STAT3 inhibits Myocardin induced cardiac hypertrophy

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28 Abstract

29 **Background:** In order to explore the molecular mechanism of cardiomyocyte-dependent
30 myocardial gene expression and cardiomyocyte differentiation in cardiac hypertrophy, and to
31 provide new insights for cardiac hypertrophy.

32 **Methods:** Cardiac myocytes were isolated from day 1-3 Sprague-Dawley rat pups.
33 Real time quantitative PCR, western blot and immunocytochemistry Assay were
34 used to detect the expression and localization of related genes. CO-IP was used to
35 detect direct protein interactions between Myocardin and STAT3. Luciferase reporter
36 assay and chromatin immunoprecipitation were used to detect the binding of
37 Myocardin to the promoter of a downstream target gene. Microinjection of zebrafish
38 embryos was used to examine the effects of STAT3 and Myocardin interactions on
39 cardiac development in vivo

40 **Results:** The N-terminus of STAT3 directly binds to the basic domain of myocardin
41 and inhibits the transcriptional activity of Myocardin-mediated cardiac-specific
42 genes ANF and α -actinin, thereby inhibiting their expression, and further inhibit
43 myocardin-mediated cardiac hypertrophy in vivo.

44 **Conclusions:** In summary, our report states that signal transduction and transcriptional
45 activation factor 3 (STAT3) are inhibitors of the major cardiac hypertrophic
46 transcription factor Myocardiin, which is required for cardiomyocyte differentiation.
47 The STAT3-cardiacin interaction identified nuclear hormone receptor-mediated and
48 cardiac-specific gene-regulated convergence sites and suggested a possible
49 mechanism for cardioprotective effects.

50 **Key words:** STAT3, Myocardin, Cardiac hypertrophy, Transcriptional regulation

51 Background

52 Myocardin play a key role in cardiovascular development and are specifically
53 expressed in the heart and smooth muscle cells. (1) In addition, Myocardiin is a
54 co-activator of serum response factor (SRF). SRF can be combined with the CArG
55 box (CC [A/T] ϵ GG), which is a DNA consensus sequence located in the control
56 region of many growth factor regulated genes and muscle-specific genes.(2)
57 Consistent with the role of Myocardiin as a transducer of hypertrophy signals, the
58 forced expression of Myocardin in cardiomyocytes is sufficient to replace the
59 hypertrophy signal and induce cardiac hypertrophy and fetal cardiac genetic
60 programs.(3) In a word, Myocardin is a key component of molecular switches that
61 regulate SRF's ability to mediate cell proliferation and muscle cell
62 differentiation.(4-6)

63 Signal transducer and activator of transcription (STAT) 3 is involved in cell survival,

64 proliferation, and immune response.(7) STAT3 has been reported as a key mediator
65 of cardiac remodeling in response to cytokines, especially glycoproteins (gp) 130
66 family, including cytokines of interleukin 6 (IL-6) and leukemia inhibitory factor
67 (LIF).(8-10)

68 IL-6 binding results in receptor dimerization, which induces tyrosine
69 phosphorylation and STAT3 activation through Janus-activated kinases. Activated
70 STAT3 is then dimerized and transported to the nucleus to activate or inhibit
71 downstream target gene expression. STAT3, as a key mediator of myocardial cell
72 survival, seems to play an essential role in inducing myocardial hypertrophy. So far,
73 there were not some researches about the effect of STAT3 in Myocardin-induced
74 cardiomyocyte hypertrophy. This study reveals a relationship between Myocardin
75 and STAT3 which are crucial transcription factors in cardiomyocyte hypertrophy
76 development.

77 **Methods**

78 **Cell culture**

79 Cardiac myocytes were isolated from day 1-3 Sprague-Dawley rat pups as described
80 previously.(11-13) COS7 cells were bought from American Type Culture Collection
81 (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen)
82 supplemented 10% fetal bovine serum (FBS) in the incubator with a humidified
83 atmosphere (5% CO₂, 37°C).

84 **Plasmid construction and transfection**

85 To overexpress Myocardin and STAT3, nucleotide fragments encoding the fusion Flag
86 tag protein and full-length of Myocardin and encoding the fusion Myc tag protein and
87 full-length of STAT3 were cloned to mammalian expression vector pCDNA3.1
88 (Invitrogen) (Flag-pCDNA3.1-Myocardin, Myc-pCDNA3.1-STAT3). Chemically
89 synthesized small interfering RNA (Si-STAT3) and scrambled negative control (NC)
90 Si-NC (Ribobio) to knockdown STAT3 expression. ANF-1000-luciferase reporter,
91 ANF-337-luciferase reporter, ANF-236-luciferase reporter and ANF-121-luciferase
92 reporter plasmid, ANF-1000-M-far-CArG, ANF-1000-M-near-CArG,
93 ANF-1000-M-GAS, ANF-1000-M-far-CArG and GAS, ANF-1000-M-GAS and
94 near-CArG and ANF-1000-M-far-CArG and near-CArG-luciferase reporter, were all
95 constructed into pGL3-basic vector (Promega). All plasmid constructs were
96 transfected into COS-7 cells or cardiomyocytes with FuGENE® HD, and the
97 experimental procedures were strictly in accordance with the manufacturer's
98 instructions.

99 **Immunocytochemistry Assay**

100 Immunofluorescence assays were performed as described previously.(14) After added
101 the primary antibodies (goat anti-rabbit ANF (Abcam), goat anti-mouse α -actinin
102 (Sigma), and then added appropriate secondary antibodies (FITC-goat anti-rabbit IgG,
103 FITC-goat anti-mouse IgG Santa Cruz), respectively. DAPI
104 (4',6'-diamidino-2-phenylindole) was used to stain nuclei, the samples were evaluated
105 under inverted fluorescence microscope (Olympus).

106 **Reverse-transcription polymerase chain reaction (RT-PCR) and real time** 107 **quantitative polymerase chain reaction (qPCR)**

108 Methods for performing RT-PCR and qPCR analysis have been described
109 previously.(14) Briefly, total RNA was isolated from cells using Trizol reagent
110 (Invitrogen), the samples were reverse-transcribed by using M-MLV reverse
111 transcriptase (Promega) according to the manufacturer's instructions. The PCR primer
112 sequences are as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): F-
113 GGAGCGAGATCCCTCCAAAAT, R- GGCTGTTGTCATACTTCTCATGG; ANF:
114 F- CAACGCAGACCTGATGGATTT, R- AGCCCCCGCTTCTTCATTC; α -actinin:
115 F-ATTGGTATGGAGTCTGCCG, R-TCCTGAGTGTAAGGTAGCCG; Myocardin:
116 F-AGAACTCAGGGGCACACGAAG, R-CCACCTTGTCAGAAGATTGTAAACC.
117 GAPDH was used as an internal control.

118 **Western Blot (WB)**

119 The operation process of Western Blot has been described previously.(14) Cells were
120 lysed in lysis buffer to extract total protein. The resulting protein was then analyzed
121 on a 12% sodium lauryl sulfate polyacrylamide gel (SDS-PAGE). The proteins were
122 transferred to a PVDF membrane and blocked for 60 min at room temperature in 5%
123 skim milk powder (wt/vol) in TBST (TBS+0.1% Tween-20, vol/vol). After mouse
124 polyclonal antibodies against Myocardin (Abcam), p-ERK (Abcam), rabbit polyclonal
125 antibodies against ANF (Abcam), mouse monoclonal antibodies against α -actinin
126 (Sigma) and appropriate secondary antibodies (HRP-goat anti-mouse IgG, HRP-goat
127 anti- rabbit IgG, HRP rabbit-anti-goat IgG, Santa Cruz) were incubated. The specific
128 proteins were visualized by odyssey detection. GAPDH expression was used as an
129 internal control.

130 **Luciferase assay**

131 The dual luciferase assay system (Promega) was used to measure luciferase activity
132 and was standardized for transfection efficiency. Normalize results by dividing

133 Firefly luciferase activity by Renilla luciferase activity of the same sample.

134 **Coimmunoprecipitation assay (CO-IP)**

135 A plasmid-based expression vector encoding Flag-Myocardin and Myc-STAT3 was
136 co-transfected into COS7 cells. Lysates were collected 48 h after transfection and
137 Flag-Myocardin was precipitated using Flag antibodies. The resulting mixture was
138 washed, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis
139 (PAGE), transferred to a polyvinylidene fluoride membrane, first developed by Myc
140 antibody to visualize Myc-STAT3, then peeled off and again with Flag antibody Probe
141 to reveal Flag-Myocardin. Myc-STAT3 was then precipitated using Myc antibody.
142 The resulting mixture was washed, subjected to PAGE, transferred to a polyvinylidene
143 fluoride membrane, first developed by Flag antibody to reveal Flag-Myocardin, then
144 peeled off and re-examined with Myc antibody to reveal Myc-STAT3.

145 **Chromatin Immunoprecipitation (ChIP) assay**

146 ChIP analysis was performed using an enzymatic chromatin IP (magnetic beads) kit
147 (CST) in Cardiac myocytes transfected with Flag-Myocardin for 24 h. Proteins
148 bound to DNA were crosslinked using formaldehyde at a final concentration of 1%
149 for 20 min at room temperature. Protein-DNA complexes were immunoprecipitated
150 using anti-Flag antibody (Santa Cruz) for Flag-Myocardin. The signal of
151 Myocardin/ANF promoter complexes was measured by qRT-PCR. The primers used
152 for amplification of the human ANF promoter were (ChIP-ANF-promoter-CArG1
153 forward: GCTGCTCAAGGCAAAGG) and (ChIP-ANF-promoter-CArG1 reverse:
154 AGAGCTGGAACCCTCCC); (ChIP-ANF-promoter-CArG2 forward:
155 ACCCACGAGGCCAATGAATC) and (ChIP-ANF-promoter-CArG2 reverse:
156 GCTCCCTCTGCTTGCACTCTCA). For the analysis of ChIP-qRT-PCR experimental
157 results, calculate the enrichment relative to the input chromatin according to the delta
158 Ct method with the percentages been calculated using the formula $2^{-\Delta Ct}$, where ΔCt
159 is Ct (ChIP-template)-Ct (Input). A standard curve from one of the dilution inputs
160 was included in the run to keep the reaction efficiency between 95% and 105%.
161 qPCR was performed separately for each ChIP assay on control areas that should not
162 bind myocardiin. The ChIP assay was considered specific if in the control region the
163 enrichment was not above the enrichment of the non-specific immunoprecipitated
164 sample made with normal rabbit immunoglobulin G.

165 **Zebrafish Strains and Microinjection**

166 Dispose in accordance with local animal welfare regulations and maintain in

167 accordance with standard procedures (www.ZFIN.org). The experiments were
168 evaluated by the Animal Experiment Committee (DierExperimenten Commissie-DEC)
169 and evaluated by the Animal Association Committee of Wuhan University of Science
170 and Technology. The experimental procedure was in accordance with Directive
171 2010/63 / EU of the European Parliament on the protection of animals. The zebrafish
172 strains used in this study were raised according to standard procedures. In order to
173 knock down endogenous expression of Myocardin and STST3 in zebrafish,
174 morpholinos (MO): Myocardin-MO, STST3-MO and control-MO targeting ATG(15)
175 were synthesized from Gene Tools (Philomath, OR). Wild-type zebrafish embryo was
176 injected at one-two cell stage with either Myocardin-MO, STST3-MO or control-MO.
177 Zebrafish phenotype was analyzed under stereo microscope as previously
178 described.(16)

179 **Whole-Mount Confocal Microscopy and Morphometric Analyses.**

180 In order to observe the shape and development of the heart, Whole-Mount Confocal
181 Microscopy and Morphometric Analyses were used, as described previously.(16)
182 Briefly, in order to examine the embryo heart, zebrafish embryos were anesthetized
183 with 0.003% tricaine, place the ventral side of the embryo in the observation chamber
184 towards the microwell, facing 45° to the right.. Confocal images were captured by
185 using a Zeiss LSM 510 Confocal Microscope System. The confocal pinholes were
186 adjusted to 1 Airy unit to achieve an optimal z resolution of 0.75 N.A. and obtain
187 continuous slices of 0.9 μm thickness. Images were acquired at 0.45 μm intervals. 3D
188 projections were constructed by using the LSM Browser software (Zeiss), and
189 analysis was performed by Image J software that reads .lsm files
190 (<http://rsb.info.nih.gov/ij>).

191 **Statistical Analysis**

192 Data were expressed as mean±SE and accompanied by the number of experiments
193 performed independently, and analyzed by t-test. Differences at $P<0.05$ were
194 considered Significant differences in statistics.

195 **Results**

196 **STAT3 antagonizes the activation of myocardial genes by Myocardin.**

197 Myocardin is reported as a key component of molecular switches that regulate
198 SRF-mediated cell proliferation and myocyte differentiation.(1) STAT3 is a critical
199 mediator for survival of cardiomyocytes and appears to be essential in the induction
200 of cardiac myocyte hypertrophy. To explore whether STAT3 synergizes with

Myocardin to promote cardiac hypertrophy, cardiomyocytes were transfected with Myocardin, STAT3 and Myocardin/STAT3 plasmids, and the mRNA and protein levels of cardiac genes were quantified by qPCR and WB. Our results indicated that overexpression of Myocardin significantly activated the expression of cardiac genes (ANF and α -actinin), while co-transfected Myocardin and STAT3 reduced cardiac gene expression induced by Myocardin (Fig. 1A-C). We also detected the ANF and α -actinin gene expression and localization by immunofluorescence, the result showed coexpression Myocardin and STAT3 can weak the Myocardin-induced cardiomyocyte hypertrophy (Fig. 1D, E). We also knocked down the expression of endogenous STAT3 using si-STAT3. Our results show that knocked down endogenous STAT3 can relieve the inhibition of STAT3 on the expression of Myocardin activation of ANF and α -actinin (Fig. 2A, B). The JAK inhibitor, AG490 was used to attenuated endothelial STAT3 activation. Our results showed that AG490 significantly relieved the mRNA and protein expression of STAT3 suppressed Myocardin activation of cardiac genes in the presence of Myocardin in cardiomyocytes (Fig. 2C, D). Taken together, these results indicate that STAT3 antagonizes the activation of myocardial genes by Myocardin.

STAT3 affects the expression of Myocardin.

To investigate the mechanism of STAT3 suppressed Myocardin activation of cardiac genes, qPCR assays were performed after overexpression of STAT3 in primary neonatal cardiomyocytes. Our data showed that overexpressing STAT3 did not affect the mRNA level of Myocardin (Fig. 3A). Then we tested whether STAT3 could regulate the stability of Myocardin protein. The primary neonatal cardiomyocytes were transfected with a STAT3 plasmid for 48 h. The expression of endogenous Myocardin has a litter decreased by overexpressing STAT3 (Fig.3B) and overexpressing STAT3 maybe phosphorylate the protein of Myocardin (see the top band in Fig. 3B). There have researches shown that phosphorylation of Myocardin by extracellular regulated protein kinases (ERK1/2) and phosphorylation of Myocardin by ERK1/2 reduces its induction of smooth muscle gene transcription.(17) Our results have shown that the STAT3 activator LIF promotes the phosphorylation of ERK but the STAT3 inhibitor AG490 inhibits ERK phosphorylation (Fig. 3C). Interestingly, the ERK activator lipopolysaccharide (LPS) promotes Myocaridn phosphorylation but the ERK inhibitor U0126 can inhibit the phosphorylation of Myocaridn (Fig. 3D). Thus, STAT3-ERK1/2-Myocardin forms a negative feedback loop regulating cardiomyocyte hypertrophy.

237 **STAT3 interacts directly with Myocardin.**

238 Next, we investigated whether myocardiin and STAT3 protein interact with
239 co-immunoprecipitation assays with transfected COS7 cells. As shown in Fig.7,
240 Myocardin specifically interacted with STAT3. To locate the domains of cardiomyocin
241 and STAT3 that mediate interactions, a series of Myocardiin and STAT3 deletion
242 mutant proteins were used by coimmunoprecipitation (Fig. 4A, C). Myocardin
243 protein deletion mutants lacking N-terminal residues up to amino acid 194 interact
244 with STAT3, while mutant proteins lacking first 268 amino acids fail to interact with
245 STAT3. The ability to interact with STAT3 was also retained when the protein of the
246 265 C-terminal residue amino acid was deleted. Thus, STAT3 interacted with a
247 distinct region near the N terminus (between amino acids 194 and 265, this is basic
248 domain) (Fig. 4B). Myocardiin was confirmed to interact with SRF through a basic
249 and glutamine-rich domain near the N-terminus.(18, 19) Because the STAT3-binding
250 region of Myocardin overlapped the SRF-binding region, we considered the
251 possibility that STAT3 may inhibit Myocardiin activity by replacing it with SRF..
252 The ability of STAT3 to inhibit the activity of Myocardin depended ANF-luciferase
253 reporter activity also reduces the potential replacement mechanism of STAT3
254 inhibition. (Fig. 6).

255 With a series of STAT3 deletion mutant proteins, we found that a mutant protein
256 lacking the 300 sequence of amino acid N still retains the ability to interact with
257 Myocardin (Fig. 4D). However, this interaction was completely eliminated when the
258 amino acid 250 was further deleted, suggesting that the region between residues 250
259 and 300 is essential for STAT3 to interact with Myocardin. C-terminal deletion
260 results indicate that residues downstream of amino acid 280 are not required to
261 interact with Myocardiin (Fig. 4D). We concluded that a Myocardin-binding domain
262 lies between amino acids 250 and 280 (coiled-coil domain) of STAT3.

263 When a STAT3 deletion mutant protein is present, there is a direct correlation
264 between its ability to interact with and inhibit its activity. Thus, STAT3 can interact
265 with and inhibit Myocardin, and only those STAT3 deletion mutant proteins that
266 contain amino acids 250 and 280. It was worth noting that the C-terminal region of
267 STAT3 (containing a transactivation domain) was not necessary for inhibiting
268 myocardiin activity (Fig. 4D). These results demonstrated a direct interaction
269 between the amino acids 250 and 280 (coiled-coil domain) of STAT3 and amino
270 acids 194 and 265 (basic domain) Myocardin.

271 **Mutation the coiled-coil domain of STAT3 and the basic domain of Myocardin**
272 **affect the interaction of Myocardin and STAT3 and its transcriptional activation**

273 This deletion mutant Myocardin basic domain [Myocardin (1-807)] abolished
274 interactions between Myocardin and STAT3 but this mutant Myocardin Q-rich
275 domain [Myocardin (1-807)] has interactions between STAT3. A construct
276 containing this basic domain [Myocardin (1-807)] also bound STAT3 (Fig. 5A, B).
277 These studies further support that the basic domain of Myocardin interacts in vitro
278 with STAT3. Since a direct interaction between the amino acids 250 and 280
279 (coiled-coil domain) of STAT3 and amino acids 194 and 265 (basic domain)
280 Myocardin, we have mutated site-directed the amino acids (KK-NQ) of Myocardin
281 basic domain (Mutation-Myocardin) (Fig. 5C) or the amino acids (DDE-GSQ) of
282 STAT3 coiled-coil domain (Mutation-STAT3) (Fig. 5D).

283 Coimmunoprecipitation assays to test the interaction mutated site-directed the amino
284 acids (KK-NQ) of Myocardin basic domain (Mutation-Myocardin) and STAT3 or
285 mutated site-directed the amino acids (DDE-GSQ) of STAT3 coiled-coil domain
286 (Mutation-STAT3) and Myocardin. Our data show that Mutation-Myocardin has no
287 interaction with STAT3 (Fig. 5E) and Mutation-STAT3 has no interaction with
288 Myocardin (Fig. 5F). Taken together, these data indicate that the interaction between
289 Myocardin and STAT3 is produced by the positive charge of the amino acids KK of
290 the Myocardin basic domain and the negative charge of the amino acids DDE of the
291 STAT3 coiled-coil domain.

292 To investigate the role of transcription activation and expression of ANF after
293 mutation the coiled-coil domain of STAT3 and the basic domain of Myocardin, the
294 qPCR and luciferase reporter and western bolt assay were used to test the
295 transcription activation and expression of ANF. As shown in Fig. 5G, H and I,
296 overexpressed Myocardin will strongly active the expression of cardiac gene (ANF)
297 but the cotransfected Myocardin and STAT3 decreased Myocardin-induced cardiac
298 gene expression compared with that in transfected Myocardin cells, Mutation-STAT3
299 significantly relieved the mRNA and protein expression of STAT3 suppressed
300 Myocardin activation of ANF in the presence of Myocardin in cardiomyocytes. More
301 importantly, Mutation-Myocardin significantly relieved the mRNA and protein
302 expression of STAT3 suppressed Myocardin activation of ANF in the presence of
303 STAT3 in cardiomyocytes. Thus, STAT3 inhibits Myocardin induced cardiac
304 hypertrophy gene expression through their interaction, mutation the interaction of the
305 site, this effect has disappeared.

306 **STAT3 interferes with the SRF-Myocardin interaction**

307 In order to understand the molecular mechanism by which STAT3 inhibits
308 myocardiin function, we asked whether STAT3 can interfere with myocardiin-SRF

interactions. To investigate this, we transfected cells with Myc-Myocardin and HA-SRF and followed their binding after silencing (Figure 6A) or over-expressing STAT3 (Fig. 6B). The former condition was significantly enhanced, while the latter significantly reduced the binding of SRF to Myocardin. Indeed, Δ coiled-coil retained substantial ANF promoter-inducing activity (Fig. 6D), whereas its combination with STAT3 was greatly reduced (Fig. 6C). Importantly, Δ coiled-coil was much less sensitive to the inhibitory action of STAT3 than the WT (Fig. 6D). These findings suggest that the key mechanism for STAT3-mediated ANF promoter inhibition is the binding of STAT3 to Myocardin.

Myocardin and STAT3 promote the transactivity of ANF depending on CArG box

Myocardin coordinate and regulate the expression of a variety of CArG-dependent cardiac genes. Previously, it was found that incorrect expression of cardiac proteins in *Xenopus* cap analysis was not sufficient to induce beating or cardiac-like coordinated contractions.(20) Although Myocardin does not directly bind to DNA, the binding of SRF to the CArG box (CC (A/T)₆GG) will cause a sharp bend in the DNA, and this bend may change with the change in base composition in the entire CArG box.(21) All STATs bind to similar SIE/GAS DNA sequences that contain the TT and AA base tandems, separated in STAT3 by 4 to 6 bases (5'-TTN₄₋₆AA-3').(22, 23) By bioinformatics analysis, we found that the ANF promoter has two CArG box sites and a GAS-like site (Fig. 7A). To further determine whether STAT3 affects the transactivation of Myocardial-mediated ANF promoters and to investigate whether this process depends on the CArG box, The ANF gene promoter luciferase reporter plasmid was constructed to contain a deleted or mutated CArG box. (Fig. 7A, B). Our data indicate that Myocardial-mediated ANF promoter activity is abolished in vitro when the CArG box is deleted or mutated in the ANF promoter, while nearby CArG boxes play an important role in Myocardial-mediated ANF promoter activity (Fig. 7C, D). The above experimental data show that the transactivation of ANF by Myocardin depends on the CArG box.

As shown in Fig. 7C and 7D, overexpressing Myocardin strongly activated the expression of ANF-1000-luc promoter. However, coexpression Myocardin and STAT3 repressed Myocardin-dependent ANF transcriptional activity compared with that in transfected Myocardin cells. These data demonstrate that STAT3 serves as a negative regulator of cardiac muscle differentiation in the presence of Myocardin through downregulation of Myocardin activity.

To further confirm the specific binding of Myocardin to ANF promoter, ChIP assays

were performed in cardiomyocytes transfected with Myocardin, Myocardin /STAT3 or vector. Immunoprecipitation of cross-linked chromatin with specific antibodies against Myocardin or without antibodies (as negative control). The precipitated chromatin DNA was then purified and PCR amplified using specific primers across the CArG box in the ANF promoter. As shown in Fig. 7E and 7F, the comparison (IgG) and the negative control (No Ab) did not show any PCR signals. Myocardiin can be combined with the CArG box of the ANF promoter (both near (CArG 1) and long-range (CArG 2) CArG boxes). Myocardiin binds to the near CArG box more than the ability to bind to the far CArG box in the ANF promoter (Fig. 7E, F). More importantly, STAT3 has the effect of inhibiting Myocardiin. (Fig. 7E, F). These data indicate that STAT3 is a potent nuclear factor that inhibits the transactivation of the Myocardium by disrupting the formation of the SRF/Myocardin/CArG complex in vivo and in vitro.

Knockdown stat3 inhibits Myocardin knockdown-mediated cardiac hypertrophy in zebrafish

To further validate the conclusions in vitro and the effects of Myocardin and STAT3 interactions on cardiac development, Myocardin-MO, STAT3-MO and control-MO targeting ATG were synthesized and microinjection into the zebrafish embryos. The developmental process of the zebrafish heart is observed by the expression of the cardiac development marker gene CMCL2. The results show that the heart cannot be circulated normally when STAT3 is knocked down. While co-injecting Myocardin-MO and STAT3-MO, most of the fish's heart showed near normal cyclization, but there were still some malformations (Fig. 8A). We also characterized cardiac hypertrophy by detecting the ANF gene. Microinjection of STAT3-MO alone induced cardiac hypertrophy. When we injected Myocardin-MO and STAT3-MO together, cardiac hypertrophy was alleviated (Fig. 8B). In addition, significant up-regulation of ANF expression could be clearly observed when knocking down STAT3 (Fig. 8B), which confirmed our conclusions in primary cardiomyocytes. These results indicated that STAT3 could antagonize Myocardin-mediated cardiac hypertrophy in vivo.

Discussion

Myocardin, which plays a key role in inducing cardiac hypertrophy and fetal cardiac genetic programs, as a co-activator of SRF,(1). However, the molecular mechanism in regulating the stability and activity of Myocardin is unclear. Despite recent evidence that STAT3 plays a cardioprotective role in the heart, there is no

information on the role of STAT3 in Myocardial-induced myocardial hypertrophy. This study revealed the relationship between Myocardin and STAT3 in the development of cardiac hypertrophy. Our study has shown that Myocardin and STAT3 have physically interaction by co-immunoprecipitation. More importantly, our data demonstrated a direct interaction between the amino acids 250 and 280 (coiled-coil domain) of STAT3 and amino acids 194 and 265 (basic domain) Myocardin (Fig. 4). So that we hypothesized that STAT3 was a coactivator for Myocardin, the regulator of cardiomyocyte hypertrophy. Interestingly, we find that co-expression of Myocardin and STAT3 can decrease Myocardin-induced cardiac gene expression compared with that in transfected Myocardin alone in cardiomyocytes (Fig. 1). Silenced STAT3 via siSTAT3 can relieve STAT3 inhibiting Myocardin activation of cardiac genes (Fig. 2A, B). Microinjection of STAT3-MO alone into zebrafish induced cardiac hypertrophy. When we injected Myocardin-MO and STAT3-MO together, cardiac hypertrophy was alleviated (Fig. 8B). In addition, significant up-regulation of ANF expression could be clearly observed when knocking down STAT3, which confirmed our conclusions in primary cardiomyocytes. The influence that AG490 could block the phosphorylation of JAK2 would restrain the phosphorylation of STAT3. AG490 would weak the effect of STAT3 inhibit Myocardin by restraining the phosphorylation of STAT3 (Fig. 2C, D). Mutation the coiled-coil domain of STAT3 and the basic domain of Myocardin affect the interaction of Myocardin and STAT3 and Myocardin activation of cardiac genes transcriptional activation (Fig. 5G, H and I).

We also provide evidence that far-CArG (CArG2) and near-CArG (CArG1) in the ANF promoter are essential for promoter function in cardiomyocytes. But the function of near-CArG is more powerful than the far-CArG (Fig. 7C, D). These results indicate that this spacing of the paired CArG elements contributes to the synergistic combination of SRF and its coactivator Myocardin with the ANF promoter. STAT3 inhibits Myocardial activation of CArG-dependent cardiac gene expression. The effect mediated by physical interruption of the Myocardin/SRF/CArG ternary complex (Fig. 7E, F). Our data demonstrate that STAT3 can behave as a negative transcriptional cofactor in a Myocardin-dependent manner to inhibit cardiomyocyte hypertrophy.

Why does STAT3 inhibit Myocardin transactivity? Firstly, STAT3 can directly inhibit Myocardin-dependent transcriptional activity, qPCR assays were performed in transfected STAT3 primary neonatal cardiomyocytes. But our data show that overexpressing STAT3 did not affect the mRNA level Myocardin. The expression of

417 endogenous Myocardin has a litter decreased by overexpressing STAT3 (Fig. 3B)
 418 and overexpressing STAT3 maybe phosphorylate the protein of Myocardin (see the
 419 top band in Fig. 3B). There have researches shown that phosphorylation of
 420 Myocardin by ERK1/2 and phosphorylation of Myocardin by ERK1/2 reduces its
 421 induction of smooth muscle gene transcription.(17, 24) Our results have shown that
 422 the STAT3 activator LIF promotes the phosphorylation of ERK but the STAT3
 423 inhibitor AG490 inhibits ERK phosphorylation (Fig. 3C). Interestingly, the ERK
 424 activator LPS promotes Myocaridn phosphorylation but the ERK inhibitor U0126
 425 can inhibit the phosphorylation of Myocaridn (Fig. 3 D). The molecular mechanism
 426 may be the phosphorylation modification of Myocardin by STAT3. And the
 427 phosphorylation modification of Myocardin might be prone to decompose by
 428 ubiquitin. Recently, Protein inhibitor of activated STAT 1(PIAS1) has been reported
 429 to regulate Myocardin transactivity via its ligase induced sumoylation. These results
 430 indicate that PIAS1 and small ubiquitin-like modifier 1 (SUMO1) enhance
 431 Myocardiin activity through SUMO-modified Myocardial proteins..(25, 26) Protein
 432 inhibitor of activated STAT represents a family of proteins originally identified
 433 through interaction with cytokine-induced STAT.(27) PIAS1 binds to STAT1 and
 434 inhibits the transcriptional activity of STAT1 by blocking its DNA-binding
 435 activity.(28, 29) STAT3 and STAT1 form complexes in response to LIF
 436 stimulation.(30) Thus, it is also possible that STAT3 inhibits Myocardin transactivity
 437 by inhibiting SUMO modification of Myocardin by PIAS1 and SUMO1. The
 438 specific binding site that both SUMO modification of Myocardin by PIAS1/SUMO1
 439 and ubiquitin modification is the site KXE (IKQE). The competition between SUMO
 440 modification and ubiquitin modification of Myocardin at the same site KXE (IKQE)
 441 regulates Myocardin transactivity.
 442 Taken together, these data demonstrate that STAT3 serves as a negative regulator of
 443 Myocardin activity to regulate cardiomyocyte hypertrophy.

444 **Conclusion**

445 The N-terminus of STAT3 directly binds to the basic domain of myocardin and
 446 inhibits the transcriptional activity of myocardin-mediated cardiac-specific genes
 447 ANF and α -actinin, thereby inhibiting their expression, and further inhibit
 448 myocardin-mediated cardiac hypertrophy in vivo. The STAT3-Myocardin interaction
 449 identifies a site of convergence for nuclear hormonereceptor-mediated and
 450 cardiac-specific gene regulation and suggests a possible mechanism for the cardiac
 451 protective effects.

452 **Abbreviations**

453 STAT3, signal transducer and activator of transcription 3; SRF, serum response
454 factor;
455 IL-6, interleukin-6; LIF, leukemia inhibitory factor; DMEM, Dulbecco's modified
456 Eagle's medium; DAPI, 4',6'-diamidino-2-phenylindole; RT-PCR, semi-quantitative
457 reverse-transcription polymerase chain reaction; qPCR, real time quantitative
458 polymerase chain reaction; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;
459 PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ChIP, Chromatin
460 Immunoprecipitation; JAK, Janus kinase; LPS, lipopolysaccharide; ERK1/2,
461 extracellular regulated protein kinases; PIAS1, protein inhibitor of activated STAT 1;
462 SUMO1, small ubiquitin-like modifier 1; MO, Morpholino.

463 **Ethics declarations**

464 **Ethics approval and consent to participate**

465 The research is approved by an ethical committee of Wuhan University of Science
466 and Technology.

467 **Consent for publication**

468 Not applicable.

469 **Availability of data and materials**

470 All data, models, and code generated or used during the study appear in the
471 submitted article. After all experiments were completed, they were executed using
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478 **Authors' contributions**

479 Xing-Hua Liao and Tong-Cun Zhang designed research; Jia-Peng Li, Yuan Xiang,
480 Hui Li, You Huang and Chao Shen performed research; Jia-Peng Li, Yuan Xiang,
481 Hui Li and Xing-Hua Liao analyzed data; Hui-Min Zhang, Xing-Hua Liao and

482 Tong-Cun Zhang wrote the paper.

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484 Not applicable.

485 **Competing interests**

486 The authors declare that they have no competing interests.

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557

558 **Figure legends**

559

560 **Fig. 1 STAT3 suppresses Myocardin activation of cardiac genes.**

561 **A and B** qPCR and RT-PCR analysis of transfected with Myocardin or
562 Myocardin/STAT3 for 48 h in cardiomyocytes. GAPDH served as loading control.

563 (**, $p < 0.01$) Data are expressed as the mean $\pm$ SEM with $N = 3$ biological

564 replicates in each group. All data are analysed using paired t-test. **C** Western blot

565 analysis of transfected with Myocardin or Myocardin/STAT3 for 48 h in

566 cardiomyocytes. GAPDH served as loading control. **D** The representative image

567 shows ANF and α -actinin expression in cardiomyocytes transfected with Myocardin

568 or Myocardin/STAT3 for 48 h. The left panels (green) show anti- ANF or α -actinin

569 antibody reactivity to demonstrate gross morphology. The middle panels (blue) show

570 the DAPI staining for nuclei. The right panels show double immunostained for ANF

571 or α -actinin and nuclei. Scale = 50 μ m.

Fig.2 Silenced STAT3 and inhibited phosphorylation of STAT3 via AG490 can relieve STAT3 inhibiting Myocardin activation of cardiac genes.

A qPCR analysis to detect the mRNA level of ANF and α-actinin in cardiomyocytes transfected with Myocardin and then treated with siSTAT3 for 48 h. GAPDH served as loading control. (**, $p < 0.01$, *, $p < 0.05$). **B** Western blot analysis to detect the protein level of ANF and α-actinin transfected with Myocardin and then treated with siSTAT3 for 48 h in cardiomyocytes. GAPDH served as loading control. **C** qPCR analysis to detect the mRNA level of ANF and α-actinin in cardiomyocytes transfected with Myocardin and then treated with AG490 for 24 h. GAPDH served as loading control. (**, $p < 0.01$, *, $p < 0.05$). **D** Western blot analysis to detect the protein level of ANF and α-actinin transfected with Myocardin and then treated with AG490 for 24 h in cardiomyocytes. GAPDH served as loading control. Data are expressed as the mean $\pm$ SEM with $N = 3$ biological replicates in each group. All data are analysed using paired t-test.

Fig.3 STAT3 affects the expression of Myocardin.

A and **B** qPCR and Western blot analysis to detect expression of Myocardin in cardiomyocytes transfected with STAT3 for 48 h. GAPDH served as loading control.

(#, $p > 0.05$) Data are expressed as the mean $\pm$ SEM with $N = 3$ biological replicates in each group. All data are analysed using paired t-test. **C** Western blot analysis of the phosphorylated ERK1/2 treated with LIF or AG490 for 24 h in cardiomyocytes. **D** Western blot analysis of the expression of Myocardin treated with LPS or U0126 for 24 h in cardiomyocytes.

Fig. 4 Direct interaction of Myocardin and STAT3 and mapping of the domains that mediate their interaction.

A Schematic diagram of Myocardin and the mutant forms used to map the STAT3-binding domain. **B** Coimmunoprecipitation assays. COS7 cells were transiently transfected with expression vectors encoding Flag-tagged Myocardin deletion mutant proteins and Myc-tagged STAT3. Flag-tagged Myocardin deletion mutant proteins were immunoprecipitated (IP) from cell lysates with a monoclonal anti-Flag antibody, and coimmunoprecipitating STAT3 was detected by immunoblotting (IB) with a monoclonal anti-Myc antibody (top parts). The membrane was reprobbed with anti-Flag antibody to reveal the total amount of Flag-tagged Myocardin proteins (bottom parts). **C** Schematic diagram of STAT3 and the mutant forms used to map the Myocardin-binding domain. **D** Coimmunoprecipitation assays. COS7 cells were transiently transfected with expression vectors encoding Myc-tagged STAT3 deletion mutant proteins and Flag-tagged Myocardin. Myc-tagged STAT3 deletion mutant proteins were immunoprecipitated (IP) from cell lysates with a monoclonal anti-Flag antibody, and coimmunoprecipitating Myocardin was detected by immunoblotting (IB) with a monoclonal anti-Flag antibody (top parts). The membrane was reprobbed with anti-Myc antibody to reveal the total amount of Myc-tagged STAT3 proteins (bottom parts).

Fig. 5 Mutation the coiled-coil domain of STAT3 and the basic domain of Myocardin affect the interaction of Myocardin and STAT3 and its transcriptional activation.

A Coimmunoprecipitation assays. COS7 cells were transiently transfected with expression vectors encoding Flag-tagged Myocardin deletion mutant (basic domain and SAP domain) proteins and Myc-tagged STAT3. Flag-tagged Myocardin deletion mutant proteins were immunoprecipitated (IP) from cell lysates with a monoclonal anti-Flag antibody, and coimmunoprecipitating STAT3 was detected by immunoblotting (IB) with a monoclonal anti-Myc antibody (top parts). The membrane was reprobed with anti-Flag antibody to reveal the total amount of Flag-tagged Myocardin proteins (bottom parts). **B** and **C** Schematic of mutated site-directed the amino acids (KK-NQ) of Myocardin basic domain (Mutation-Myocardin) and mutated site-directed the amino acids (DDE-GSQ) of STAT3 coiled-coil domain (Mutation-STAT3). **E** and **F** Coimmunoprecipitation assays in mutated site-directed the amino acids (KK-NQ) of Myocardin basic domain (Mutation-Myocardin) and STAT3 or mutated site-directed the amino acids (DDE-GSQ) of STAT3 coiled-coil domain (Mutation-STAT3) and Myocardin. **G**, **H** and **I** QPCR, Luciferase assay and Western blot analysis to detect expression of ANF in cardiomyocytes transfected with Mutation the coiled-coil domain of STAT3 and

637 Myocardin or the basic domain of Myocardin and STAT3 for 48 h. GAPDH served
638 as loading control (**, $p < 0.01$, #, $p > 0.05$) Data are expressed as the
639 mean $\pm$ SEM) with N=3 biological replicates in each group. All data are
640 analysed using paired t-test.

641

642 **Fig. 6 STAT3 interferes with Myocardin-SRF interaction.**

643 **A** Cells were transfected with Myc-Myocardin and HA-SRF along with NC or
644 STAT3 siRNA. Association of Myocardin and SRF was analyzed by
645 coimmunoprecipitation. STAT3 silencing was detected from whole cell lysates
646 (WCL). Controls for the immunoprecipitation were reaction without antibody (No
647 AB) or Myc transfection (No Tx). (right) Densitometric analysis of three
648 experiments is shown. **B** Myc-Myocardin and HA-SRF were cotransfected with
649 empty vector or STAT3. Myocardin was immunoprecipitated with anti-Myc antibody
650 as in A from control or LCM-treated (1 h) cells. **C** Coimmunoprecipitation shows
651 decreased association of STAT3 to Δcoiled-coil compared with WT. **D** Coiled-coil
652 domain mutant shows reduced sensitivity to inhibition by STAT3. Cells were
653 transfected with ANF-Luc, Δcoiled-coil, or WT-Myocardin along with empty vector
654 or STAT3. Luciferase assay was performed 48 h later. Results are normalized to the
655 control (top; fold increase over control) or expressed as a percentage of the maximal
656 effect of the given Myocardin construct (bottom). **, $p < 0.01$. Error bars indicate
657 mean $\pm$ SEM.

Fig. 7 STAT3 inhibits Myocardin-induced the transactivity of ANF depending on CARG box.

A Schematic of the -1000 ANF promoter, containing CARG box element was linked to a luciferase reporter. Mutation or truncations that remove the CARG box element. -337, -236 and -121 ANF promoter, a truncated promoter. **B** The -1000 ANF promoter with mutations in CARG box. **C** Cardiomyocytes were transfected with the Wild-Type-1000 ANF promoter, or a truncated promoter -337, -236 or -121 and transfected with Myocardin or STAT3 for 48 h. Then the luciferase reporter assays were used to test the transactivity of ANF. **D** Cardiomyocytes were transfected with the Wild-Type-1000 ANF promoter, or -1000 ANF promoter with mutations in CARG box and transfected with Myocardin or STAT3 for 48 h. Then the luciferase reporter assays were used to test the transactivity of ANF. (**, $p < 0.01$, *, $p < 0.05$). **E and F** Cardiomyocytes were transiently transfected with a Myocardin, Myocardin /STAT3 or a control vector (pCDNA3.1) 48 h, and ChIP assays were performed by PCR with primers associated with the genes for ANF as described in Materials and Methods.

674 Sheared DNA/protein complexes were immunoprecipitated by using an
 675 anti-Flag-Myocardin Ab. Then, PCR was carried out to detect the endogenous CArG
 676 regions in immunoprecipitated chromatin fragments. The amount of DNA in each
 677 sample (2% input) is shown at the second land. Immunoprecipitations were
 678 performed without primary antibody (No Ab) as a control and IgG as a negative
 679 control (**, $p < 0.01$). Data are expressed as the mean $\pm$ SEM with $N = 3$
 680 biological replicates in each group. All data are analysed using paired t-test.

681
 682 **Fig.8 STAT3 antagonizes Myocardin-mediated cardiac hypertrophy.**
 683 Microinjection of Myocardin-MO, STAT3/Myocardin-MO and control-MO
 684 separately into the zebrafish embryos, and then the zebrafish embryos were
 685 subjected to in situ hybridization of the entire embryo. **A** Expression of CMLC2 2
 686 days (upper) and 3 days (bottom) after fertilization. ($n > 30$). **B** Expression of ANF 2
 687 days (upper) and 3 days (bottom) after fertilization. ($n > 30$).

688
 689

A

	All experiments			
Condition	1	2	3	4
Pre-treatment	None	None	None	None
Post-treatment	None	None	None	None
Pre-treatment	None	None	None	None
Post-treatment	None	None	None	None

B

	All experiments			
Condition	1	2	3	4
Pre-treatment	None	None	None	None
Post-treatment	None	None	None	None
Pre-treatment	None	None	None	None
Post-treatment	None	None	None	None

C

	All experiments			
Condition	1	2	3	4
Pre-treatment	None	None	None	None
Post-treatment	None	None	None	None
Pre-treatment	None	None	None	None
Post-treatment	None	None	None	None

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