N-cadherin induction by ECM stiffness and FAK overrides the spreading requirement for proliferation of vascular smooth muscle cells

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Atomic force microscopy

Intracellular stiffness was measured by atomic force microscopy (AFM) as previously described (Bae et al., 2014).

In vivo vascular injury

N-cadherin$^{fl/fl}$ (Radice et al., 1997) or FAK$^{fl/fl}$ mice (Beggs et al., 2003) were bred to SM-MHC-Cre-ERT2 mutant mice in which the smooth muscle cell-specific myosin heavy chain promoter drives expression of a Tamoxifen-inducible Cre recombinase/mutant ER fusion protein (Wirth et al., 2008). Both mouse lines were backcrossed to C57BL/6 for at least 6 generations. The inducible Cre transgene is integrated into the Y chromosome, so all experiments were performed with male mice. Tamoxifen (4 mg in 0.2 ml 98% corn oil, 2% ethanol) or vehicle was given to 3-5 month old male N-cadherin$^{fl/fl}$;iCre or FAK$^{fl/fl}$;iCre mice by oral gavage for 5 consecutive days. Fourteen days after the last injection, wire-mediated femoral artery injury was performed as previously described (Kothapalli et al., 2007). In vivo cell cycling was examined by administering 200 μg BrdU (in 1.5 ml PBS) over three intraperitoneal injections at 72, 48 and 24 hours prior to sacrifice. Mice were sacrificed 14 days after vascular injury. Mouse procedures and protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. In some experiments, femoral artery injuries were performed on male wild-type (C57Bl/6) mice.

Injured arteries and uninjured contralateral controls were isolated, fixed, embedded in paraffin, sectioned and stained for elastin (Accustain Elastin Stain; Sigma-Aldrich) or BrdU (Cell Proliferation Labeling Reagent; Roche) according to the manufacturer's recommendations except that sections analyzed for BrdU were treated with 0.7 U/μl DNase (Sigma D4527) in 0.1 M Tris-HCl, pH7.6, for 40 min before blocking. Images were visualized using a Nikon Eclipse 80i

microscope and captured at 20X magnification with either a QImaging MicroPublisher 5.0 RTV camera or Hamamatsu C4742-95 digital camera.

Cell culture, infection and transfection

Spontaneously immortalized mouse embryonic fibroblasts (MEFs), Src/Yes/Fyn (SYF)-null MEFs, c-Src reconstituted SYF-null MEFs and primary N-cadherin$^{fl/fl}$ MEFs were maintained in DMEM containing 10% FBS. Primary mouse vascular smooth muscle cells (SMCs) were isolated from the aortae of 3-5 month C57BL/6 mice or N-cadherin$^{fl/fl}$ mice as described previously described (Cuff et al., 2001). SMCs were maintained in DMEM/F12 with 10% FBS and used at passages 2-5. For serum starvation, near confluent cells were incubated for 48 h in serum-free media containing 1mg/ml heat-inactivated, fatty-acid free BSA.

Cells were treated with 10 μM Y-27632 (Calbiochem) in DMSO, 150 μM NSC23766 (Santa Cruz Biotechnology) in water, 1-20 μM PF573228 (Toccris) in DMSO, 20 μM JNK inhibitor II SP600125 (EMD) in DMSO, or 10 ng/ml TGF-β3 (R&D systems) for 20-24 h. Asynchronous cells were infected with recombinant adenoviruses overnight. For experiments using serum-starved cells, the adenoviruses were added for the last 24 hours of serum starvation. Adenoviruses encoding Cre, FRNK, FAK^{Y397F}, Rac^{N17}, β2-chimaerin, N-cadherin, GFP and LacZ were used at 600 -1000 MOI. Cells were transfected with 200 nM siRNA for 4-5 h using Lipofectamine 2000 (Invitrogen) in OPTI-MEM as described (Bae et al., 2014; Klein et al., 2007). A non-specific siRNA was used as a control. FAK, p130Cas and paxillin siRNAs were obtained from Ambion: FAK siRNA_1 ID: 157448 sense: 5'-CCUAGCAGACUUUAACCAAtt-3', antisense: 5'-UUGGUUAAAGUCUGCUAGGtg-3'; FAK siRNA_2 ID: 61352 sense: 5'-GGCAUGGAGAUGCUACUGAtt-3', antisense: 5'-UCAGUAGCAUCUCCAUGCCtg-3'; p130Cas siRNA ID: 161328 sense: 5'-GCCAAUCGGCAUCUUCCUUtt-3', antisense: 5'-AAGGAAGAUGCCGAUUGGCtg-3'; p130Cas siRNA ID: 161329 sense: 5'-

GCUGAAACAGUUUGAGCGAtt-3', antisense: 5'-UCGCUCAAACUGUUUCAGCtg-3'; paxillin siRNA ID: 87164 sense: 5'-GGAGAUUGGAUCCCGAAACtt-3', antisense: 5'-GUUUCGGGAUCCAAUCUCCtc-3'; paxillin siRNA ID 87349 sense: 5'-GGACGGCAAAGCGUACUGtt-3', antisense: 5'-ACAGUACGCUUUGCCGUCCtt-3'. Twist siRNAs sequences are from Yang et al. (Yang et al., 2004). Twist siRNA_1: AAGCTGAGCAAGATTCAGACC; Twist siRNA_2: AGGTACATCGACTTCCTGTAC. Following siRNA transfection, cells were incubated overnight in DMEM (MEFs) or DMEM/F12 (SMCs) containing 10% FBS. Experiments with siRNA typically used 30-50 cells/μm^2.

Fibronectin-coated polyacrylamide hydrogels were prepared as previously described (Klein et al., 2007). The acrylamide concentration remained constant at 7.5% while the bis-acrylamide concentration was 0.03% for soft hydrogels (2-4 kPa), 0.15% for intermediate stiffness hydrogels (10-12 kPa) and 0.3% for stiff hydrogels (22-24 kPa).

Real-time qPCR (RT-qPCR)

Total RNA was extracted with TRIzol (Invitrogen) according to manufacturer's instructions, and reverse transcribed (RT) as described (Klein et al., 2007). The RT product (5 ng) was analyzed by RT-qPCR. Taqman assays were used for N-cadherin (Mm00483213_m1, Applied Biosystems), Twist (Mm04208233_g1*, Applied Biosystems) and 18S rRNA (Klein et al., 2007). mRNA expression for each gene was determined by the standard curve or ddCt methods using 18S rRNA as reference.

Immunoblotting

SDS-gel electrophoresis and western blot analysis was performed using standard conditions and the following antibodies: N-cadherin (BD Transduction, 610921), FAK (BD Transduction, 610087), FAK^{pY397}(Life Technologies, 44-624G), FAK^{pY576} (Cell Signaling Technology, 3281), FAK^{pY861} (Abcam, ab81293), p130Cas (BD Transduction, 610271), p130Cas^{pY410} (Cell Signaling

Technology, 4011), paxillin (Santa Cruz, sc-5574), Src (Millipore), Src ^{pY416} (Cell Signaling Technology, 2101), GAPDH (Santa Cruz, sc-25778) and tubulin (Sigma, T9026). For western blots, protein was collected by inverting 40-mm diameter hydrogels onto 50-µl drops of 5X SDS sample buffer containing β-mercaptoethanol. Western blot signals were detected using enhanced chemiluminescence. Graphs show quantified relative signal intensities that have been normalized to the loading control (GAPDH or tubulin).

Immunohistochemistry and immunofluorescence staining of cells and tissue

Mouse SMCs cultured on micropatterned adhesive islands were fixed in 4% paraformaldehyde for 15 min, washed in PBS, permeabilized in 0.2% Triton-X for 15 min, and blocked in 2% BSA, 0.2% Triton-X in PBS for 1 hr. SMCs were then incubated in 30-µl mouse anti-N-cadherin (BD Transduction, 610921; diluted 1:50) or rabbit anti-FAK^{pY397} (Abcam, ab4803; diluted 1:50) overnight at 4^{o}C, washed three times with PBS, and incubated with 30 µl Alexa-594 anti-mouse or Alexa-488 anti-rabbit secondary antibodies for 1 h at room temperature. Primary and secondary antibodies were diluted in 2% BSA, 0.2% Triton-X in PBS.

Isolated arteries were embedded in paraffin and cut into 5-µm sections. The sections were deparaffinized in xylene, rehydrated, and incubated in antigen retrieval buffer (0.1 M sodium citrate, Tween-20, pH 6.0) for 20 min at near boiling. For immmunohistochemical staining of N-cadherin and immunofluorescent staining of FAK^{pY397}, the sections were blocked with 1% goat serum and 2% BSA in 0.1 M Tris-HCl, pH 7.6 for 1 h at room temperature. Primary and secondary antibodies were diluted in 1% goat serum with 2% BSA in 0.1 M Tris-HCl, pH 7.6. The samples were incubated with 25-50 µl mouse anti-N-cadherin (BD Transduction, 610921; diluted 1:300), rabbit anti-FAK^{pY397} (Abcam, ab4803; diluted 1:100), or rabbit anti-p130Cas^{pY410} (Sigma SAB4503824; diluted 1:100) overnight at 4^{o}C, washed three times with 0.1 M Tris-HCl, pH 7.6, and then incubated for 1 h at room temperature with a biotin-labeled anti-mouse IgG

(Vector Laboratories) for N-cadherin, Alexa-488 chicken anti-rabbit IgG (Invitrogen) for anti-FAK^{pY397}, or Alexa-594 goat anti-rabbit IgG (Invitrogen) for anti-p130Cas^{pY410}. Immunohistochemical staining for FAK^{pY397} was performed similarly except that PBS replaced 0.1 M Tris-HCl. Immunohistochemical signals were developed using VIP (Vector Laboratories). The FAK^{pY397} and p130Cas^{pY410} signals in the media of injured vehicle- and Tamoxifen-treated N-cadherin$^{fl/fl}$;iCre mice were quantified using Image J.

Image analysis

Immunofluorescence and Western blot images were quantified using ImageJ. For immunofluorescence, serial sections of injured N-cadherin$^{fl/fl}$;iCre mouse femoral arteries were immunostained with FAK^{pY397}, p130Cas^{pY410} or an isotype-matched control antibody. The media and neointima of each immunostained tissue section was traced using the polygon drawing tool in ImageJ, and the raw integrated density and area were measured. The raw integrated density was normalized to the area to obtain the relative fluorescence from each section. Background relative fluorescence was subtracted from the FAK^{pY397} and p130Cas^{pY410} signals. The net relative fluorescence intensity for FAK^{pY397} and p130Cas^{pY410} were averaged from at least 4 different mice and reported as mean + standard error. Western blots were quantified similarly except that the polygon tool was used to outline bands of interest, and the background signal was obtained from areas of the blot adjacent to each band of interest. Signal intensities were averaged from at least 3 separate experiments and reported as mean + standard error.

Analysis of confocal microscopy images

Confocal images from fluorescently stained SMCs cultured on unpaired and paired micropatterned adhesive islands of different areas (2,500 μm^2 and 10,000 μm^2) were acquired using a Leica TCS SP8 laser scanning confocal with a 20X, 0.75 NA air immersion objective. FAK^{pY397} and N-cadherin fluorescence signal intensity for each unpaired cell or pair of cells was

summed from three consecutive peak sections using ImageJ software. The total signal intensity for FAK^{pY397} was then normalized to the spread area of each cell or pair of cells. Total N-cadherin signal intensity was measured from traced regions of cell-cell contact for paired cells; (n=5-9).

Bioinformatics

We used the Ingenuity Pathway Analysis Path Explorer tool to identify experimentally observed, direct or indirect, signaling intermediates reported to be downstream of BCAR (p130Cas) and upstream of CDH2 (N-cadherin). As p130Cas is an intermediate in the FAK-Rac dependent induction of N-cadherin mRNA, we excluded protein-protein interactions directly upstream of CDH2.

Statistical analysis

Significance of the in vitro and in vivo results was determined using a 2-tailed t-test and 2-tailed Mann-Whitney test, respectively. Outliers in the in vivo datasets were identified using a ROUT test (Graphpad). *p*-values are shown for statistically significant differences.

SUPPLEMENTAL REFERENCES

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Figure S1, Related to Figure 1

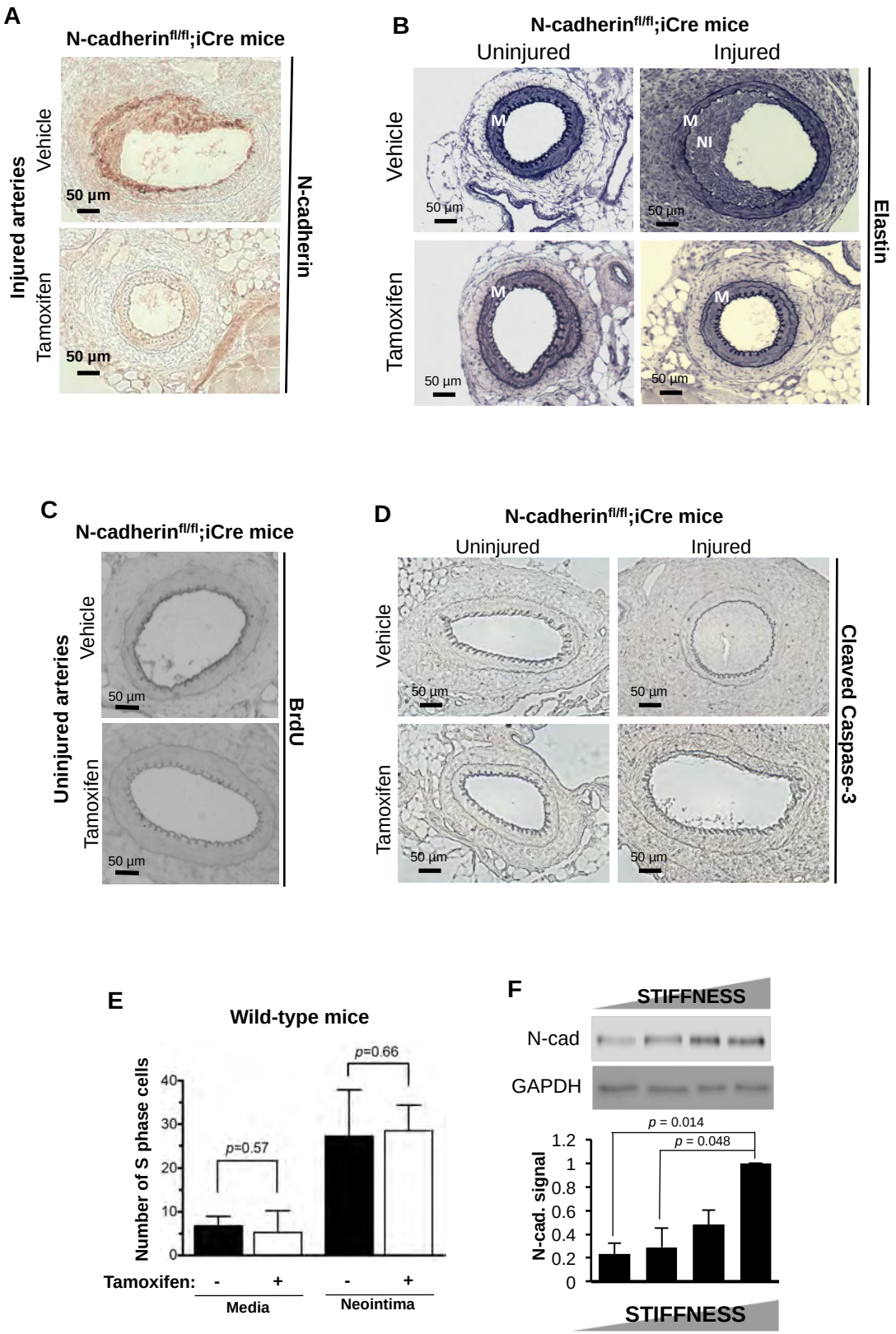

Figure S1. N-cadherin is essential for in vivo SMC proliferation, Related to Figure 1. (A) Representative images from injured femoral artery sections of vehicle- (n=2) and Tamoxifen-treated (n=3) N-cadherin$^{fl/fl}$;iCre mice stained for N-cadherin. (B) Representative images from uninjured (n=5-6) and injured (n=10-11) femoral artery sections of vehicle- and Tamoxifen-treated N-cadherin$^{fl/fl}$;iCre mice stained for elastin. (C) Representative images showing the absence of S phase-labeled nuclei in uninjured arteries of N-cadherin$^{fl/fl}$;iCre mice (n=5). (D) Femoral artery sections from uninjured and injured, vehicle- (n=3) and Tamoxifen-treated (n=3) N-cadherin$^{fl/fl}$;iCre mice were stained for cleaved caspase-3. (E) Femoral artery injuries were performed on wild-type mice that had been treated with vehicle (n=4) or Tamoxifen (n=4). The number of S phase nuclei in the neointimal and medial layers was determined by in vivo EdU labeling and manual counting, and plotted as mean + SD. (F) Total cell lysates from MEFs cultured on FN-coated polyacrylamide hydrogels (2, 6, 11, 24 kPa) for 24 h in 10% FBS were analyzed by western blot for N-cadherin and GAPDH (loading control). Graph shows mean + SE; n=3.

Figure S2, Related to Figure 2

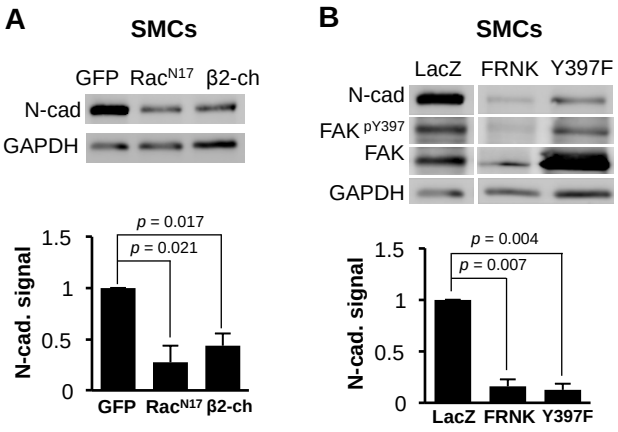

Figure S2. N-cadherin is regulated by Rac, Related to Figure 2. (A-B) SMCs in 10% FBS were infected with (A) adenoviral GFP, RacN17 or β2-chimaerin or (B) adenoviral LacZ, FRNK or FAK^{Y397F} (Y397F) for 24 h. Total cell lysates were collected for western blot analysis. The vertical white line in D indicates removal of extraneous lanes. Graphs show mean + SE; n=3.

Figure S3, Related to Figure 3

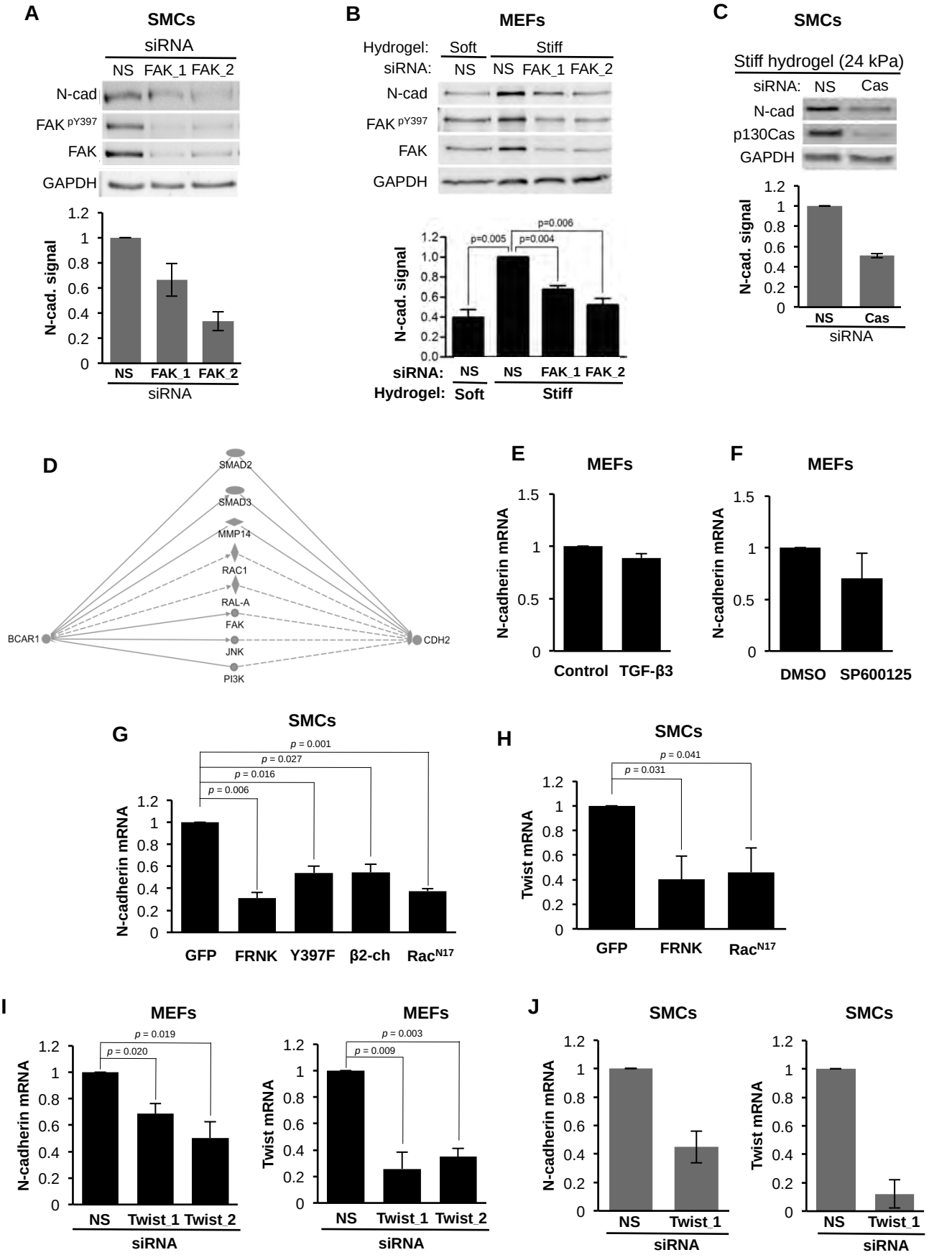

Figure S3. N-cadherin is induced by FAK-Src-Cas signaling, Related to Figure 3. (A) SMCs were transfected with 200 nM non-specific (NS) siRNA, or two distinct FAK siRNAs, seeded with 10% FBS for 24 h and examined by western blot; n=3. (B) Serum-starved MEFs were transfected with 200 nM non-specific (NS) siRNA, or two distinct FAK siRNAs, cultured on stiff FN-coated hydrogels with 10% FBS for 24 h and examined by western blot; n=3. (C) Serum-starved SMCs were transfected with 200 nM non-specific (NS) siRNA or p130Cas siRNA_1, incubated with 10% FBS, and analyzed by western blot. Bar graphs below western blots show mean + SE (B); n=3 or mean ± range (A,C); n=2. (D) The Ingenuity Pathway Analysis Path Explorer tool was used to identify experimentally observed direct or indirect signaling intermediates downstream of BCAR (p130Cas) and upstream of CDH2 (N-cadherin mRNA). Symbol codes can be found at http://ingenuity.force.com/ipa/IPATutorials#. (E-F) MEFs in 10% FBS were treated with 10 ng/ml TGF-β3 (E) or 20 μM JNK inhibitor II SP600125 (F) for 24 h and analyzed by RT-qPCR; n=3. (G-H) SMCs in 10% FBS were infected with adenoviral GFP, FRNK, Y397F, RacN17 or β2-chimaerin for 24 h. Total RNA was isolated, and N-cadherin (G) and Twist (H) mRNA expression was determined by RT-qPCR; n=3. (I-J) MEFs (I) and SMCs (J) in 10% FBS were transfected with non-specific (NS) siRNA or Twist siRNA for 24 h and analyzed by RT-qPCR; n=3.

Figure S4, Related to Figure 4

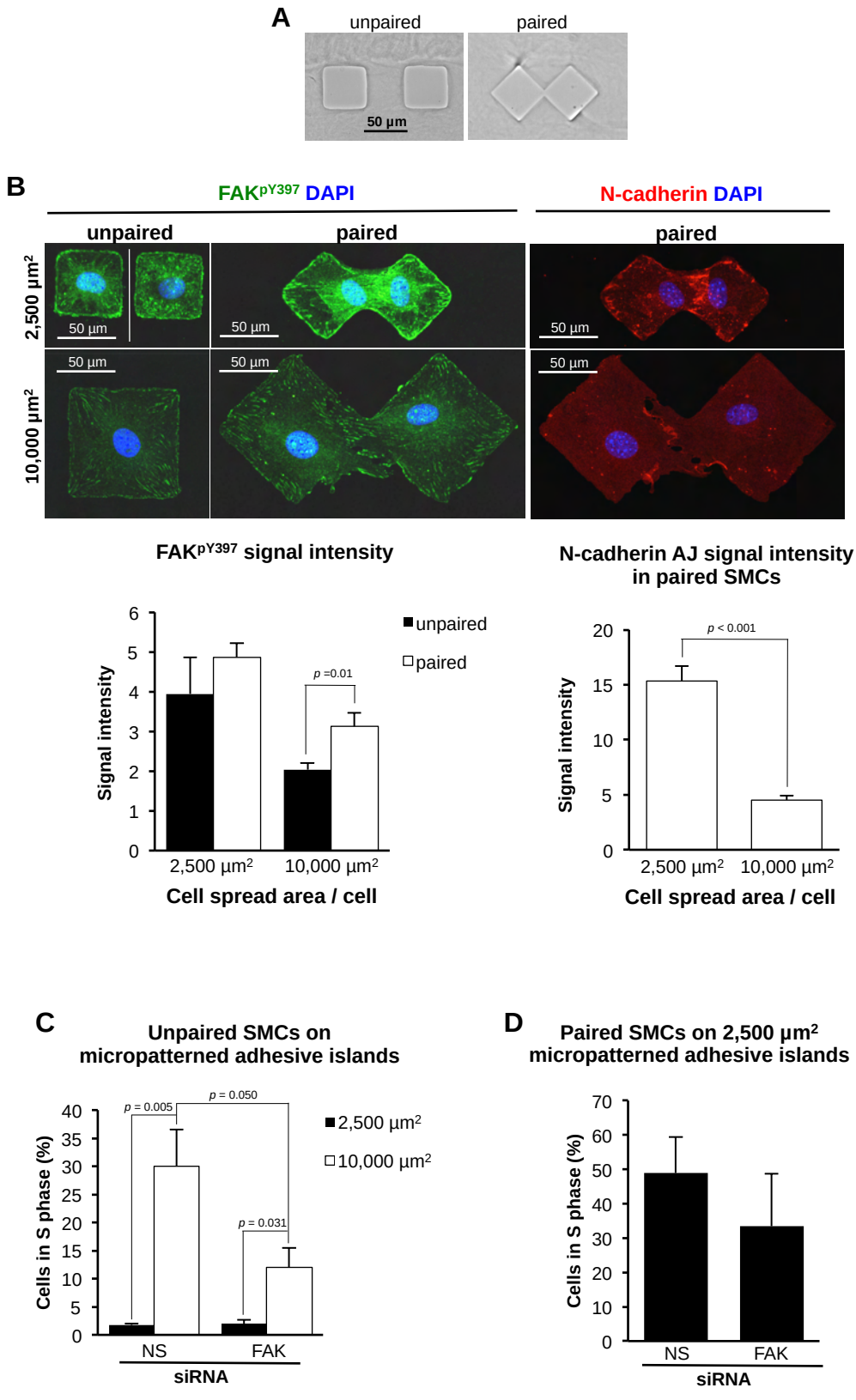

Figure S4. N-cadherin determines the spreading requirement for proliferation, Related to Figure 4. (A) Unpaired and paired micropatterned adhesive islands imaged by phase contrast microscopy. (B) SMCs were cultured on unpaired and paired micropatterned adhesive islands (2,500 and 10,000 μm^2) with 10% FBS for 24 h. Cells were then fixed, immunofluorescently stained for FAK^{pY397} and N-cadherin, and imaged by confocal microscopy. The images show a sum of signals from 3 consecutive confocal sections per condition. Signal intensity is plotted as mean + SE from at least 5 different cells. *p*-values were determined by 2-tailed t-test. The vertical white line in the top left panel of figure S3B indicates removal of extraneous space between the cells. (C-D) Serum-starved SMCs were transfected with 200 nM non-specific (NS) siRNA or FAK siRNA_2. The cells were seeded on (C) 2,500 μm^2 and 10,000 μm^2 unpaired or (D) 2,500 μm^2 paired micropatterned adhesive islands with 10% FBS for 48 h and analyzed for S phase entry. Results in C and D are plotted as mean + SE; n=3.

Figure S5, Related to Figure 5

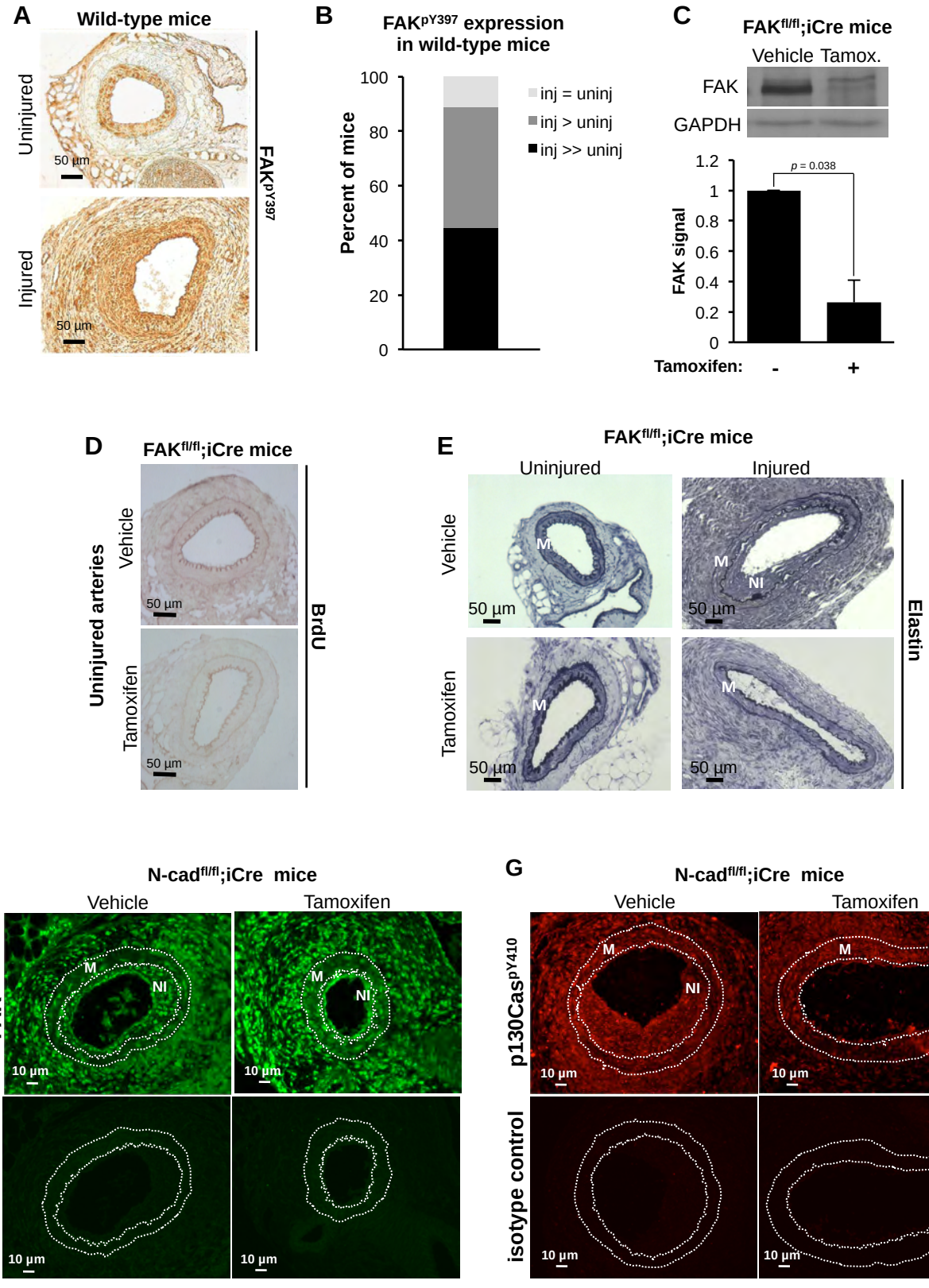

Figure S5. N-cadherin is essential effector of FAK-regulated SMC cycling in vivo, Related to Figure 5. (A) Representative images of FAK^{pY397} expression in uninjured (n=5) and injured (n=8) femoral artery sections from wild-type mice were obtained by immunohistochemical staining. (B) FAK^{pY397} expression (injured relative to contralateral uninjured femoral arteries for each mouse) was scored by blinded grouping. (C) Aortas were isolated from $FAK^{fl/fl}$;iCre mice 4 weeks after being treated with vehicle or Tamoxifen (Tamox.). The isolated aortas were cleaned, cut into pieces, homogenized in SDS sample buffer using a pellet pestle motor, boiled, fractionated on SDS gels, and western blotted. The graph shows quantification of FAK levels in 3 aortae. (D) Representative images showing the absence of S phase-labeled nuclei in uninjured arteries of $FAK^{fl/fl}$;iCre mice (n=5). (E) Uninjured femoral arteries (vehicle; n=5 or Tamoxifen; n=5) and injured femoral arteries (vehicle; n=7 or Tamoxifen; n=8) from $FAK^{fl/fl}$; iCre mice were stained for elastin. (F-G) Images in 5F and 5H have been reproduced and shown alongside of corresponding sections stained with isotype-matched control antibody.