

Lamin A/C Regulates Endothelial Glucocorticoid Receptor Nuclear Translocation in Response to Cyclic Stretch

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Abstract: The glucocorticoid receptor (GR) has multiple phosphorylation sites that can be activated by MAPKs, which have been previously shown to be activated in response to cyclic stretch in endothelial cells. It is possible therefore that physiological and/or pathological degrees of cyclic stretch may also initiate phosphorylation-induced changes in GR subcellular localization as we previously showed with shear stress. However, little is known about the effects of cyclic stretch on glucocorticoid receptor activity in endothelial cells. We used control and lamin shRNA BAECs and subjected them to ligand (dexamethasone) treatment, physiological stretch (10% at 1 Hz), or pathological stretch (20% at 1 Hz or 10% at 2 Hz), in order to evaluate GR nuclear translocation in endothelial cells with and without lamin A/C as well as potential upstream protein regulators of GR subcellular movement during cyclic stretch. Upon exposure to pathological degrees of stretching, control shRNA BAECs showed greater nuclear concentration of GR at each time point compared to when they were stretched at physiological parameters. The response of GR in lamin-deficient cells to cyclic stretching was relatively non-existent compared to that observed in control shRNA cells. Our results suggest that in cells with lamin A/C, cyclic stretch activates GR through the JNK pathway, and ERK has some inhibitory role on GR nuclear translocation. DUSP proteins become upregulated in response to stretch as a result of GR activation (DUSP1) or by stretch-induced MAPK signaling. In lamin-deficient cells, only the combination of cyclic stretch and p38 inhibition was able to induce marginal nuclear translocation. Increased MAPK phosphorylation due to lamin A/C absence could drive DUSP expression as a negative feedback mechanism. Upregulation of the cytoplasmic DUSP6 suggests a significant role of ERK in reducing GR sensitivity to mechanical strain.

Keywords: Glucocorticoid receptor, cyclic stretch, nuclear lamin, MAPK, DUSP.

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1 Introduction

In addition to shear stress, cyclic circumferential stretch is another important hemodynamic force that influences endothelial cell function. Its effects at the cellular and molecular levels are dependent on the rate, duration, and magnitude of the strain [Califano JP & Reinhart-King CA (2010)]. Cyclic strain of the blood vessel wall occurs as a result of distension and relaxation during the cardiac cycle, with the force perpendicular to the vessel wall due to the tensile component of blood pressure. In response to this force, the constituents of the blood vessel experience circumferential and longitudinal forces [Califano JP & Reinhart-King CA (2010), Cummins PM, *et al.* (2007), Cummins PM, *et al.* (2007), Liu HB, *et al.* (2013)]. Similar to shear stress, cyclic strain has direct effects on both endothelial cell health and remodeling of the vessel. Also, stretching of the vessel wall induces endothelial cells to proliferate, changes phenotype and alignment, signals to underlying smooth muscle cells, and remodels their native extracellular matrix [Califano JP & Reinhart-King CA (2010)].

Physiological cyclic strain is considered to be between 6-10%, whereas pathological strain, such as those observed in hypertension, is approximately 20% and has been shown to lead to the development of atherosclerosis [Califano JP & Reinhart-King CA (2010), Cummins PM, *et al.* (2007), Ando J & Yamamoto K (2011)]. Moreover, pathological strain can be due to an elevated frequency of vessel wall stretching as a result of a rapid heart rate such as arrhythmia. The most common type of arrhythmia is atrial fibrillation, which has been linked to high mortality risk and morbidity [Freestone B & Lip GY (2008), Krishnamoorthy S, Lim SH, & Lip GY (2009)]. High frequency cyclic stretch increases stress and fatigue on the endothelium, leading to its dysfunction and ultimately coronary atherosclerosis. Furthermore, the diastolic period is reduced, resulting in susceptibility of endothelial cells to atherogenic low and oscillatory shear stress [Freestone B & Lip GY (2008), Giannoglou GD, *et al.* (2008), Guazzi M & Arena R (2009)].

Integrins, G proteins, stretch-sensitive ion channels, and other transmembrane mechanosensors on the cell surface are involved in converting mechanical stimuli into chemical signals within the cell [Cummins PM, *et al.* (2007), Cummins PM, *et al.* (2007), Liu HB, *et al.* (2013), Giannoglou GD, *et al.* (2008), Wang JH & Thampatty BP (2006)]. Mechanical force on integrins leads to their clustering and formation of focal adhesions. Focal adhesion kinase is then phosphorylated prior to activating downstream proteins. Second messengers such as MAPKs (p38, JNK, ERK) can then induce other downstream proteins and transcription factors [Cummins PM, *et al.* (2007), Cummins PM, *et al.* (2007), Kakisis JD, Liapis CD, & Sumpio BE (2004), Lehoux S & Tedgui A (2003)]. Moreover, the cytoskeleton, which is directly linked to integrins and focal adhesions complexes, is also critical in the transmembrane mechanotransduction. Changes of its components (microfilaments, microtubules, and intermediate filaments) not only modulate tension within the cell, but also initiate intracellular signaling pathways [Ando J & Yamamoto K (2011), Wang JH & Thampatty BP (2006), Lehoux S & Tedgui A (2003)]. Ultimately, anti- or pro-inflammatory genes can then be regulated depending on the state of the endothelium while under the influence of cyclic stretch. Previous studies have shown cyclic stretch modulates expression of genes related to proliferation, migration, angiogenesis, cell-cell communication, as well as endothelial cell orientation and remodeling of the actin cytoskeleton [Cummins PM, *et al.* (2007), Cummins PM, *et al.* (2007), Wang JH & Thampatty BP (2006)].

Transcription factor activation in response to mechanical strain has been demonstrated in activator protein 1 and 2 (AP-1/-2), cAMP response element (CRE), early growth response protein (Egr-1), and NF- κ B [Cummins PM, *et al.* (2007), Cummins PM, *et al.* (2007), Ando J & Yamamoto K (2011), Kakisis JD, Liapis CD, & Sumpio BE (2004)]. The glucocorticoid receptor has multiple phosphorylation sites that can be activated by MAPKs [Gallagher-Beckley AJ & Cidlowski JA (2009)], which have been previously shown to be activated in response to cyclic stretch in endothelial cells [Cummins PM, *et al.* (2007), Aikawa R, *et al.* (2002), Gawlak G, *et al.* (2014), Hirayama Y & Sumpio BE (2007), Hsu HJ, Lee CF, Locke A, Vanderzyl SQ, & Kaunas R (2010), Kaunas R, Usami S, & Chien S (2006)]. It is possible therefore that physiological and/or pathological degrees of cyclic stretch may also initiate phosphorylation-induced changes in GR subcellular localization as we previously showed with shear stress [Nayebosadri A, Christopher L, & Ji JY (2012), Nayebosadri A & Ji JY (2013)]. However, little is known about the effects of cyclic stretch on GR activity in endothelial cells.

Mechanical strain on lamin-deficient mouse embryonic fibroblasts have resulted in reduced viability, increased nuclear deformation, defective mechanotransduction, as well as abnormal nuclear-cytoskeletal coupling [Lammerding J, *et al.* (2004), Lee JS, *et al.* (2007), Dahl KN, Ribeiro AJ, & Lammerding J (2008)]. We previously showed that silencing of lamin A/C via shRNA did not inhibit GR nuclear translocation under the presence of physiological magnitudes of shear stress, but at 5 dynes/cm² cells showed significantly different amounts of nuclear GR after two hours [Nayebosadri A & Ji JY (2013)]. Moreover, it has been reported that the megakaryoblastic leukemia 1 protein (MKL1) in lamin-mutated myoblasts did not show nuclear translocation in response to cyclic stretching compared to wild-type cells [Bertrand AT, *et al.* (2014)]. However, studies on transcription factor subcellular localization in lamin A/C deficient cells are still limited. Cyclic stretch experiments are done with cells on a flexible substrate (*i.e.* silicone membrane) while shear stress experiments are carried out on a more rigid substrate, a glass slide. Thus, these mechanical cues may elicit different responses in endothelial cells, such as time required for nuclear translocation, compared to our previous results.

This work incorporates the effects of both physiological and pathological strain parameters as well as lamin deficiency in understanding endothelial GR activity. We used control and lamin shRNA BAECs and subjected them to ligand (dexamethasone) treatment, physiological stretch (10% at 1 Hz), or pathological stretch (20% at 1 Hz or 10% at 2 Hz), in order to evaluate GR nuclear translocation in endothelial cells with and without lamin A/C as well as potential upstream protein regulators of GR subcellular movement during cyclic stretch.

2 Materials and methods

2.1 Reagents

Dexamethasone (Sigma) was dissolved in ethanol at a stock concentration of 25 mM and stored at -20 °C. PD98059 (Promega and Cell Signaling) was dissolved in DMSO to make a stock concentration of 20 mM or 50 mM and stored at -20 °C. SP600125 (Sigma) was dissolved in DMSO to make a stock concentration of 10 mM and stored at 4 °C. SB203580

(Sigma and Cell Signaling) was dissolved in DMSO at a stock concentration of 10 mM and stored at -20 °C. Anisomycin (Sigma) was dissolved in DMSO at a stock concentration of 10 mg/mL and stored at 4 °C.

2.2 Cell culture

Following selection of control and lamin A/C shRNA positive cells as previously described [Nayebosadri A & Ji JY (2013)], BAECs, passages 12 or less, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo HyClone), 1% L-glutamine, 2% penicillin streptomycin, and 2 µg/mL Puromycin (all Sigma) and incubated at 37°C in a humidified 5% CO₂ environment. Cells were seeded on 1.4 cm x 7.5 cm silicone membranes (Specialty Manufacturing) coated with fibronectin (1 µg/cm², Becton Dickinson), at a density of 4 ×10⁵ cells per membrane. For pharmacological drug studies, cells were treated with 50 µM PD98059, 10 µM SP600125, 5 µM SB203580, or 250 ng/mL anisomycin for six hours under static conditions or for 1 hour prior to stretching.

2.3 Cyclic stretching

BAECs were stretched in a custom designed device at IUPUI and subjected to various strain rates and percentages (1 Hz (60 cycles/min) at 10% or 20% strain, or 2 Hz (120 cycles/min) at 10% strain). Four silicone membranes are capable of being stretched simultaneously in four-well rectangular dishes (Nunc). The device was stretched using a Cool Muscle motor (Myostat Motion Control) and controlled through CoolWorks Lite 4.1.7.4 software.

2.4 Protein analysis

After static or stretching experiments, BAECs were lysed and separated into cytoplasm and nuclear fractions using a nuclear extraction kit (Active Motif) following manufacturer's instruction. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Protein samples were separated by SDS-PAGE on 4-12% Bis-Tris NuPAGE gels (Life Technologies), transferred to a nitrocellulose membrane, and blocked for 1 hour at room temperature with 5% nonfat milk in TBS with 0.1% Tween (Sigma) (TBS-T). Incubation with the primary rabbit monoclonal anti-GR antibody (Cell Signaling D6H2L) at 1:1000 ratio or mouse monoclonal anti-lamin A/C (Cell Signaling 4777) at 1:2000 ratio was done in blocking solution for 2 hours at room temperature or overnight at 4 °C while shaking gently. The blot was then washed 3 times (5 minutes each) with TBS-T prior to incubating with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Bio-Rad) at 1:4000 dilution for 1 hour. Mouse monoclonal Transcription Factor IID (Santa Cruz sc-374035, 1:300 ratio in 5% non-fat dry milk/PBS-T) primary antibody served as the nuclear control, while goat polyclonal GAPDH (Santa Cruz sc-48166, 1:300 ratio in 3% Bovine Serum Albumin/PBS-T) primary antibody served as the cytoplasmic control. Membranes were illuminated using SuperSignal West Pico Chemiluminescent Substrate Reagents (Thermo). Imaging was done using Bio-Rad Molecular Imager ChemiDoc XRS+ System and acquired using Quantity One Image Analysis Software.

2.5 Quantitative real-time polymerase chain reaction

Immediately following static or cyclic stretching treatment, RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). RNA concentration and purity were measured on a NanoDrop 2000 (Thermo Scientific). 1 μ g RNA was turned into cDNA using the qScript cDNA SuperMix (Quanta Biosciences) per manufacturer's protocol. Quantitative PCR using 100 ng cDNA was performed using iTaq Universal SYBR Green Mastermix (Bio-Rad) and analyzed on the 7500 Real Time PCR system (Applied Biosystems). Primer kits for bovine DUSP1 (539175, BTR_DUSP1_2), DUSP6 (515310, BTR_DUSP6_2), DUSP10 (541175, BTR_DUSP10_2), and DUSP16 (618644, BTR_DUSP16_1) were purchased from Sigma Life Science and used in amplification conditions of 95 °C (15 seconds) denature, 58 °C (30 seconds) anneal, and 72 °C (30 seconds) extend, 40 cycles, according to primer manufacturer protocol. Data was analyzed using the delta-delta C_t method.

2.6 Statistics

All experiments were carried out for a minimum of three times, and each time sample was prepared and analyzed independently. Results are expressed as mean \pm standard error. Standard error of each group was calculated to verify the statistical significance of the results. Paired Student *t*-test was used to compare one condition between two sample sets. One-way ANOVA was performed to compare cyclic stretching at two, four, and six hours as well as for comparisons between MAPK drugs. *P* value less than 0.05 was considered sufficient for statistical significance.

3 Results

3.1 Lamin A/C is not required for GR translocation by dexamethasone on silicone substrate

BAECs were plated on silicone membranes coated with fibronectin and grown to confluency before addition of dexamethasone. In both control and lamin shRNA cells, Western blots reveal GR being predominately cytoplasmic (Figure 1A) under control condition, specifically a $60.2 \pm 3.4/39.8 \pm 3.4\%$ and $79.0 \pm 2.0/21.0 \pm 2.0\%$ cytoplasmic/nuclear percent distribution, respectively. Quantitative analysis of Western blots revealed that the GR cytoplasmic presence in lamin A/C deficient cells was significantly more than that of those with intact lamin A/C (Figure 1B, $P < 0.01$). Following treatment with dexamethasone for 30 minutes, GR translocated into the nucleus in both cell types similarly. Control and lamin shRNA cells had an $8.2 \pm 1.3/91.8 \pm 1.3\%$ and $12.8 \pm 1.5/87.2 \pm 1.5\%$ cytoplasmic/nuclear distribution, respectively. Our results on dexamethasone-induced GR translocation for cells on silicone membranes agree with cells plated on glass [Nayebosadri A & Ji JY (2013)] Interestingly, we found a significantly greater cytoplasmic presence in static, non-treated lamin A/C deficient cells compared to control cells.

3.2 Physiological stretch induces nuclear translocation of GR after several hours

Control and lamin shRNA BAECs were initially stretched under physiological conditions

of 10% strain at 1 Hz for 2, 4, and 6 hours. As time progressed, GR gradually became more nuclear and less cytoplasmic in control shRNA cells (Figure 2A). Quantitative analysis of Western blots revealed significant nuclear translocation after being stretched for a duration of 6 hours compared to static cells ($P < 0.05$), but not 2 or 4 hours (Figure 2C). From 2 to 6 hours, GR cytoplasmic presence decreased from 58.5 ± 4.7 to $49.4 \pm 1.5\%$, and nuclear presence increased from 41.5 ± 4.7 to $50.6 \pm 1.5\%$. However, there was no significant difference in GR subcellular distribution when the three time points were compared. On the contrary, physiological stretch on lamin-silenced BAECs had no significant effect on inducing GR nuclear translocation (Figure 2B). At 2, 4, and 6 hours, GR had cytoplasmic and nuclear distributions of $73.0 \pm 3.8/27.0 \pm 3.8\%$, $79.1 \pm 5.6/20.9 \pm 5.6\%$, and $77.6 \pm 3.1/22.4 \pm 3.1\%$, respectively (Figure 2C). This data suggests that cyclic stretching does not induce GR nuclear translocation in lamin-deficient cells compared to control shRNA cells.

3.3 20% strain at 1 Hz induces greater GR translocation in control shRNA BAEC compared to 10%, but not when lamin A/C is absent

We next proceeded to double the strain magnitude to 20% while maintaining the frequency at 1 Hz, which is considered pathological and has been previously used to mimic hypertension in several studies [Liu HB, *et al.* (2013), Hurley NE, *et al.* (2010), Kou B, Zhang J, & Singer DR (2009)]. Figures 3A and 3B show Western blots of GR distribution after stretching for the indicated duration in each cell type. Control shRNA BAECs again showed significant differences in GR subcellular localization compared to static cells ($P < 0.05$). After 2, 4, and 6 hours of cyclic stretch, cytoplasmic and nuclear distributions were $43.0 \pm 6.0/57.0 \pm 6.0\%$, $47.9 \pm 1.1/52.1 \pm 1.1\%$ and $39.5 \pm 3.4/60.5 \pm 3.4\%$, respectively (Figure 3C). When GR localization at these time points was compared to that observed when stretched at 10% 1Hz, we found a significant difference to be present at 4 and 6 hours ($P < 0.05$), but not 2 hours. For lamin shRNA cells, 20% strain again had no effect on inducing GR nuclear localization. After 2, 4, and 6 hours, GR cytoplasmic and nuclear distributions were $74.5 \pm 1.6/25.5 \pm 1.6\%$, $77.4 \pm 2.4/22.6 \pm 2.4\%$, and $80.4 \pm 3.8/19.6 \pm 3.8\%$ (Figure 3C), and nearly identical to that of both static and physiological stretch conditions. Thus, at a stretching frequency of 1 Hz, our results suggest that GR in lamin-silenced endothelial cells does not actively respond to either 10% or 20% strain.

3.4 10% stretching at 2 Hz induces greater GR activity in control shRNA cells, but not in lamin deficient cells

Control and lamin shRNA BAECs were also subject to 10% strain at 2 Hz, another pathological stretching condition that mimics an arrhythmia, specifically tachycardia, and generates stress and fatigue within endothelial cells [Freestone B & Lip GY (2008), Giannoglou GD, *et al.* (2008), Guazzi M & Arena R (2009)] Figures 4A and 4B show Western blots of GR distribution after stretching for the indicated durations in both cell types. Again, BAECs with intact lamin showed significant GR nuclear translocation after 2, 4, and 6 hours of stretching ($P < 0.05$) compared to static cells. Cytoplasmic and nuclear distributions at these times were $35.0 \pm 0.3/65.0 \pm 0.3\%$, $42.5 \pm 1.7/57.5 \pm 1.7\%$ and $38.4 \pm 1.9/61.6 \pm 1.9\%$, respectively (Figure 4C). Furthermore, GR subcellular

localization at each of these time points was significantly more nuclear than that induced by physiological stretch ($P < 0.01$). For lamin shRNA cells, however, analysis of Western blots once again suggests no changes in GR translocation compared to cells in static conditions. At 2, 4, and 6 hours, GR cytoplasmic and nuclear distributions were $74.0 \pm 7.1/26.0 \pm 7.1\%$, $78.6 \pm 2.8/21.4 \pm 2.8\%$, and $75.5 \pm 4.4/24.5 \pm 4.4\%$, respectively (Figure 4C). These results therefore suggest that doubling the frequency of cyclic stretch had no effect on inducing GR nuclear translocation in lamin-deficient cells. Lamin-intact cells did respond to pathological stretching conditions by inducing greater GR movement into the nucleus.

3.5 JNK and ERK inhibition exert opposite effects on GR nuclear transport under pathological stretch conditions in BAECs with lamin A/C

We hypothesized that MAPK signaling cascades are altered in lamin-silenced BAECs, thus interfering with GR activation and nuclear translocation. All three MAPKs (ERK, JNK, and p38) have previously been shown to phosphorylate various sites on GR, leading to different responses [Gallagher-Beckley AJ & Cidlowski JA (2009), Bodwell JE, *et al.* (1991), Bouazza B, *et al.* (2014), Chen W, *et al.* (2008), Krstic MD, Rogatsky I, Yamamoto KR, & Garabedian MJ (1997)]. We pretreated control shRNA BAECs with inhibitors specific to all three MAPKs for 1 hour prior to stretching at 10% and 2 Hz for 6 hours. This stretching condition was chosen because it induced significantly greater GR nuclear localization compared to physiological stretch. It is also more relevant to patients with dilated cardiomyopathy. Figures 5A and 5C show Western blots of GR subcellular localization after treatment with each drug under static or cyclic stretch conditions for 6 hours, respectively.

PD98059 (50 μ M), an inhibitor of MEK1/2 upstream of ERK1/2, was the only inhibitor to induce significant nuclear-localized GR in static cells ($P < 0.01$). Compared to static cells without inhibitor treatment, GR cytoplasmic and nuclear distributions were 39.1 ± 3.9 and $60.9 \pm 3.9\%$, respectively, after 6 hours treatment (Figure 5B). Western blot analysis for stretched cells further revealed that ERK inhibition induced significantly greater nuclear localization of GR than without the inhibitor ($30.0 \pm 1.8/70.0 \pm 1.8\%$ cytoplasmic/nuclear localization, respectively, $P < 0.05$).

Inhibiting the activity of p38 with 5 μ M SB203580 showed no significant changes in GR localization compared to when BAECs were stretched without the drug (Figures 5C). The cytoplasmic and nuclear distributions after p38 inhibition were $40.3 \pm 2.6/59.7 \pm 2.6\%$ for stretched cells (Figure 5D). Under static control conditions, p38 inhibition with SB203580 instead normalized GR distribution fairly evenly between the cytoplasm and nucleus. The cytoplasmic and nuclear distributions after p38 inhibition were $50.6 \pm 5.4/49.4 \pm 5.4\%$ for static cells (Figures 5B).

The JNK inhibitor SP600125 (10 μ M) also yielded no changes in GR distribution after 6 hours of treatment (Figure 5A). This was confirmed after quantifying the respective Western blots, which showed a $51.9 \pm 5.6/48.1 \pm 5.6\%$ cytoplasmic and nuclear distribution, respectively (Figure 5B). However, when JNK was inhibited prior to stretch at 10% and 2 Hz, GR did not nuclear localize, as was the case without drug inhibition (Figure 5C). Rather, stretched cells had a cytoplasmic and nuclear distribution nearly

identical to that of static cells ($56.5 \pm 2.7/43.5 \pm 2.7\%$, $P > 0.05$ compared to static control) (Figure 5D). In summary, these results suggest that inhibition of JNK or ERK prior to stretch desensitizes and enhances, respectively, ligand-independent GR nuclear localization in cells with lamin A/C.

3.6 Inhibition of p38 in lamin-deficient BAECs prior to cyclic stretch alters the nuclear to cytoplasmic GR ratio

Previous studies found all three MAPKs in heart tissue from lamin-mutated mice to be significantly more phosphorylated compared to those from wild-type mice [Muchir A, *et al.* (2007), Muchir A, *et al.* (2012), Muchir A, Wu W, & Worman HJ (2009)]. We hypothesized that this may be a characteristic feature of cells with defective A-type lamins, and we used the MAPK inhibitors on lamin shRNA BAECs in order to see if pharmacological inhibition of MAPK activity would affect GR subcellular distribution under both static and stretching conditions. For static cells, treatment with SP600125, SB203580, or PD98059 did not yield any changes in GR localization compared to no inhibitors (Figure 6A). In all cases, GR remained mainly in the cytoplasm. Quantitative analysis of Western blots revealed cytoplasmic and nuclear distributions of $75.2 \pm 1.8/24.8 \pm 1.8\%$, $75.0 \pm 4.7/25.0 \pm 4.7\%$, and $77.2 \pm 4.1/22.8 \pm 4.1\%$ for SP600125, SB203580, and PD98059, respectively (Figure 6B).

We next pretreated lamin deficient BAECs with each inhibitor drug for 1 hour prior to exposing them to 10% and 2 Hz cyclic stretch for 6 hours. Figure 6C shows western blots of GR from cells treated with each inhibitor and stretched, and Figure 6D shows the densitometry analysis. Contrary to control shRNA BAECs, lamin shRNA cells showed no changes in GR localization compared to stretching without the inhibitor for two of the three drugs. While treatment of control shRNA BAECs with the JNK inhibitor SP600125 failed to allow GR nuclear translocation, the same treatment in lamin shRNA cells yielded significant cytoplasmic GR presence: $78.0 \pm 3.3\%$ vs. $22.0 \pm 3.3\%$ between cytoplasmic and nuclear distribution.

The effect of PD98059 treatment was also strikingly different compared to that observed in control shRNA cells since inhibition of ERK1/2 did not allow increased nuclear translocation in either static or stretched case. After inhibitor treatment and stretch, the GR cytoplasmic and nuclear distributions of 75.6 ± 2.9 and $24.4 \pm 2.9\%$, respectively, remained similar to that of static lamin-deficient cells. Interestingly, however, inhibition of p38 with SB203580 allowed for a slight yet significant increase in GR nuclear presence ($P < 0.05$ compared to stretching without inhibitor). GR in the nucleus reached $37.7 \pm 4.6\%$ while that within the cytoplasm decreased to $62.3 \pm 4.6\%$ after 6 hours of stretching. This result suggests that mechanical strain following p38 inhibition in cells without lamin A/C is likely to induce intracellular responses that release GR from remaining predominately in the cytoplasm. Overall, our data suggest that the effect of disrupting the nuclear lamin supersedes the effects of most MAPKs signaling inhibition on GR distribution in BAEC under static or stretch condition.

3.7 Anisomycin induces changes in GR subcellular localization only in the presence of cyclic stretch and lamin A/C

We also treated BAECs with anisomycin, an antibiotic that inhibits protein synthesis,

induces apoptosis, and is an agonist for stress-activated protein kinase (SAPK) signaling, specifically the JNK and p38 pathways. We hypothesized that, regardless of lamin presence, the initiation of stress-induced signaling pathways and activation of inflammatory transcription factors (such as AP-1) may directly and indirectly, respectively, induce GR anti-inflammatory activity. In the case of the latter, GR may translocate into the nucleus and act via transrepression to inhibit transcription factor function.

Interestingly, although 6 hours treatment with 250 ng/mL anisomycin induced cell death in both static control and lamin shRNA BAECs, no difference in GR translocation was observed compared to no treatment (Figures 5A, 6A). GR cytoplasmic and nuclear distribution was $60.3 \pm 0.3/39.7 \pm 0.3\%$ and $77.7 \pm 3.9/22.3 \pm 3.9\%$ in control and lamin shRNA cells, respectively (Figures 5B, 6B). However, the addition of 10% and 2 Hz stretch increased GR nuclear translocation in control shRNA cells after pretreatment with anisomycin for one hour, but this was not the case in lamin shRNA cells (Figures 5C, 6C). After 6 hours of stretching, cytoplasmic and nuclear distributions were $29.1 \pm 1.5/70.9 \pm 1.5\%$ and $70.7 \pm 3.4/29.3 \pm 3.4\%$ for control and lamin shRNA cells, respectively (Figures 5D, 6D). The subcellular distribution of GR in anisomycin and stretch-treated BAECs with intact lamin A/C was identical to that observed when PD98059 was used ($P > 0.05$). Taken together, these data suggest a dominating inhibitory mechanism preventing GR nuclear localization that is related to presence of functional lamin A/C.

3.8 Lamin A/C deficiency significantly alters MAPK phosphatase gene expression

We found an inability of GR in lamin-deficient BAECs to change its subcellular distribution in response to physiological or pathological cyclic stretch. We hypothesized that a part of the signal transduction cascade, either upstream or downstream of GR, could be desensitized to the mechanical stimulus. We focused on MAPK phosphatases (MKPs), which comprise the dual-specificity protein phosphatase (DUSP) family and dephosphorylate phosphotyrosine and phosphothreonine on their target MAPK substrates [Huang CY & Tan TH (2012)]. Previous studies showed a significant upregulation of DUSP4 in hearts from lamin-mutated mice as a response to elevated ERK1/2 activity [Choi JC, *et al.* (2012)]. We have also shown that in response to dexamethasone and fluid shear stress, DUSP1 is significantly upregulated in lamin-deficient BAECs compared to cells with intact lamin subjected to the same stimuli [Nayebosadri A & Ji JY (2013)]. Here again, we examined the expression of DUSP1 or MKP-1, which resides predominately in the nucleus and targets all three members of the MAPK family. We also looked at DUSP6 (MKP-3), which is localized in the cytoplasm and targets ERK1/2 for dephosphorylation, as well as DUSP10 (MKP-5) and DUSP16 (MKP-7), both of which target JNK and p38 and are localized in the cytoplasm (DUSP16) or cytoplasm and nucleus (DUSP10). Of these four DUSPs, only DUSP1 contains a glucocorticoid response element, which GR may recognize and bind to during transactivation of anti-inflammatory genes [Shipp LE, *et al.* (2010)].

Figure 7A shows mRNA fold induction differences between static control and lamin-deficient BAECs. Lamin shRNA cells had over a 2-fold increase in DUSP1 mRNA expression compared to control cells, while GR was predominately localized in the cytoplasm based on

Western blot data shown above. DUSP6 mRNA expression was even greater in lamin-deficient cells compared to control cells – a 2.7-fold increase. Similarly, DUSP10 expression was almost 2-fold higher in lamin shRNA cells than control cells. Interestingly DUSP16 expression was decreased in lamin shRNA cells: 64% of expression in control BAECs with intact lamin A/C. These data show that DUSP gene expression is affected by the presence of nuclear lamin. Thus, the resultant elevated phosphatase activity may be involved in the difference in GR sub-cellular localization under static conditions.

We next compared the expression of each of the four DUSPs in control and lamin shRNA BAECs after being stretched at 10% and 2 Hz for 6 hours to that of static conditions (Figure 7B). In control shRNA cells, there was a significant upregulation in the expression of all genes ($P < 0.01$) after stretching. DUSP1 expression increased over 5-fold, which can be related to GR translocation into the nucleus and its binding to glucocorticoid response elements on the DUSP1 gene. DUSP6 was upregulated over 2.5-fold. Even though it does not contain a GR binding site, it is possible that the pathological stretching stimulus initiates its upregulation through increased ERK activity. The JNK and p38-specific DUSP10 and DUSP16 also exhibited elevated expression, approximately 2- and 4-fold, respectively.

In lamin shRNA cells, upregulation of DUSP1, DUSP6, and DUSP10 was much less: 0.9, 1.4 ($P < 0.05$), and 0.9-fold respectively, compared to static cells. DUSP16, however, did not appear to be affected by lamin-deficiency as much as the others since it was increased by 4.2 fold, similar to the fold increase in control cells. DUSP16 may therefore be more susceptible to the effects of cyclic stretch than to the lack of lamin A/C. This data further supports results from previous studies documenting defective mechanotransduction in the presence of mechanical stimuli as a result of lamin knockout or mutation [Lammerding J, *et al.* (2004)]. Moreover, with respect to GR sensitivity to cyclic strain, our results suggest that the upregulated DUSP1, DUSP6, and DUSP10 genes in static lamin-deficient cells supersede any extracellular and ligand-independent induction of GR by the MAPK pathway, thus explaining its cytoplasmic retention.

4 Discussion

We previously showed that laminar shear stress induces GR translocation into the nucleus within two hours in bovine aortic endothelial cells with and without the nuclear structural protein lamin A/C [Nayebosadri A, Christopher L, & Ji JY (2012), Nayebosadri A & Ji JY (2013)]. In this study, another hemodynamic force, cyclic strain, was utilized to study its effects on the subcellular response of GR for the first time. This force is due to the pulsatile nature of blood pressure and is exerted on endothelial, smooth muscle, and fibroblast cells in the intima, media, and adventitia layers, respectively, of the blood vessel [Chien S (2007)]. Under physiological conditions of 10% strain at a frequency of 1 Hz, we observed a gradual shift in GR subcellular localization in control shRNA cells compared to static controls. Even after six hours of stretching, the cytoplasmic presence of GR was approximately 30% more than that observed following 2 hours of shear stress [Nayebosadri A, Christopher L, & Ji JY (2012), Nayebosadri A & Ji JY (2013)]. This is likely due to a difference in the magnitude of the response of mechanoreceptors. Whereas shear stress activates numerous mechanosensors on both the apical and basal surface of endothelial cells and is sustained as long as flow is present, cyclic stretch predominately

activates stretch-activated ion channels, integrins bound to the basement membrane, as well as G-proteins and receptor tyrosine kinases on the apical surface [Cummins PM, *et al.* (2007), Katsumi A, Orr AW, Tzima E, & Schwartz MA (2004)]. Furthermore, MAPK responses to extracellular mechanical stimuli is dependent on the substrate to which the cells are attached [Lehoux S & Tedgui A (2003)]. For our shear stress studies, cells were plated directly onto glass slides, whereas fibronectin-coated silicone membranes were used for this work which better resembles that of the *in vivo* vessel wall. Regardless, it is well known that GR is activated at specific amino acid residues by all three members of the MAPK family [Gallagher-Beckley AJ & Cidlowski JA (2009)], and therefore it is possible that differences in the magnitude of MAPK activation between the two hemodynamic forces, as well as the effects of substrate stiffness, predominate in explaining the contrasting behaviors of GR subcellular movement.

Upon exposure to pathological degrees of stretching (10% stretch at 2 Hz or 20% strain at 1 Hz), control shRNA BAECs showed greater nuclear concentration of GR at each time point compared to when they were stretched at physiological parameters (Figures 3A, 4A). It is likely that both conditions induced a greater activation response on the mechanosensors bound to the extracellular matrix, thus amplifying downstream signaling that induces a potentially higher MAPK phosphorylation state and perhaps ultimately activating a greater number of GR. As mechanical force applied to the ECM increases in magnitude, mature focal adhesions are formed and consist of a greater number of integrins, likely contributing to increased integrin-mediated signaling [Kuo JC (2013)]. Previous work has also shown that in response to 1 Hz uniaxial cyclic stretch, all three MAPKs (JNK, ERK, and p38) in BAECs demonstrated a significantly higher phosphorylation state compared to static, and this is maintained for minutes to hours prior to returning back to baseline [Hsu HJ, Lee CF, Locke A, Vanderzyl SQ, & Kaunas R (2010), Kaunas R, Usami S, & Chien S (2006), Goldschmidt ME, McLeod KJ, & Taylor WR (2001)]. Moreover, MAPK phosphorylation was greatest at a stretching frequency of 1 Hz compared to 0.1 or 0.01 Hz [Hsu HJ, Lee CF, Locke A, Vanderzyl SQ, & Kaunas R (2010), Goldschmidt ME, McLeod KJ, & Taylor WR (2001)].

The response of GR in lamin-deficient cells to cyclic stretching was relatively non-existent compared to that observed in control shRNA cells (Figures 2B, 3B, 4B). The initial distribution of GR in lamin shRNA cells was significantly more concentrated in the cytoplasm – another difference observed compared to our previous data with cells adhered on glass. The combination of a softer substrate and lamin deficiency may both play a role in the observed differences in GR subcellular localization. However, after treatment with dexamethasone, a GR agonist, the localization of GR was nearly identical to that of cells with lamin A/C (Figure 1A), in agreement with past results [Nayebosadri A, Christopher L, & Ji JY (2012), Nayebosadri A & Ji JY (2013)]. While the ability of GR to become activated by its ligand agonist still exists, ligand-independent activation is thwarted in lamin-deficient BAEC. The differences observed in this study with the introduction of another hemodynamic force, stretch, again suggests that lamin-deficient cells may be dependent on substrate for intracellular signaling in response to mechanical stimuli. Moreover, the difference in strain-induced signaling between lamin and control shRNA cells must lie upstream of GR since no stretching condition was able to alter the intracellular distribution in lamin deficient cells. These results correlate with that observed with the megakaryoblastic leukemia 1 protein by another group who also incorporated

cyclic stretch and lamin mutations [Bertrand AT, *et al.* (2014)].

The MAPK proteins ERK, JNK, and p38 have all been previously shown to phosphorylate various sites on GR, specifically Ser134, Ser203, Ser211, and Ser226 [Gallagher-Beckley AJ & Cidlowski JA (2009), Beck IM, *et al.* (2009) Kadmiel M & Cidlowski JA (2013), Moutsatsou P & Papavassiliou AG (2008), Nicolaides NC, Galata Z, Kino T, Chrousos GP, & Charmandari E (2010)]. We first hypothesized that altered phosphorylation of upstream MAPK proteins in lamin-deficient BAECs may explain why GR failed to respond to any of the cyclic stretch conditions. Inhibition of JNK and ERK in control shRNA cells had the greatest yet opposite effects. Whereas SP600125 seemed to significantly inhibit translocation of GR compared to stretching without the drug, PD98059 allowed for greater translocation of GR.

The effects of ERK inhibition, using the MEK1/2 inhibitor PD98059, on GR translocation in both static (Figure 5A) and stretching (Figure 5C) conditions more closely agrees with results published by other groups who used dexamethasone to activate GR [Takabe S, Mochizuki K, & Goda T (2008), Rogatsky I, Logan SK, & Garabedian MJ (1998), Blind RD & Garabedian MJ (2008)]. ERK inhibition increased the nuclear presence of GR, suggesting that phosphorylation by ERK functions to sequester GR within the cytoplasm. A decrease in phosphorylation of the target site would thus allow GR to migrate into the nucleus. Indeed, it has been determined that the Ser203 location on GR is a target for ERK, and phosphorylation at this specific site is associated with predominately cytoplasmic GR that is transcriptionally inactive, whereas its dephosphorylation allows for nuclear transport and transcription of target genes [Gallagher-Beckley AJ & Cidlowski JA (2009), Gawlak G, *et al.* (2014)].

There is conflicting data on the role of p38 phosphorylation of GR. Whereas some groups have found that it does in fact have a role in phosphorylating GR at its transcriptional activation site Ser211 or at Ser134 [Gallagher-Beckley AJ & Cidlowski JA (2009) Gallagher-Beckley AJ, Williams JG, & Cidlowski JA (2011) Miller AL, *et al.* (2005)], others have reported the opposite or an inhibitory effect on its function [Rogatsky I, Logan SK, & Garabedian MJ (1998) Adcock IM & Caramori G (2001) Szatmary Z, Garabedian MJ, & Vilcek J (2004) Irusen E, *et al.* (2002) Wang X, Wu H, & Miller AH (2004) Mercado N, *et al.* (2012)]. Indeed, the role of p38 on the state of GR appears to be dependent on both the cell-type and physiological or pathological state. A recent study by Bouazza *et al.* found that inhibition of p38 with SB203580 or its silencing through siRNA significantly altered the phosphorylation status of GR and enhanced its nuclear translocation under both basal and agonist-induced conditions [Bouazza B, *et al.* (2014)]. Our data from static and stretched BAECs with lamin A/C suggests that p38 does not have a significant role in either induction or repression of GR nuclear translocation (Figures 5A, C).

It was interesting that the stress-inducing and apoptotic effects of anisomycin had no significant effect on GR subcellular movement when static BAECs were treated with the drug for six hours (Figure 5A). We hypothesized that GR would counteract the effects of anisomycin by translocating into the nucleus and function by either transactivation (upregulate anti-inflammatory genes) or transrepression (tether to nuclear-localized inflammatory transcription factors to inhibit transcription of their target immediate-early genes). However, based on our results, it is possible that its concentration was not high enough for GR to respond when cells were static. Combining the effects of cyclic stretch and anisomycin likely amplified the activation of JNK and p38, thus inducing significantly more GR to translocate compared to

without the drug (Figure 5C). Since the GR subcellular distribution after 6 hours of cyclic stretch was similar to that following ERK inhibition with PD98059, it is likely that a hyper-activation of the stress-activated protein kinases overpowered the inhibitory ERK effect on GR.

Recent work by Worman and others have shown that phosphorylation of JNK, ERK, and p38 in cells from mice with mutated lamin A/C are in fact all significantly greater than those from wild-type mice [Muchir A, et al. (2007) Muchir A, et al. (2012) Muchir A, Wu W, & Worman HJ (2009) Wu W, Muchir A, Shan J, Bonne G, & Worman HJ (2011) Wu W, Shan J, Bonne G, Worman HJ, & Muchir A (2010)]. The same group also showed that pharmacological inhibitors of the MAPKs improved heart function in mice with cardiomyopathy after four weeks, and in addition ameliorated skeletal muscle in the presence of muscle dystrophy [Wu W, Muchir A, Shan J, Bonne G, & Worman HJ (2011) Muchir A, et al. (2013)]. We proceeded to apply our MAPK inhibitors used above, as well as anisomycin, in lamin shRNA BAECs to see if any of them could shift GR into an active state under both static and stretching stimuli. Although the amount of nuclear GR following p38 inhibition and stretching was not identical to that observed in BAECs with intact lamin A/C, it was still significant compared to stretching without the inhibitor. Our results resemble those published by groups who found reduced GR sensitivity to glucocorticoids as a result of increased p38 activity, and inhibiting its activity restored GR sensitivity [Irusen E, et al. (2002) Wang X, Wu H, & Miller AH (2004)]. Furthermore, our data correlates to those published recently by Bouazza et al. who found increased nuclear translocation (as well as transcription of a GRE promoter) following p38 inhibition. However, they observed this under basal and agonist-induced conditions, whereas we only observed this under stretching [Bouazza B, et al. (2014)].

We had expected ERK inhibition to give us similar results to those of control shRNA cells (*i.e.*, more nuclear translocation as a result of reduced phosphorylation at the cytoplasmic retention site). However, our data suggests that p38 may prevail over the three MAPKs in lamin-deficient BAECs as a possible inhibitory factor. Since p38 has been shown to be significantly upregulated in lamin-mutated cells previously, the likely mechanism is by phosphorylating either Ser203 or another site that keeps GR in its inactive conformation [Bouazza B, et al. (2014) Muchir A, et al. (2012) Irusen E, et al. (2002)]. This specific MAPK is increasingly activated in response to stressful and inflammatory stimuli [Kadmiel M & Cidlowski JA (2013) Roux PP & Blenis J (2004)], and the absence of lamin A/C has been shown to induce such necessary conditions [Sieprath T, Darwiche R, & De Vos WH (2012), Dubinska-Magiera M, Zaremba-Czogalla M, & Rzepecki R (2013)]. Future work will have to further investigate differences in cyclic stretch-induced phosphorylation of GR at the primary activation sites between control and lamin-deficient cells in order to better explain the variations in response to mechanical strain.

Finally, we hypothesized that an inhibitory protein was likely impeding the mechanotransduction cascade upstream of both GR and MAPK. We previously studied DUSP1, a nuclear MAPK dual-specificity phosphatase that contains a glucocorticoid response element and is capable of suppressing both p38 and JNK, in response to dexamethasone and fluid shear stress. We found that it was expressed to a greater degree in lamin shRNA BAECs compared to control shRNA cells under both conditions [Nayebosadri A & Ji JY (2013)]. Since all MAPKs in cells with aberrant lamin A/C have been shown by others to be increasingly phosphorylated

compared to wild-type cells [Muchir A, et al. (2007) Muchir A, et al. (2012)], we chose to pursue DUSPs that are specific to ERK, p38, and JNK. We narrowed our focus to four DUSP proteins: DUSP1 (MKP-1), which dephosphorylates nuclear MAPKs and is directly targeted by GR [Muchir A, et al. (2007) Tchen CR, et al. (2010) Nunes-Xavier C, et al. (2011) Nicoletti-Carvalho JE, et al. (2010) Furst R, et al. (2007) Vandevyver S, et al. (2012)]; DUSP6 (MKP-3), a cytoplasmic MAPK phosphatase that only targets ERK1/2 and has previously been shown to be upregulated in endothelial cells as well as in cardiac pathologies [Rossig L, et al. (2000) Rossig L, et al. (2002) Yang D, Xie P, & Liu Z (2012) Bermudez O, Pages G, & Gimond C (2010)]; as well as the cytoplasmic and nuclear DUSP10 (MKP-5) and cytoplasmic DUSP16 (MKP-7), phosphatases that are specific to JNK and p38, which have also been far less studied in endothelial cells compared to cancerous and immune cells [Nizamutdinova IT, Kim YM, Lee JH, Chang KC, & Kim HJ (2012)].

Previous work by others has shown increased nuclear accumulation of phosphorylated JNK and ERK in response to defective lamin A/C [Muchir A, Wu W, & Worman HJ (2009)]. The two-fold increase in expression of DUSP1 in static lamin shRNA cells was therefore not due to increased nuclear GR compared to control shRNA cells, but likely due to a relatively greater nuclear localization of MAPKs that are targeted by DUSP1 for inactivation (primarily JNK and p38), or as a result of an immediate-early gene target by a hyper-activated nuclear transcription factor, such as AP-1, in lamin-deficient cells [Boutros T, Chevet E, & Metrakos P (2008)]. Furthermore, whereas control shRNA cells showed a significant increase in expression of DUSP1 after stretching (likely due to elevation in MAPK activity and subsequent GR nuclear localization), lamin shRNA cells did not show this trend. It is possible that DUSP1 activity was sufficiently high enough in the latter that the addition of mechanical stimulus had little effect on the gene. Expression of DUSP6 is mediated solely by ERK activity, in contrast to DUSP1, which can also be induced as an immediate-early gene target by other transcription factors [Boutros T, Chevet E, & Metrakos P (2008) Jeffrey KL, Camps M, Rommel C, & Mackay CR (2007)]. Inactive ERK is primarily localized within the cytoplasm, whereas the phosphorylated form translocate into the nucleus to induce expression of its target transcription factors [Nunes-Xavier C, et al. (2011) Calvo F, Agudo-Ibanez L, & Crespo P (2010) Rodriguez J & Crespo P (2011)]. DUSP6 is then expressed and functions to retain ERK within the cytoplasm in an inactive state by competing with its upstream activator, MEK1/2 [Bermudez O, Pages G, & Gimond C (2010) Owens DM & Keyse SM (2007)] Elevated ERK activity within the cytoplasm that is targeted by DUSP6 would also correlate to inhibition of GR at the inhibitory Ser203 site. Thus, our DUSP6 data from static cells likely suggests that upraised ERK activity is why GR fails to respond to the stretching stimulus. DUSP6 upregulation likely indicates a mechanism by the cell to regulate increased ERK activity as a result of lamin A/C absence.

DUSP10 and DUSP16 are both inducible MAPK phosphatases that block enzymatic activation of p38 and JNK about equally albeit with much greater selectivity compared to ERK [Bermudez O, Pages G, & Gimond C (2010) Jeffrey KL, Camps M, Rommel C, & Mackay CR (2007) Theodosiou A, Smith A, Gillieron C, Arkininstall S, & Ashworth A (1999) Farooq A & Zhou MM (2004)]. However, they vary in their expression depending on the type of tissue they reside in [Nunes-Xavier C, et al. (2011) Theodosiou A, Smith A, Gillieron C, Arkininstall S, & Ashworth A (1999) Lawan A, et al. (2012)]. Here we show

for the first time both their expression in bovine aortic endothelial cells, and their response to both lamin deficiency as well as cyclic stretch (Figure 7). Our results suggest that the inhibitory effect of DUSP10 was more present in lamin-deficient cells compared to control shRNA cells under static conditions, a similar trend to that observed with DUSP1 and DUSP6. However, following cyclic stretch, only cells with intact lamin A/C exhibited an increase in expression. This is likely due to the increase in JNK and p38 activity while cells are under the influence of strain [Hsu HJ, Lee CF, Locke A, Vanderzyl SQ, & Kaunas R (2010) Chien S (2007)], and DUSP10 functions by negative feedback to dephosphorylate these specific stress-activated MAPKs. Again, it is possible that since DUSP10 was already upregulated in lamin shRNA BAEC, the signaling cascade that it targets was almost nonexistent during cyclic strain, and thus expression of the gene was unchanged. If JNK does induce GR nuclear translocation, as was apparent following its inability to do so following inhibition with SP600125 or activation with anisomycin, then its dephosphorylation by DUSP10 may further support another manner in which GR is unable to respond to cyclic stretch.

DUSP16, however, exhibited opposite effects in lamin shRNA BAECs under both static and stretch conditions compared to all other DUSPs. We had expected the opposite to occur, since it was previously observed in several cell lines that elevated ERK activity, which we indirectly observed to take place via increased DUSP6 expression, could induce DUSP16 expression to inhibit JNK as a crosstalk signaling mechanism [Fey D, Croucher DR, Kolch W, & Kholodenko BN (2012) Masuda K, Shima H, Katagiri C, & Kikuchi K (2003)]. Due to DUSP16 and DUSP10 both having substrate specificity for the same MAPKs, one hypothesis may be that DUSP10 dominates in the inhibitory effect. JNK and p38 may be activating genes that could indirectly induce DUSP10 expression over that of DUSP16. In response to cyclic stretch, cells with and without lamin A/C upregulated DUSP16 expression similarly. Its characteristic ability to shuttle MAPKs out of the nucleus may be activated in response to extracellular mechanical stimuli, but it is uncertain how this may have any effect on GR differences in subcellular distribution when linked to the nuclear lamina. We hypothesize that DUSP16 may not have as much of a significant effect on GR subcellular concentration in cells without lamin A/C compared to other DUSPs under both static and stretching conditions. Rather, it may be more susceptible to the effects of mechanical stimuli than lamin deficiency.

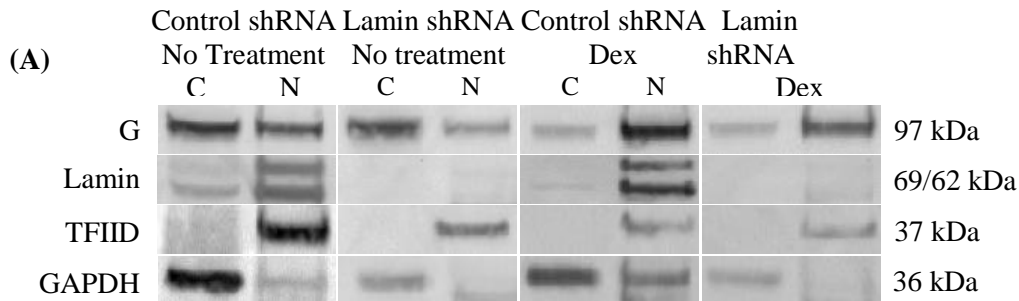
Figure 8A shows a proposed schematic of the intracellular signaling cascade that incorporates the effects of both MAPK inhibitors and DUSP proteins on GR. Our results suggest that in cells with lamin A/C, cyclic stretch activates GR through the JNK pathway, and ERK has some inhibitory role on GR nuclear translocation. DUSP proteins become upregulated in response to stretch as a result of GR activation (DUSP1) or by stretch-induced MAPK signaling. Figure 8B shows a proposed representation of the same network in lamin-deficient cells. Only the combination of cyclic stretch and p38 inhibition was able to induce marginal nuclear translocation. Increased MAPK phosphorylation due to lamin A/C absence could drive DUSP expression as a negative feedback mechanism. Upregulation of the cytoplasmic DUSP6 suggests a significant role of ERK in reducing GR sensitivity to mechanical strain. Finally, Figure 8C summarizes the qRT-PCR results from Figure 7 on DUSP expressions.

Our group has now shown that GR exhibits nuclear translocation in response to two hemodynamic forces, shear stress and cyclic stretch. Future work will delve deeper into the upstream mechano-transduction responses that activate GR in order to further understand differences in activation that arise from various physiological and pathological forces. Indeed, this study has brought novel insight into bridging the linkage between nuclear lamina and GR sensitivity to extracellular mechanical strain.

5 Conclusions

Physiological and/or pathological degrees of cyclic stretch may initiate phosphorylation-induced changes in GR subcellular localization as we previously showed with shear stress [Nayebosadri A & Ji JY (2013)]. To incorporate the effects of both physiological and pathological strain parameters as well as lamin deficiency in understanding endothelial GR activity, we used control and lamin shRNA BAECs and subjected them to ligand (dexamethasone) treatment, physiological stretch (10% at 1 Hz), or pathological stretch (20% at 1 Hz or 10% at 2 Hz). We evaluated GR nuclear translocation in endothelial cells with and without lamin A/C as well as potential upstream protein regulators of GR subcellular movement during cyclic stretch.

Upon exposure to pathological degrees of stretching, control shRNA BAECs showed greater nuclear concentration of GR at each time point compared to when they were stretched at physiological parameters. The response of GR in lamin-deficient cells to cyclic stretching was relatively non-existent compared to that observed in control shRNA cells. Our results suggest that in cells with lamin A/C, cyclic stretch activates GR through the JNK pathway, and ERK has some inhibitory role on GR nuclear translocation. DUSP proteins become upregulated in response to stretch as a result of GR activation (DUSP1) or by stretch-induced MAPK signaling. In lamin-deficient cells, only the combination of cyclic stretch and p38 inhibition was able to induce marginal nuclear translocation. Increased MAPK phosphorylation due to lamin A/C absence could drive DUSP expression as a negative feedback mechanism. Upregulation of the cytoplasmic DUSP6 suggests a significant role of ERK in reducing GR sensitivity to mechanical strain.



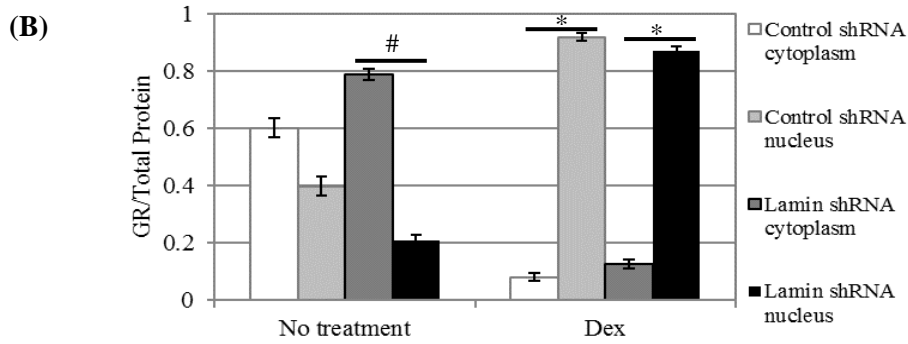
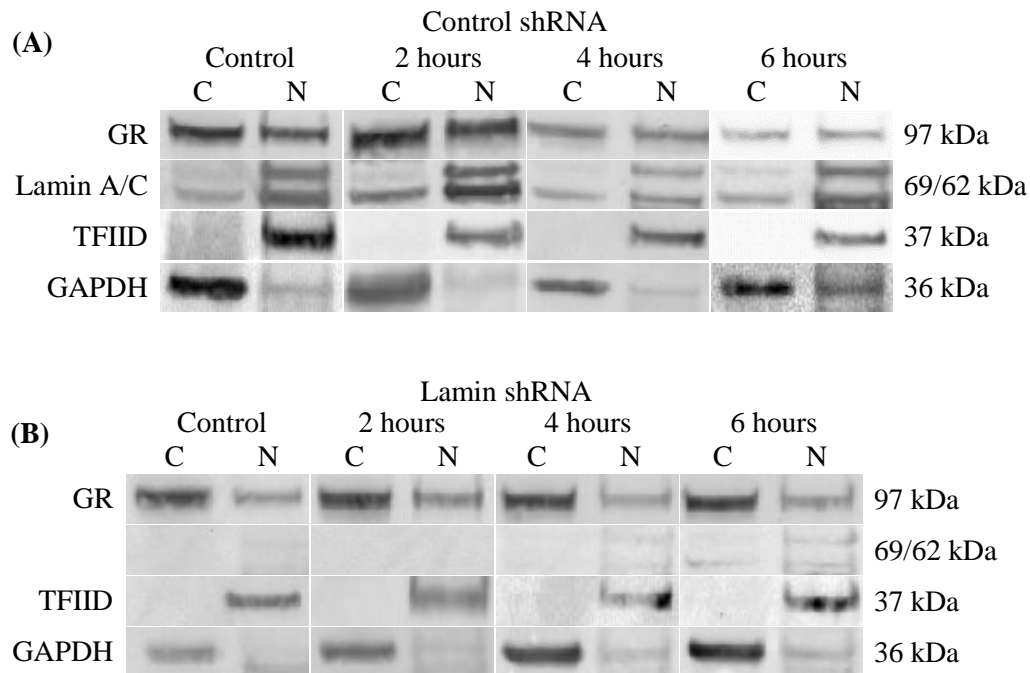


Figure 1: GR subcellular distribution in non-treated and dexamethasone (Dex)-treated control and lamin shRNA BAECs.

(A) Western blot of endogenous GR from control and lamin-deficient BAECs shows greater cytoplasmic (C) and less nuclear (N) concentration when lamin A/C is absent, but activation and subsequent nuclear translocation of GR by Dex leads to identical distribution between both cell types. Lamin A/C and TFIID served as the nuclear protein controls, and GAPDH was used as the cytoplasmic loading control. (B) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots ($n > 3$) reveals significant differences in GR distribution in lamin shRNA non-treated BAECs compared to control shRNA cells, as well as following Dex treatment in both cell types compared to non-treatment (* $P < 0.001$ compared to no treatment; # $P < 0.01$ compared to control shRNA no treatment).



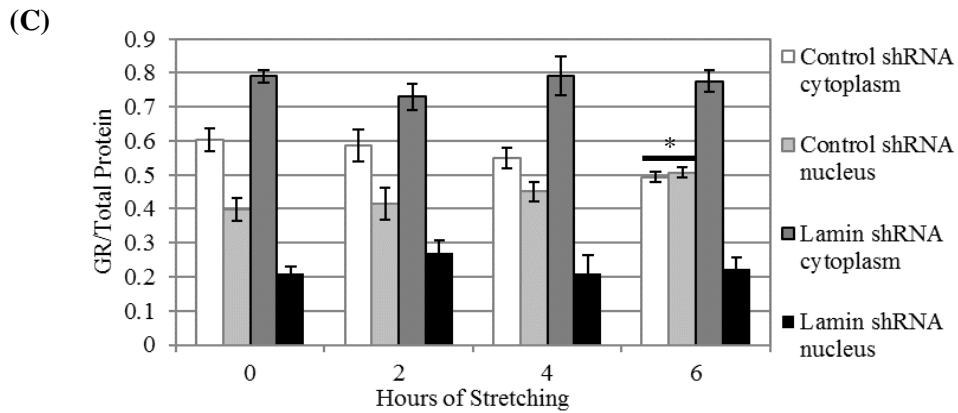
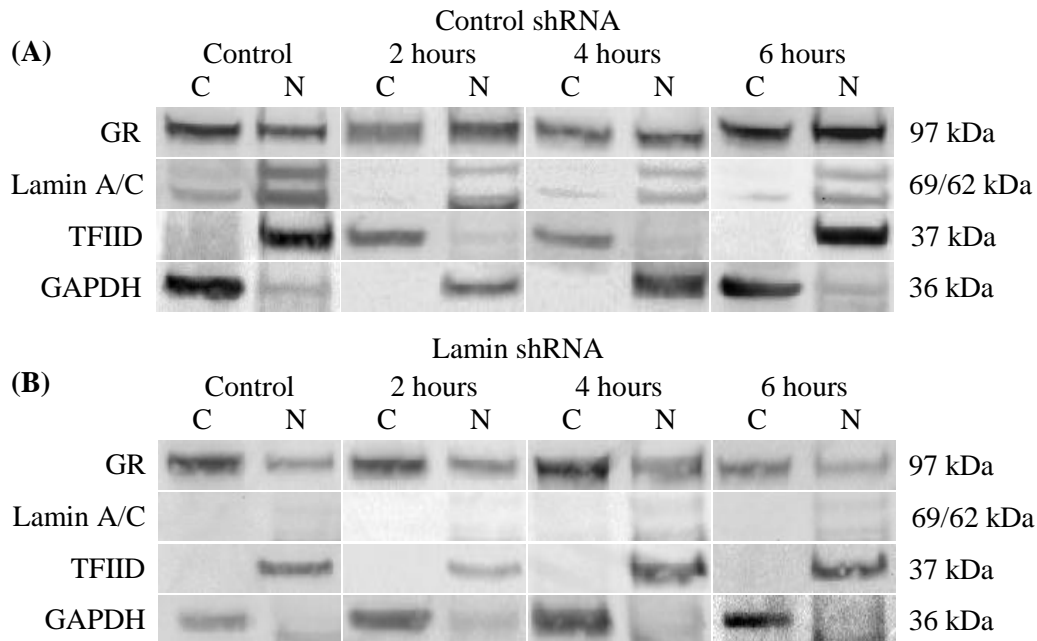


Figure 2: Physiological stretch induces GR nuclear translocation only under the presence of lamin A/C.

(A) Western blot of endogenous GR from control shRNA BAECs subject to zero, two, four, and six hours of physiological stretch (10% 1 Hz) shows gradual translocation of GR from the cytoplasm (C) fraction into the nuclear (N) fraction. (B) Western blot of GR from lamin shRNA BAECs exposed to physiological stretch shows no changes in distribution compared to control condition. Lamin A/C and TFIID served as the nuclear protein controls, and GAPDH was used as the cytoplasmic loading control. (C) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots ($n > 3$) show a significant difference in GR distribution in control shRNA BAECs after six hours of stretching. GR in lamin-deficient BAECs showed no significant changes in distribution at any stretching duration (* $P < 0.05$ compared to control).



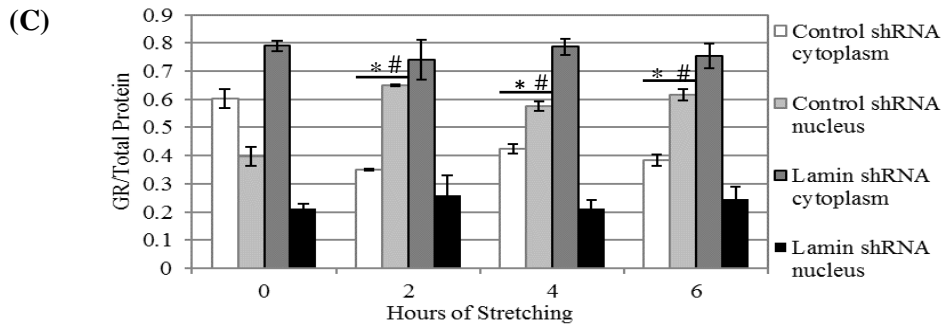


Figure 4: Increased stretching frequency affects GR subcellular distribution only when lamin A/C is present.

(A) Western blot of endogenous GR from control shRNA BAECs exposed to pathological stretch at a higher frequency (10% 2 Hz) shows greater concentration within the nuclear (N) compared to cytoplasmic (C) fraction at two, four, and six hours. (B) Western blot of GR from lamin shRNA BAECs suggests no effect of stretching on inducing changes in subcellular distribution. Lamin A/C and TFIID served as the nuclear protein controls, and GAPDH was used as the cytoplasmic loading control. (C) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots ($n > 3$) reveals a significant shift in GR distribution in control shRNA BAECs at all stretching durations, as well as a significant difference compared to physiological stretching. High frequency strain did not induce any significant shift in GR distribution in lamin-deficient BAECs (* $P < 0.01$ compared to control; # $P < 0.01$ compared to physiological stretch).

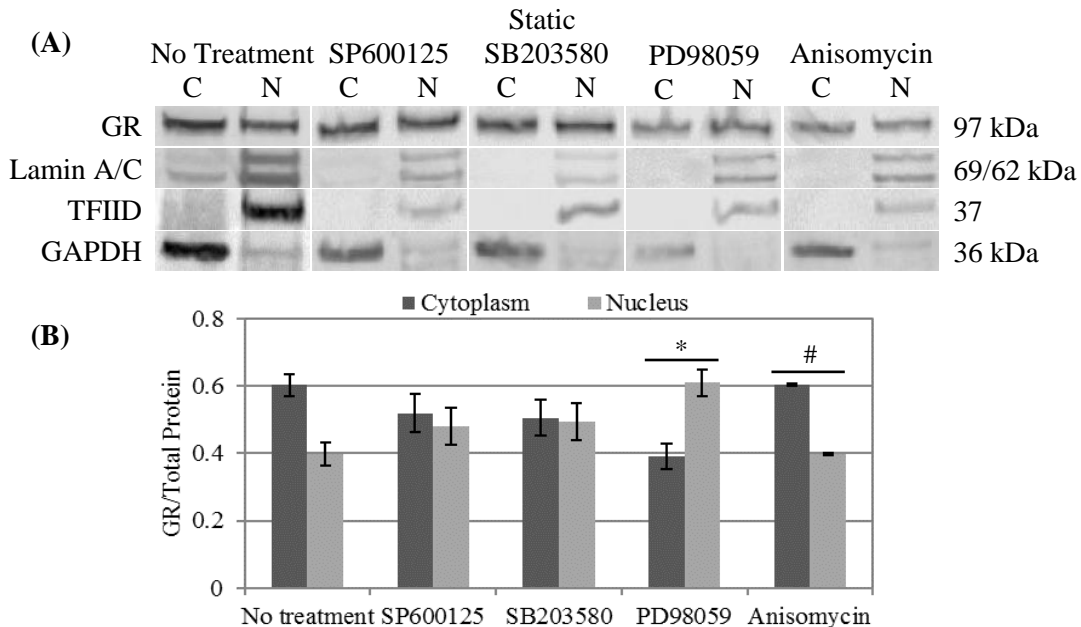
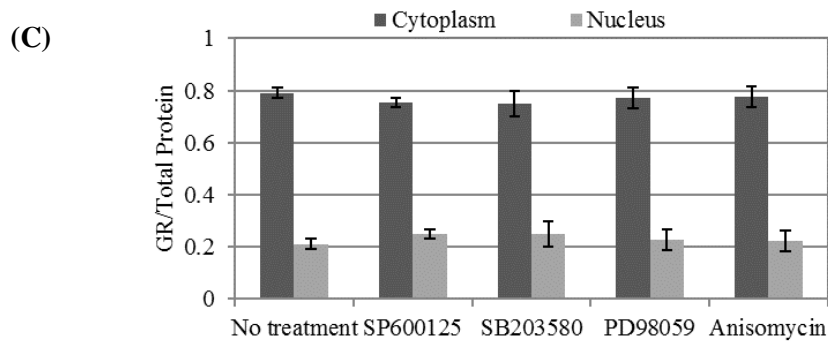
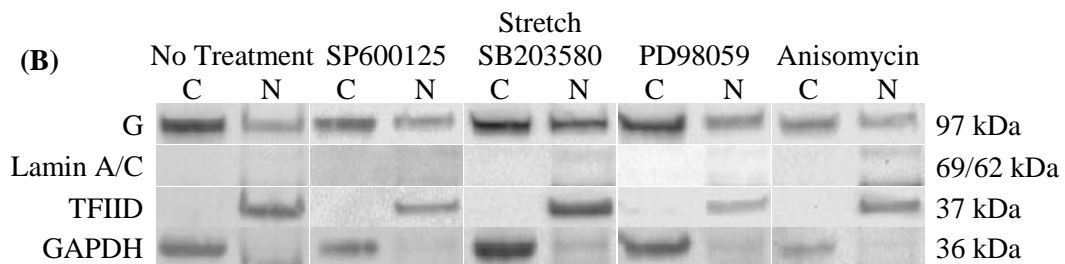
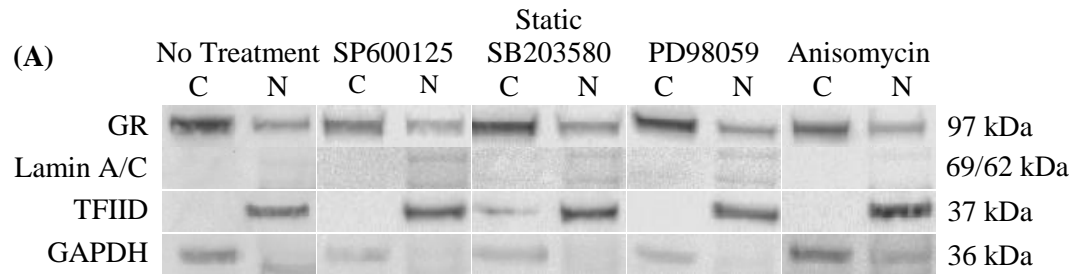


Figure 5: JNK or ERK inhibition significantly alters GR distribution under the presence of static and/or pathological cyclic stretch conditions in control shRNA BAECs.

(A) Western blot of endogenous GR from static cells treated with 10 μ M SP600125, 5 μ M SB203580, 50 μ M PD98059, or 250 ng/mL anisomycin for six hours shows a nuclear (N) shift from the cytoplasmic (C) fraction only when treated with PD98059. Lamin A/C and TFIID served as the nuclear protein controls, and GAPDH was used as the cytoplasmic loading control. (B) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots of static cells ($n > 3$) further supports a significant shift in GR distribution in BAECs when treated with PD98059 but no other drug (* $P < 0.05$ compared to control; # $P < 0.05$ compared to PD98059). (C) Western blot of endogenous GR from stretched cells (10% 2 Hz) pretreated with drugs for one hour shows retention of GR within the cytoplasm, similar to non-treated stretched cells, with SP600125. PD98059 treatment induced greater nuclear translocation. (D) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots of stretched cells ($n > 3$) reveals a significant shift in GR distribution compared to non-treated cells when treated with SP600125, PD98059, or anisomycin (* $P < 0.05$ compared to no treatment; # $P < 0.05$ compared to SP600125; % $P < 0.05$ compared to SB203580).



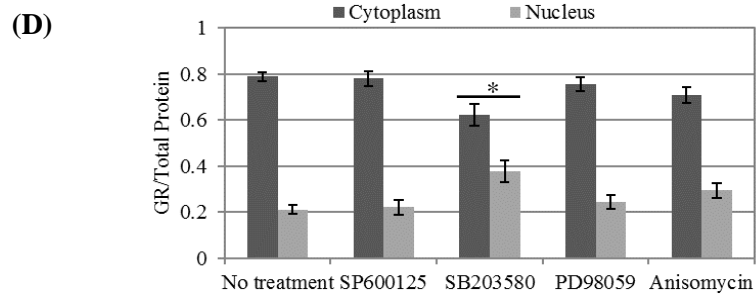


Figure 6: Inhibition of p38 with SB203580 induces movement of GR into the nucleus under pathological stretch in lamin-deficient BAECs.

(A) Western blot of endogenous GR from static cells treated with 10 μ M SP600125, 5 μ M SB203580, 50 μ M PD98059, or 250 ng/mL anisomycin for six hours reveals no changes in the cytoplasmic (C) and nuclear (N) fractions compared to non-treated cells. Lamin A/C and TFIIID served as the nuclear protein controls, and GAPDH was used as the cytoplasmic loading control. (B) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots of static cells ($n > 3$) shows no significant shifts in GR distribution following drug treatment. (C) Western blot of endogenous GR from stretched cells (10% 2 Hz) pretreated with drugs for one hour shows identical subcellular distributions across treatments, with SB203580 inhibition of p38 inducing a greater nuclear concentration compared to the others. (D) Quantitative analysis of cytoplasmic and nuclear GR fractions from western blots of stretched cells ($n > 3$) reveals a significant shift in GR distribution compared to non-treated cells when treated with SB203580 (* $P < 0.05$ compared to no treatment).

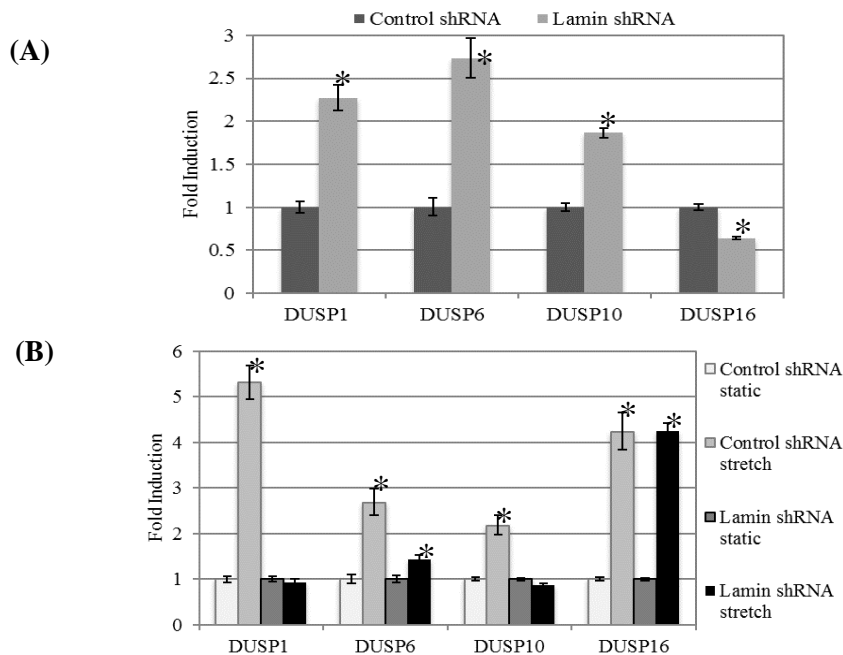


Figure 7: Expression of DUSP genes is significantly altered in lamin shRNA BAECs under both static and stretching conditions.

(A) Fold induction of DUSP1 (2.27 ± 0.15 , $n = 4$), DUSP6 (2.72 ± 0.23 , $n = 3$), and DUSP10 (1.87 ± 0.06 , $n = 3$) mRNA is significantly higher in static lamin-deficient BAECs compared to cells with intact lamin A/C, whereas DUSP16 expression (0.64 ± 0.02 , $n = 3$) was significantly less (* $P < 0.05$ compared to control shRNA). (B) In cells with lamin A/C, expression of all DUSP genes increased significantly after six hours of cyclic stretch at 10% strain and 2 Hz (5.31 ± 0.37 , 2.67 ± 0.29 , 2.17 ± 0.21 , and 4.22 ± 0.40 -fold for DUSP1, DUSP6, DUSP10, and DUSP16, respectively, $n = 4$ for each). In cells without lamin A/C, cyclic stretch induced a significant upregulation in expression compared to static in only the DUSP6 (1.42 ± 0.10 fold, $n = 4$) and DUSP16 (4.25 ± 0.16 fold, $n = 4$) genes, whereas DUSP1 (0.93 ± 0.08 fold, $n = 4$) and DUSP10 (0.86 ± 0.05 fold, $n = 4$) expression was not affected (* $P < 0.05$ compared to static).

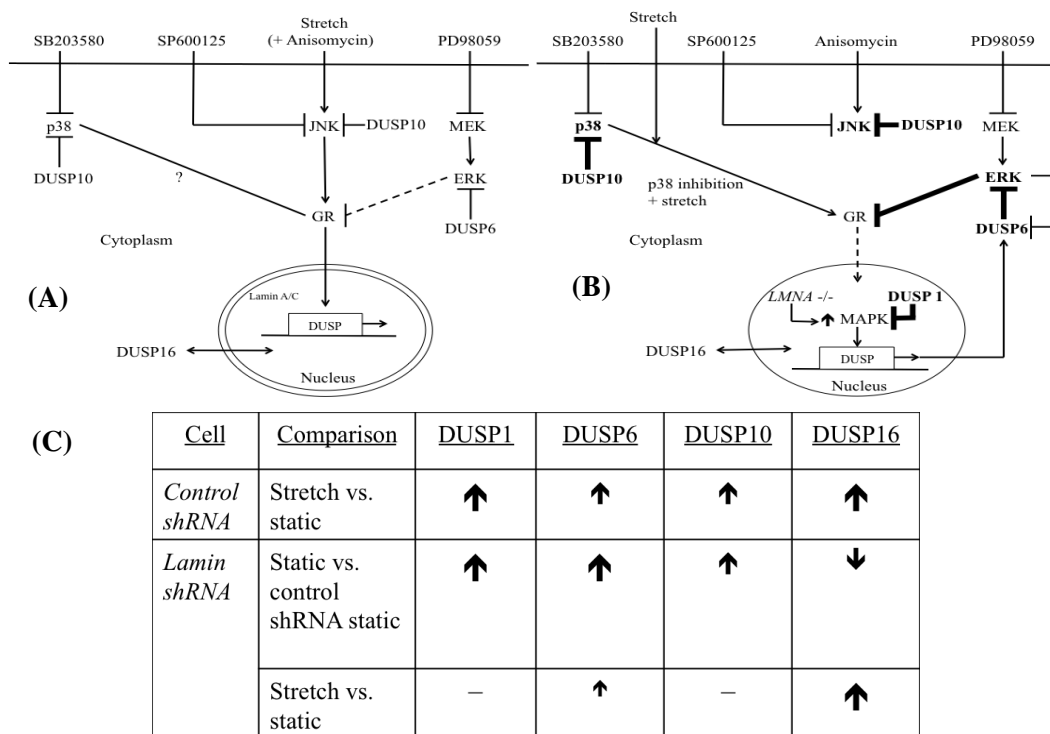


Figure 8: Proposed schematics of intracellular MAPK and DUSP effects on GR and summary of DUSP activity in control and lamin shRNA BAEC.

(A) In BAECs with intact lamin A/C, cyclic stretch induces activation of GR through the JNK pathway. ERK has some inhibitory effect on GR (- - -), and the addition of PD98059 overcomes this by inhibiting ERK phosphorylation. It is unclear whether p38 has any effect. DUSP proteins are expressed while stretching, likely a result of increased GR nuclear translocation that targets DUSP1 as well as increased MAPK activity that induces activation of other DUSPs. (B) In BAECs with lamin deficiency, MAPKs are hypothesized to be increasingly active and phosphorylated (bold). This drives the expression of DUSP proteins and their inhibitory, negative feedback effects on MAPKs (bold). Neither SP600125 nor PD98059 induced any effect on GR, but SB203580 in

combination with cyclic stretch induced marginal nuclear translocation (- - ->). We postulate that ERK may have a significant role in reducing GR sensitivity due to the high expression of DUSP6. (C) In control shRNA BAECs, DUSP expression increases after cells have been stretched for six hours (10% 2 Hz). In lamin shRNA static cells, DUSP expression is greater compared to control shRNA static cells, except for DUSP16, which is downregulated. In stretched lamin-deficient cells, DUS P6 had slightly greater expression compared to static, whereas DUSP16 increased much more.

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