The molecular regulation of GADD153 in apoptosis of cultured vascular smooth muscle cells by cyclic mechanical stretch

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Aims The expression of GADD153 (growth arrest and DNA damage-inducible gene 153), an apoptosis-regulated gene, increases during endoplasmic reticulum (ER) stress. How mechanical stretch affects the regulation of GADD153 in vascular smooth muscle cells (VSMCs) during apoptosis is not fully understood. We aimed to test the hypothesis that mechanical stretch induces GADD153 expression in VSMCs undergoing apoptosis.

Methods and results Rat VSMCs grown on a flexible membrane base were stretched by vacuum to 20% of maximum elongation, at 60 cycles/min. An in vivo model of aorta-caval shunt in adult rats was used to investigate GADD153 expression. Cyclic stretch significantly increased GADD153 protein and mRNA expression after 18 h of stretch. Addition of c-jun N-terminal kinase (JNK) inhibitor SP600125, JNK siRNA, tumour necrosis factor-α (TNF-α) and TNF-α receptor antibody 30 min before stretch inhibited the induction of GADD153 protein. Gel shift assay showed that DNA-binding activity of activating factor 1 (AP-1) increased after stretch. SP600125, JNK siRNA and TNF-α antibody abolished the binding activity induced by stretch. Stretch increased while GADD153-Mut plasmid, SP600125, and c-jun antibody abolished the promoter activity. Both conditioned media from stretched VSMCs and exogenous administration of TNF-α recombinant protein to the non-stretched VSMCs increased GADD153 protein expression similar to that seen after stretch. An in vivo model of aorta-caval shunt in adult rats also demonstrated the increased GADD153 protein expression in the aorta.

Conclusion Cyclic stretch enhanced GADD153 expression in cultured rat VSMCs. The stretch-induced GADD153 is mediated by TNF-α, at least in part, through the JNK and AP-1 pathway. These findings suggest that GADD153 plays a role in stretch-induced VSMC apoptosis.

1. Introduction

In recent years, vascular smooth muscle cells (VSMCs) apoptosis has been increasingly implicated in both development and outcome of atherosclerotic disease. Apoptosis of VSMCs is sufficient to induce features of plaque vulnerability in atherosclerosis. Apoptosis pathways include death initiated by ligation of membrane-bound death receptor, release of proapoptotic factors from mitochondria or stress at the endoplasmic reticulum (ER). Protein folding in the ER is impaired under various physical and pathological conditions, called ER stress. In order to overcome ER stress, the organelle has a specific signalling pathway termed the unfolded protein response (UPR). When the accumulation of protein aggregates is overwhelming or their clean-up somehow hampered, the stress cannot be resolved and the cell dies by apoptosis. One of the components of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP), also known as GADD153.

GADD153 plays a critical role in cell survival or cell death as part of the UPR. Previously, JNK/AP-1 was reported to contribute significantly to GADD153 gene transcriptional activity by oxidative stress. Moreover, GADD153 gene promoter activity is negatively regulated by nuclear factor-1 in VSMCs. GADD153 is expressed at undetectable levels in growing mammalian cells but is markedly increased by treatment with genotoxic agents, calcium ionophore, lipopolysaccharide, and nutrient deprivation. Microinjection of GADD153 protein into NIH3T3 cells induces G1/S arrest of the cell cycle.

The application of cyclic stretch to cultured VSMCs has been used as an in vitro experimental approach to study molecular events in response to mechanical overload. Cells in the cardiovascular system are continually subjected to mechanical forces due to changes in pressure and volume.

KEYWORDS
GADD153; Stretch; Smooth muscle cells; Apoptosis

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VSMCs are the major cellular components of the blood vessel wall and are subjected to a dynamic mechanical environment modulated by pulsatile pressure and oscillatory shear forces. The cyclic strain model system subjects cultured cells to repetitive stretch-relaxation at rates comparable to dynamic stretch overload in vivo. It has been reported that cyclic mechanical stretch-induced apoptosis in VSMCs. However, there is still much debate and controversy concerning the role of apoptosis in heart failure. There is a good evidence to show that GADD153 plays an important role in VSMCs apoptosis. However, there is no conclusive proof on how cyclic mechanical stretch affects the GADD153 on the apoptosis in VSMCs. Thus, in this study, we first investigated the mechanism and signal pathways of GADD153 by cyclic mechanical stretch and secondly, investigated the effect of GADD153 induced by cyclic stretch on the apoptosis in VSMCs.

2. Methods

2.1 Vascular smooth muscle cells culture

Primary cultures of vascular smooth muscle cells (VSMCs) were grown by the explant technique from the thoracic aorta of 200-250 g male Sprague-Dawley rats, as described previously. Cells were cultured in medium 199 containing 20% foetal calf serum, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 4 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5%CO2/95% air in a humidified incubator. When confluent, VSMC monolayers were passaged every 6–7 days after trypsination and were used for experiment from the third to sixth passages. These third to sixth passage cells were then cultured in Flexcell I flexible membrane dish in medium 199 containing 0.5% foetal calf serum, and the cells were incubated for a further 2 days to render them quiescent before the initiation of each experiment. The study conforms to Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was reviewed and approved by the Institutional Animal Care and Use Committee of the Shin Kong Wu Ho-Su Memorial Hospital.

2.2 In vitro cyclic stretch on cultured vascular smooth muscle cells

The strain unit Flexcell FX-2000 (Flexcell International Co., NC, USA) consists of a vacuum unit linked to a valve controlled by a computer program. VSMCs cultured on the flexible membrane base were subjected to cyclic stretch produced by this computer-controlled application of sinusoidal negative pressure as characterized and described in detail previously. A frequency of 1 Hz (60 cycles/min) was used for cyclic stretch.

2.3 Western blot analysis

Western blot was performed as previously described. Mouse monoclonal anti-GADD153 antibody (Santa Cruz Biotechnology, CA, USA) was used.

2.4 Reverse transcription polymerase chain reaction and northern blot analysis

Total RNA was isolated from VSMCs using the single-step acid guanidium thiocyanate/phenol/chloroform extraction method. The cDNA produced by reverse transcription (RT) was used to generate GADD153 probes by polymerase chain reaction (PCR) as described previously. The primers for GADD153 were 5'-CCCTCCACCCACATCG-3' and 5'-GCCACTCAGAGTCC-3'. The PCR products were run on 2% agarose gel for DNA fragment size verification, then eluted and served as a probe in the northern blot analysis. The northern blot was performed as described previously.

2.5 Electrophoretic mobility shift assay

Nuclear protein concentrations from cultured VSMCs were determined by Biorad protein assay. Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labelled by polynucleotide kinase incorporation of [γ-32P]dATP. Oligonucleotide sequences included the activating factor 1 (AP-1) consensus 5'-CGTTGATGACCTCACCAGGAA-3'. The AP-1 mutant oligonucleotides sequences were 5'-CGCTTGAAGGTGCAGGGAA-3'. Electrophoretic mobility shift assay was performed as described previously. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to compete with labelled sequence.

2.6 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (CHIP) assays were carried out with the CHIP kit (Upstate Biotech, Temecula, CA, USA). The assay was performed as the manufacturer's instruction. One-third of the cell lysate from stretched VSMCs was immunoprecipitated by anti-c-jun monoclonal antibody (Cell Signalling, Beverly, MA, USA) and then was analysed by PCR of the GADD153 promoter and the remaining two-thirds cell lysate were added with anti-acetylated histone H3 antibody and analysed by PCR of GADD153 promoter. The primers for the GADD153 promoter were 5'-CTTCCACCACCATCG-3' and 5'-GCCAT-CTTCCACCGGAC-3'. The primers for the GAPDH were 5'-CATCACCATCTTCCAGGAC-3' and 5'-GGATGATGTCTCGGCTGCC-3'.

2.7 RNA interference

VSMCs were transfected with 800 ng GADD153 annealed siRNA oligonucleotide or siRNA of JNK1 (Dharmacon, Lafayette, CO, USA). GADD153 siRNA is a target-specific 21 nt siRNA according to a computer program provided by Dharmacon. The GADD153 targeted base sequences were sense: 5'-GGUGAAGGAGAUCUGGAGAUU; antisense: 5'-UGUCUGCAGAUCUCUACUU. The sequence of the scramble siRNA for GADD153 were 5'-GGUGAAGGAGAUCUGGAGAUU; antisense: 5'-UGUCUGCAGAUCUCUACUU. The sequence of JNK1 siRNA and negative control, a non-targeting siRNA (control siRNA) were used as described previously.

2.8 Promoter activity assay

A -845 to +85 bp rat GADD153 promoter construct was generated as follows. Rat genomic DNA was amplified with forward primer, CTCAGAGGAGGGCA TAAGACGATCA and reverse primer, CCGCTTCTGAGTGGTTCTCGGT. The amplified product was digested with MluI and BgII restriction enzymes and ligated into pGL3-basic luciferase plasmid vector (Promega, Madison, WI, USA) digested with the same enzymes. The GADD153 promoter contains AP-1 conserved sites (TGACTCA) at -246 to -240 bp. For the mutant, the AP-1 binding sites were mutated using the mutagenesis kit (Stratagene, La Jolla, CA, USA). Site-specific mutations were confirmed by DNA sequencing. Plasmids were transfected into VSMCs using a low pressure-accelerated gene gun (Bioarray Technologies, Taipei, Taiwan) essentially following the protocol from the manufacturer. In brief, 2 μg of plasmid DNA was suspended in 5 μL of PBS and was delivered to the cultured VSMCs at a helium pressure of 15 psi. The transfection efficiency using this method is 30%. Following 12 h of cyclic stretching, cell extracts were prepared using Dual-Luciferase Reporter Assay System (Promega) and measured for dual luciferase activity by luminometer (Turner Designs, Sunnyvale, CA, USA).
2.9 Measurement of TNF-α concentration by enzyme-linked immunosorbent assay
Conditioned medium from VSMCs subjected to cyclic stretch and those from control (unstretched) cells were collected for TNF-α measurement. The level of TNF-α was measured by a quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, MN, USA). The lowest limit of TNF-α ELISA kit was 52 pg/mL.

2.10 Real-time PCR
The real-time PCR was performed as described previously.22 The primers used were as follows: TNF-α, 5′-d(CCGAGTGGCTGACAAAC)-3′ (forward) and 5′-d(CGGACTCCGTGATGTC)-3′ (reverse); GAPDH, 5′-d(CATCACCATCCTCA GGAGC) (forward) and 5′-d(GGATGATGTCTGGGTGCTGCC)-3′ (reverse).

2.11 Cytotoxicity study
Cytotoxicity study was performed as described previously.20 For detection of cell injury induced by stretch, cell viability after application of cyclic stretch was monitored by trypan blue staining.

2.12 Flow cytometric analysis for apoptotic quantitation
Apoptotic cells were quantified as the percentage of cells with hypodiploid DNA (sub-G1). VSMCs were fixed with 70% ethanol and treated with RNase. Then nuclei were stained with propidium iodide. The DNA content was measured by using a FACSCalibur flow cytometer and Cell Quest software (Becton–Dickinson, Franklin Lakes, NJ, USA). For all assays, 10 000 cells were counted.

2.13 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay
DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, CA, USA). At the end of cyclic stretch, VSMCs were fixed in 4% paraformaldehyde for 10 min followed by a staining procedure according to the manufacturer's protocol.

2.14 Rat model of aorta-caval shunt
The aorta-caval shunt was produced as previously described.24 In brief, the aorta was punctured at the union of the segment two-thirds caudal to the left renal artery and one-third cephalic to the aortic bifurcation, with an 18-gauge disposable needle held with a plastic syringe. The induced aorta-caval shunt caused a ratio of 1.7 of pulmonary flow to systemic flow. Sham-operated control animals were prepared in a similar manner, except that the aorta was not punctured.

2.15 Statistical analysis
All results were expressed as means ± SEM. Statistical significance was evaluated using of variance (GraphPad Software Inc., San Diego, CA, USA). The Dunnett’s test was used to compare multiple groups to a single control group. Tukey-Kramer comparison was used for pairwise comparisons between multiple groups after the ANOVA. A value of P < 0.05 was considered as significant. A value of P < 0.05 was considered to denote statistical significance.

3. Results

3.1 Cyclic stretch enhances GADD153 protein and mRNA expression in vascular smooth muscle cells
The level of GADD153 protein began to increase as early as 6 h after stretch to 20% elongation was applied, reached a maximum of 4.5-fold over the control by 18 h, remained elevated up to 24 h and tended to decline at 48 h. When VSMCs were stretched at 10% elongation, the level of GADD153 protein was similar to that of control without stretch (Figure 1A and B). The northern blots showed that GADD153 messages increased maximally after 18 h of stretch at 20% elongation (Figure 1C and D). These results indicated that cyclic stretch-induced GADD153 expression in VSMCs.

3.2 Stretch-induced GADD153 protein expression in vascular smooth muscle cells is mediated by JNK
To investigate the possible signal pathway mediating the stretch-induced GADD153 in VSMCs, the VSMCs were stretched 20% for 18 h in the presence and absence of inhibitors or siRNA. As shown in Figure 2, the stretch-induced increases of GADD153 proteins were significantly blocked...
after the addition of SP600125 (20 μM) 30 min before stretch. The GADD153 proteins induced by stretch were not affected by the addition of PD98059 (50 μM), but partially blocked by the addition of SB203580 (3 μM) and N-acetylcysteine (500 μM). SP600125 is a potent, cell-permeable, selective, and reversible inhibitor of JNK. PD98059 is a specific and potent inhibitor of ERK kinase. SB203580 is a highly specific, cell permeable inhibitor of p38 kinase. N-Acetylcysteine is a free radical scavenger.

To test the specific effect of JNK MAP kinase pathway mediating the expression of GADD153, JNK siRNA was transfected to VSMCs before cyclic stretch. Moreover, JNK siRNA also completely blocked the GADD153 expression induced by stretch. JNK siRNA knocked down the expression of total JNK protein (Figure 2C). The DMSO alone as a vehicle control and control siRNA did not affect the GADD153 expression induced by cyclic stretch. All the inhibitors used on non-stretched cells did not change the GADD153 protein expression (Figure 2D). These findings implicated that JNK pathway, but not p42/p44 MAP kinases, mediated the induction of GADD153 proteins by stretch in VSMCs. The conditioned medium from stretched VSMCs could induce the same increase in GADD153 protein expression in non-stretched VSMCs. These findings suggested that cyclic stretch regulated GADD153 protein in VSMCs possibly via autocrine or paracrine mechanisms.

**Figure 2** Effects of MAPK inhibitors on GADD153 protein expression induced by cyclic stretch in vascular smooth muscle cells (VSMCs). (A) Representative western blots for GADD153 protein levels in VSMCs subjected to cyclic stretch in the absence or presence of inhibitors, siRNA, and vehicle (DMSO 0.1%). CM, conditioned medium; NAC, N-acetylcysteine. (B) Quantitative analysis of GADD153 protein levels. The values from stretched VSMCs have been normalized to values in control cells (n = 3 per group). *P < 0.01 vs. control. (C) JNK siRNA knocked down total JNK protein expression (n = 3). (D) GADD153 protein expression in non-stretched cells treated with different inhibitors (n = 3).
3.3 Cyclic stretch increases AP-1 binding activity

Cyclic stretch of VSMCs for 6–18 h significantly increased the DNA-protein binding activity of AP-1 (Figure 3A). An excess of unlabelled AP-1 oligonucleotide competed with the probe for binding AP-1 protein, whereas an oligonucleotide containing a 2 bp substitution in the AP-1 binding site did not compete for binding. Addition of SP600125 and TNF-α antibody (5 μg/mL, purchased from R&D Systems) 30 min before stretch abolished the DNA-protein binding activity induced by cyclic stretch. JNK siRNA, similar to SP600125, also abolished the DNA-protein binding activity induced by stretch (Figure 3B). Exogenous administration of TNF-α to the VSMCs without stretch increased the AP-1-DNA binding activity. Addition of c-jun antibody shifted the DNA-protein binding site. Moreover, CHIP assay confirmed that stretch enhanced AP-1 binding activity in VSMCs (Figure 3C). These results demonstrated that stretch enhanced AP-1 binding activity in VSMCs.

3.4 Cyclic stretch increases GADD153 promoter activity through AP-1

To study whether the GADD153 expression induced by stretch is regulated at the transcriptional level, we cloned the promoter region of rat GADD153 (−845 to +85) and constructed a luciferase reporter plasmid (pGL3-Luc). The GADD153 promoter construct contains SP-1, NF-IL6, NF1 and AP-1 binding sites. As shown in Figure 4, transient transfection experiment in VSMC using this reporter gene revealed that stretch for 12 h significantly caused GADD153 promoter activation. This result indicated that GADD153 expression is induced at transcriptional level during cyclic stretch in VSMCs.

When the AP-1 binding sites were mutated, the increased promoter activity induced by stretch was abolished. Moreover, addition of c-jun antibody and SP600125 caused an inhibition of transcription. These results suggested that AP-1 binding site in the GADD153 promoter is essential for the transcriptional regulation by cyclic stretch.

3.5 Cyclic stretch stimulates secretion of TNF-α from vascular smooth muscle cells

As shown in Figure 5A, cyclic stretch significantly began to increase the TNF-α secretion from VSMCs at 6 h after stretch and reached a maximum at 12 h and remained elevated for 24 h. Cyclic stretch also significantly enhanced the expression of TNF-α mRNA (Figure 5B). These results indicated that stretch causes secretion of TNF-α for VSMCs.

3.6 Exogenous TNF-α increases GADD153 protein expression

To investigate the direct effect of TNF-α on GADD153 expression in VSMCs, TNF-α at different concentrations was administrated to the cultured medium for 18 h. As shown in Figure 6, the effect of TNF-α on GADD153
protein expression was dose-dependent. Addition of TNF-α and TNF-α receptor monoclonal antibody 30 min before stretch significantly blocked the expression of GADD153 induced by cyclic stretch. These findings suggested that TNF-α enhances GADD153 expression by cyclic stretch.

3.7 Cyclic stretch-induced apoptosis is mediated by GADD153 in vascular smooth muscle cells

As shown in Figure 7A and B, cyclic stretch not only enhanced the death rate but also decreased the viability of VSMCs measured by a cell counter and MTT assay. This result suggested that cyclic stretch-induced cell death of VSMCs.

As shown in Figure 7C, apoptosis was assessed by PI staining and FACS analysis. The percentage of cells in the sub-G1 fraction stained with PI was elevated after stretch for 18 h and addition of TNF-α. The TUNEL assay was used to confirm the presence of apoptotic nuclei after cyclic stretch (Figure 7D). A significant increase in TUNEL-positive nuclei was present after stretch for 18 h and addition of TNF-α. These increases of apoptosis in VSMCs induced by stretch were significantly reversed by GADD153 siRNA and transient transfection of GADD153-Mut plasmid. These findings demonstrated that GADD153 mediates stretch-induced apoptosis of VSMCs. To further investigate the mechanism of GADD153-induced cell death in stretched VSMCs, caspase activity was measured by colorimetric activity assay kit (Chemicon International). As shown in Supplementary material online, Figure S1, mechanical stretch increased caspases 3 and 8 activity. GADD153 siRNA and mutant GADD153 abolished the increase of caspase 3 induced by stretch but not caspase 8. TNF-α alone also induced caspases 3 and 8. These findings indicate that caspase 3 is involved in the GADD153-induced apoptosis of VSMCs under mechanical stretch.

3.8 In vivo aorta-caval shunt increases aortic GADD153 protein expression

Aorta-caval shunt was performed to explore whether GADD153 expression was increased under volume-overload in vivo. As shown in Figure 8, the GADD153 protein expression in rat aorta significantly increased at 5 day after induction of aorta-caval shunt. It reached a maximum of 4.3-fold over the sham and remained elevated up to 7 days. The in vivo aorta-caval shunt also induced caspase 3 activity and increased apoptosis by TUNNEL.
assay (see Supplementary material online, Figure S2) in the aorta. Treatment with SP600125 abolished the induction of caspase 3 and apoptosis.

4. Discussion

In this study, we demonstrated several significant findings. First, cyclic stretch upregulates GADD153 expression in rat VSMCs; secondly, cyclic stretch induces TNF-α expression in VSMCs; thirdly, TNF-α acts as an autocrine factor to mediate the increased GADD153 expression induced by cyclic stretch; Fourthly, JNK MAP kinase and AP-1 transcription factor are involved in the signalling pathway of GADD153 induction; fifthly, cyclic stretch induces VSMCs apoptosis via GADD153; and sixthly, in vivo acute haemodynamic overload increases aortic GADD153 expression. GADD153 was upregulated in both a time- and load-dependent manner by cyclic stretch. Cyclic stretch of VSMCs increased both GADD153 protein and mRNA expression.

In our study, exogenous addition of TNF-α to non-stretched VSMCs is sufficient to induce a similar GADD153 protein expression as observed in stretched VSMCs. These results provide the first evidence that TNF-α mediates cyclic stretch-induced expression of GADD153 in VSMCs. Our study revealed that TNF-α acts as an autocrine mediator in response to cyclic stretch in VSMCs. Previously, we have demonstrated that cyclic stretch-induced TNF-α secretion and mRNA expression in HUVECs. In this study, we found that cyclic stretch also enhanced TNF-α expression in VSMCs. However, another study showed no increased concentration of TNF-α in the medium collected from pulmonary VSMCs at 24 and 48 h after 25% stretch. Sotoudeh et al. used pulmonary VSMCs, whereas our study used rat aortic VSMCs. Different species, stretch intensity and stretch time may explain the discrepancy.

Our results suggested that TNF-α is responsible for AP-1-DNA binding in VSMCs. In this study, we demonstrated that cyclic stretch stimulation of AP-1-DNA binding activity required at least phosphorylation of JNK since JNK inhibitor and JNK siRNA abolished the AP-1 binding activity. SP600125, a potent and specific inhibitor of JNK MAP kinase, inhibited the GADD153 expression induced by stretch. SB203580, a potent and specific inhibitor of p38,
had partial inhibitory effect, whereas inhibitors of p42/p44 MAP kinase did not have the inhibitory effect. JNK and MAP kinases are important intracellular signalling pathways that regulate GADD153.26,27 We also demonstrated that the JNK1 siRNA significantly inhibited GADD153 expression induced by stretch. AP-1, a well-characterized downstream target of JNK is needed for GADD153 promoter activity in rat VSMCs.28 Oxidative stress resulting in the induction of GADD153 expression is mediated by an AP-1 site in GADD153 promoter.28,29 N-Acetylcysteine had partial inhibitory effect on the stretch-induced GADD153 expression. Since N-acetylcysteine is a free radical scavenger and mechanical stretch is known to affect the production of reactive oxygen species, the findings in our study may indicate a potential role of reactive oxygen species in the induction of GADD153 by mechanical stretch. In this study, we demonstrated that increased transcriptional activity of GADD153 promoter by cyclic stretch was AP-1 dependent via reporter gene assay. These data implicated that the JNK MAP kinase pathway, but not the p42/p44 MAP kinase pathway, is the potential role of reactive oxygen species in the induction of GADD153 protein expression after acute haemodynamic overload as in aorta-caval shunt. It has been reported that GADD153 protein expression increased in the carotid artery balloon injury model in rats.34 It suggested that GADD153 may be enhanced during acute haemodynamic overload in vivo. The increased GADD153 protein expression after acute haemodynamic overload may contribute to the regulation of vascular repair and remodelling, which involves VSMC proliferation and apoptosis.

In summary, our study reports for the first time that cyclic mechanical stretch enhances GADD153 expression in cultured rat VSMCs. The stretch-induced GADD153 is mediated by TNF-α, at least in part, through JNK MAP kinase and AP-1 pathway.

Supplementary material
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References
Regulation of GADD153 by stretch


