Mechanical stretch via transforming growth factor-beta1 activates microRNA-208a to regulate hypertrophy in cultured rat cardiac myocytes

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Abstract
Purpose: MicroRNAs (miRs) and mechanical stress play a key role in cardiac hypertrophy. MiR208a is essential for expression of the genes involved in cardiac hypertrophic growth. The relationship between miR208a and mechanical stress in cultured cardiomyocytes has not been investigated yet. The molecular mechanisms underlying miR208a involved hypertrophy of cardiomyocytes by mechanical stress is poorly understood. We sought to investigate whether miR208a is a critical regulator in cardiomyocyte hypertrophy under mechanical stretch.
**Methods:** Neonatal rat cardiomyocytes grown on a flexible membrane base were stretched via vacuum to 20% of maximum elongation at 60 cycles/min. TaqMan® microRNA real-time quantitative assay was used to quantitate microRNA expression. Western blot was used to measure hypertrophic protein expression. Quantitative sandwich enzyme immunoassay was used to measure transforming growth factor-beta1 (TGF-β1) in the culture medium. Protein synthesis of cultured cardiomyocytes was measured by 3H-proline incorporation assay.

**Results:** Mechanical stretch significantly enhanced miR208a expression after 4 h of stretch. Stretch significantly induced cardiomyocyte hypertrophic protein expression such as β myosin heavy chain (MHCβ), thyroid hormone receptor-associated protein 1, myostatin, connexin 40, GATA4 and brain natriuretic peptide. MHCα was not induced by stretch. Overexpression of miR208a significantly increased MHCβ protein expression while Pretreatment with antagomir208a significantly attenuated the MHCβ protein expression induced by stretch and overexpression of miR208a. Mechanical stretch significantly increased the secretion of TGF-β1 from cultured cardiomyocytes. Exogenous addition of TGF-β1 recombinant protein significantly increased miR208a expression and pretreatment with TGF-β1 antibody attenuated the miR208a expression induced by stretch. Mechanical stretch and overexpression of miR208a increased protein synthesis while antagomir208a attenuated the protein synthesis induced by stretch and overexpression of miR208a.
Figure 1 legend

Mechanical stretch induces the expression of miR208a in cultured cardiomyocytes.


B. TaqMan microRNA real-time quantitative PCR assays (Applied Biosystems) for miR208a, miR208b and miR133 in cardiomyocytes subjected to stretch 20% for various periods of time (n=3 per group). All fold changes between samples were determined using the ΔΔCT method. *P<0.001 vs. control.
Fig. 1

miRNA relative expression level (fold of control)

A-1

A-2

Base pairs

B

miRNA relative expression level (fold of control)

- miR208a
- miR208b
- miR133

Stretch 20%

control 2hr 4hr 6hr 8hr
Figure 2 legend

Mechanical stretch enhances the protein level of MHCβ, TRAP100, BNP, GATA-4 and connexin 40 in cultured cardiomyocytes in a time-dependent manner except MHCα.

(A) Representative western blot for target proteins in cardiomyocytes after stretch for various periods of time.

(B) Quantitative analysis of target proteins. The values from stretched cardiomyocytes have been normalized to matched α-tubulin (n=3 per group). *P<0.001 vs. control. **P<0.01 vs. control.
Figure 3 legend

Construct of miR208a and miR208 antagomir expression vector.

(A) The 165bp amplified miR208a precursor was digested with EcoRI and BamHI restriction enzymes and ligated into pmR-ZsGreen1 plasmid vector (coexpression miR208a and green fluorescent protein, Clontech) digested with the same enzymes.

(B) MiR208a antagomir precursor construct was generated in pmR-ZsGreen1 plasmid vector.
Fig. 3

A.png

B.png
MiR208a acts as a regulatory factor in mediating stretch-induced MHCβ expression in cardiomyocytes, but not at MHCα, TRAP100, BNP, GATA-4 and connexin 40.

(A) Representative western blots for the level of targets protein in cardiomyocytes stretch alone, in the presence of overexpression of miR208a wild and miR208a mutant type, stretch plus antogomir208a or miR208a wild type plus antogomir208a.

(B) Quantitative analysis of target protein levels. The values from stretched cardiomyocytes have been normalized to values in control cells (n=3 per group). *P<0.001 vs. control. §P<0.01 vs. stretch. +P<0.01 vs. miR-208a.
### A

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<th>Protein</th>
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<th>Stretch</th>
<th>mir-208a</th>
<th>Mut-208a</th>
<th>Stretch +Antagomir-208a</th>
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</table>

### B

![Bar chart showing protein levels](chart.png)

Fig. 4
Figure 5 legend

TGF-β1 mediates the expression of miR208 by mechanical stretch in cultured cardiomyocytes.

A. TaqMan microRNA real-time quantitative PCR assays (Applied Biosystems) for miR208a in cardiomyocytes subjected to stretch 20% for 4h in the presence of TGF-β1 (5 μg/ml), TNF-α (5 μg/ml) or Ang II antibody (5 μg/ml) or exogenous addition of TGF-β1 (200 pg/mL), TNF-α (500 pg/mL) or Ang II (10 ng/mL). *P<0.001 vs. control. +P<0.01 vs. stretch. (N=4 per group).

B. Mechanical stretch increases release of TGF-β1 from cultured cardiomyocytes subjected to stretch at 20 % elongation for various periods of time. Cultured cardiomyocytes were stretched for various periods of time and the cultured medium was collected for measurement of TGF-β1 via ELISA. *P<0.001 vs. control. **P<0.01 vs. control. (n=3 per group).
**Fig. 5**

**A**

Graph showing the relative expression level of Mir208a under different conditions. Treatments include Control, TGF-β1 4hr, Ang II 4hr, TNF-α 4hr, Stretch, TGF-β1 Ab, Ang II Ab, TNF-α Ab, and Control IgG. The y-axis represents the relative expression level (fold of control), and the x-axis lists the treatment groups. Symbols * and + indicate statistical significance.

**B**

Graph showing the concentration of TGF-β1 (pg/ml) over time (1-24 hours) with a stretch of 20%. The y-axis represents TGF-β1 concentration, and the x-axis represents time in hours. Significant differences are indicated by * and **.
Mechanical stretch increases protein synthesis in cultured cardiomyocytes. Quantitative analysis of protein synthesis of cultured cardiomyocytes was measured by $^3$H-proline incorporation assay. (n=4 per group). *P<0.001 vs. control.
+P<0.01 vs. stretch 16 h. ++P<0.01 vs. miR208a.
Fig. 6
Conclusions

Cyclic mechanical stretch enhances miR208a expression in cultured rat cardiomyocytes. MiR208a plays a role in stretch-induced cardiac hypertrophy. The stretch-induced miR208a is mediated by TGF-β1.