrained control (C), 5 min strain (S), 5 min strain after 2h pretreatment with 10μM PD98059 (S+PD), or 5 min strain after 2h pretreatment with 10μM UO126 (S+UO). Cells were subjected to SDS-PAGE and probed for phosphorylated and total p42/44 MAP kinase. Representative western blot of N=3.
Figure 6: Transfection with dominant negative MEK1 blocks FBS-induced p42/44 MAP kinase phosphorylation. MLE15 cells were plated as described, with or without transfection of dominant negative MEK1 DNA. Cells were exposed to serum free or 10% FBS media for 5 min, and assessed by SDS-PAGE and western blotting for phospho p42/44 MAP kinase. Actin was used to demonstrate equal loading. Lanes labeled: C: nontransfected control cells; FBS: nontransfected cells exposed to FBS for 5 min; Transfected FBS: cells transfected with dominant negative MEK1 exposed to FBS for 5 min. Transfected cells demonstrated inhibition of FBS-induced p42/44 MAP kinase phosphorylation. Representative western blot of N=3.

Figure 7: Dominant negative MEK1 cells do not demonstrate increased cell number after strain. Non-transfected and transfected MLE15 cells were incubated for 24h under unstrained or strained conditions followed by cell counting. Cells transfected with MEK1 dominant negative DNA demonstrated inhibition of strain-induced increase in cell number. *p<0.05 compared to unstrained controls. Data are Mean ± SEM for N=3.

Figure 8: EGF and strain do not show a synergistic proliferative effect. MLE15 cells were plated as described and exposed to the conditions noted for 24h, followed by cell counting. Strain and EGF increase cell number, but do not have a synergistic effect. *p < 0.05 compared to unstrained control. Data are Mean ± SEM for N=4.
Figure 9: Strain-induced SP135 phosphorylation is not inhibited by pretreatment with NAC. Untreated and NAC pretreated H441 cells were strained for 0-30 minutes, then subjected to SDS-PAGE. Western blotting for phosphotyrosines revealed that strain-induced tyrosine phosphorylation of the 135 kDa protein (SP135) is not inhibited by 24h pretreatment with NAC. Representative western blot of N=3

Figure 10: Strain-induced SP135 phosphorylation is not inhibited by pretreatment with gadolinium. Untreated and 2h gadolinium pretreated H441 cells were strained for 0-30 minutes, then subjected to SDS-PAGE. Western blotting for phosphotyrosines revealed that strain-induced tyrosine phosphorylation of the 135 kDa protein (SP135) is not inhibited by 2h pretreatment with gadolinium. Representative western blot of N=3.
Figure 1

A

B

C

D

E

F

R

Relative fluorescent intensity

- GsMtx-4
+ GsMtx-4

0 15 30

0.E+00 5.E+05 1.E+06 2.E+06 2.E+06 3.E+06

time strained (minutes)
Figure 2

![Bar graph showing relative number of fluorescing cells at different time points and treatments.](image)

- **X-axis:** Time (0 min, 15 min, 30 min)
- **Y-axis:** Relative # fluorescing cells
- **Legend:**
  - Black bars: 0 min
  - Light grey bars: 15 min
  - Dark grey bars: 30 min

Key treatments and inhibitors:
- No inhibitor
- Gadolinium
- PD98059
- UO126
- Allopurinol
- Genistein
- Cyclohexamide

Significance indicated by asterisks (*) for comparisons between time points.
Figure 3

The bar chart shows the cell number comparison between unstrained and strained conditions with and without NAC treatment.}

- **- NAC** (black bars)
- **+ NAC** (white bars or error bars)

The chart indicates a significant increase in cell number with NAC treatment under strained conditions, as indicated by the asterisk (*).
Figure 4
Figure 5

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Figure 6

phospho p42/44 MAP kinase
actin

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Figure 7

- **Control strain**
- **Cell number (% control)**

- **Nontransfected**
- **Transfected**

* indicates a significant difference.
Figure 8

![Bar chart showing cell number (% control) for control, strain, EGF, and EGF+ strain.

- Control: 40
- Strain: 100
- EGF: 200
- EGF+ strain: 200

Significant differences indicated by asterisks (*)]
Figure 9

- NAC  +NAC

SP135

actin

0  15  30  0  1  30
time strained (minutes)
Figure 10

SP135

actin

0  15  30  0  15  30

time strained (minutes)

-GAD  +GAD