

SUPPLEMENTAL INFORMATION

Supplemental Information includes an expanded Methods section and additional data including: seven figures and legends, one table, and reference citations.

Supplemental Methods

Antibodies, Reagents, and Constructs

The Pyk2 and phospho-specific ERK1/2 antibodies were purchased from Cell Signaling. Anti-vinculin, SM α -actin, SM γ -actin, and α -tubulin antibodies were purchased from Sigma. The N-term specific anti-FAK, C-term specific anti-FAK, cortactin, Rac1, ERK2, and anti-phospho-PDGF receptor β antibodies were purchased from Upstate. Paxillin antibody was purchased from BD Transduction Laboratories and anti-Cyclin D1 was purchased from Santa Cruz. The anti-phospho ^{Y397}FAK antibody was purchased from BioSource and the Texas-Red phalloidin was purchased from Molecular Probes. SM-22 antibody was a generous gift from Mario Gimona and SM-MHC antibody was obtained from U. Groeschel-Stewart. PDGFBB and TGF- β were purchased from Calbiochem. Ad5CMV Cre adenovirus was purchased from the University of Iowa Gene Transfer Vector Core and Ad5CMV LacZ adenovirus was purchased from the University of North Carolina-Chapel Hill Viral Core. Both viruses were expanded using Puresyn ϕ Adenopure adenovirus purification kit according to manufacturer's protocol. Flag-FAK variants were a generous gift from Dr. Tom Parsons (University of Virginia) ¹.

Primary SMC culture and treatment

Aortic SMC were isolated from 4-8 week old male *fak*^{flox/flox} mice as previously described ². In brief, thoracic aortas were stripped of the endothelial and adventitial layers. Primary SMC were then isolated by trypsin and collagenase digestion. Only the cell preparations at least 85% pure are utilized for further experiments. Cells were maintained in DMEM: F12 (1:1) supplemented with 10% FBS and 1% penicillin-streptomycin and were used between passages 5 and 18. After infection with Cre or LacZ adenoviruses for 72 hr, cells were serum starved for 24 hr and treated with PDGF-BB (20ng/ml) for indicated times. For cortactin silencing experiments, cortactin specific short-interfering RNA (siRNA) oligos were obtained from Invitrogen with the following sequences:

GCUCUUCCCAGCCAACUAUTT[dT][dT] and östealthö
GGAGAAGCAUGAGUCUCAGAAAGAU[dT][dT]. SMC maintained in growth conditions were transfected with 150 nM control or a mixture of the two cortactin-specific siRNAs according to manufacturer's specifications using Dharmafect 1.

Transwell migration assay

SMC were plated on either 10 µg/ml fibronectin-coated or matrigel-coated transwell membranes (8µm; Bio-Coat) with the lower chamber containing serum-free media, 10% serum, or PDGF-BB (20 ng/ml, Calbiochem). After incubation at 37°C for 7 h, the upper surface of the membrane was scraped gently to remove non-migrating cells. The remaining cells were stained with 1% crystal violet and counted. Data represent the total number of cells in four separate fields for each condition.

Western Blotting

Western blots were performed using specific primary antibodies at a 1/1000 dilution (see Supplemental Methods for reagent details). Blots were washed in TBS-T followed by incubation with horseradish peroxidase conjugated secondary antibody at a 1/2000 dilution. Signals were visualized by using chemiluminescence reagents (Amersham).

Immunostaining

Tissue sections (10 µm) were deparaffinized, rehydrated, and antigen-retrieved in 10 mmol/L citrate buffer or 0.1% trypsin. After blocking in 10% goat serum, slides were incubated with primary antibodies at 1/200 dilution at 4°C overnight. Signals were detected using either biotinylated-, fluorescent or HRP linked secondary antibodies coupled with ABC detection kit (Vector) or diaminobenzidine.

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with primary antibodies for 1 hr (1/250 for Rac1, all others 1/1000). Signals were detected using fluorescent-conjugated secondary antibodies. Filamentous actin and nuclei were visualized by staining

with Alexa 488-conjugated phalloidin or DAPI. To quantify polarized Rac1 and cortactin labeling, lines were drawn through the nucleus to divide the cell into quartiles. Membrane localized protein in 1-2 quartiles was considered polarized (if the lamella was continuous), whereas labeling in 3-4 quartiles was considered random. All cells were scored in a blinded fashion.

Kymographic Analysis

Kymography was performed by using an Olympus IX70 inverted microscope that is equipped with a programmable Delta T motorized x,y,z stage and is encased in Plexiglas housing to control the internal environment (37 C, 5% CO₂ and a relative humidity of 60%). Images were acquired at 40x magnification every 5 seconds (for kymography) or every 2 min (for random motility) by an Optronix DEI 750 CCD camera using OpenLab software (Improvision). Kymographic measurements were taken using the Multiple Kymograph plugin for ImageJ and analyzed as described previously³.

Rac GTPase Activity Assays

Cells were lysed in Buffer A (50 mM Tris pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM MgCl₂) containing 100 nM leupeptin, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 20 µg/mL soybean trypsin inhibitor, and 0.05 trypsin inhibitory units/ml aprotinin. Lysates (200-500 µg/condition) were rotated with 30 µg immobilized GST-p21 binding domain (PBD) for 30 minutes at 4°C, then washed three times with Buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂) containing the protease inhibitors listed above. Complexes were pelleted by centrifugation, boiled in SDS-PAGE buffer, electrophoresed (15% SDS-PAGE), transferred to PVDF membrane and immunoblotted for total Rac1.

Epicardial Explant Assays

Proepicardial organs were isolated from HH16-17 quail embryos and placed into explant culture as described previously⁴. After attachment (3-8 hrs post plating), explants were infected with 2.2×10^8 pfu of GFP- or GFP-FRNK (FAK related non-kinase) adenoviruses for the

indicated times. Explants were fixed in 4% paraformaldehyde and processed using immunocytochemistry as described above.

Cell proliferation and apoptosis

fak^{flox/flox} SMC (pre-treated with Cre or LacZ adenovirus for 72 hr) were trypsinized and plated onto a 96-well microplate (5×10^3 cells/well). Cells were serum starved for 24 hr and treated with PDGFBB (20ng/ml) or EGF (100 ng/ml) for 48 hr. Cells were incubated with the formazan dye, WST-1 (10 μ l; Roche) for 4 hr and the absorbance was read at 450 nm as per manufacturer's instructions. Alternatively, BrdU (Sigma, 30 μ g/mL) was administered to SMC grown on chamber slides. After 4 hrs, cells were fixed in 4% paraformaldehyde and stained using a BrdU detection kit (Invitrogen). Caspase activity was determined using the Caspase-Glo kit (Promega).

Matrix degradation assays

fak^{flox/flox} SMC (pre-treated with Cre or LacZ adenovirus for 72 hr) were trypsinized and plated in serum-free medium onto glass chamber slides pre-coated with FN(10 μ g/ml) plus 0.2% Oregon Green 488-Gelatin (Invitrogen) as previously described⁵. After 4 hr, cells were treated with vehicle or PDGF-BB for 90 min at 37°C. Cells were stained as described above and fluorescent images of cells were evaluated for patches of degradation. Alternatively, Cre- or LacZ-treated SMC were plated onto 96 well plates (4×10^3 cells/well) and incubated with DQ-gelatin (100 μ g/well; Molecular Probes) for 90 min in the presence of PDGF-BB. Fluorescence was read on a Polarstar plate reader (BMG Lab Technologies, Durham NC) at Ex/Em 495/515 nm.

Supplemental Table 1. Viable FAK^{wnt} and FAK^{cSMC} offspring (expect 25%).

Age	E10.5	E12.5	E13.5	P0	P4	P14
FAK^{wnt} (viable/total)	8/31	14/60	12/50	18/88	n.d.	0/43
% viable	26%	23%	24%	20%	n.d.	0%
FAK^{cSMC} (viable/total)	n.d.	n.d.	n.d.	8/42	0/29	n.d.
% viable	n.d.	n.d.	n.d.	19%	0%	n.d.

E, embryonic day; P, postnatal day; n.d., not determined.

Supplemental References

1. Richardson A, Malik RK, Hildebrand JD, Parsons JT. Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol.* 1997;17:6906-6914.
2. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest.* 1983;49:327-333.
3. Cai L, Marshall TW, Uetrecht AC, Schafer DA, Bear JE. Coronin 1B coordinates Arp2/3 complex and cofilin activities at the leading edge. *Cell.* 2007;128:915-929.
4. Landerholm TE, Dong XR, Lu J, Belaguli NS, Schwartz RJ, Majesky MW. A role for serum response factor in coronary smooth muscle differentiation from proepicardial cells. *Development.* 1999;126:2053-2062.
5. Berdeaux RL, Diaz B, Kim L, Martin GS. Active Rho is localized to podosomes induced by oncogenic Src and is required for their assembly and function. *J Cell Biol.* 2004;166:317-323.

Supplemental Figure Legends

Supplemental Figure 1. Conditional deletion of *fak* in *wnt-1* and *nkx2-5* expressing cells

leads to PTA that is not associated with defective SM differentiation. **A.** Similar SMC differentiation is observed surrounding the pharyngeal arches (PA) in E11 Control and FAK^{wnt} mice as shown by α -SMA IHC (brown). **B.** Cross sections through *wnt*-derived regions of the left common carotid artery of E13 embryos stained with SM-22 antibody reveal similarly intense staining in FAK^{wnt} vessels in comparison to genetic controls. **C.** P0 neonates were fixed and stained with X-gal (blue) to identify *wnt*-derived cells. Red arrows denote septated pulmonary and aortic arteries in genetic control (left) and un-septated OFT in FAK^{wnt} neonate (right). Yellow arrows reveal β -galactosidase expression throughout the common carotids in the FAK^{wnt} neonate. **D.** Heart sections of P0 genetic control or FAK^{wnt} neonates showed similarly intense SM-22 staining (brown) in coronary arteries (not targeted for FAK deletion). **E.** Transverse sections through the cardiac outflow tracts of P0 genetic control or FAK^{nk} neonates stained with α -SMA. Data are representative of at least 5 mice that exhibited PTA (approximately 13% of the FAK^{nk} mice analyzed). DAo-dorsal aorta, PT-pulmonary trunk, OFT-outflow tract, DA-ductus arteriosus, Ao-aorta, A-atrium, E-esophagus. **F.** Alcian blue/alizarin red stain of P0 FAK^{wnt} and genetic control P0 neonate reveals grossly normal skeleton and cranial facial development.

Supplemental Figure 2. *wnt1*^{Cre} induces coronary-restricted recombination. *wnt1*^{Cre} and Rosa26R^{LacZ} reporter mice were intercrossed and double positive P0 transgenic hearts were subjected X-gal staining, embedded, sectioned and imaged. Data are representative of at least 5 hearts.

Supplemental Figure 3. Depletion of FAK does not affect cardiac growth or other major vessels that are not targeted for FAK deletion in the FAK^{cSMC} neonates. **A.** Transverse sections of H&E stained P0 FAK^{cSMC} and genetic control hearts. Note similar heart size and shape. Arrows demarcate distended coronary vessels apparent in FAK^{cSMC} heart. **B.** Quantification of ventricular cross-sectional area showing no significant difference in heart size between P0 FAK^{cSMC} and genetic control hearts (mean +/- SE; n=8). **C.** Left carotid artery of P0 FAK^{cSMC} and genetic control mice revealed similar SMC coverage as measured by α -SMA staining.

Supplemental Figure 4. Depletion of FAK does not affect cell proliferation or apoptosis in the FAK^{cSMC} hearts. **A.** Confocal images of E15.5 FAK^{cSMC} (*right*) and genetic control hearts (*left*) co-stained for β -gal (green) and the proliferation marker phosphor-Histone H3 (Ser 10) (*top*, red) or the apoptotic marker TUNEL (*bottom*, red). Nuclei are labeled with DAPI (blue). Scale bar = 20 μ m. **B.** Quantification of cell proliferation and apoptosis in E15.5 FAK^{cSMC} and genetic control hearts (n = 4 each). Proliferation is presented as a percentage of phosphor-Histone H3 (Ser 10)⁺ / β -Gal⁺ double-positive cells to β -Gal⁺ cells. Apoptosis is presented as a percentage of TUNEL⁺ / β -Gal⁺ double-positive cells to β -Gal⁺ cells. **C.** Quantification of cell proliferation and apoptosis in P0 FAK^{cSMC} and genetic control hearts (n = 4 each).

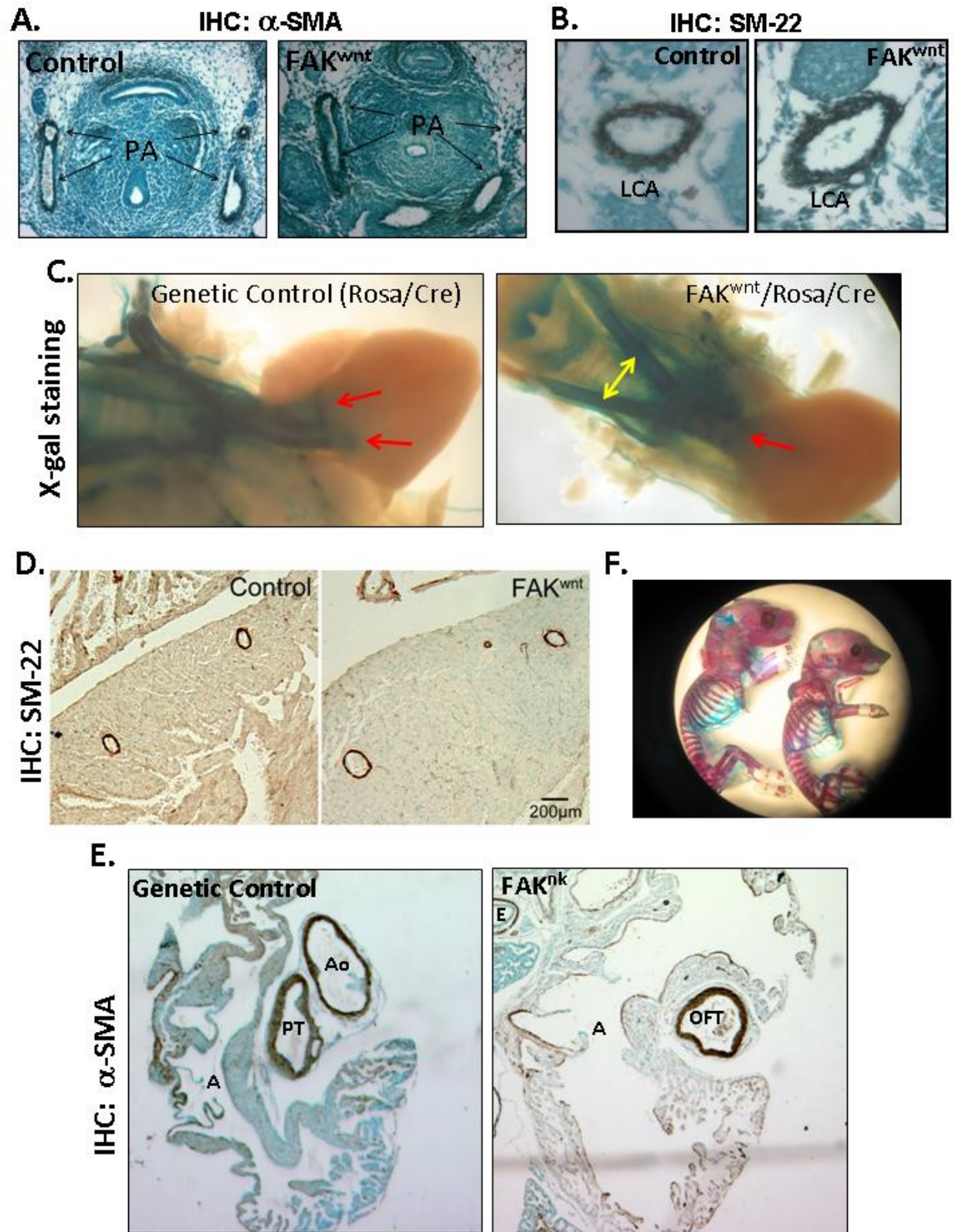
Supplemental Figure 5. FAK activity is not required for coronary SMC differentiation from pro-epicardial cells. HH stage 17 quail pro-epicardial explants were incubated with GFP- or GFP-FRNK adenoviruses and cultured in serum-containing medium for 24-96 hours. **A, B)** Phase and fluorescent images were collected and area of the explants was assessed. Lines in

panel A show the radii of each colony. Data are presented as mean \pm SE (N=3, n=10-16) in panel B. C, D) Explants were fixed 72 hr following treatment with viruses and stained with anti SM γ A antibody.

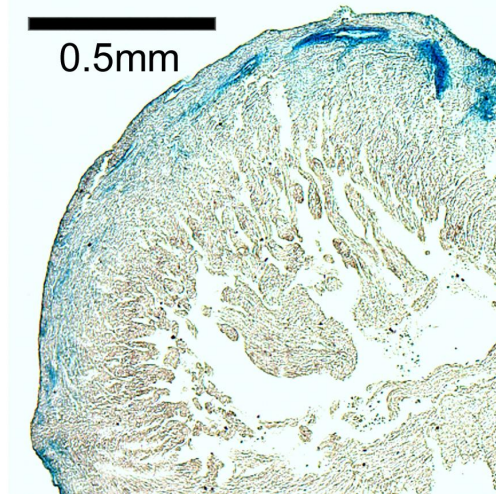
Supplemental Figure 6. FAK is not required for growth or PDGFBB-dependent mitogenic

signaling in SMC. A. *fak*^{flox/flox} SMC maintained in 10% serum were infected with either LacZ or Cre adenovirus for 72 hrs and cell lysates were immunoblotted with indicated antibodies. B. Cells treated as above were fixed and stained with phalloidin and indicated antibodies. Data are representative of at least four experiments. C. *fak*^{flox/flox} SMC were serum starved for 24 hrs prior to treatment with PDGFBB (20 ng/ml) for 15 min and cells were stained with pY397 FAK. Note activation and redistribution of active FAK to nascent leading-edge focal adhesions D. LacZ- or Cre-pretreated *fak*^{flox/flox} SMC were serum starved for 24 hrs prior to treatment with either PDGFBB (20 ng/ml) or EGF (100 ng/ml) for 48 hrs. Cell growth was assessed by WST-1 activity as described in the Materials and Methods section. Data represent mean \pm SE of three separate experiments E. Cre or LacZ infected *fak*^{flox/flox} SMC were serum starved for 24 hrs prior to treatment with PDGFBB (20 ng/ml) for the times indicated. Cells were lysed, electrophoresed and Western blotting was performed with anti-FAK, anti-active pERK1/2 and anti-ERK antibodies. F. *fak*^{flox/flox} SMC were infected with either Cre or LacZ adenovirus for 72 hrs. Cells were serum starved for 4 hrs prior to treatment with either 10% serum (SM) or PDGFBB (20 ng/ml) for 24 hrs. Cells were lysed, electrophoresed, and Western blotting was performed with anti-FAK, anti-Cyclin D1 and anti-ERK antibodies. G. LacZ or Cre infected *fak*^{flox/flox} SMC were treated with 20 ng/ml PDGF-BB for the times indicated and total Rac1 activity was determined using the GST-PBD precipitation assay.

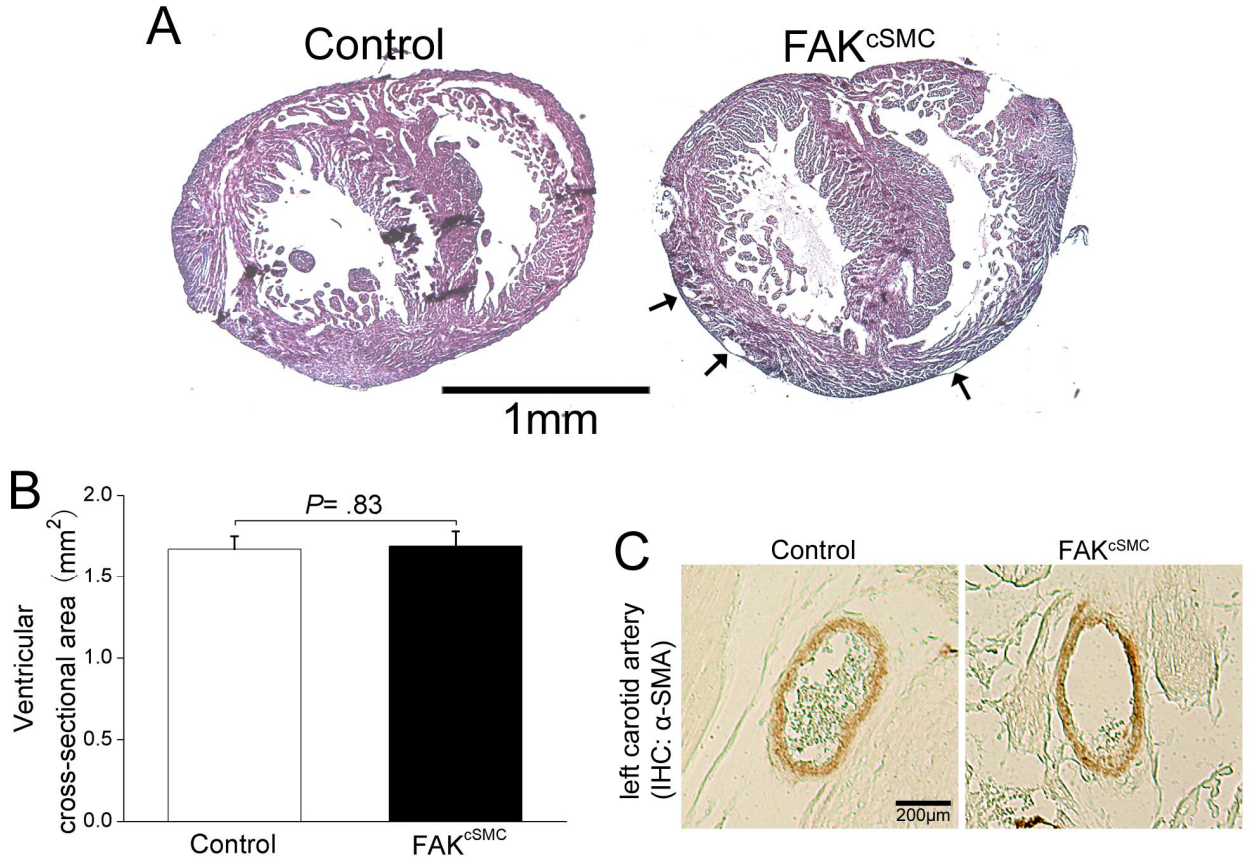
Supplemental Figure 7. Rac1 and cortactin are required for PDGF-stimulated leading edge formation and chemotaxis. **A,B** SMC were treated with NSC23766 (10 μ M, Calbiochem) for 15 min. prior to treatment with 20 ng/ml PDGF-BB for 2.5 min (A) and 15 min (B). Cells were stained with anti-cortactin antibodies and scored for dorsal ruffle and leading edge formation. Results are mean \pm SEM of 200-250 cells from 3 independent experiments. $*p < .05$. **C.** Cells treated as above were plated on fibronectin-coated inserts (10 μ g/ml; Bio-Coat) in serum-free media using PDGF-BB (20 ng/ml) as the chemoattractant (mean \pm SEM of cells counted in 4 fields from 3 independent experiments; $*p < .05$). **D.** SMC were treated with control siRNA (cSi) or cortactin siRNAs (CTNSi; see Methods) for 72 hr and cell extracts were blotted with antibodies directed against cortactin and ERK (loading control). **E-G.** SMC pre-treated with cSi (not shown) or CTNSi for 72 hr were treated with 20 ng/ml PDGF-BB for 2.5 min (E,F) , 15 (E,G), or 30 min (E). Cells were co-stained with pY118-paxillin (pPAX, red), phalloidin (green) and anti-cortactin (blue) antibodies and scored for dorsal ruffle and leading edge formation. Results are mean \pm SEM of 150-200 cells from 3 independent experiments. $*p < .05$. **H.** CSi or CTNSi pre-treated cells were plated on fibronectin-coated inserts (10 μ g/ml; Bio-Coat) in serum-free media using PDGF-BB (20 ng/ml) as the chemoattractant (mean \pm SEM of cells counted in 4 fields from 3 independent experiments; $*p < .05$).



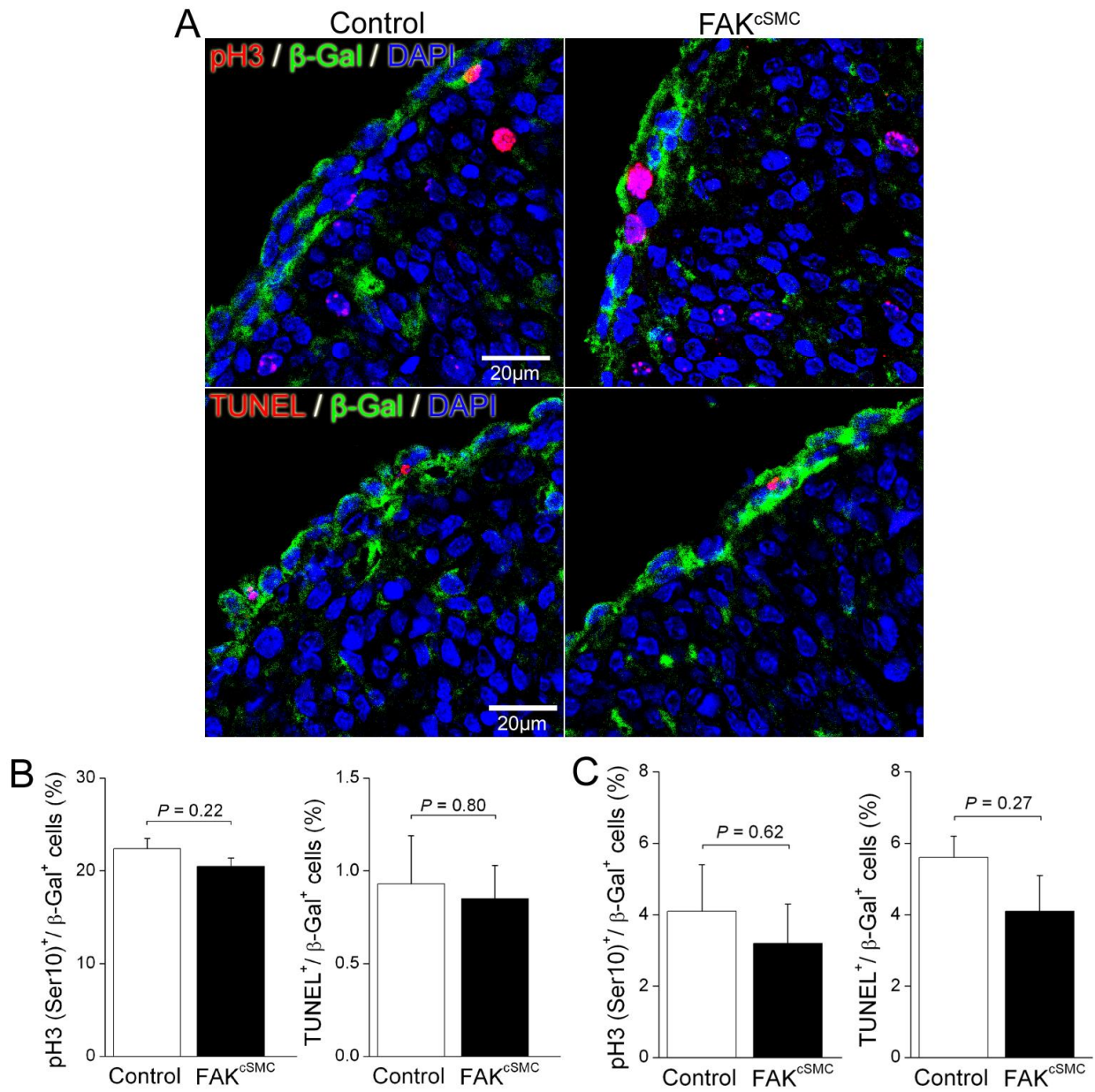
Cheng *et al.* Supplemental Figure 1



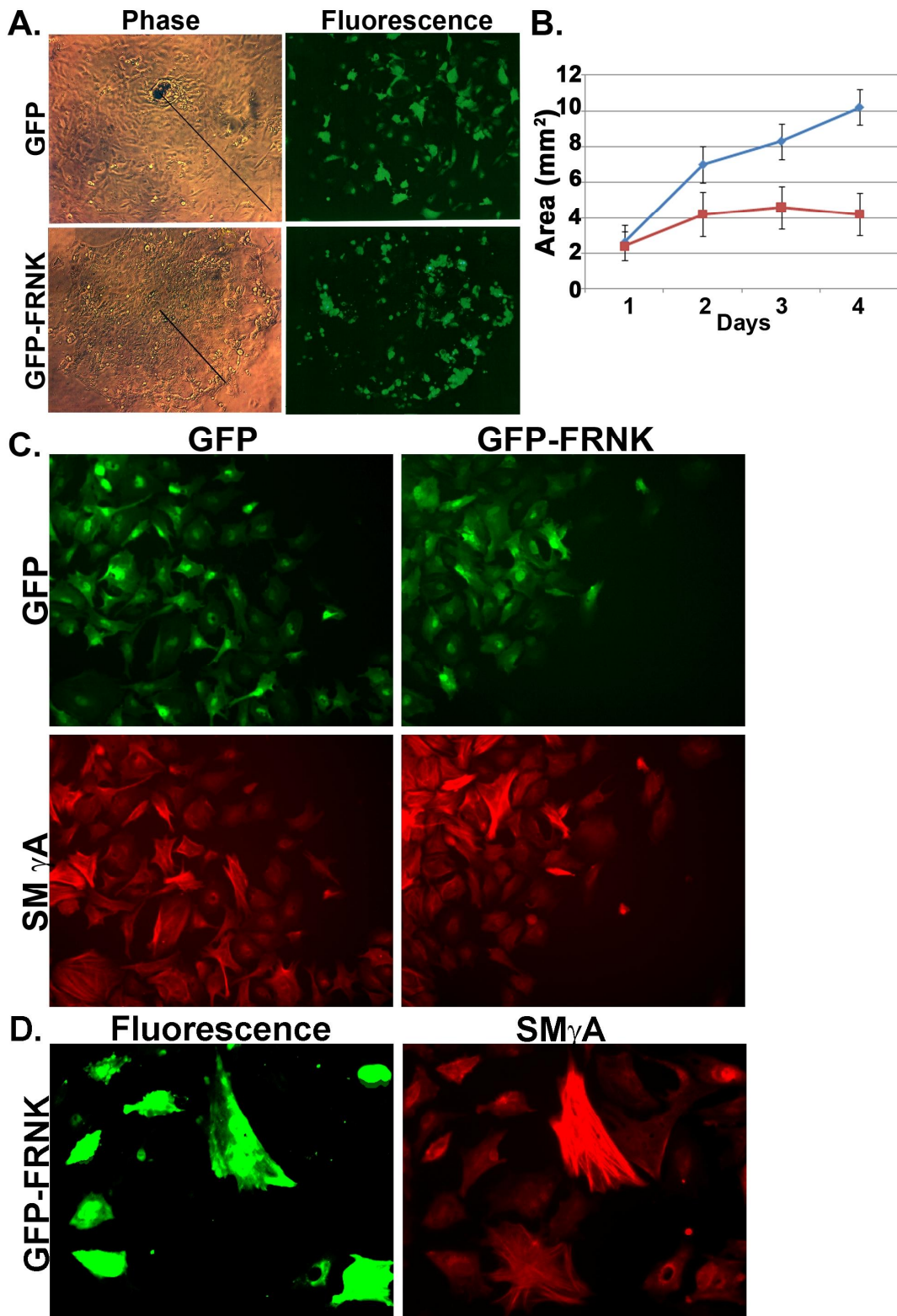
Cheng *et al.* Supplemental Figure 2



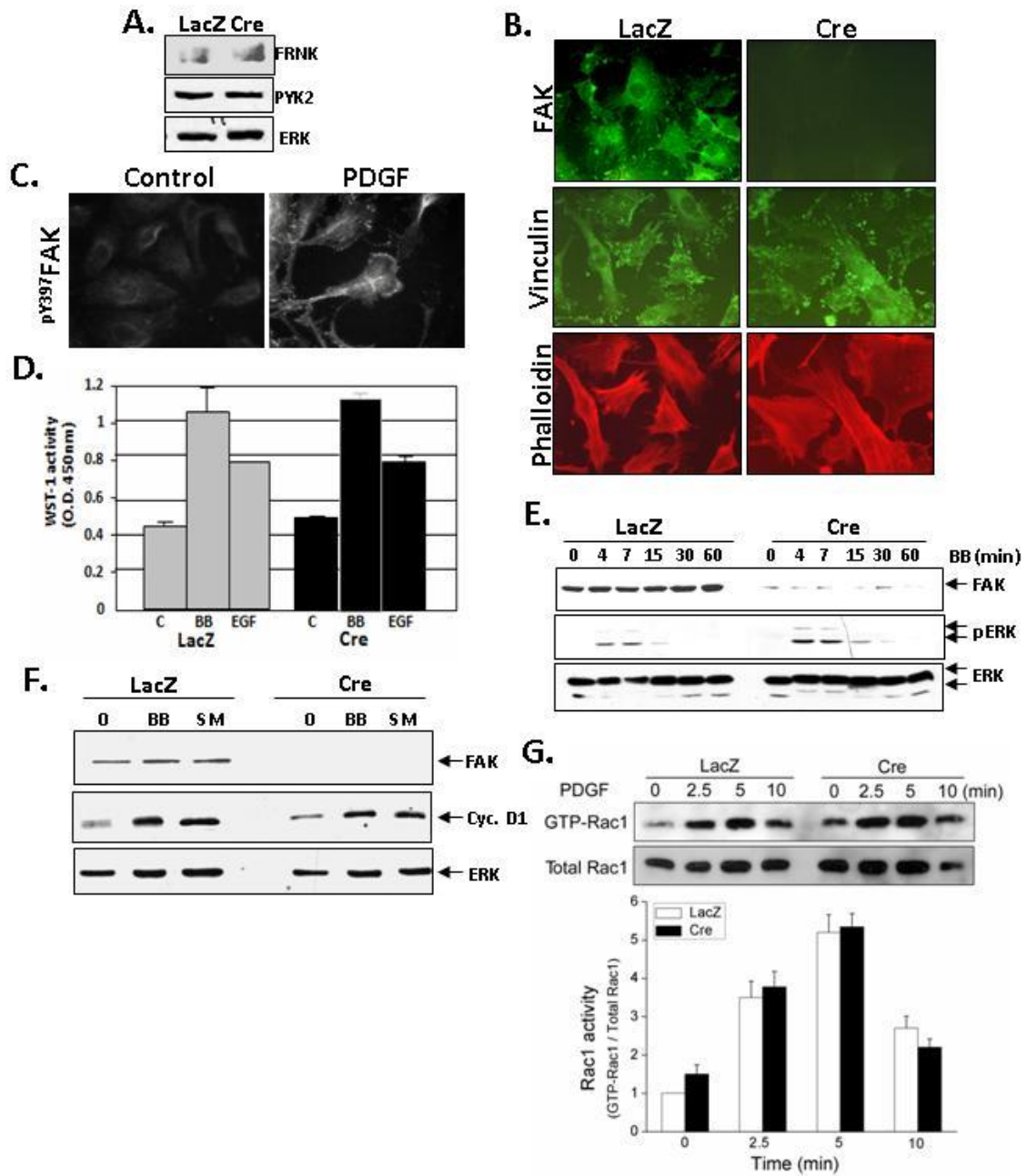
Cheng *et al.* Supplemental Figure 3



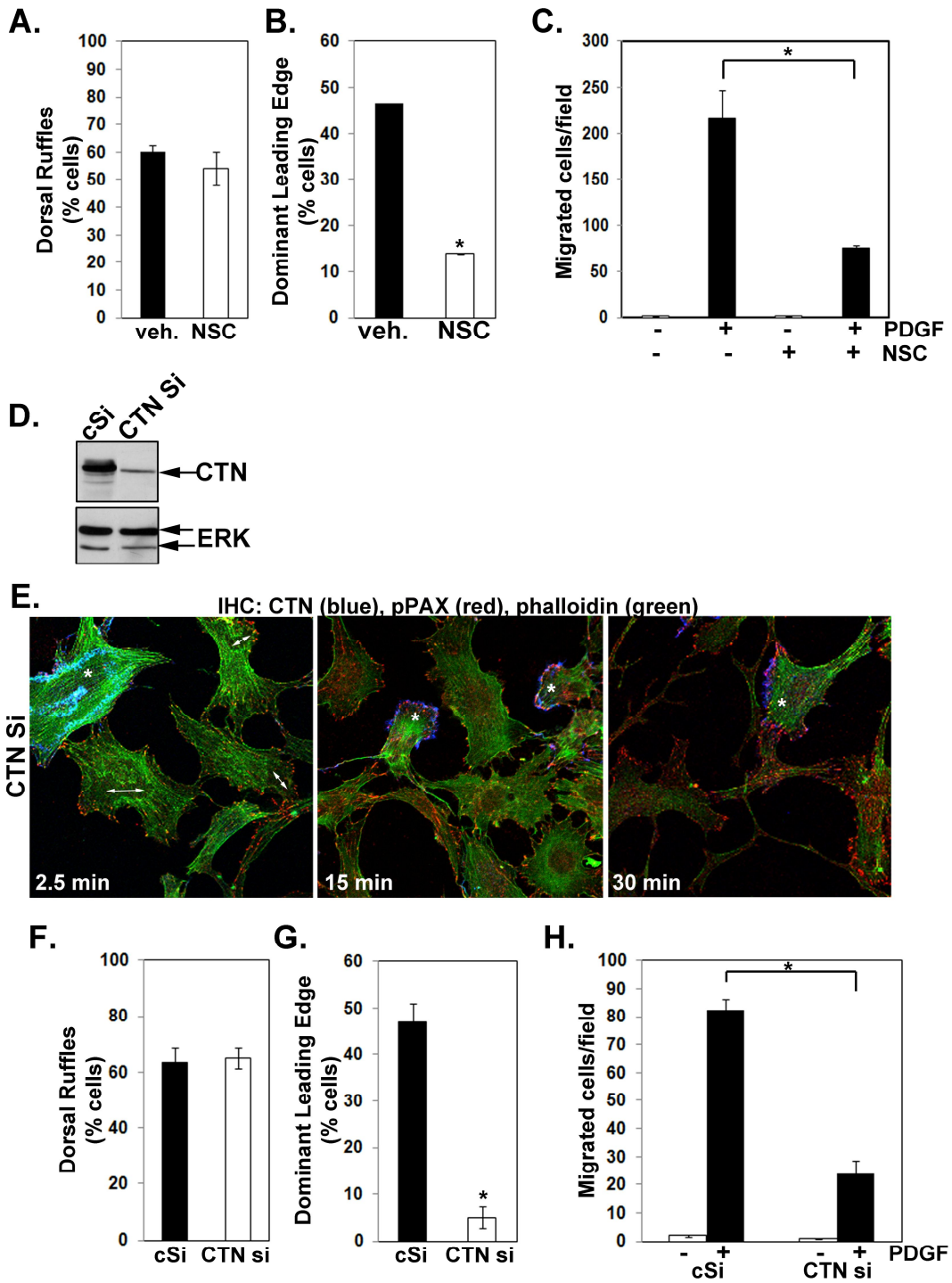
Cheng *et al.* Supplemental Figure 4



Cheng *et al.* Supplemental Figure 5



Cheng *et al.* Supplemental Figure 6



Cheng *et al.* Supplemental Figure 7