

Mechanical Inhibition of RANKL Expression Is Regulated by H-Ras-GTPase*

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Janet Rubin^{‡1}, Tamara C. Murphy[‡], Jill Rahnert[‡], Hannah Song[§], Mark S. Nanes[‡], Edward M. Greenfield[¶], Hanjoong Jo^{‡§}, and Xian Fan[‡]

From the [‡]Department of Medicine, Emory University School of Medicine and Veterans Affairs Medical Center, Atlanta, Georgia 30033, the [¶]Department of Orthopaedics, Case Western Reserve University, Cleveland, Ohio 44106, and the [§]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30322

Mechanical input is known to regulate bone remodeling, yet the molecular events involved in mechanical signal transduction are poorly understood. We here investigate proximal events leading to the ERK1/2 activation that is required for mechanical repression of RANKL (receptor activator of NF- κ B ligand) expression, the factor that controls local recruitment of osteoclasts. Using primary murine bone stromal cells we show that dynamic mechanical strain via substrate deformation activates Ras-GTPase, in particular the H-Ras isoform. Pharmacological inhibition of H-Ras function prevents strain activation of H-Ras as well as the downstream mechanical repression of RANKL. Furthermore, small interfering RNA silencing of H-Ras, but not K-Ras, abrogates mechanical strain repression of RANKL. H-Ras-specific inhibition of mechanorepression of RANKL was also demonstrated in a murine pre-osteoblast cell line (CIMC-4). The requirement of cholesterol for H-Ras activation was probed; cholesterol depletion of rafts using methyl- β -cyclodextrin prevented mechanical H-Ras activation. That the mechanical repression of RANKL requires activation of H-Ras, a specific isoform of Ras-GTP that is known to reside in the lipid raft microdomain, suggests that spatial arrangements are critical for generation of specific downstream events in response to mechanical signals. By partitioning signals this way, cells may be able to generate different downstream responses through seemingly similar signaling cascades.

Biophysical input generated during normal physiologic loading is a major determinant of bone mass and morphology. Removing skeletal load acutely leads to osteoclast recruitment and bone loss. We have previously shown that biophysical factors generated during loading, such as mechanical strain/deformation and pressure, inhibit osteoclastogenesis *in vitro* (1, 2). The inhibitory effect of dynamic strain on osteoclastogenesis arises via mechanical control of at least two genes: the inhibition of RANKL² expression, unarguably the dominant regulator of osteoclastogenesis, and the induction of eNOS expression (3), leading to secondary effects of nitric oxide to decrease the RANKL/osteoprote-

gerin ratio, thus enhancing bone formation (4). Both gene responses to strain require activation of ERK1/2 signaling (3).

Activation of MAPK signaling cascades is a common early step in translating mechanical force into intracellular signals. ERK1/2 activation follows strain and shear force not only in osteoblasts (5–7) but in endothelial cells (8). Despite a deceptive commonality of the signal cascade, cells appear to be able to differentiate the incoming signal, assigning a panel of responses to specific inputs. Thus, response to mechanical signals is reminiscent of the variable response to soluble factors that share, for example, the ability to activate the downstream cAMP cascade and yet cause different responses. Changes in the temporal application of stimuli also cause variable responses; for example the response of osteoblasts to continuous parathyroid hormone differs from that seen after intermittent parathyroid hormone (9). Similarly, the cellular responses to ERK1/2 activation are not uniform: different levels of force, or different types of force, initiate variable arrays of cellular response. In the case of strain, for instance, when osteoblastic cells are deformed at 8% magnitude (*i.e.* induction of 8% change in cell length), collagenase-2 expression is induced, an effect predicted to stimulate bone resorption (10). This osteoblast response contrasts with observations that lower strain levels (<2%) are anabolic and antiresorptive (2, 5, 11).

One possible solution to the differential responses to incoming signals, mechanical or soluble, that activate MAPK cascades may be that the molecules involved in transmitting the MAPK signal are aligned in specific scaffolds within the cell membrane such that MAPK activation at one site might be linked to a unique set of downstream responders (12). To investigate early mechanically activated events involved in strain regulation of RANKL that might confer specificity, we turned our attention to signaling molecules proximal to ERK1/2, concentrating on the Ras-GTPase located in the deforming membrane.

The Ras subfamily includes the classical Ras proteins H-, K-, and N-Ras (13). While these Ras proteins share downstream targets, they undergo isoform-specific posttranslational modification of the hyper-variable COOH termini that is responsible for membrane targeting. Modifications include prenylation (farnesylation and/or geranylgeranylation), proteolysis of the carboxyl-terminal CAAX motif and esterification (14). H- and N-Ras proteins are also palmitoylated (14), while the polybasic domain of K-Ras is not further prenylated. These modifications direct H- and N-Ras to discrete locations of the plasma membrane, while K-Ras reaches alternate membrane and cytosolic domains through a different transport system (15). The palmitoyl group of H-Ras laterally associates with the cholesterol and glycosphingolipid of lipid rafts allowing it to become part of the caveolar signaling domain (14), and indeed H-Ras is predominantly associated with lipid raft microdomains (13, 16). As such, H-Ras has been shown to be a key member of the “signaling centers” associated with the organized membrane system, cholesterol-rich microdomains that can be either lipid rafts or caveolae.

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¹ To whom correspondence should be addressed: VAMC-151, 1670 Clairmont Rd., Decatur, GA 30033. Tel.: 404-321-6111 (ext. 2080); E-mail: janet.rubin@emory.edu.

² The abbreviations used are: RANKL, receptor activator of NF- κ B ligand; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; FBS, fetal bovine serum; GST, glutathione S-transferase; dsRNA, double-stranded RNA; siSCR, siRNA-scrambled; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; GEF, guanine exchange factor; GTP- γ S, guanosine 5'-O-(thiotriphosphate); MEM, minimal essential medium.

In the case of H-Ras, the membrane thus can serve as a platform where MAPK signaling events are both generated and modulated. K-Ras, which is anchored to the membrane through its charged polybasic domain, is not often found in organized membrane (12, 17).

Here we probe the generation of signal proximal to ERK1/2 activation that follows application of strain to osteoprogenitor cells. We show that downstream regulation of RANKL expression requires activation of a specific isoform of Ras-GTPase, the H-Ras isoform. Finally we show that cholesterol depletion of the lipid membrane inhibits mechanical activation of H-Ras.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Antibodies to total ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and those to phosphorylated ERK1/2 from New England BioLabs (Beverly, MA). Reagents for siRNA were purchased from Ambion, Inc. (Austin, TX) and those for the Ras activation assay from Upstate Biotechnology (Lake Placid NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Other chemicals and supplies were purchased from Sigma. Monoclonal antibody against caveolin-1 was from BD Biosciences.

Cell Culture—To generate primary stromal cell cultures, murine marrow cells collected from the tibiae and femurs of 3–5-week-old male C57BL/6 mice were plated in 6-well plates at 1.6×10^6 cells/cm² as published previously (18). After 60 min non-adherent cells containing the stromal elements were transferred to Bioflex collagen I-coated plates (Flexcell Corp., McKeesport, PA) in α -MEM, 10% FBS. The next day non-adherent cells were discarded; adherent stromal cells were cultured with 10 nM 1,25-dihydroxyvitamin D added on day 4 to stimulate RANKL expression. Strain regimens were applied on day 6.

Conditionally immortalized murine calvarial cells (CIMC-4, clone 4) cells cloned from calvarial osteoblasts (19) and used prior to passage 21 were maintained in permissive conditions at 33 °C in α -MEM with 10% FBS and 100 units/ml interferon- γ (PeproTech, Rocky Hill, NJ). Prior to experiments, cells were cultured for 1 week in non-permissive conditions (37 °C in MEM, 10% FBS, 1.25 mM glutamine, and 100 mg/ml penicillin/streptomycin). For experiments, cells were plated at a density of $1-2 \times 10^4$ cells/cm² in 6-well strain plates for 3 days and treated with 10 nM 1,25-dihydroxyvitamin D for the final 48 h.

Application of Mechanical Strain—Uniform equibiaxial mechanical strain was generated using a Flexcell Bioflex instrument (Flexcell Corp., McKeesport, PA) as described previously (20). Strain magnitudes were as noted from 0.5 to 2% with strain frequency fixed at 10 cycles per minute (0.17 Hz). Similar plates containing control cultures were kept in the same incubator but not subjected to strain regimens.

Assessment of Ras Isoforms and Activation—To measure activation of Ras isoforms, cells were treated with GTP (to activate Ras) and GDP (to inactivate Ras) in the presence or absence of strain. Lysates were cleared after microcentrifugation and allowed to bind with 5–10 μ g of immobilized GST-Raf-RBD (Raf's Ras binding domain fused to GST), (Upstate Biotechnology); only activated Ras binds to the Raf-1 beads. The beads were collected, washed, re-suspended in reducing sample buffer, and boiled for 5 min. After gel separation of captured Ras-GTP, the activated Ras isoforms were determined using specific anti-Ras antibodies (Santa Cruz Biotechnology) for immunoblotting.

Design of Ras Isoform-specific Silencing RNAs—To assess the role of specific Ras isoforms we used two silencing RNA strategies. With the first method, a single 21-nucleotide sequence was selected that effectively knocked down the gene of interest. Four possible silencing sequences for each Ras in their hypervariable regions (12) were chosen using the Ambion algorithm (www.ambion.com). siRNAs were pre-

pared with the Silencer siRNA construction kit (Ambion). Briefly, three sets of 29-mer oligonucleotides representing the sense and antisense strands of three prospective silencer regions were synthesized. These were complementary to the 21-nucleotide DNA sequence at the 5' end and contain an 8-nucleotide sequence at the 3' end of each oligonucleotide complementary to the T7 promoter primer provided in the kit. The 21-nucleotide oligonucleotides were annealed to their complementary oligonucleotide, and dsRNA was prepared by *in vitro* transcription. The resulting dsRNA was then subjected to RNase A and DNase I treatment as per protocol to achieve the final siRNA product. siRNAs were also prepared to GAPDH (positive control, Ambion), as well as an oligonucleotide sequence known *not* to silence any mammalian gene (negative control #1, Ambion) that we refer to as "siRNA-scrambled" or siSCR. Individual siRNAs were transfected into cells at a concentration of 40 nM.

A second siRNA strategy was also used to generate "mixtures" of siRNA using the RNase III mixture kit from Ambion. Briefly, a 200-base pair target region of specific Ras genes was amplified using 40-mer gene-specific/T7 promoter PCR primers and gel-purified before overnight transcription of dsRNA with a T7 polymerase. The resultant 200-base pair dsRNA was digested with RNase A and DNase I, column-purified, and further digested with RNase III. The final reaction mix was purified with Ambion's siRNA purification unit and the resulting 15–18-base pair siRNAs used at 50 nM.

All siRNAs were transfected into cells at 50% confluence with 2.5 μ l of Oligofectamine (Invitrogen) per well of 6-well plates.

Real-time PCR to Assess mRNA Species—Analysis of RANKL, GAPDH, and 18 S mRNA were performed as described in Refs. 18 and 21 using the iCycler (Bio-Rad). H-Ras and K-Ras mRNA species were also relatively quantified by RT-PCR using primers specific for each isoform. For H-Ras primers were as follows: forward, tac att gga aca tca gcc aag; reverse, cag gac agc aca cat ttg ca. For K-Ras primers were as follows: forward, aag tgt gat ttg cct tct ag; reverse, atg ttt tcg aat ttc tcg gac t.

Statistical Analysis—Results are expressed as the mean \pm S.E. Statistical significance was evaluated by two-tailed *t* test, with significance at < 0.05 (GraphPad Prism).

RESULTS

Mechanical Strain Activates Ras-GTPase—In an effort to characterize relevant molecules proximal to ERK1/2, we set out to characterize the strain-initiated GTPase activation step in primary murine marrow-derived stromal cells. As shown in Fig. 1A, dynamic strain (2%, 10 cpm) activated total Ras as determined by a pull-down assay for GTP-bound Ras. Cells subjected to 10 min of dynamic strain showed activation of total Ras to nearly the same degree as that caused by addition of saturating amounts of GTP. To define the Ras isoforms responsive to strain at this time point, Ras-GTP was probed with isoform specific antibodies to delineate Ras isoforms. Shown in Fig. 1B, at 10 min, there was significant stimulation of the H-Ras isoform, but N-Ras was unaffected. Mechanical activation of K-Ras activation was present but consistently less than that of H-Ras.

Because ERK1/2 is fully rapidly activated by mechanical strain by 10–15 min (18), we examined Ras activation at earlier time points. As shown in Fig. 2A, appreciable activation of both H- and K-Ras was seen after application of strain for 2 min. The amount of activated H-Ras typically showed a greater change than that for K-Ras, and the K-Ras appears to be deactivated sooner than H-Ras. In many experiments, K-Ras activation was simply not appreciable.

Mechanical Inhibition of RANKL Requires H-Ras

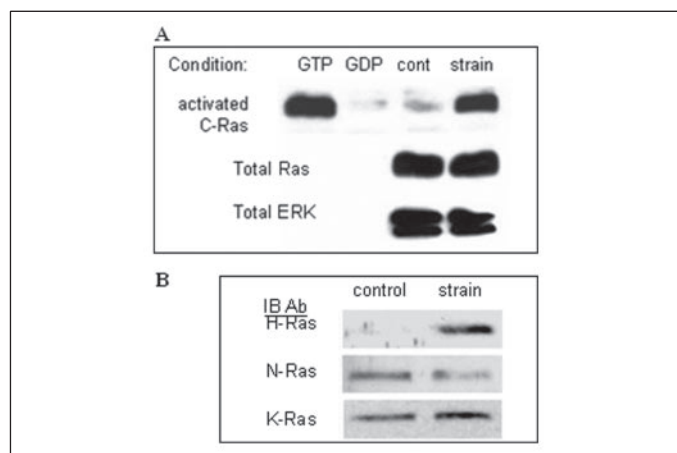


FIGURE 1. Strain activates Ras-GTPase in stromal cells. *A*, primary stromal cells were treated with GTP- γ S (100 μ M) or GDP (1 mM) or static or strain conditions for 10 min as noted. Activated total RAS (t-Ras) is shown in the top row; both GTP and strain activate Ras, while GDP inhibits Ras. The second row shows total Ras and the third row total ERK1/2, to confirm equal loading prior to binding to the Raf beads. *B*, primary stromal cells were strained or kept in static conditions for 10 min. Activated RAS was collected as described for *A*. The eluate from the Raf beads was probed with antibodies specific for Ras isoforms. H-Ras activation increased after strain as shown in the figure.

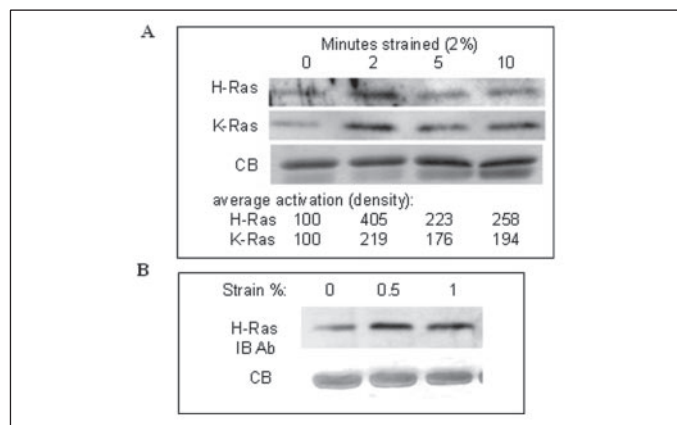


FIGURE 2. Strain activation of H-Ras-GTPase occurs rapidly and at low magnitude strain. *A*, strain (2%, 10 cpm) was applied to stromal cells, and activated Ras was measured as described in the legend to Fig. 1. At 2 min after strain initiation, both H-Ras and K-Ras were activated. By 5 min the amount of activation decreased, with near complete return to basal levels of K-Ras. Equal loading of Ras beads is shown by a Coomassie Blue stain of the lysate starting material (CB). *B*, activation of H-Ras was seen after 2 min of strain as low as 0.5%. Equal loading of Ras beads is shown by a Coomassie Blue stain of the lysate starting material (CB).

Lower strain magnitudes also effectively stimulated H-Ras. In Fig. 2*B* cells were studied after 2-min strain at 0.5 and 1%, resulting in activation of the H-Ras isoform.

Ras Activation Is Necessary for Transmitting the Strain Effect to RANKL—Ras activation is dependent on the location of the Ras-GTPase. As Ras proteins are targeted to their various membrane locations by posttranslational modifications of their carboxyl-terminal CAAX domains, inhibition of palmitoylation can be used as a means of inhibiting Ras function. The farnesyltransferase (FTase) inhibitor FTI277 prevents farnesylation of H-Ras and subsequent inactivation at levels 100-fold less than those needed to inhibit K-Ras (22). This probably occurs not only because H-Ras is a more efficient substrate for FTase but because K-Ras may be alternatively geranylgeranylated when cellular FTase is inhibited (23).

FTI277 was given 24 h prior to straining the cells to allow turnover of membrane Ras. In Fig. 3*A*, total Ras-GTP is shown in strained and unstrained cells that treated with FTI277. The addition of FTI277 to

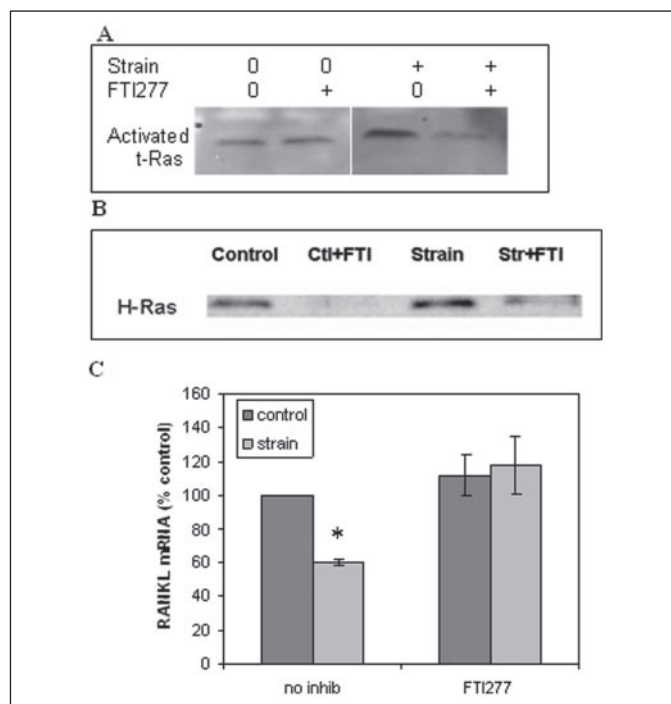


FIGURE 3. Pharmacologic inhibition of H-Ras prevents strain initiated events. *A*, stromal cells were treated with or without farnesyltransferase inhibitor (FT277) prior to strain. There was clear activation of total Ras after 10 min of strain (lane 3). There was no difference in basal Ras activation in the presence of FT277, but the strain effect was entirely abrogated in the presence of the inhibitor (lane 2 equivalent to lane 4). *B*, stromal cells were treated as in 3*A* and strained for 5 min prior to lysing cells for loading onto the GST-Raf-RBD beads. The bound (active) Ras-GTP was probed for H-Ras. As shown, strain activated H-Ras. When FTI277 was present, H-Ras activation by mechanical strain did not occur. *C*, stromal cells were treated with FTI277 for 24 h prior to initiating dynamic strain. After overnight strain, total RNA was extracted and RT-PCR was performed for RANKL and 18 S. RANKL mRNA expression was compared with levels in control cells (unstrained, no FTI277). Strain inhibited RANKL expression (dark bars, unstrained; gray bars, strain) (asterisk = $p < 0.05$). In the presence of FTI277, strain did not inhibit RANKL expression.

stromal cells did not change the basal activation state of total Ras, which would be consistent with this state being dependent on K-Ras, which is largely insensitive to this level of FTI277 (22). As shown in Fig. 3*B*, however, strain activation of H-Ras was completely prevented in the presence of the farnesyltransferase inhibitor.

The effect of inhibiting farnesyltransferase on the downstream RANKL expression target was examined next. As shown in Fig. 3*C*, strain decreased RANKL mRNA expression by 40% in a series of three experiments in cultures not treated with FTI277. In cultures with 24-h pretreatment with FTI277 prior to application of strain, unstrained cultures expressed RANKL at a level not significantly different from untreated cells. However, in cultures treated with FTI277, the strain effect was completely abrogated. This result would be expected if a necessary molecule, *i.e.* H-Ras, was disengaged from the mechanical signaling cascade.

siRNA Inhibition of H-Ras Prevents Strain Effects on RANKL—To further confirm the farnesyltransferase inhibition data supporting a role for H-Ras in the mechanorepression of RANKL gene expression, silencing RNA technology was used (24). To explore the requirement for Ras proteins in transmitting a strain-initiated signal resulting in down-regulation of RANKL, we designed siRNAs against H- and K-Ras using two complementary methodologies.

In the first method, a single 21-nucleotide sequence, which effectively knocked down the gene of interest, was used, with nonsense siRNA (here called siSCR) used in transfections as a negative control. As shown in Fig. 4*A*, the siRNA sequence against H-Ras ("siHRas": aac cag gtc gtc

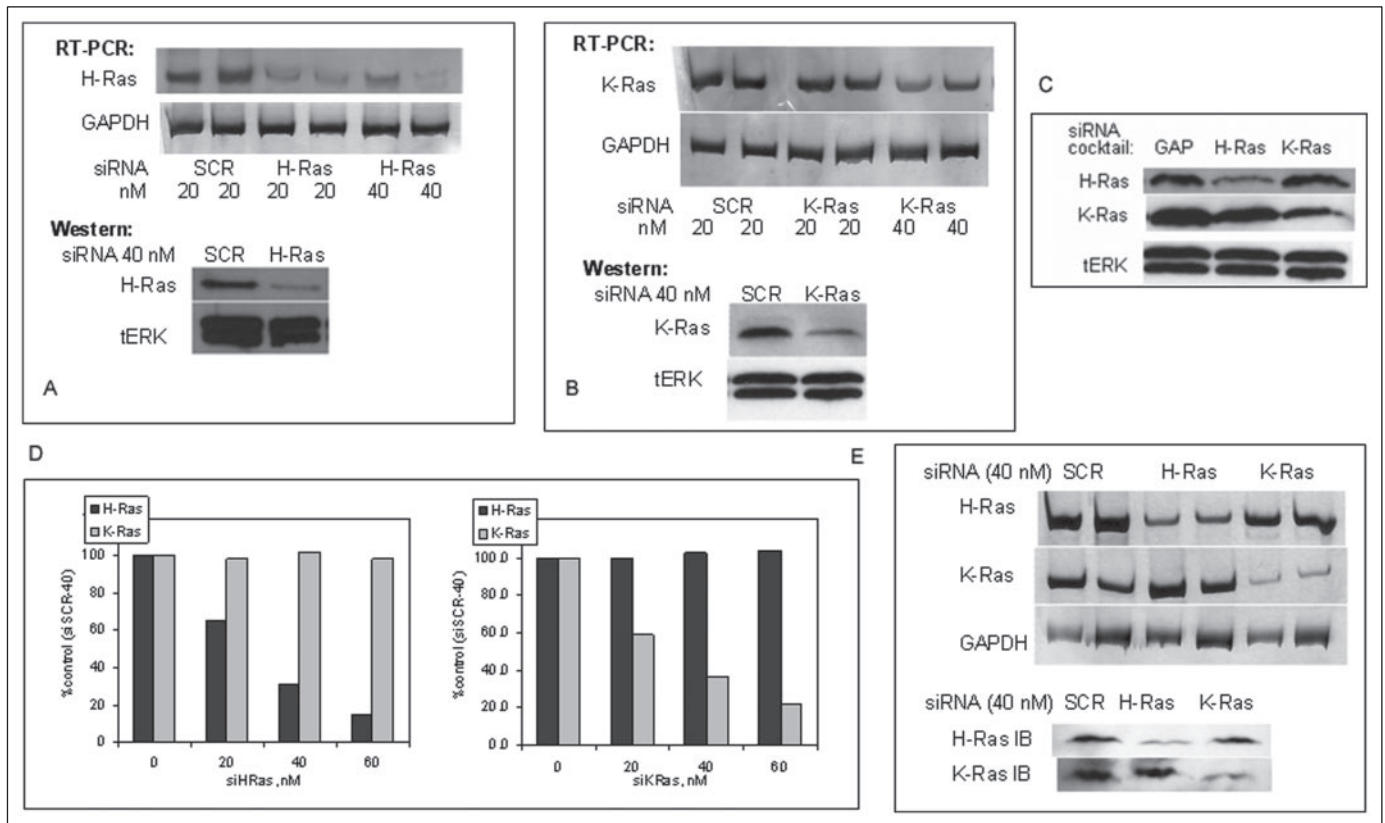


FIGURE 4. Effect of specific siRNA for H and K-Ras. *A*, a single siRNA designed to silence H-Ras in primary stromal cells showed efficacy at 20 nM in the upper RT-PCR gel, with no effect on GAPDH mRNA. An accompanying Western blot shows that 40 nM siHRas knocked down H-Ras protein at 48 h, with total ERK probed to confirm equal loading. *B*, a single siRNA designed against K-Ras also effectively knocked down K-Ras mRNA and protein when used at 40 nM. *C*, primary stromal cells were treated with siRNA mixtures (see methods) designed to silence either GAPDH, H-Ras, or K-Ras at 50 nM. Western blots (immunoblot) were probed with antibodies specific for H-Ras (top row) or K-Ras (middle row). The third row shows equal loading, as probed by tERK antibodies. siHRas and siKRas specifically decreased their respective protein targets without affecting the other member of the Ras family. *D*, CIMC-4 cells were treated with either 40 nM siSCR and siHRas (left graph) or siKRas (right graph) in the doses as shown. RT-PCR for H-Ras (dark bars) and K-Ras (light bars) was performed, showing that the siRNAs, in a dose-dependent fashion, specifically targeted the appropriate Ras isoform. *E*, CIMC-4 cells were treated with 40 nM single siRNAs. The top gel set shows that compared with control siRNA (SCR), siRNA targeting H-Ras, and K-Ras specifically decreased appropriate mRNA and protein targets, without affecting the alternate Ras isoform.

att gat ggg) decreased steady state mRNA levels for H-Ras at 24 h after addition of siHRas, resulting in knockdown of the H-Ras protein at 72 h when cells were treated with 40 nM siHRas. Fig. 4*B* shows that a similar strategy designed to silence K-Ras ("siKRas": aac tgg gga ggg ctt tct ttg) was also effective at the mRNA and protein level when cells were treated with 40 nM siHRas; 20 nM was not effective for siKRas at the mRNA level.

A secondary method was also used to generate specific siRNA mixtures for H- and K-Ras proteins (25). Fig. 4*C* shows that the siRNA mixtures designed against H- and K-Ras resulted in specific knockdown of the desired targets. The efficacy of the 40 nM dose was further analyzed by real-time RT-PCR as shown in Fig. 4*D*. In the left panel, increasing doses of siHRas dose-dependently inhibited H-Ras mRNA but had no effect on K-Ras mRNA. Similarly, siKRas dose-dependently inhibited its designated target without affecting H-Ras mRNA.

siRNA at a dose of 40 nM also effectively knocked down specific mRNA and protein in the CIMC-4 cell line, as shown in Fig. 4*E*.

Downstream effects of the siRNA silencing regimens were examined first in primary stromal cells. For these experiments, siRNA was transfected 24 h prior to application of strain to culture wells, and expression of RANKL and 18 S mRNAs analyzed 24 h after continuous application of dynamic strain (18, 20). In Fig. 5*A*, the single siRNA strategy was used to specifically silence either H- or K-Ras. The data show the expression of RANKL in each condition compared with the unstrained controls treated with siSCR. siHRas did not change basal expression of RANKL (first bar in the middle set of bars). Silencing of H-Ras completely abro-

gated the strain effect on RANKL. Importantly, silencing of K-Ras, despite the significant increase in basal RANKL in unstrained cultures, did not prevent the mechanical repression of RANKL by 50% compared with that in unstrained cells. These data reinforce the results gathered from the studies of H-Ras activation in the presence of farnesyltransferase inhibition (Fig. 3*C*).

To complement these data, and that of the effect of the farnesyltransferase inhibition studies, we utilized the siRNA mixtures designed against H- and K-Ras. Again, siRNA against H-Ras did not change the basal expression of RANKL in unstrained cultures, as compared with control cultures that were treated with siGAPDH mixtures that did not affect Ras (Fig. 5*B*). The effects of mechanical strain in cultures treated with the siRNA mixtures were equivalent to those seen using the single siRNA strategy: siRNA against H-Ras completely abrogated the strain effect, whereas the strain effect was fully preserved in cultures treated with siRNA targeting K-Ras. These results suggest a specific role of H-Ras in strain-induced RANKL repression.

Strain Has Analogous Effects on RANKL in the CIMC-4 Pre-osteoblast Cell Line—To inquire whether the requirement for H-Ras transmitting the strain signal to inhibit RANKL was present in another mechanically responsive RANKL-expressing cell, we tested effects of strain in the immortalized pre-osteoblast cell line, CIMC-4. This pre-osteoblast line was generated from the calvariae of the large TAG transgenic mouse calvariae (26). Calvarial cells from this mouse have been shown to support osteoclast formation from pre-osteoclast cells after treatment with osteotropic cytokines and vitamin D (19, 27). For

Mechanical Inhibition of RANKL Requires H-Ras

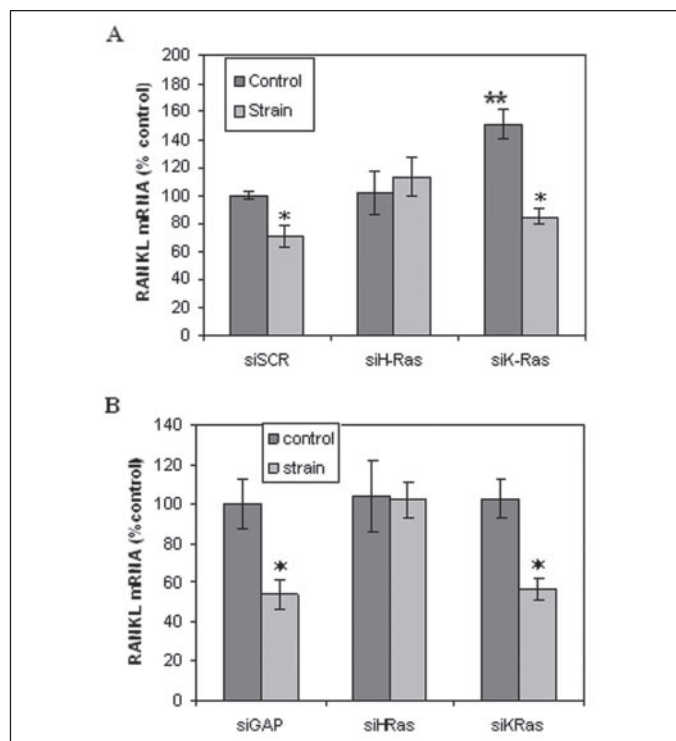


FIGURE 5. Knockdown of H-Ras, but not K-Ras, prevents strain inhibition of RANKL. *A*, primary stromal cells were treated with single siRNAs against H-Ras and K-Ras and compared with control cells (siSCR for nonsense siRNA). Dynamic strain was initiated 24 h after treatment with siRNA, and real-time RT-PCR for RANKL and 18 S (for normalization of RANKL) was performed on total RNA isolated the next day. The first set of *gray bars* shows that strain significantly decreased RANKL expression by 35% in a series of four experiments ($* = p < 0.05$). In cells treated with siRNAs, siHRas prevented strain decreases in RANKL, while the strain effect was in the presence of siKRas was equivalent to that in cultures treated with siSCR siRNA. There was a statistically significant difference between basal levels of RANKL comparing siSCR and siKRas (*dark bars*, first and third set; $** = p < 0.05$) suggesting that silencing of K-Ras may affect basal RANKL expression. *B*, real-time RT-PCR for RANKL was performed in cells treated with siRNA mixtures (50 nM) specific for GAPDH (siGAP), H-Ras, and K-Ras for 48 h prior to application of strain. In this series of 4 compiled experiments, strain inhibition of RANKL expression was seen in the presence of siGAP and siKRas mixtures (first and third set of *bars*, respectively). Basal RANKL was unchanged from control (siGAP) in all conditions. The siHRas mixture completely abrogated the strain effect, while RANKL in cells treated with siGAP and siKRas was decreased by strain ($* = p < 0.05$ compared with unstrained cells treated with siGAP).

experiments, cells were plated in strain dishes at 37 °C and γ -interferon removed; under these non-permissive conditions there was less than a doubling in the cell population (data not shown). Cells were then subjected to the strain protocol at day 7: strain application caused a similar decrease in RANKL, indeed at a very similar degree to $45 \pm 2\%$ that of unstrained cells, as shown in Fig. 6A.

Cells responded to strain with an activation of Ras-GTPase. H-Ras was the predominant responding isoform (data not shown). K-Ras levels were quite low in the CIMC-4 cells 1 week after moving them to non-permissive conditions, perhaps consistent with the cessation of cell proliferation.

Treatment of CIMC-4 cells with siHRas and siKRas reproduced the effects seen in the primary stromal cells. As shown in Fig. 6B, only siRNA treatment of H-Ras abrogated the ability of the strain to decrease RANKL expression, while cells treated with siKRas continued display strain-induced RANKL repression.

Cholesterol Depletion Blunts H-Ras Activation during Mechanical Strain—Since H-Ras has been shown to reside in cholesterol-rich lipid raft microdomains (28), we inquired whether a spatial arrangement dependent on cholesterol might be important to the ability of mechanical strain to activate the Ras isoforms in bone cells. Cells were treated

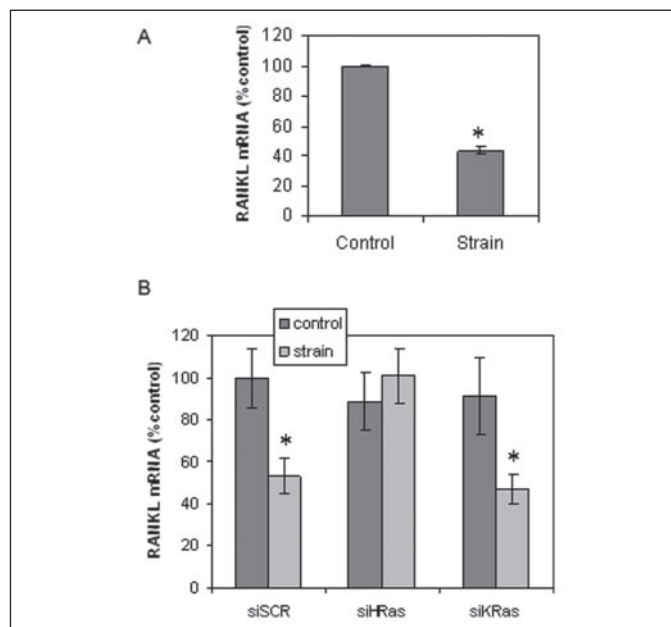


FIGURE 6. Strain has analogous effects in the CIMC-4 osteoblast cell line. *A*, CIMC-4 cells that were plated at near-confluence and cultured for 1 week after removal from permissive conditions were subjected to dynamic strain (10 cpm, 2% magnitude). The next day, total RNA was extracted for real-time RT-PCR assay of RANKL and 18 S levels. Strain significantly inhibited RANKL expression ($* = p < 0.05$, different from control). *B*, CIMC-4 cells were treated with single siRNAs against H-Ras and K-Ras and compared with control cells (siSCR for nonsense siRNA). Dynamic strain was initiated 24 h after treatment with siRNA and total RNA isolated the next day for real-time RT-PCR. The first set of *bars* shows that strain (*light bar*) significantly decreased RANKL expression by 45% in a series of 4 experiments ($* = p < 0.05$ different from unstrained cells). siHRas prevented the strain effect, while siKRas had no effect on strain inhibition of RANKL; mechanorepression of RANKL was preserved in the presence of siKRas, *i.e.* it was equivalent to that seen in the negative control (cells treated with siSCR).

with methyl- β -cyclodextrin, a membrane-impermeable cholesterol-binding agent that leaches cholesterol out of membranes, destroying lipid raft domains (29, 30). After application of strain, stromal cell homogenates were first run over GST-Raf-RBD agarose to pull-down activated Ras and then probed with an antibody recognizing all isoforms of Ras. As shown in Fig. 7A, mechanical activation of total Ras at 10 min is inhibited when cholesterol-sensitive compartments in the plasma membrane are depleted. It is perhaps not surprising that in the presence of M β CD that there is increased activation of H-Ras, since alteration of membrane cholesterol is associated with a mild degree of MAPK activation (29, 31). Despite this, mechanical strain was unable to further activate the MAPK signaling molecule.

To separate the requirement of these compartments to specific Ras isoforms, repeated experiments were probed for H-Ras, as shown in a representative experiment in Fig. 7B. Full strain activation of H-Ras was prevented by M β CD pretreatment.

DISCUSSION

The multiplicity of signals that are initiated by mechanical forces at the cell membrane is reminiscent of the many signals generated by ligand-receptor binding. How a cell mounts a specific response to a barrage of incoming signals that share intracellular pathways has been enigmatic. One clue to deciphering the use of common signals to generate specific response has been recent understanding that signal encryption can arise from spatial organization, *e.g.* the site of signal activation can be critical (12, 16). In explicating the response of the osteoprogenitor cell to mechanical strain in terms of the repression of the osteoclastic signal RANKL, we have here described a very specific intracellular response that emanates from the H-Ras isoform of Ras-

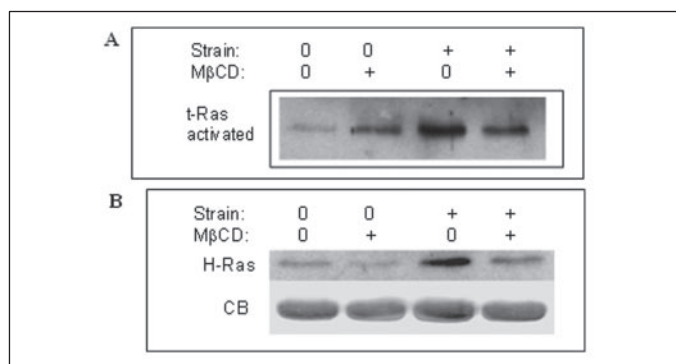


FIGURE 7. Cholesterol depletion blunts H-Ras activation during mechanical strain. A, stromal cells treated with 5 mM methyl- β -cyclodextrin (M β CD) as noted for 1 h were strained for 10 min. Activation of Ras was determined as described in the legend to Fig. 1. The blot was probed for total Ras, which was shown to be activated by strain at 10 min. Incubation with methyl- β -cyclodextrin completely inhibited strain activation of Ras. B, stromal cells were treated as described for A, strained for 2 min, and then probed for H-Ras. The lower row shows Coomassie Blue (CB) staining of the blot. These experiments were repeated at least two times.

GTPase. This Ras isoform has been shown to be located within lipid rafts (16, 32), and thus organization of membrane microdomains may be critical to the specific mechanical response.

Strain, along with shear, is known to activate Ras, and inhibition of Ras function prevents some downstream mechanical effects (8, 33, 34). Ras activation, however, can lead to many different cellular effects, including proliferation (5) or change in phenotype (35). These variable effects may be explained by activation of specific Ras isoforms. We have here proved that the H-Ras isoform is specifically required for mechanorepression of RANKL gene expression. Inhibition of farnesyltransferase, which is necessary for proper trafficking of H-Ras to its membrane destination (15), prevents both mechanical activation of H-Ras and the downstream effect of strain on RANKL expression. Pharmacologic inhibitor data are supported by the use of siRNA knockdown of H-Ras, which completely blocks strain inhibition of RANKL expression. Importantly, siRNA knockdown of the K-Ras isoform has no effect on the strain repression of RANKL.

The requirement for H-Ras is also true for mechanorepression of RANKL in the pre-osteoblast CIMC-4 cell line. The CIMC-4 line was harvested from a transgenic mouse containing the large T-Ag (26) and responds to osteoclastogenic signals by increasing its ability to support osteoclast differentiation (19). The pro-osteoclastogenic nature of the line is largely through up-regulation of RANKL expression; 1,25(OH)₂D₃ induces RANKL in these cells (data not shown), and application of strain represses this expression, as we have shown here. This is the first indication that this cell line is responsive to mechanical forces.

Studies of Ras signaling have shown that while the Ras family shares protein effectors, the spatial distribution directed by the individual Ras family member's carboxyl-terminal domain creates non-redundant regulatory pathways. Practically, H-Ras and K-Ras operate in different membrane microdomains. K-Ras is found largely in the disorganized plasma membrane, while H-Ras circulates between cholesterol-rich lipid rafts (*i.e.* organized membrane) and, upon activation, into the cytosol where a GTP-dependent segregation from lipid rafts is required for biologic activity (16, 30, 32). Our finding that H-Ras is involved in regulation of RANKL suggested that the site of mechanical signal generation might be within a lipid raft (12). Indeed, our data showing that H-Ras cannot be activated by strain after membrane cholesterol depletion points to the lipid raft as a required spatial context for translation of mechanical input into diminished RANKL expression. K-Ras signaling may not be affected by depletion of membrane cholesterol, since this

isoform does not associate with organized membrane (36–38), and inhibition of K-Ras activity in our *in vitro* systems does not affect the mechanical regulation of RANKL. This suggests that the plasma lipid membrane might underwrite specialization of Ras isoforms by allowing compartmentalization through scaffolded systems.

What guanine exchange factor (GEF) is involved in the H-Ras activation, or indeed, how a “ligand-less” process is initiated, is unknown. Most GEFs can activate many members of the Ras subfamily, and even GTPases outside of a subfamily may be affected (13). There is some evidence for that GEFs may be specific in some cases, for instance Ras-GRF 1 and 2 may target only H-Ras (39). In our experiments, the GEF does not appear to be specific for H-Ras, as indeed K-Ras is activated by mechanical strain. SOS1 (40) and Gab1 (41) are activated by shear stress and would be candidates for strain induced signaling. The specific process initiated at the membrane by strain remains an open question.

Organized membrane may serve to regulate H-Ras function. In the case of endothelial cells, caveolae have been shown to be necessary to mediate shear-dependent ERK1/2 and eNOS activation (29, 42, 43), another case where membrane microdomains are involved in generating mechanical responses. Indeed multiple mechanical signals, for instance both strain/stretch and shear, may activate the same downstream signals, *e.g.* ERK1/2, as do receptors also present in the organized membrane, but without requiring a receptor. Thus, the relational geometry of signaling proteins within the membrane may represent a mechanotransducer.

In summary, our data show that the location of H-Ras within the organized membrane is critical to the mechanical regulation of RANKL. Whether this strict signal processing is required for other mechanically activated genes, or indeed, required to generate clustered gene responses, will be of great interest to our future studies.

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