SUPPLEMENT MATERIAL

Materials: Antibodies against activated β1 integrin(clone 12G10) were from Serotec and Chemicon and those directed againstT788/789 of β1 integrin cytoplasmic tail, and phosho FAK pY-397 were from Biosource International/ Millipore. Alexa-conjugated phalloidin and secondary antibodies were from Molecular Probes/ Invitrogen. Antibodies against phospho AKT Ser-473, AKT, phospho-ERK1/2, ERK1/2 , FAK and PARP were from Cell Signaling, and those against vinculin and actin were from Sigma. The polyclonal antibodies against ki-67were obtained from Thermo Scientific. Human fibronectin was obtained from BD Biosciences. Gadolinium Chloride, 4-α-PDD and ruthenium red were purchased from Sigma; LY294002 was from Calbiochem. The polyclonal antibodies against TRPV4 were obtained from Affinity BioReagents and MBL International Corporation. The primers used for RT-PCR were: TRPV2 (human: Forward- CAAACCGATTTGACCGAGAT; Reverse- GTTCAGCACAGCCTTCATCA and bovine: Forward- CAGCTGGGAGGAAAACTCAG; Reverse- GGGAGGAAGTCCTTTTCCAG), TRPV4 (human: Forward- GACGGGGACCTATAGCATCA; Reverse- AACAGGTCCAGGAGGAAGGT and bovine: Forward-GACTACCTGCGGCTGGC; Reverse- TTCATCCAGCCCAGGAC), TRPC1 (human: Forward- CACTCGTTCATTGGCACCTGCTTT; Reverse- GCAGCTTCGTCAGCACAATCACA; bovine: Forward- CCATTCGTTCATCGGCACTTGCTT; Reverse- TTATGAAGCATTGCCACCAGCAGC) and GAPDH (Forward- ACCACAGTCCATGCCATCAC; Reverse- TCCACCACCCTGTTGCTGTA)

Morphological and Immunofluorescence Studies: Cells were transfected with GFP-AKT-PH domain (kind gift of Dr. Martin Schwartz) using Effectene (Qiagen, Chatsworth, CA). Cells adherent to flexible ECM substrates and subjected to mechanical stretch were washed in PBS, fixed in 4% paraformaldehyde for 30 min either mounted on glass slides (for visualizing GFP-AKT-PH translocation) or permeabilized with 0.25% Triton-X 100/PBS for 5 min for immuno staining. After blocking with DMEM containing 10% FBS, cells were incubated for 1h with Alexa-phalloidin to visualize stress fibers, washed and mounted on glass slides using fluoromount-G (Southern biotech). For staining focal adhesions, cells were incubated with antibodies against vinculin for 1h followed by rinsing and incubation with Alexa-conjugated secondary antibodies; activated β1 integrins were detected using 12G10 antibody. For measuring proliferation, cells were incubated with antibodies against ki-67 for 1h followed by rinsing and incubation with Alexa-conjugated secondary antibodies. Images were acquired on a Leica Confocal SP2 microscope and processed using Leica software and Adobe Photoshop. GFP-AKT-PH domain translocation to the plasma membrane was quantified measuring either ratio of the perimeter of whole cell membrane and the membrane that contains the GFP-AKT-PH domain or the ratio of GFP fluorescence intensity of translocated GFP-AKT-PH domain and cytosol adjascent to the membrane.

Biochemical Analysis: Our methods for Western blotting have been published previously [1]. Membranes containing transferred protein were blocked in 3% BSA/TBST for 1h and incubated overnight with primary antibodies against AKT and phospho Ser-473 AKT (1:1000), phospho-Thr 788/789 of β1 integrin cytoplasmic tail (1: 300-1000), ERK1/2 and phospho ERK1/2 (1:1000), FAK and phospho FAK-pY397 (1:1000), actin (1:1000), PARP (1:1000) and TRPV4 (1:300) at 4^{o}C. The membranes were subsequently washed incubated with HRP-

conjugated secondary antibodies (1:5000) for 1h and washed and incubated with West-Pico ECL reagent from Pierce (USA) and exposed to Kodak X-ray film (Sigma).

Calcium Imaging: CE cells adherent to the flexible substrates were loaded with Fluo-4/AM (1-4 μM) for 30 min, washed 3 times in calcium medium (136 mM NaCl, 4.7 mM KCl,1.2 mM MgSO4, 1.1 mM CaCl2, 1.2 mM KH2PO4, 5 mM NaHCO3, 5.5 mM glucose, and 20 mM Hepes. pH 7.4) and then exposed to static stretch (15% elongation) for 3-4 sec using a 'Stage Flexer' (FlexCell International) apparatus that is fixed on a Nikon upright microscope equipped with CCD camera (Spot-RT slider, Diagnostics Corp, USA). Images were acquired for every 4 seconds and analyzed using IP lab software and Microsoft Excel as described[2]. Calcium imaging with TRPV channel activators was performed on cells cultured on MatTek glass bottomed dishes on Leica Confocal Microscope and analyzed using Leica software and Microsoft Excel.

Integrin Activation Assay: β1 integrin activation was measured using a glutathione S-transferase (GST) fusion protein consisting of of FN III repeat 8-11 domains or 12G10 antibodies[3]. Briefly, cells subjected to mechanical stretch were incubated with either 5 μg/ml of GST-FN III $_{8\text{-}11}$ protein or 12G10 antibodies in PBS containing Ca^{2+} and Mg^{2+} for 30 min at 37ºC, washed and lysed in SDS-sample buffer. The samples were separated on SDS-PAGE and the bound reagents were assessed on Western blots using anti GST-antibodies and HRP-conjugated secondary antibodies.

FACS analysis: Activated β1 integrin expression was measured with specific antibodies (12G10) using flow cytometry [4]. Briefly, cells were incubated with 12G10 antibody in FACS buffer (PBS containing 1% bovine serum albumin) for 20 min on ice and fixed in 4% paraformaldehyde for 15 min. After fixation, the cells were washed twice with FACS buffer and

incubated with PE-conjugated secondary antibodies (Vector Laboratories, USA) for 20 min on ice. The cells were then washed twice and analyzed on Guava Personal Cytometer (Guava Technologies). Isotype-matched IgG and secondary antibodies alone were used as controls.

Whole-Cell Patch Clamp Experiments: One day after bovine CE cells were transfected with TRPV4-EGFP, they were plated on gelatin-coated glass coverslips and allowed to grow for ~24 h prior to recording. Cells were recorded in the whole-cell mode using borosilicate glass pipettes (1-3 MΩ) containing (in mM): 120 $CsMeSO_3$, 10 EGTA, 2 $MgCl_2$, 10 HEPES; pH and osmolarity were adjusted with CsOH or $HMeSO_3$, as needed, to 7.2 (23°C) and ~300 mOsm, respectively. Cells were bathed in a solution containing (in mM): 138 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES; pH and osmolarity were adjusted with NaOH or HCl, as needed, to 7.4 (23°C) and ~310 mOsm, respectively. Cells were held at -40 mV for 3-5 min to allow for intracellular dialysis. Bath superfusion was stopped prior to initiating the recording of currents resulting from the indicated voltage protocol applied every 5 s. Immediately before use, a 4-α-PDD stock solution (2 mM in EtOH, on ice) was diluted 1:50 in bath solution under vortex and 50 μl was subsequently added to a still bath (0.5 ml) by pipette (final concentrations of 4 μM 4-α-PDD and 0.2% EtOH) and mixed by 3 gentle up/down pipette strokes. Once a clearly discernable 4-α-PDD-induced current was observed (typically after a 10-30 sec delay), the bath solution was changed to a Na^+- and Ca^{2+}-free solution (in mM: 145 N-methyl-D-glucamine·Cl, 10 HEPES, adjusted to pH 7.4 and ~310 mOsm with HCl) by re-starting the superfusion. In some cases, the original bath solution was later re-introduced as a wash.

Supplementary References

1. Mammoto A, Huang S, Ingber DE. Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts. *J Cell Sci.* 2007;120:456-467.

2. Matthews BD, Overby DR, Mannix R, Ingber DE. Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels. *J Cell Sci.* 2006;119:508-518.

3. Orr AW, Ginsberg MH, Shattil SJ, Deckmyn H, Schwartz MA. Matrix-specific suppression of integrin activation in shear stress signaling. *Mol Biol Cell.* 2006;17:4686-4697.

4. Kawaguchi N, Sundberg C, Kveiborg M, Moghadaszadeh B, Asmar M, Dietrich N, Thodeti CK, Nielsen FC, Moller P, Mercurio AM, Albrechtsen R, Wewer UM. ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating beta1 integrin function. *J Cell Sci.* 2003;116:3893-3904.

Supplementary Figure Legends

Online Figure I. 12G10 antibody detects β1 integrin activation by manganese on bovine CE cells. Flow cytometric analysis of activated β1 integrin expression on bovine CE cells detected using the 12G10 antibody in the absence and presence of manganese (Mn^{2+}). Note that the expression of activated β1 integrin is

increased following treatment with manganese. The istoype-matched control IgG is shown as a red peak.

Online Figure II. Mechanical strain-induced FAK activation is dependent on PI3 kinase activity. Representative Western blot showing static mechanical strain (15%, 15 min) dependent tyrosine phosphorylation of FAK (FAK-pY397 antibody) in bovine CE cells in the absence and presence of the PI3 kinase inhibitor, LY 294002 (LY, 40 μM).

Online Figure III. Stretch-activated (SA) calcium channels are upstream of mechanical strain-induced β1 integrin phosphorylation. A) Relative change in cytosolic calcium in Fluo-4 loaded CE cells in response to applied static strain (15%, 3 sec, arrow) in the absence (♦) and presence (○) of gadolinium chloride (25 μM;Gd) (F/Fo = ratio of normalized Fluo-4 fluorescence intensity relative to time 0). B-C) Representative Western blots showing cyclic strain dependent binding of GST-$FNIII_{8-11}$ fragment in bovine and human (HCE) CE cells (B), and β1-integrin phosphorylation in CE cells (C) in the absence and presence of gadolinium. D) Percentages of CE cells displaying GFP-AKT-PH domain translocation to the plasma membrane when subjected to 0 or 15% static stretch in the absence and presence of gadolinium chloride ($p < 0.022$). E) Percentage of CE cells oriented 90 ± 30^{o} degrees (aligned) relative to the direction of applied cyclic strain in the absence and presence of gadolinium chloride ($p < 0.0002$).

Online Figure IV. β1 integrin activation is required for cyclic strain-induced CE cell reorientation. Immunofluorescence photomicrographs of CE cells exposed to 0 or 10% uniaxial cyclic strain (arrow indicates the direction of applied strain) in the

absence and presence of the function-blocking anti-β1 integrin antibody P5D2 and stained for vinculin (green) and stress fibers (magenta). Colocalization of vinculin and stress fibers is shown in white. Scale bar: 25 μm.

Online Figure V. siRNA knock down of TRP channels in human CE cells. A) Repre-sentative RT-PCR results confirming knockdown of TRPV4, TRPV2 and TRPC1 mRNA levels in human CE cells using specific siRNAs. B) Western blotting analysis showing that the same TRPV4 siRNA produced comparable suppression of protein expression.

Online Figure VI. TRPV4 channels are functionally expressed in CE cells. Relative changes in cytosolic calcium measured in Fluo-4 loaded bovine (a) or human (b) CE cells in response to the specific TRPV4 activator 4-α-PDD (2 μM) or the TRPV4 blocker ruthenium red (RR, 2 μM) in the absence or presence of extracellular calcium. Arrows denote the time drugs were added to cells.

Online Figure VII. Activation of TRPV4 currents by 4-α-PDD in bovine CE cells transiently transfected with TRPV4-EGFP. Conventional whole-cell patch clamp recording shows activation of an outwardly rectifying current by 4-α-PDD (4 μM) in bovine CE cells expressing TRPV4-EGFP. Traces shown were recorded 10 s (control, black line), 20 s (4-α-PDD, blue line), 50 s (NMDG, red line) or 135 s (wash, cyan line) after 4-α-PDD addition. Note that when NMDG was substituted for cations in the bathing solution, it inhibited activation of inward, but not outward, currents induced by 4-α-PDD.

Online Figure VIII. TRP siRNA treatment does not affect human CE cell morphology or viability. Phase contrast photomicrographs of human CE cells transfected with indicated siRNA shown in cell culture. Scale bar : 100 μm.

Online Figure IX. Cyclic strain did not affect CE cell proliferation or apoptosis. CE cells were exposed to cyclic strain as mentioned in materials and methods and either fixed and stained using ki67antibody and positive cells were quantified (A) or cells were lysed and subjected to SDS-PAGE and Western blot analysis for PARP (poly (ADP-ribose) polymerase) cleavage to assess apoptosis (B).

Online Figure X. Representative full Western blots for TRPV4 expression in bovine (A) and human (B) CE cells, which correspond to the blots shown in Fig. 4C (bovine) and Online Fig.V (human), respectively.

Online Figure XI. Representative full Western blots for β1 integrin activation and actin levels corresponding to the blots shown in Fig. 2A (β1 integrin phosphorylation), Online Fig.IIIB (GST-FN-binding) and Fig.5A (binding of 12G10 antibody).

Online Figure XII. Representative full Western blots for ERK activation (phospho and total ERK1/2) in bovine CE cells, which correspond to the blots shown in Fig. 2C.

Online Figure XIII. Representative full Western blots for AKT phopshorylation (phospho and total AKT) in bovine CE cells, which correspond to the blots shown in Fig. 3D.

Online Figure XIV. Representative full Western blots for FAK phopshorylation (phospho and total FAK) in bovine CE cells, which correspond to the blots shown in Online Fig. II.