Online Supplement

NEPRILYSIN REGULATES PULMONARY ARTERY SMOOTH MUSCLE CELL PHENOTYPE THROUGH A PDGF RECEPTOR DEPENDENT MECHANISM

.

Vijaya Karoor PhD[*§], Masahiko Oka MD PhD[#], Sandra J. Walchak[*], Louis B. Hersh PhD[∞], York E. Miller MD[§ǂ], Edward C. Dempsey MD[*§†ǂ]

Cardiovascular Pulmonary Research Laboratory[*], Division of Pulmonary Sciences and Critical Care Medicine[§] and University of Colorado Cancer Center[†], University of Colorado Anschutz Medical Campus, Aurora, Colorado; University of Kentucky[∞], Lexington, Kentucky; University of South Alabama, Mobile, Alabama; and Pulmonary and Critical Care Section[ǂ] , VA Eastern Colorado Health Care System , Denver, Colorado

Running title: Neprilysin and PDGFR in PASMC Phenotype

To whom correspondence should be addressed:
Vijaya Karoor PhD,
Cardiovascular Pulmonary Research Laboratory,
RC-2 Room 8118, University of Colorado Anschutz Medical Campus,
12700 E 19th Ave; RC-2, Aurora CO 80045.
Tel: (303)-724-3685;
Fax: (303)-724-3693;
E-mail: vijaya.karoor@ucdenver.edu

Supplement Methods

NEP-/- mice: NEP-/- mice on a C57BL/6 and FVB/n background were routinely outbred with mice obtained from Jackson Laboratories (Bar Harbor, ME) (21). NEP+/+ and NEP-/- mice were obtained by heterozygous mating and identified by PCR of genomic DNA obtained from tail samples as previously described (17). Approval of animal protocols was obtained from the University of Colorado and Denver VA Medical Center Institutional Animal Care and Use Committees.

Isolation and characterization of PASMC: Mice were anesthetized with sodium pentobarbital (100mg/kg i.p.). Proximal medial tissue was microdissected from individual age matched 13-17wk old NEP+/+ and NEP-/- littermate mice. Tissue was digested with collagen and cells were grown in DMEM-F-12 medium containing 10% fetal bovine serum (FBS) as described (10) . PASMCs were characterized by light microscopic appearance, growth characteristics, and stained for α-SM-actin, SM-myosin, Factor VIII and LDL between passage 3 and 6. PASMCs were used for studies between passage 6 and 13. In all our experiments 'n' represents the number of population of cells each isolated from different matched NEP+/+ and NEP-/-mice.

Inhibitor treatments: 1000X stocks of PDGFRI inhibitor III and PP2 were prepared in DMSO. Phosphoramidon, Ambrisentan and Atrasentan were water soluble. Appropriate vehicle controls were used for comparison.

ET_AR antagonist treatment in mice: NEP+/+ and -/- mice were treated with ET_AR antagonist Atrasentan (10mg/Kg) for 7d in drinking water. The dose was pre-determined to be sufficient to block Erk activation to acute ET-1 challenge in these mice. Lungs were harvested and lysates analyzed for PDGFR, Src and PTEN phosphorylation.

Densitometry: A Bio-Rad gel scanner and densitometer (Gel DocXR with Quantity 1 program) were used to assess the intensity of the bands obtained by Western blots. Samples from NEP+/+ and NEP-/- PASMCs were run on the same gel. The arbitrary units obtained were normalized to GAPDH and the ratios for NEP+/+ to NEP-/- PASMCs were calculated. Data obtained from 3 to 6 different isolates were used for statistical analysis.

Semi quantitative RT-PCR: Total cellular RNA was extracted from NEP+/+ and NEP-/- PASMCs using a kit from Qiagen (Valencia, CA) as per manufacturer suggestions. cDNA was generated from RNA extracts using a reverse transcription (RT) kit from Applied Biosystems/Ambion (Austin, Texas). Polymerase chain reaction (PCR) was done using the following primers obtained from Primer bank MGH Harvard (SM-markers) and PDGFR primer sequence was from (24).

PDGFR-α

Forward 5'-CAAACCCTGAGACCACAATG-3'; Reverse 5'-TCCCCCAACAGTAACCCAAG-3'

PDGFR-β

Forward 5'-TGCCTCAGCCAAATGTCACC-3'; Reverse 5'-TGCTCACCACCTCGTATTCC-3'

GAPDH
Forward 5'-GCCAAGGTCATCCATGACAAC-3'; Reverse 5'-GTCCACCACCCTGTTGCTGTA-3'
Annealing temperatures for the PCR reactions were as follows: 55°C for α-SM-actin, 58°C for SM-22α and 50°C for SM-myosin; 55°C for PDGFR alpha, PDGFR beta and GAPDH. Extension time for all reactions was 3 min and 35 cycles were performed.

SiRNA transfection: We tested three different siRNA (Novus, Santa Cruz and Sigma) for knockdown of NEP. NEP siRNA from Sigma decreased protein expression by > 90% at 48h. PASMC were transfected with mouse specific siRNA for NEP or universal siRNA from Sigma Aldrich, using Dharmafect Reagent® from Dharmacon (Denver, CO) as per manufacturer recommendations. SiRNA for PDGFRα, PDGFRβ and Src were obtained from Sigma. The final concentration of siRNA was 10nM. Universal siRNA was used as a negative control. Cells were used 48h after transfection for migration and proliferation assays. Protein lysates were analyzed by Western blotting to determine efficiency of knockdown.

Supplement Table 1

Antibody	NEP+/+		NEP-/-	
Flow cytometry	% cells	Mean intensity	% cells	Mean Intensity
PDGFRα	89.1±2.4	7.1±1.4	84.8±10.2	10.4±1.2*
PDGFRβ	98.7±0.4	7.8±1.0	97.1±1.2	12.4±1.5*
PDGFRβ^{y751}	28±5.0	1.4±0.0	48±2.0*	1.9±0.1*
Western Blot	Fold increase in NEP-/- compared to NEP+/+			
PDGFRα	1.3*			
PDGFRβ	1.6*			
PDGFRβ^{y751}	1.8*			

Average mean intensity of binding of PDGFRα, β, and PDGFR^{y751} in NEP+/+ and NEP-/- cells by flow cytometry and fold change in levels by Western blot. *indicates $P \leq 0.05$ for comparison between NEP+/+ and NEP-/- PASMCs.

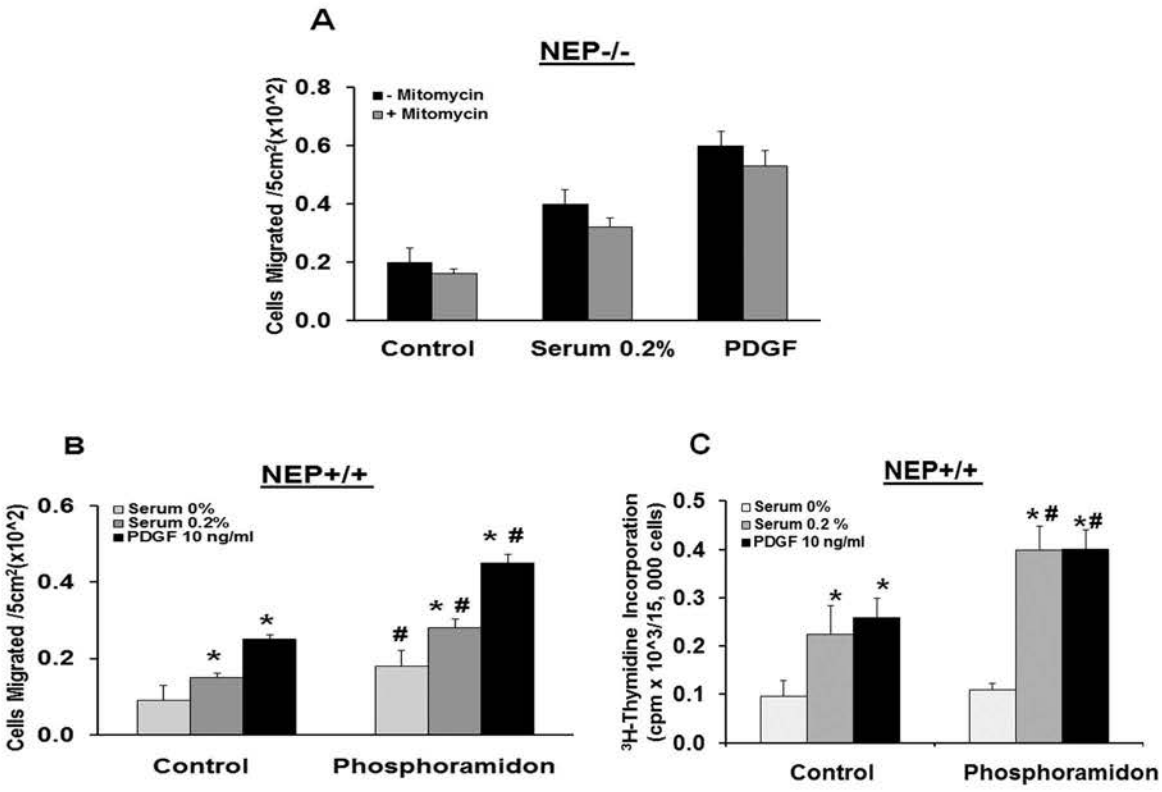

Figure S1 Inhibition of NEP activity by phosphoramidon increases migration and proliferation in NEP+/+ PASMCs. NEP+/+ PASMCs were treated with (10nMole/L) of the NEP inhibitor phosphoramidon, and migration and proliferation were assessed. Panel A shows migration of NEP-/- cells treated without or with mitomycin (10μM) in response to serum (0.2%) and PDGF (10ng/ml). Panel B shows average number of cells migrated in 5cm^2 area for 6h and Panel C shows effect on thymidine incorporation in 3 different PASMC populations. (*) represents p= 0.05 for comparison of treatments in a group and (#) represents p= 0.05 for comparisons between control and phosphoramidon treatments.

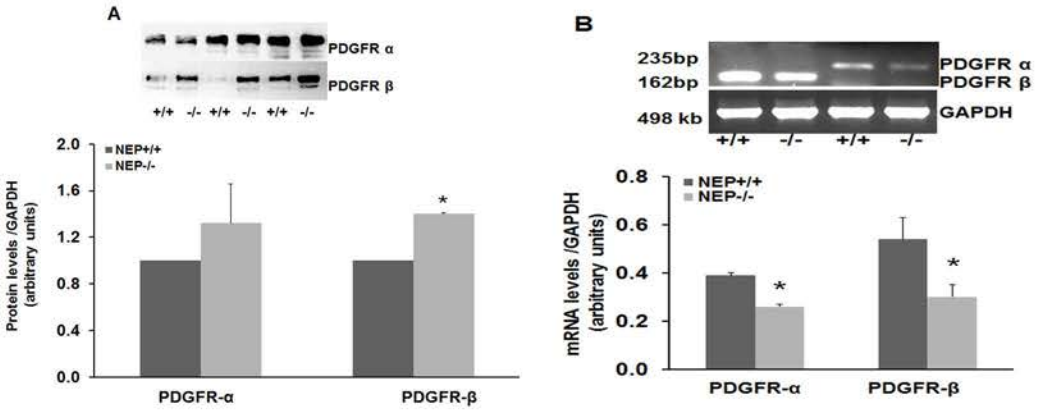

Figure S2 Loss of NEP increases PDGFR expression in SMCs: Levels of PDGFR α and β were measured at the protein level by Western blotting and mRNA level by semi quantitative RTPCR. Quantification of protein from 3 different isolates normalized to GAPDH is shown in Panel A and Panel B shows normalized mRNA levels. (*) represents p= 0.05 for comparisons of NEP+/+ to NEP-/- (n=3).

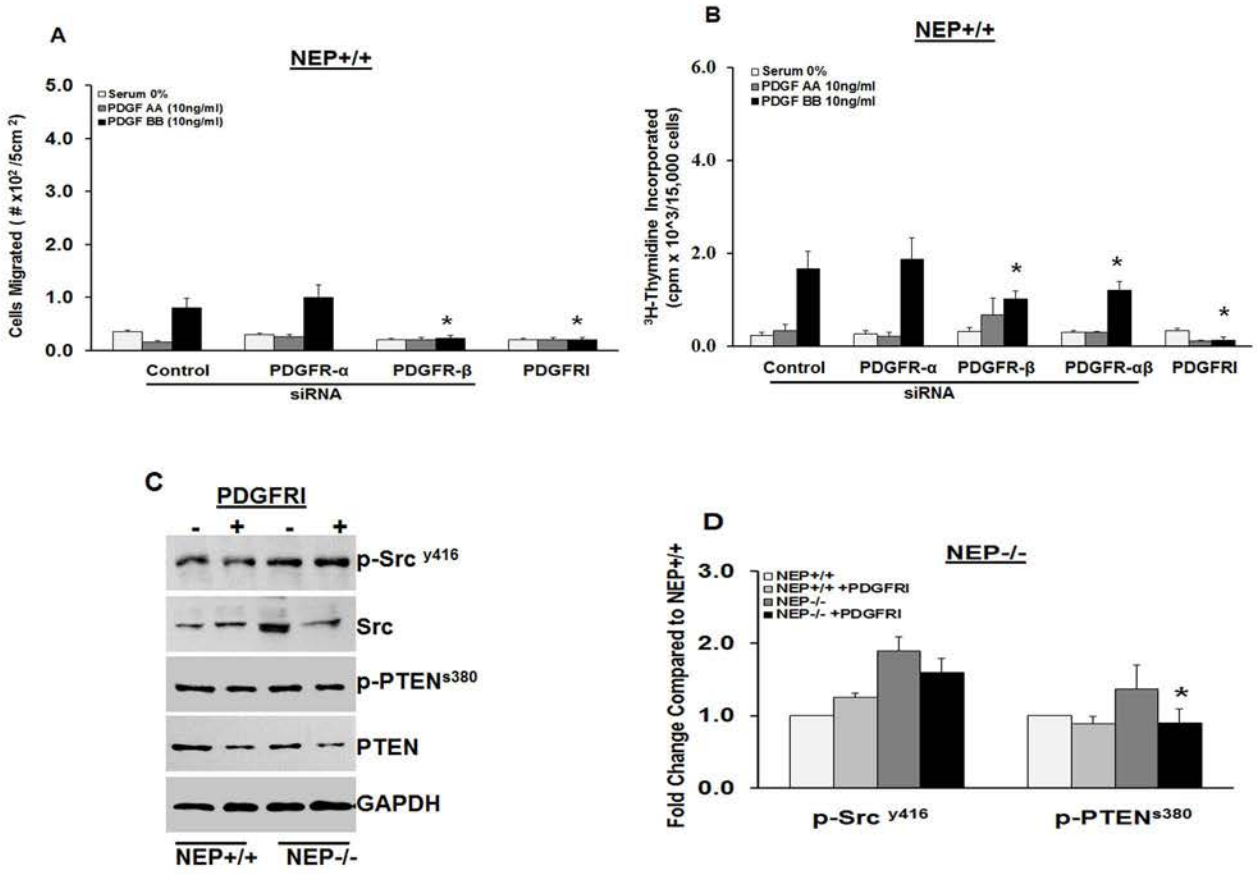

Figure S3 Inhibition of PDGFR attenuates migration and proliferation in PASMCs: NEP+/+ PASMCs were treated with siRNA (10 nM) to PDGFRα, PDGFRβ or αβ and migration and proliferation were measured after 48h. PDGF AA ligand specific for PDGFRα and PDGF BB specific for PDGFRβ were used to assess the contribution of each receptor to migration and Panel A shows effects of siRNA or PDGFR inhibitor III (PDGFRI,500 nM/L) on migration and Panel B on thymidine incorporation in NEP +/+ cells. Effect of PDGF inhibition on levels of phospho and total Src and PTEN is shown in Panel C and levels from 3 different isolates normalized to GAPDH is shown in Panel D. (*) represents p= 0.05 for comparisons of control to PDGF in a group for Panel A and B and control to PDGFRI in Panel D (n=3).

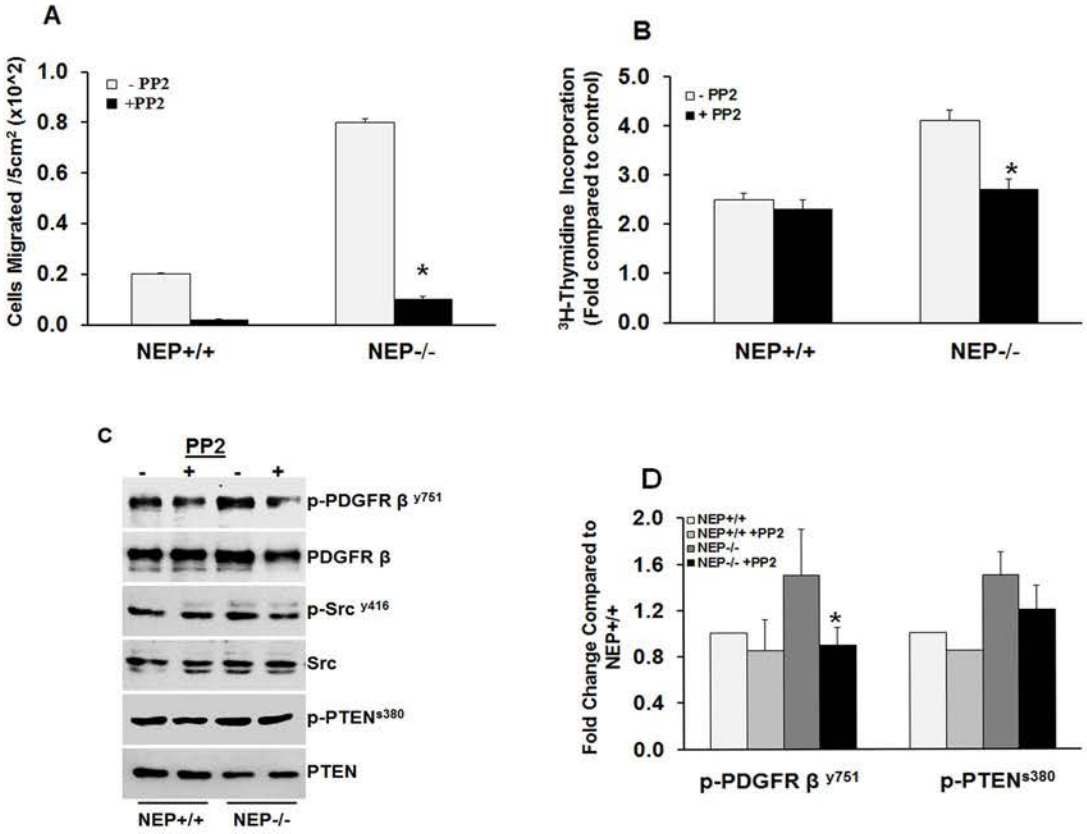

Figure S4 Inhibition of Src kinase attenuates migration, proliferation and PDGFR signaling: Cells were treated with Src kinase inhibitor PP2 (10 μM) for 6h in migration experiments shown in Panel A and for 24h for proliferation experiments shown in Panel B. Effect of Src kinase inhibition on phospho- and total levels of PDGFR, Src and PTEN is shown in Panel C. Panel D shows quantitation of p-PDGFR and p-PTEN in null cells from 3 different isolates. (*) represents p= 0.05 for comparisons of control to PP2 in a group (n=3).

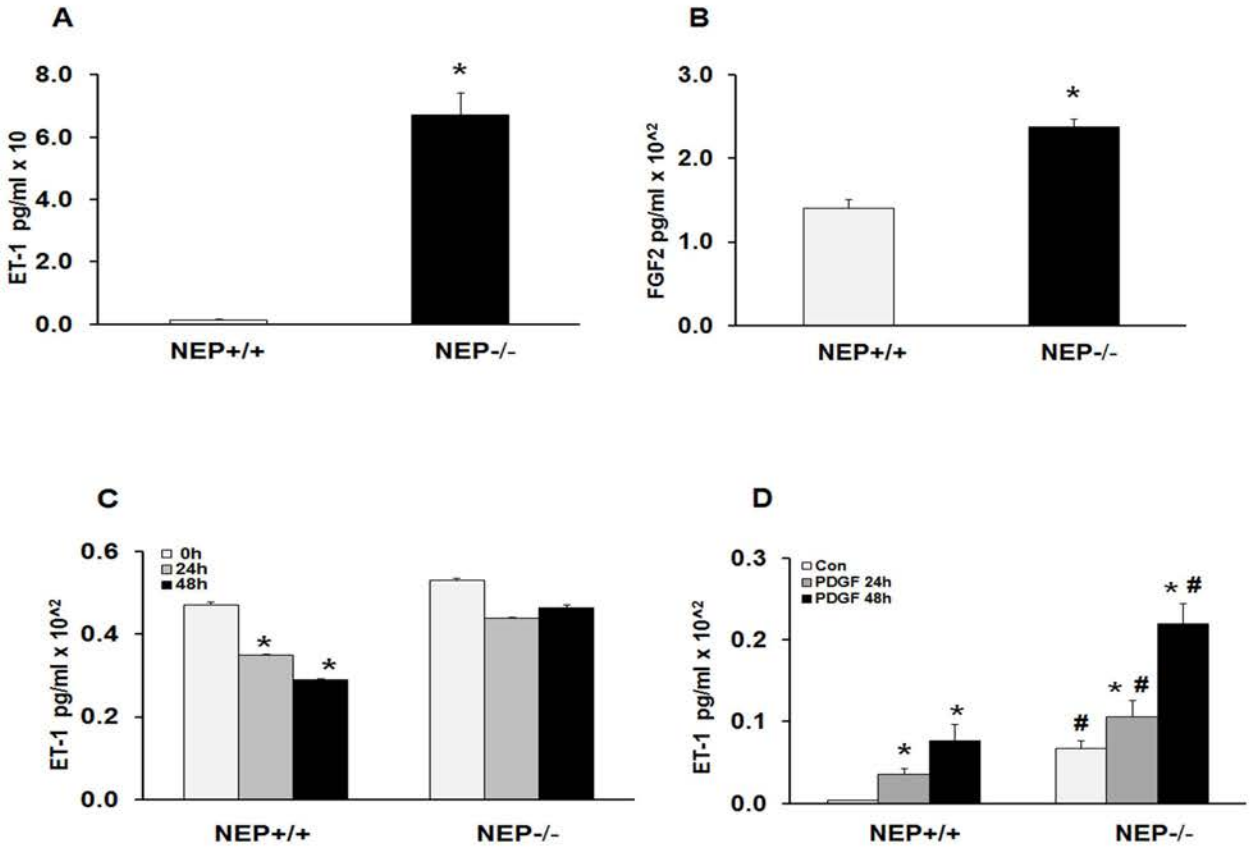

Figure S5 Increased stability of ET-1 and FGF2 in NEP null PASMCs : NEP+/+ and NEP-/-PASMCs (30,000/well) were dispensed in 24 well plates, serum starved overnight and incubated with either serum free media, or PDGF 10ng/ml for 24 and 48h. Cell culture supernatant and lysates were collected and analyzed for levels of ET-1 and FGF2. To assess differences in stability of the peptides cells were incubated with ET-1 (5nM) and FGF2 (10ng/ml) for 0, 24 and 48h and levels of ET-1 and FGF2 at different time points measured. Panel A shows ET-1 levels in cell culture supernatant at 48h and Panel B levels of FGF2 in cell lysates. Panel C shows levels of remaining ET-1 at 24 and 48h and Panel D shows PDGF induced ET-1 in cell culture supernatants. Data was obtained from 3 different populations.. *represents p= 0.05 for comparisons of treatments in a group (n=3).# represents p= 0.05 for comparisons of NEP+/+ and NEP-/-.

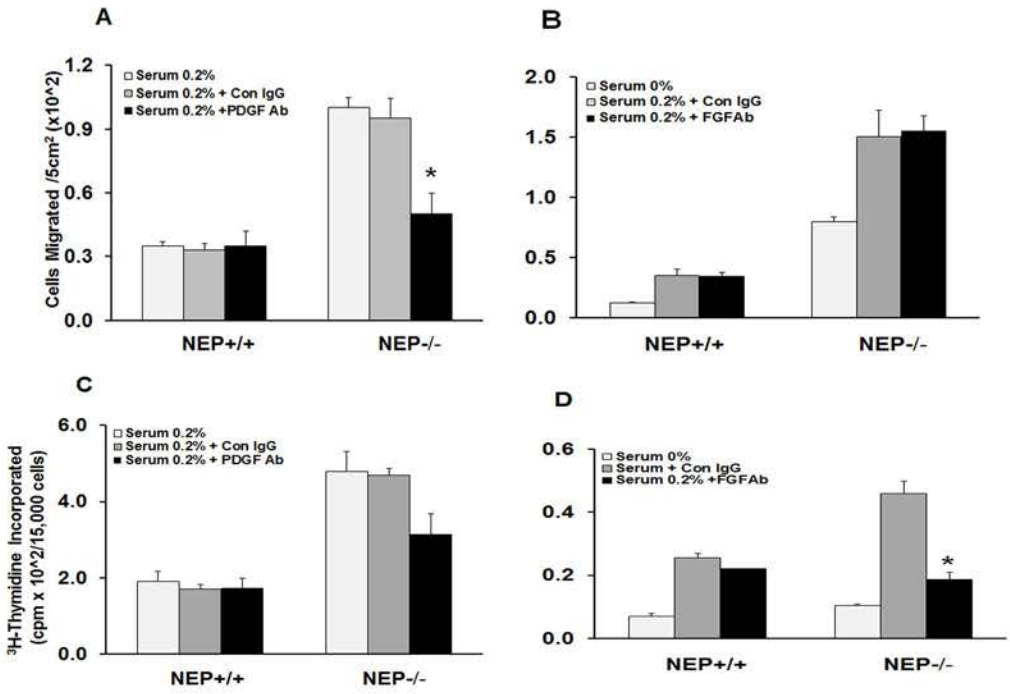

Figure S6 Neutralizing antibody to FGF2 inhibits proliferation not migration in NEP-/- cells: NEP+/+ and NEP-/- PASMCs were treated with either control IgG, antibody to FGF2 or PDGF and migration and proliferation measured as described in Figure 3. Figure 6A shows the effect of PDGFAb and Figure 6B of FGFAb on migration and Panel C and D on proliferation. Data was obtained from 3 different populations. (*) represents p= 0.05 for comparisons of control Ab to specific Ab in a group (n=3).

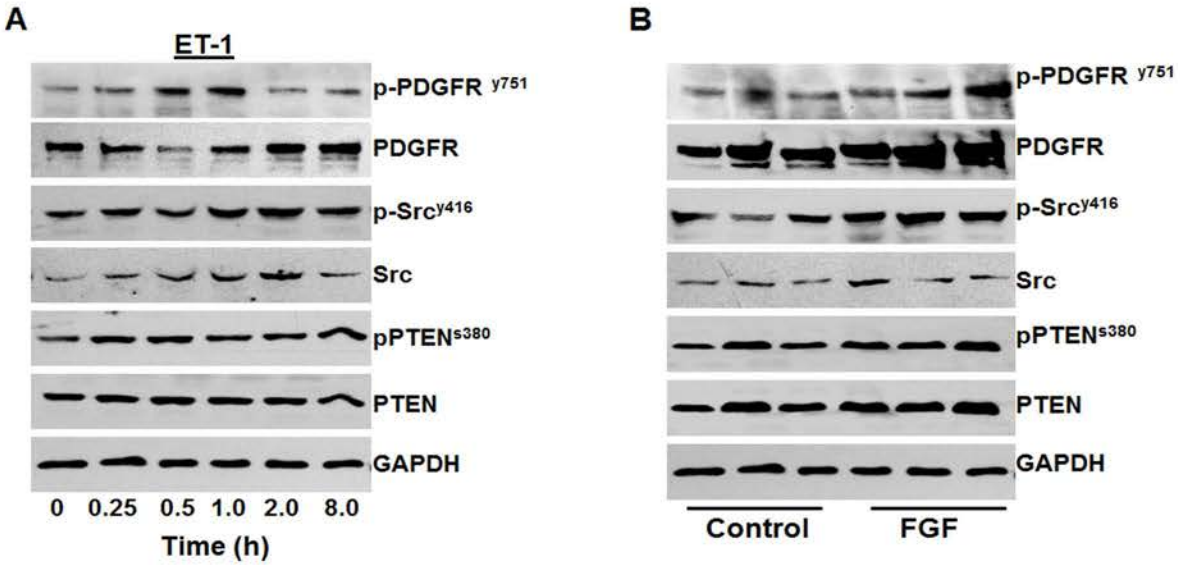

Figure S7 NEP substrates increases phopshorylation of Src and PDGFR in NEP+/+ PASMCs NEP+/+ PASMCs were treated with ET-1(10nM) for 0-8h and lysates analyzed for phospho and total PDGFR, Src and PTEN shown in Panel A. Panel B shows effect of FGF2 (10 ng/ml) treatment for 24h on levels of phospho and total PDGFR, Src and PTEN (n=3).