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Sustained Activation of RhoGTPases Promotes a Synthetic Pulmonary Artery Smooth Muscle Cell Phenotype in Neprilysin Null Mice

Vijaya Karoor, PhD^{*,§}, Mehdi A. Fini, MD^{*,§}, Zoe Loomis, MS^{*}, Timothy Sullivan, BS^{*}, Louis B. Hersh, PhD[∞], Evgenia Gerasimovskaya, PhD^{*}, David Irwin, PhD^{*}, and Edward C. Dempsey, MD^{*,§,‡}

^{*}Cardiovascular Pulmonary Research Laboratory, University of Colorado Anschutz Medical Campus, Aurora, Colorado

[§]Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado

[∞]University of Kentucky, Lexington, Kentucky

[‡]Denver VA Medical Center, Denver, Colorado

Abstract

Objective—Pulmonary artery smooth muscle cells (PASMCs) from Neprilysin (NEP) null mice exhibit a synthetic phenotype and increased activation of RhoGTPases compared to their wild type (wt) counterparts. Although RhoGTPases are known to promote a contractile SMC phenotype, we hypothesize that their sustained activity decreases SM-protein expression in these cells.

Approach and Results—PASMCs isolated from wt and NEP^{-/-} mice were used to assess levels of SM-proteins (SM-actin, SM-myosin, SM22 and calponin) by Western blotting, and were lower in NEP^{-/-} PASMCs compared to wt. Rac and Rho levels and activity were higher in NEP^{-/-} PASMCs, and ShRNA to Rac and Rho restored SM-protein, and attenuated the enhanced migration and proliferation of NEP^{-/-} PASMCs. SM-gene repressors, p-Elk-1 and Klf4, were higher in NEP^{-/-} PASMCs and decreased by shRNA to Rac and Rho. Co-stimulation of wt PASMCs with PDGF and the NEP substrate, ET-1, increased Rac and Rho activity and decreased SM-protein levels mimicking the NEP KO phenotype. Activation of Rac and Rho and downstream effectors was observed in lung tissue from NEP^{-/-} mice and humans with COPD.

Conclusions—Sustained Rho activation in NEP^{-/-} PASMCs is associated with a decrease in SM-protein levels and increased migration and proliferation. Inactivation of RhoGDI and RhoGAP by phosphorylation may contribute to prolonged activation of Rho in NEP^{-/-} PASMCs. RhoGTPases may thus have a role in integration of signals between vaso peptides and growth

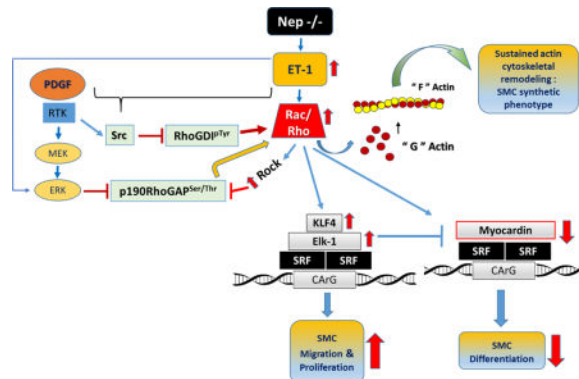
To whom correspondence should be addressed: Vijaya Karoor PhD, Cardiovascular Pulmonary Research Laboratory, RC-2 Room 8480, University of Colorado Anschutz Medical Campus, 12700 E 19th Ave; RC-2, Aurora CO 80045. Tel: (303)-724-3687; Fax: (303)-724-3693; vijaya.karoor@ucdenver.edu.

Materials and Methods are available in the online-only data supplement

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factor receptors and could influence pathways that suppress SM-proteins to promote a synthetic phenotype.

Graphical abstract



Keywords

Rho; Pulmonary artery smooth muscle cells; Signaling; Neprilysin

Subject Code

Smooth muscle cell proliferation and differentiation

INTRODUCTION

Neprilysin (NEP), a cell-surface endopeptidase expressed in vascular cells, cleaves and inactivates neuropeptides important for vasopressor responses^{1, 2}. NEP^{-/-} mice show increased pulmonary vascular remodeling in response to hypoxia³. We have shown in NEP^{-/-} pulmonary artery smooth muscle cells (PASMCs), persistent ET-1 signals synergize with PDGF to promote migration and proliferation⁴.

RhoGTPases maintain vascular tone and a differentiated SMC phenotype by regulating Srf-mediated transcription of SM-genes by contractile agonists⁵. In pathological states, such as pulmonary hypertension (PHTN) and COPD, elevated levels of contractile agonists such as ET-1, promote vasoconstriction and activation of RhoGTPases⁶. Prolonged vasoconstriction promotes inward remodeling of resistance arteries^{7, 8}. In animal models of PHTN, inhibition of RhoGTPases decreases neointima formation suggesting a role in vascular remodeling^{9, 10}.

Increased Rho activity inhibits myogenic differentiation of skeletal muscle and maintains bronchial SMCs in a mesenchymal phenotype^{11, 12, 13, 14}. Whether sustained RhoGTPase activity enhances remodeling of pulmonary arteries by maintaining a mesenchymal/synthetic phenotype of SMCs is not known. In this study, we will begin to test this concept and examine mechanisms by which sustained Rho may suppress SM-protein levels in NEP^{-/-} PASMCs thereby promoting a dedifferentiated phenotype.

Our study will identify how sustained levels of a contractile agonist like ET-1 in NEP^{-/-} PSMCs could synergize with PDGF to reduce SM-contractile protein levels. Understanding the role of RhoGTPases in modulating the SMC phenotype may help explain their role in increased vascular remodeling observed in PHTN associated with COPD where co-expression of PDGF and ET-1 occurs^{15,16}.

RESULTS

Decreased levels of SM-proteins in NEP^{-/-} PSMCs

Phenotypic switching of SMC is accompanied by down-regulation of SM-proteins (α -SMA (alpha-smooth muscle actin), SM-myosin(SM-MHCs), SM22, and calponin)¹⁷. Compared to wt, NEP^{-/-} PSMCs had significantly lower levels of α -SMA, SM22, and calponin, which were restored by lentiviral expression of NEP (Figure 1A–1C), or recombinant NEP (Supplement Figure IA and IC). SiRNA-induced knockdown of NEP (90% reduced) or treatment with the NEP inhibitor, phosphoramidon, decreased levels of SM-proteins in wt PSMCs (Figure 1D–E, Supplement Figure IB and ID). mRNA for SM-genes (α -SMA, SM-MHCs, and calponin) were lower in NEP^{-/-} PSMCs (Supplement Figure IE–F). Decrease in SM-actin was confirmed by immunohistochemistry (Figure 1F). Although SM-MHCs levels were lower they did not reach significance. The antibody for SM-myosin used recognizes both SM1 and SM2 isoforms. The levels in NEP^{-/-} PSMCs may be the SM1 isoform of SM-myosin as SM2 levels are lost during de-differentiation.

An increase in non-muscle (NM)-myosin and CRBP-1 (markers for synthetic phenotype) was observed in NEP^{-/-} PSMCs as shown in Supplement Figure II. Light microscopy images for NEP^{+/+}, NEP^{-/-} and NEP^{-/-} + lentivirus-NEP are shown in Supplement Figure IIC. Taken together, these data suggest that loss of NEP decreases SM-protein levels in PSMCs.

Increase in baseline activation of RhoGTPases in NEP^{-/-} PSMCs

RhoGTPase (Rac and Rho) signaling plays a critical role in maintaining SM-protein levels through actin remodeling-induced Srf activity⁵. We measured Rac and Rho activity and downstream effectors (Rock2, p-cofilin, and p-Mlc) in wt and NEP^{-/-} PSMCs at baseline. Rac and RhoA activity were 1.3–1.4 fold higher in NEP^{-/-} PSMCs (Figure 2A–B) compared to wt. Downstream effectors of Rho GTPases, were 1.2–1.5 fold higher at baseline in NEP^{-/-} PSMCs compared to wt (Figure 2C–D). The level of MYPT1^{Thr696} was 2 fold higher in NEP^{-/-} PSMCs confirming increased Rho activity. In four of the 15 isolates of NEP^{-/-} PSMCs tested we observed a decrease in phospho and total cofilin levels compared to wt. Src mediated phosphorylation of cofilin at Tyr86 targets it for degradation and has been suggested as an alternate mechanism for controlling actin dynamics¹⁸.

Decrease in cofilin activity by phosphorylation leads to stabilization of F-actin¹⁹. Phalloidin (F-actin) and DNase I (G-actin) stain, were used to visualize F- and G-actin levels. NEP^{-/-} PSMCs showed increased F-actin staining compared to wt (Figure 2E). F/G actin ratio measured by differential centrifugation, showed 2 fold higher levels of F-actin in NEP^{-/-}

cells compared to wt (Figure 2F). Our results show that NEP^{-/-} PSMCs exhibit sustained activation of Rac and Rho and downstream effectors.

ShRNA mediated knockdown of Rac and Rho restores SM-protein levels

To determine whether increased Rac and Rho activity suppresses SM-protein, we used shRNA to Rac and Rho, and examined effects on levels, in serum and PDGF-treated NEP^{-/-} PSMCs. There was 70–80% knockdown of Rac and Rho proteins by shRNA treatment (Supplement Figure III A–B). Cofilin phosphorylation was decreased by shRNA to Rho, and Mlc phosphorylation by Rac and Rho (Supplement Figure III A–3D).

In wt PSMCs, shRNA to Rho decreased cofilin phosphorylation. Mlc can be phosphorylated by Rac and Rho. Mlc was weakly phosphorylated at baseline in wt cells and shRNA to either Rac or Rho caused compensatory increase in phosphorylation (Supplement Figure III A–B).

ShRNA to Rac restored SM-proteins levels both at baseline and in PDGF-treated NEP^{-/-} PSMCs. (Figure 3A and Supplement Figure IV). ShRNA to Rho restored α -SMA and SM-myosin levels but suppressed SM-22 and calponin (Figure 3A and Supplement Figure IV). These results suggest that Rho activity is required to maintain baseline expression of SM-22 and calponin in NEP^{-/-} PSMCs.

In wt PSMCs, shRNA to Rac and Rho restored α -SMA levels decreased by PDGF treatment. ShRNA to Rho decreased SM-myosin and calponin levels (Supplement Figure VA–B) suggesting that Rho activity is required for their expression. However, SM-22 levels were increased with Rho shRNA in wt cells suggesting that Rac activity could contribute to expression of SM22. We were unable to knock down both Rac and Rho to confirm role on SM22 levels because the double KO cells did not grow.

RhoGTPases regulate cell migration and proliferation and contribute to mechanisms of vascular remodeling¹². ShRNA to Rac and Rho attenuated baseline and serum and PDGF-induced migration and decreased proliferation to wt levels in NEP^{-/-} PSMCs (Figure 3B–C). Similar results were obtained using the Rho kinase inhibitor, Y27362 (Supplement Figure VI). The decreases in proliferation were due to a reduction in cell number. ShRNA to Rac and Rho decreased migration, and PDGF stimulated proliferation was inhibited by Rho shRNA in wt cells (Supplement Figure VIIA–B).

In summary, results from shRNA studies show that sustained activation of Rac and Rho contribute to suppression of SM-protein levels, and increase migration and proliferation of NEP^{-/-} PSMCs. Taken together, the results from shRNA in wt and NEP^{-/-} PSMCs suggest that Rac and Rho have differential effects on SM-proteins under normal physiological conditions as seen in wt cells and in pathological context as seen in NEP^{-/-} cells.

Increased levels of SM-gene repressors, p-Elk-1 and Klf4, in NEP^{-/-} PSMCs

A decrease in mRNA levels for SM-proteins suggested transcriptional repression may contribute to decreased SM-protein levels in NEP^{-/-} PSMCs²⁰. Nuclear levels of SM-gene

transcription factors (Srf and myocardin) were 0.5 fold lower, and repressors (Elk1 and Klf4) were 1.5 fold higher in NEP^{-/-} PSMCs compared to wt (Figure 4A–C). Expression of NEP using a lentiviral vector in NEP^{-/-} PSMCs restored levels of Srf and myocardin, while decreasing levels of Klf4 and Elk-1 (Figure 4B–C). SiRNA to NEP significantly decreased Srf and myocardin and increased Klf4 levels in wt PSMCs (Figure 4D–E). Nuclear localization of Srf and p-Elk-1 in wt and NEP^{-/-} PSMCs assessed by immunofluorescence staining is shown in Figure 4F.

Elk-1 and Klf4 inhibit SM-gene expression by competing with myocardin for binding with Srf²⁰²¹. We examined the levels of Srf associated with Elk-1, Klf4, and myocardin, by co-immunoprecipitation. There was a 2 fold increase in Srf associated with Elk-1 and Klf4 and a 0.5 fold decrease for myocardin in NEP^{-/-} PSMCs compared to wt (data not shown).

ShRNA to Rac increased Srf expression, and shRNA to Rac and Rho significantly decreased levels of the repressors, p-Elk and Klf4, in NEP^{-/-} PSMCs treated with serum or PDGF (Figure 4G). Baseline levels were not significantly changed in these cells (Figure 4G). These results suggest that enhanced Rac and Rho activity increases levels of SM-gene repressors in PDGF treated NEP^{-/-} PSMCs. In NEP^{+/+} PSMCs, shRNA to Rho decreased Srf and Klf4 levels (Supplement Figure VIIC–D).

NEP substrate, ET-1, synergizes with PDGF to activate Rac and RhoA and decreases SM-protein levels

Neuropeptide substrates of NEP and Src transactivate growth factor receptors and enhance PDGF-induced migration and proliferation of PSMCs⁴²². We tested whether simultaneous treatment with PDGF and ET-1 would suppress SM-protein levels in wt PSMCs, mimicking the NEP^{-/-} phenotype. Cells were treated with PDGF in the absence or presence of ET-1, and activation of Rac and RhoA and downstream effectors, and levels of SM-proteins were measured. PDGF treatment increased RhoGTP levels by 2 fold and ET-1 + PDGF caused a 2-fold increase in Rac and RhoGTP levels at 24h (Figure 5A–B).

A time course (0–6h) for activation of downstream effectors of RhoGTPases in response to ET-1, PDGF, and ET-1 + PDGF is shown in Figure 5C and fold change compared to control at 6h in Figure 5D. ET-1 + PDGF increased total levels of Rac, Rho and p-cofilin (Figure 5C–D). ET-1 treatment increased phosphorylation of cofilin and PDGF increased phosphorylation of cofilin and Mlc at times tested (Figure 5C–D). ET-1 and PDGF cause sustained activation of Rac and Rho and downstream effectors. Levels of α -SMA, calponin, and SM22 were also decreased with ET-1+PDGF, as was observed in NEP^{-/-} PSMCs (Figure 5E–F). Surprisingly, ET-1 + PDGF treatment significantly increased SM-myosin which needs further investigation.

Taken together, an NEP substrate, ET-1, synergized with PDGF to increase Rac and Rho activation and cofilin phosphorylation. The increase in Rac and Rho activation coincided with a decrease in SM-proteins as was seen in NEP^{-/-} PSMCs. Similar treatment of NEP^{-/-} PSMCs caused a further increase in p-cofilin and p-Mlc (Supplement Figure VIII). Ambrisentan, an ETR_A inhibitor, decreased p-Elk and Klf4 levels in NEP^{-/-} PSMCs,

suggesting that ET-1 contributes to the increase in levels of these proteins (Supplement Figure IX).

The presence of NEP in wt PSMCs decreases half-life of ET-1 and is a limitation of this experiment. Whether effects of treatment with ET-1 + PDGF on SM22 in NEP^{+/+} PSMCs may require prolonged presence of ET-1 is not known.

Altered phosphorylation of Rho regulatory proteins in NEP^{-/-} PSMCs

To further understand the mechanism for sustained activation of Rac and Rho we examined levels and phosphorylation status of their regulators. RhoGTPases are positively regulated by GEFs, and negatively by GAPs and GDIs¹¹²³. GAPs and GDI activities are regulated by phosphorylation by Src, Erk and Rock²⁴ and activity of these kinases is increased in NEP^{-/-} PSMCs.

Tyrosine phosphorylation of RhoGDI by Src inhibits its activity resulting in activation of Rac and Rho. We assessed RhoGDI^{p-Tyr} levels by immunoprecipitation of RhoGDI and probing with a pan-phospho-tyrosine (p-Tyr) antibody due to lack of a commercial site specific one (Figure 6A). Increased p-Tyr levels observed in RhoGDI from NEP^{-/-} PSMCs, and in wt PSMCs treated with ET-1+PDGF suggest that activated Src may inactivate RhoGDI. Serine phosphorylation of RhoGDI inactivates Rho activity. RhoGDI^{Ser174} levels were lower in NEP^{-/-} PSMCs and were restored significantly by shRNA to Rac and modestly by shRNA to Rho (Supplemental Figure X).

Serine phosphorylation of p190RhoGAP by Rock or Erk inhibits its activity, leading to sustained activation of Rho²⁴. We measured p190RhoGAP^{pSer/pThr} by immune-precipitation with p190RhoGAP antibody and probed with pan-antibodies to p-Thr and p-Ser/Thr. p190RhoGAP was constitutively phosphorylated at Ser/Thr residues in NEP^{-/-} PSMCs suggesting inactivation. As seen in Figure 6B, p-Thr and p-Ser/Thr levels were also increased 2 fold in ET-1 and ET-1+PDGF treated wt PSMCs.

Tyrosine phosphorylation of p190RhoGAP at Y1105 by Src increases its activity causing inhibition of RhoGTPases. Levels of p190RhoGAP^{Y1105} were significantly lower in NEP^{-/-} PSMCs compared to wt. ShRNA to Rac increased p190-RhoGAP^{Y1105} levels 4–6 fold and shRNA to Rho 2–3 fold (Supplement Figure X). We did not detect alterations in the levels of Vav or p115 GEF in these cells.

Rac and Rho and their downstream effectors are increased in lung tissue from NEP^{-/-} mice and humans with COPD

We examined lungs from NEP^{-/-} mice to determine whether RhoGTPases and downstream effectors are activated in vivo. Similar to the observations in SMC, lungs from NEP^{-/-} mice showed increased levels of RhoGTPases and activation of downstream effectors, cofilin and Mlc, which was reversed by treatment with fasudil a Rho kinase inhibitor (Supplement Figure XI). RhoGDI^{pTyr} and RhoGAP^{pSer} levels were higher in lung lysates from NEP^{-/-} mice and is shown in Figure 6C. Levels of RhoGDI^{Ser174} and p190RhoGAP^{Y1105} were also decreased in NEP^{-/-} lungs (Supplement Figure XI).

Activity of RhoA/Rho-kinase pathway has been shown to higher in pulmonary arteries from patients with hypoxemic COPD. We examined activation of RhoGTPases and downstream effectors in lungs from COPD patients with FEV₁>80% and FEV₁<50%. Lungs with FEV₁<50% showed increased Rac and Rho and downstream effectors p-cofilin and p-Mlc (Supplement FigureXI). RhoGDI^{P^{Tyr}} and p190RhoGAP^{p^{Ser}} levels were higher and RhoGDI^{Ser174} and p190RhoGAP^{Y1105} were lower in FEV₁<50% (Figure 6, Supplement FigureXII and XIII). Lungs of COPD patients with FEV₁<50% also showed decrease in NEP (Supplement FigureXI) and increased levels of PDGF and ET-1 compared to lungs from COPD FEV₁>80% (data not shown).

Taken together our results suggest that inactivation of RhoGTPase regulators by increase in phosphorylation of RhoGDI^{P^{Tyr}} and p190RhoGAP^{p^{Ser/Thr}} may contribute to sustained activation of RhoGTPases. We speculate that sustained ET-1 and PDGF levels in pathological conditions inactivate RhoGAP and RhoGDI by phosphorylation therefore increasing activities of Rac and Rho, and a dedifferentiated SMC phenotype as shown in the abstract Figure.

DISCUSSION

Differentiated SMCs exist in a quiescent, contractile state and switch to a proliferative one in pathological conditions. The regulation of SMC phenotype is complex and involves transcriptional repressors and signaling pathways, resulting in the downregulation of SM-proteins^{20, 21}. GPCR-coupled contractile agonists including angiotensin II, norepinephrine and ET-1, stimulate RhoA and its downstream effector Rock to maintain vascular tone, and promote a differentiated contractile SMC phenotype. However, studies have shown that prolonged contractile activity leads to muscularization of arteries due to increased SMC proliferation, and contributes to the pathogenesis of systemic and pulmonary hypertension^{25,26,27}.

Our results show NEP^{-/-} PSMCs, and wt PSMCs treated with ET-1 and PDGF have decreased expression of SM-proteins with increased Rac and Rho expression/activity (Figures 1, 2 and 5). Rac and Rho activation is sustained by phosphorylation- dependent inactivation of RhoGDI and RhoGAP in NEP^{-/-} PSMCs and in their wt counterparts treated with ET-1 and PDGF (Figure 6 and graphic abstract). These results suggest that sustained Rac and Rho activity can promote a synthetic SMC phenotype (Figures 1 and 2) and contribute to mechanisms important in vascular remodeling (abstract Figure). Rho and downstream effectors were also activated in lungs from NEP^{-/-} mice.

GWAS studies have reported variants within the *FAM13A* (family with sequence similarity 13, member A) gene are associated with normal lung function (FEV₁/forced vital capacity, FVC) and reduced risk of COPD²⁸. Sequence analysis has identified a RhoGAP domain in the protein suggesting that Rho pathway is involved in lung diseases. Our results show that RhoGDI and RhoGAP were inactivated by phosphorylation in lungs of COPD patients with reduced function (Figure 6D). There was increased activation of downstream effectors cofilin and Mlc (Supplemental FigureXI).

Studies in transgenic mice have suggested a role for both Rac and Rho in vascular remodeling. Prolonged Rho activity is central to increased neointima formation in *MLK3*^{-/-} mice⁹. The prevention of medial thickening with Rho inhibitors observed in animal models of hypertension also support a role for Rho in vascular remodeling²⁹. Mice with SM-specific deletion of Rac have enhanced Rho activation and are hypertensive³⁰. Studies with mice overexpressing Vav, a common GEF for Rac and Rho, show that increased Rac activity contributes to vascular remodeling while increased Rho contributes to hypertension³¹. Kailirin, a GEF for Rac in SMCs, promotes intima formation in atherosclerotic mice¹⁰. Our results demonstrate that sustained Rac in *NEP*^{-/-} PSMCs is more efficient than Rho in suppressing SM-protein expression (Figure 3).

Rho mediated expression of SM-proteins is mediated by transcription factor, Srf, and repressed by Elk-1 and Klf4²⁰. *NEP*^{-/-} PSMCs express higher levels of SM-gene repressors, Elk-1 and Klf4. Transcriptional repression of SM-genes in response to injury and disease states plays an important role in vascular disease³². Klf4 is a transcription factor that is upregulated in human forms of pulmonary hypertension and in proliferating SMCs³³⁻³⁶. Modulation of Klf4 levels by Rho has not been reported in SMCs. However, sustained Rho activity is required for the repression of protein kinase G (PKG) by Klf4 in SMCs³⁷. Whether increased Klf4 and sustained Rac/Rho activity is required for suppression of SM-proteins expression in *NEP*^{-/-} PSMCs will need further investigation. Ambrisentan, an *ETRA* inhibitor, decreased p-Elk and Klf4 levels in *NEP*^{-/-} PSMCs, suggesting that *ET-1* contributes to the increased levels of these proteins (Supplement Figure IX). Muscularization of distal arteries contributes to the pathogenesis of PHTN and other vascular disorders. Understanding SMC phenotypes that contribute to this process maybe important in developing therapeutic strategies.

The Rho/Rho kinase pathway can be activated by several mechanisms involving receptor tyrosine kinases, GPCRs or integrins. It has been suggested that the mechanism by which activity of RhoGTPases is increased, may depend on the stimulus and pathological context³¹. It is not known if the outcome and coupling to downstream effectors in the activation of Rac/Rho activity may vary depending on whether the mechanism of activation involved GEFs, GAPs or GDIs. Alternatively, matrix proteins like fibronectin activate Rac and Rho and suppress SM-protein expression^{13, 38}. It is possible that alterations in the extracellular matrix of *NEP*^{-/-} PSMCs couples Rac/Rho to different effectors to promote dedifferentiation compared to that in wt cells. This possibility needs further investigation. Our data show that inactivation of RhoGDI and RhoGAPs by phosphorylation leads to sustained Rac/Rho activity and suppression of SM-contractile protein levels promoting a synthetic SMC phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NEP	Neprilysin
Rho	Ras homology family member
RhoGDI	Rho guanine dissociation inhibitor
RhoGAP	Rho GTPase activating protein
Srf	Serum response factor
Klf4	Kruppel lung factor 4

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HIGHLIGHTS

- Under physiological conditions Rho is required for expression of SM-contractile proteins and maintains vascular tone.
- Pulmonary artery smooth muscle cells isolated from Neprilysin null mice exhibit a synthetic phenotype with decreased levels of SM-proteins and increased Rac and Rho activity and levels.
- ShRNA to Rac and Rho restore SM-protein levels suggesting a role for the GTPases in decreased expression.
- The NEP^{-/-} phenotype could be mimicked by treating wild type PSMCs with PDGF in the presence of the NEP substrate, ET-1.
- Rho may play an important role in integrating signals from growth factors like PDGF, and GPCRs, like ET-1, to promote a synthetic SM phenotype in these cells.

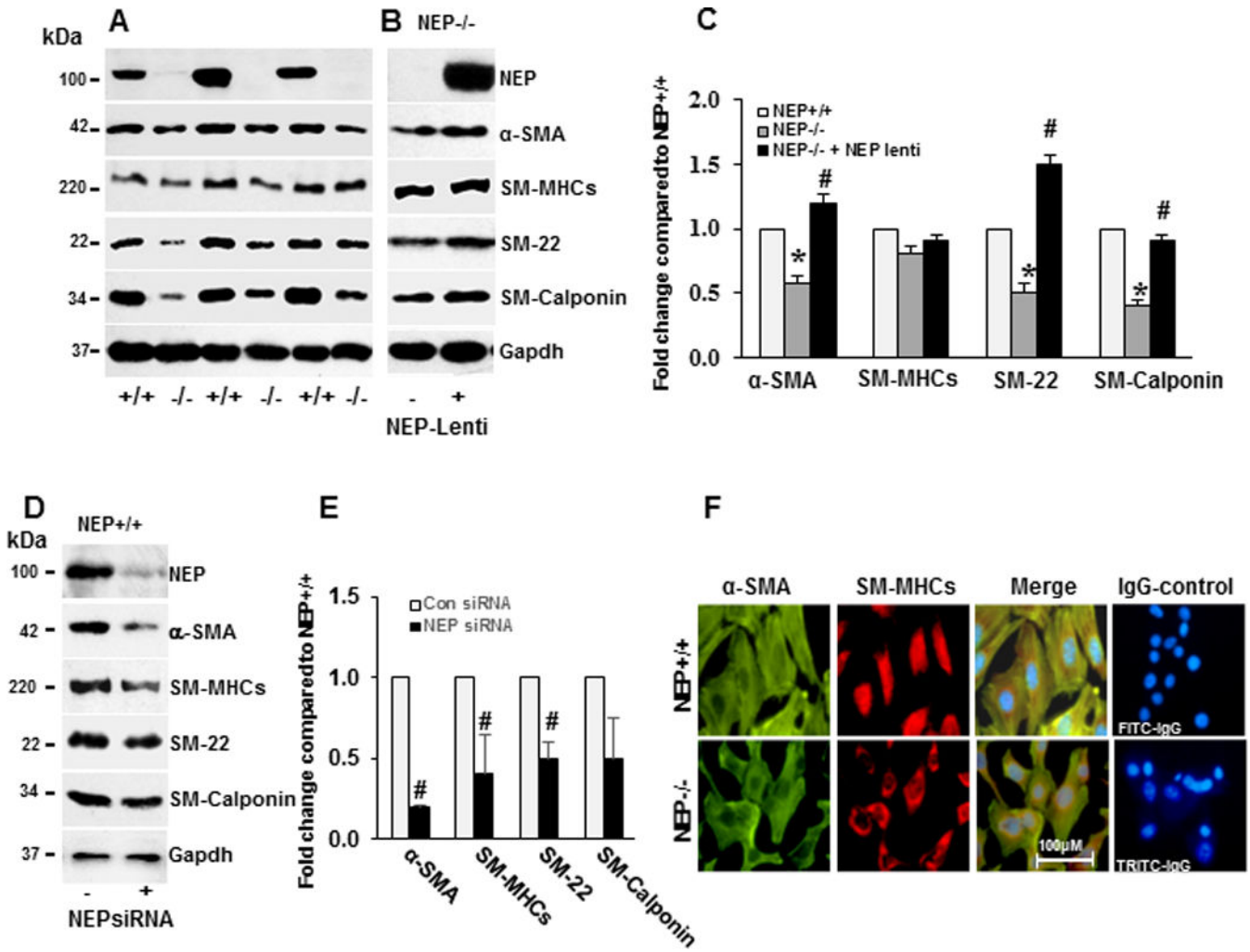


Figure 1. Levels of SM-proteins are lower in NEP^{-/-} PSMCs compared to NEP^{+/+} PSMCs
 Levels of SM-proteins (α-SMA, SM-myosin, SM-22 and calponin) were measured in lysates from NEP^{+/+} and ^{-/-} PSMCs by Western blotting or immunohistochemistry. Panel A shows levels by Western blotting. Panel B shows effect of expressing NEP in NEP^{-/-} PSMC, on SM-protein levels in NEP^{-/-} PSMCs. Panel C shows average fold change in NEP^{-/-} compared to NEP^{+/+} PSMCs normalized to Gapdh and effect of NEP expression (n=6). Effect of NEP siRNA (10 nMole/L) on SM-protein levels in NEP^{+/+} PSMCs is shown in Panel D. Panel E shows average fold change from 3 different transfections normalized to Gapdh. Panel F shows immunostaining for α-SMA and myosin along with IgG controls. (*) represents p < 0.05 for comparison between NEP^{+/+} and NEP^{-/-} PSMC and (#) for comparisons between NEP^{-/-} control and lentivirus-expressing NEP treated cells.

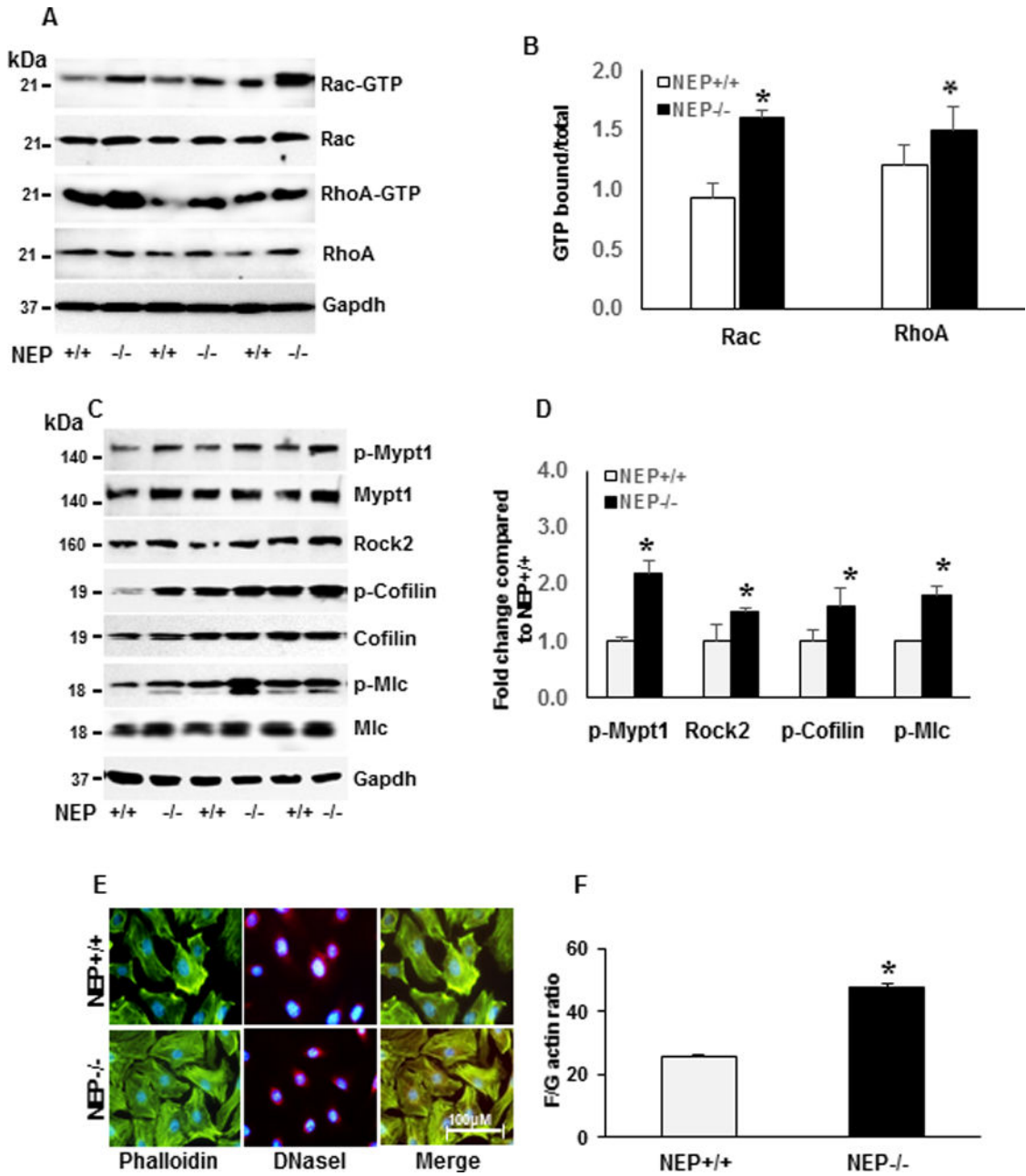


Figure 2. Increased activation of Rho GTPases and downstream effectors in NEP^{-/-} PSMCs
 Rac and Rho activity was assessed by measuring levels bound to GTP. Levels from NEP^{+/+} and ^{-/-} PSMCs at baseline are shown in Panel A, and average GTPbound/total from 6 different paired isolates is shown in Panel B. Panel C shows levels of p-Mypt1, Rock2, p-Cofilin, and p-Mlc at baseline. Average levels from 3 different isolates normalized to Gapdh is shown in Panel D. Representative staining for F actin with phalloidin and G-actin with DNaseI in NEP^{+/+} and ^{-/-} PSMCs is shown in Panel E. Levels of F- and G-actin assessed by differential centrifugation is shown in Panel F. *represents p < 0.05 for comparison between NEP^{+/+} and ^{-/-} PSMCs.

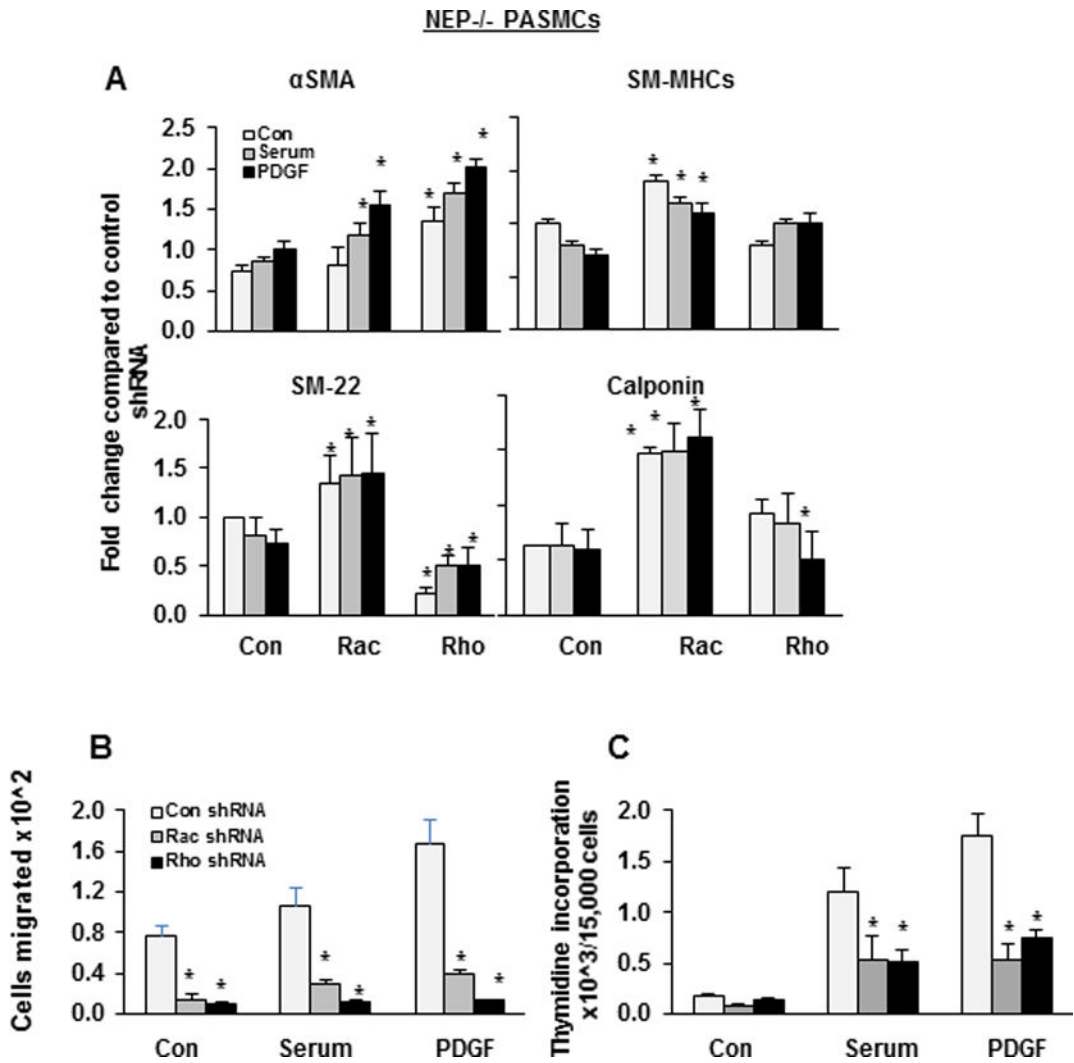


Figure 3. ShRNA to Rac and RhoA, restores SM-protein levels and attenuates migration and proliferation in NEP^{-/-} PASMCS
 NEP^{-/-} PASMCS were infected with either control shRNA or shRNA to Rac or Rho and selected with puromycin. Cells were treated with serum (0.2%) or PDGF (10ng/ml) for 24h and lysates were analyzed for SM-proteins. Panel A shows effect of shRNA on SM-protein levels from 3 different isolates normalized to Gapdh. Panel B shows the effect of shRNAs on migration and Panel C on proliferation of NEP^{-/-} PASMCS. (*) represents p < 0.05 for comparison between control and shRNA treatment.

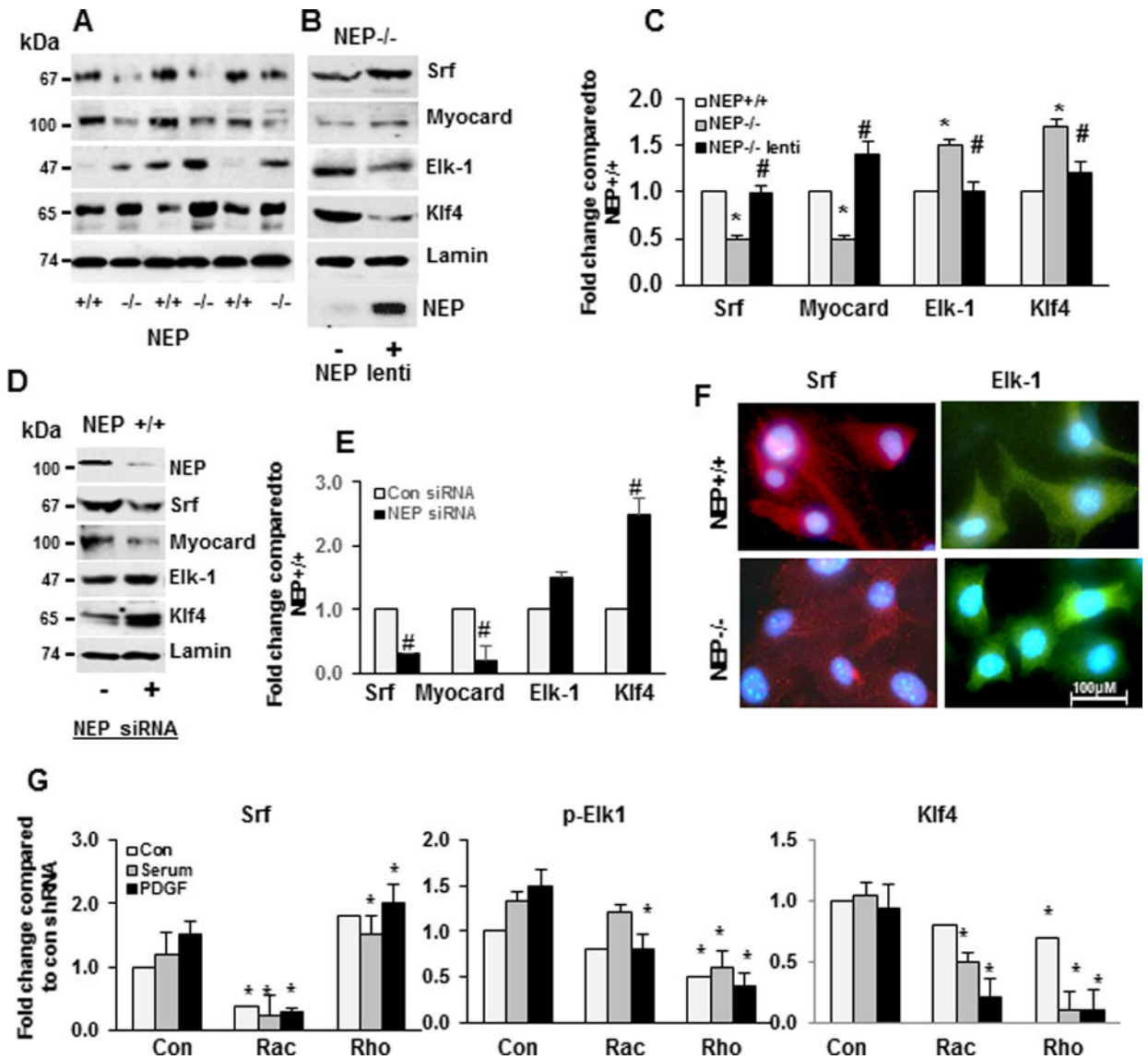


Figure 4. NEP-/- PSMCs express higher levels of p-Elk-1 and Klf4 compared to wt
 Levels of transcription factors (Srf, myocardin, Elk-1 and Klf4) were measured in nuclear lysates from NEP+/+ and -/- PSMCs and are shown in Panel A. Panel B shows effect of NEP lentivirus on transcription factor levels in NEP -/- PSMCs. Panel C shows average fold change in transcription factor levels in NEP-/- compared to NEP+/+ PSMCs normalized to laminin (n=6). Panel D shows effect of NEP siRNA on transcription factor levels in NEP+/+ PSMCs and Panel E shows average fold change compared to control NEP+/+ PSMCs (n=6). Panel F shows localization of Srf and Elk-1 in NEP+/+ and NEP-/- PSMCs by immunocytochemistry. Panel G shows effect of shRNAs on Srf, p-Elk-1 and Klf4 from 3 different isolates. Protein levels were normalized to Gapdh. (*) represents p < 0.05 for comparison between NEP+/+ and NEP -/- PSMC, and for comparisons between control and shRNA (#) for comparisons between NEP-/- control and lentivirus-expressing NEP treated cells.

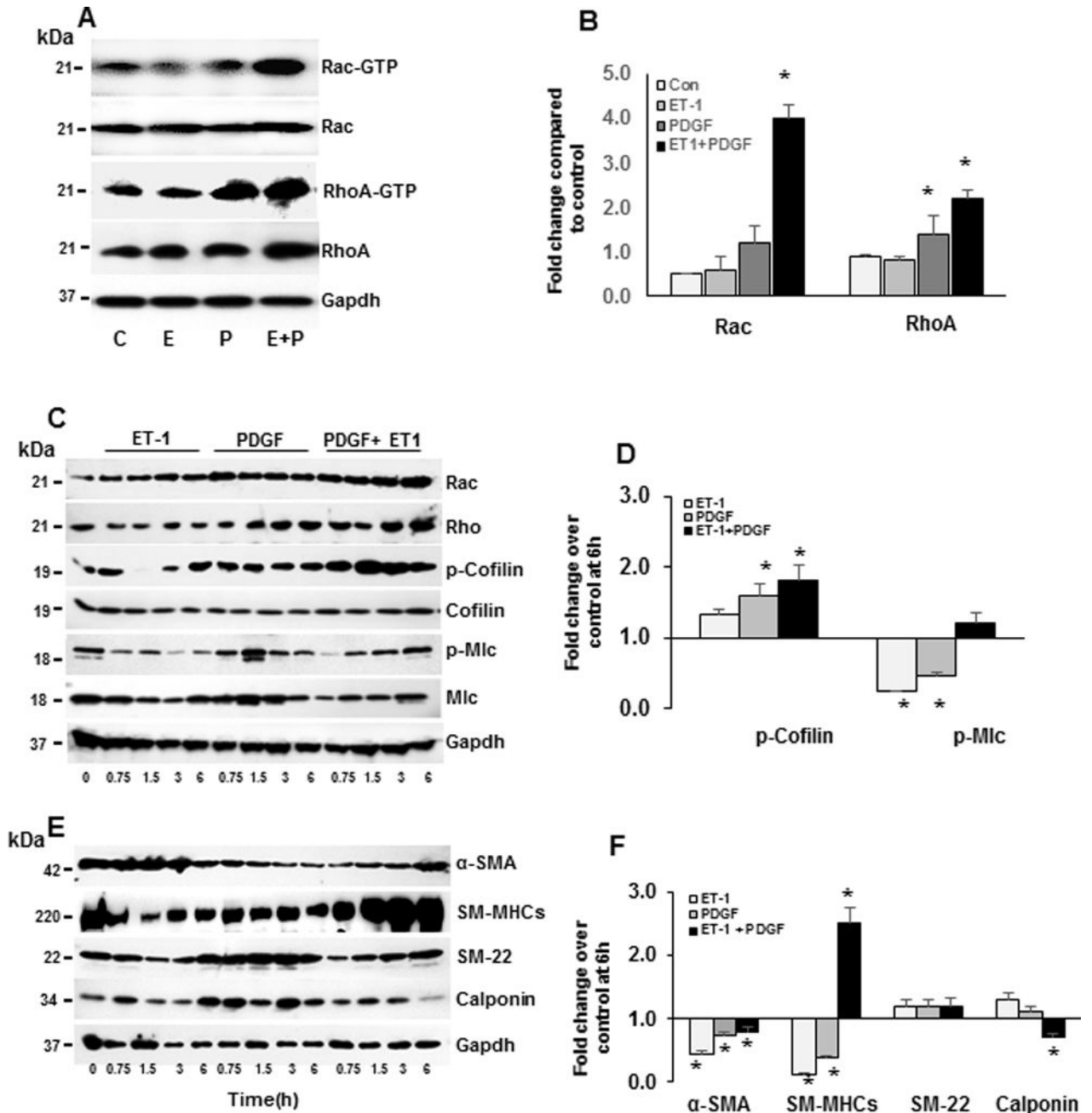


Figure 5. PDGF treatment in the presence of NEP substrate, ET-1, increases Rac and Rho activity and decreases SM-protein levels in NEP+/+ PSMCs

NEP+/+ PSMCs were treated with saline (C) PDGF (P)(10 ng/ml), ET-1(E) (100 nM) and PDGF+ET1 for different time points (0.75h, 1.5h, 3h, and 6h) and lysates probed for downstream effectors of Rho, transcription factors and SM-proteins. Panel A shows levels of Rac and Rho GTP bound form after 24h treatment and graphical representation of average levels of GTPbound/total from 6 isolates is shown in Panel B. Panel C shows effect of agonists on levels of downstream effectors of Rho at different time points and Panel D average fold change at 6h compared to control from 3 different isolates normalized to Gapdh. Panel E shows effect on SM-proteins at different time points and Panel F average

fold change at 6h compared to control normalized to Gapdh. *p < 0.05 for comparisons between control and treated (n=3).

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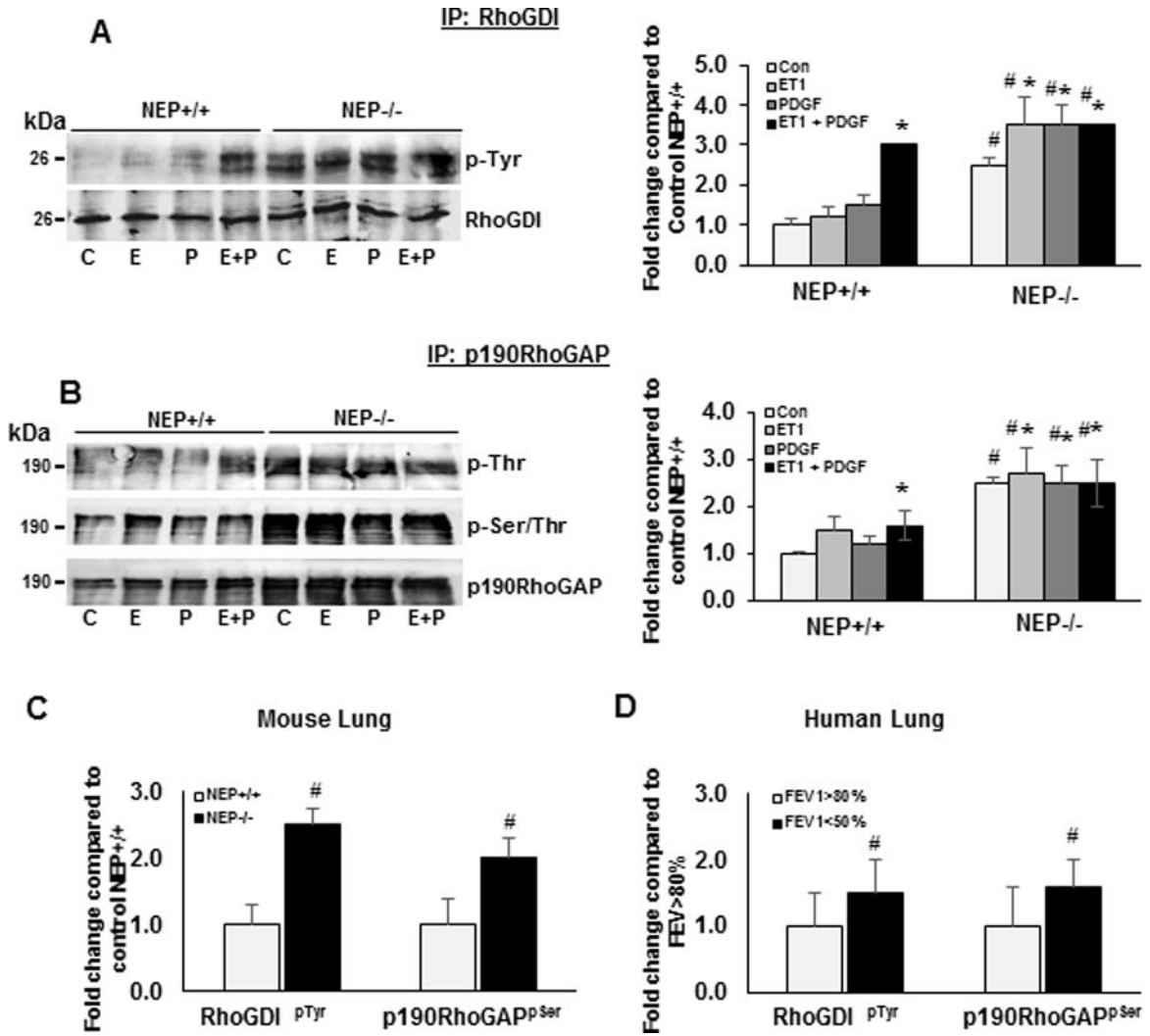


Figure 6. Decreased tyrosine phosphorylation of RhoGDI and Serine phosphorylation of p190RhoGAP in NEP^{-/-} PSMCs, NEP^{-/-} mouse lungs and human lungs with Copd
 NEP^{+/+} and ^{-/-} PSMCs were treated with saline (C) PDGF (P) (10 ng/ml), ET-1(E) (100 nM), PDGF+ET-1 (E+P) for 24h. Lysates (500 ug) were prepared from lungs of NEP^{+/+} and ^{-/-} mice and Copd patients. Lysates were immunoprecipitated with antibodies to RhoGDI and p190RhoGAP and probed with antibodies to Pan p-Tyr, p-Ser and p-Thr and total RhoGDI and p190RhoGAP. Panel A shows representative Western blot of IP with RhoGDI probed with p-Tyr and fold change from 3 different isolates of PSMCs. Panel B shows representative Western blot of IP with p190RhoGAP probed with p-Thr and p-Ser/Thr blot and fold change from 3 different isolates of PSMCs. Panel C shows levels of p-Tyr in RhoGDI IP and p-Ser in p190RhoGAP IP from lungs of NEP^{+/+} and ^{-/-} mice and Panel D from Copd lungs (n=3). (*) represents p < 0.05 for comparison between control and treated and (#) between NEP^{+/+} and NEP^{-/-} and FEV1>80% to FEV1<50%.