Methods

Sample harvest and histological analysis

Age and sex matched TG and NTG mice were randomly selected and echocardiogram was performed in a blind fashion. After examination, the mice were sacrificed with an overdose of pentobarbital (50 mg/kg i.p.) and the hearts were harvested and rinsed with saline. Heart weight (HW) was measured and the ratio to Body Weight (HW/BW) was calculated. The hearts were divided into four groups (n=3-6 in each group at each time point) for total RNA or protein extraction, for nuclear/cytosol fractionation, or for histological examination.

Hearts were fixed with 4% formalin, embedded in paraffin, and sectioned at 4-6 μm thickness. Cells for immunocytochemical were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100. Standard protocols for immunofluorescence and immunocytochemical staining were performed using antibodies as indicated in the results. Laser confocal images were collected with a Leica TCS SP2 microscope (Leica Microsystems Heidelberg GmbH).

Western blot and Immunoprecipitation

Total proteins were extracted from cultured cells or mouse hearts using Total Protein Extraction Kit (Biochain Institute, Hayward, CA). Nuclear and cytosolic fractions were prepared using Ne-Per Nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL) according to the protocols provided by the manufacturer. Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Forty to sixty

micrograms of total protein was used for Western blotting assay using appropriate antibodies. Membranes were stripped and re-blotted for other antibodies if necessary.

For immunoprecipitation of STAT3, the EZview Red ANTI-FLAG M2 Affinity Gel system (Sigma-Aldrich) was used exactly as in the protocol provided by the manufacturer. For immunoprecipitation of p300 and p65, protein G agarose (Cell Signaling Technology) was used.

Monoclonal HA antibody was purchased from Roche (Pleasanton, CA). Antibodies to Tyr-705 phosphorylated STAT3, Ser-727 phosphorylated STAT3 and p300 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to OPN, RGS2, and NF-κB p65 were from Santa Cruz Biotechnologies (Santa Cruz, CA). STAT3 from Cell Signaling Technology and Santa Cruz Biotechnologies was used. Alexa Fluor 488 or 568 conjugated goat anti-rabbit, or mouse IgG, or rabbit anti-mouse IgG antibodies were from Invitrogen (Carlsbad, CA). Anti-GAPDH antibody was from Ambion (Austin, TX). Peroxidase conjugated donkey anti-goat IgG (H+L), Vectashield Mounting Medium with DAPI and Vectastain ABC kit was from Vector Laboratories, Inc (Burlingame, CA). Mouse anti-actin antibody was from Research Diagnostics, Inc (Pleasanton, CA). Anti-Flag M2 monoclonal antibody was from Sigma (St. Louis, MO).

Real-time RT-PCR

One microgram of total RNA was treated with DNase (Sigma) and used for first-strand cDNA synthesis using the First Strand cDNA Synthesis Kit for RT-PCR (AMV+) (Roche Diagnostics). Real-time RT-PCR was performed on ABI Prism 7700 Sequence Detection System (Amplied Biosystems, CA) using QuantiTect SYBR Green PCR kit (Qiagen),

with the primers as described below. Data are presented as ratio of target gene expression to GAPDH gene expression.

The following oligonucleotide primers were used in this study.

Mouse GAPDH,

Sense 5’-GAAGGTCGGTGTGAACGGATTTGG - 3’

Antisense 5’-CCGTTGAATTTGCCGTGAGTGGA- 3’

Human GAPDH,

Sense 5’-AGCCAAAAGGGTCATCATCTCTG - 3’

Antisense 5’-CATGAGTCCTTCCACGATACCAAA- 3’;

Mouse ANP,

Sense 5’- CCCTGGGCTTCTTCCTCGTC- 3’

Antisense 5’-CTACCGGCATCTTCTCCTCCAG- 3’

Mouse BNP,

Sense 5’-AAGAGAAAAGTCGGAGGAAAT - 3’

Antisense 5’-CTCTTTTTGGGTGTTCTTTTG- 3’

Mouse ACTA1,

Sense 5’- AGATCTGGCACCACACCTTC- 3’

Antisense 5’-GAGGCATAGAGGGACAGCAC- 3’

Human RGS2,

Sense 5’-CCCAAAAGCTGTCCTCAAAA - 3’

Antisense 5’-TTCTGGGCAGTTGTAAAGCA- 3’

Mouse RGS2,

Sense 5’-GCAAGAAAAGCAAACAGCAAA - 3’

Antisense 5’-CTTTAAAAACGCCCTGAATGC- 3’

Mouse STAT3,

Sense 5’- GCCCCGTACCTGAAGACCAAGTT- 3’

Antisense 5’-GGGCTCAGCACCTTCACCGTTAT- 3’

Human STAT3,

Sense 5’-TGCAAGATCTGAATGGAAACAAC - 3’

Antisense 5’-ACGTACTCCATCGCTGACAAAA- 3’

Mouse SPP1

Sense 5’-GTGATTTGCTTTTGCCTGTTT- 3’

Antisense 5’-CATGTGGCTATAGGATCTGGG- 3’

Human SPP1,

Sense 5’-AAGCCAATGATGAGAGCAATGAG - 3’

Antisense 5’-TTGGGGTCTACAACCAGCATATC- 3’.

Plasmid vector constructions, gene transfection, and gene knock-down

HA tagged human AT1R cDNA was cloned into pcDNA3 vector which is driven by a cytomegalovirus promoter and containing neomycin resistance gene. Flag tagged mouse STAT3 (Flag-mSTAT3) and Flag tagged Y705F mutant mouse STAT3 (Flag-Y705F-mSTAT3) were purchased from Riken (Tokyo, Japan) and sub-cloned into pcDNA6 at the *Sal I* site which is under control of cytomegalovirus promoter and contains blasticidin resistance gene. The plasmid DNA for experiments was prepared

from TOP 10F' *Escherichia coli* (Invitrogen) by using an endotoxin-free plasmid extraction kit (Qiagen).

HEK293 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FCS). When cells reached 60-65% confluence, 6 μg of plasmid vector was transfected into the cells using Fugene 6 Transfection Reagent (Roche). Stably transfected cell lines were generated from a single cell and cultured in culture media in the presence of 1.0 mg/mL G418 (HEK-AT1R) and/or 10 μg/ml blasticidin (HA-AT1R and Flag-mSTAT3 or Flag-Y705F-mSTAT3 double transfected HEK293). The expression of the interest protein was confirmed by immunoblotting assay using anti-HA or -Flag antibodies.

To define effect of STAT3 gene knockdown on identified gene expression, pre-designed siRNA targeting human STAT3 (Ambion) was transfected into HEK-AT1R cells using Amine transfection reagent from Silencer siRNA Transfection II Kit (Ambion). Gene knockdown efficiency was evaluated by immunoblotting assay. All experiments were conducted in the absence of serum at 60-80% confluence. Cells were stimulated by 10^{-7} mol/L Ang II in the presence or absence of Losartan for different times and then used for extraction of proteins, or RNA using the relevant kit as described previously.

Chromatin immunoprecipitation assay

AT1R-Flag-Y705F-STAT3 HEK293 cells and AT1R-Flag-WT -STAT3 HEK293 cells were used for Chromatin Immunoprecipitation (ChIP) assay with the EZ-Chip™ kit

(Millipore, Catalog #17-371), based on the introduction of the manufacturer. Briefly, **c**ells were allowed to grow to 80-90% confluence in 150 mm culture dish in DMEM supplemented with 10% FBS. The cells were cross-linked with 1% formaldehyde at room temperature for 10 min, and then sonicated in SDS lysis buffer for five times and 10 seconds for each (Microson^{TM} Ultrasonic Cell Disruptor, Misonix, Millipore). Immunoprecipitation was performed with anti- Flag antibody and isotype control Ig G for overnight at 4°C with rotation. The precipitates were washed and eluted twice with 1% SDS and 0.1 mol/l $NaHCO_3$. The cross-linked protein–DNA complexes was reversed by adding NaCl and incubated at 65°C for at 6 hrs followed by treatment with RNase and proteinase K. The DNA was purified and then subjected to PCR analysis. The PCR products were separated by 1.2% agarose gel electrophoresis.

Supplementary Table 1. Angiotensin II type 1 receptor (AT1R) transgenic animal model and their signaling mechanisms.

Target	Transgene	Phenotype	Strain	Signaling mechanism	References
Cardiac myocyte	αMHC-AT1R	Lethal Myocytes hyperplasia Heart block	FVB	Not defined	[S1]
Cardiac myocyte	αMHC-AT1R	Cardiac hypertrophy Cardiac remodeling Heart failure	C57BL/6 X C3H mixed	Not defined	[14]
Cardiac myocyte	αMHC-AT1R	Hypertrophy after volume and pressure overload	SD rat	Ca^{++}	[s2]
Cardiac myocyte	αMHC-AT1R	Cardiac hypertrophy Cardiac fibrosis Apoptosis	FVB	Gq, PKC	[s3, S4]
Cardiac myocyte	αMHC-AT1R - second intracellular loop mutant	Cardiac hypertrophy Bradycardia Low cardiac function	FVB	Src, ERK	[s3]
Cardiac myocyte	α-MHC-AT1R - YIPP motif mutant	No or less cardiac hypertrophy, fibrosis, and apoptosis	FVB	EGFR	[S4]
Cardiac myocyte	αMHC-AT1R αMHC-N111G-AT1R	Compensated hypertrophy adverse ventricular remodeling following Ang IV injection	C57BL/6 C57BL/6	not defined not defined	[24]
Whole body	AT1R-knock-in	Hypertension Renal and cardiac fibrosis Diastolic dysfunction	C57BL/6	not defined	[S5]
Endothelial cell	tie-AT1R-mutant	Hypotension Bradycardia	B6/CBA	eNOS	[15]
Podocyte	nephrin-AT1R	Protein leakage Glomerulosclerosis	SD rat	not defined	[S6]
Neuron	neuron-AT1R	Enhanced cardiovascular sensitivity	C57BL/6	not defined	[S7]

Supplementary references:

S1. Hein L, Stevens ME, Barsh GS, Pratt RE, Kobilka BK, Dzau VJ. Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. *Proc Natl Acad Sci U S A.* 1997;**94**:6391-6396.

S2. Hoffmann S, Krause T, van Geel PP, Willenbrock R, Pagel I, Pinto YM et al. Overexpression of the human angiotensin II type 1 receptor in the rat heart augments load induced cardiac hypertrophy. *J Mol Med.* 2001;**79**:601-608.

S3. Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, et al. Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest* 2005;**115**:3045-56.

S4. Zhai P, Galeotti J, Liu J, Holle E, Yu X, Wagner Tet al. An angiotensin II type 1 receptor mutant lacking epidermal growth factor receptor transactivation does not induce angiotensin II-mediated cardiac hypertrophy. *Circ Res* 2006;**99**:528-536.

S5. Billet S, Bardin S, Verp S, Baudrie V, Michaud A, Conchon S, et al. Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice. *J Clin Invest* 2007;**117**:1914-25.

S6. Hoffmann S, Podlich D, Hähnel B, Kriz W, Gretz N. Angiotensin II type 1 receptor overexpression in podocytes induces glomerulosclerosis in transgenic rats. *J Am Soc Nephrol* 2004;**15**:1475-87.

S7. Lazartigues E, Dunlay SM, Loihl AK, Sinnayah P, Lang JA, Espelund JJ, et al. Brain-selective overexpression of angiotensin (AT1) receptors causes enhanced cardiovascular sensitivity in transgenic mice. *Circ Res* 2002;**90**:617-24.

Supplementary Table 2. Echocardiograph analysis of AT1R transgenic and non-transgenic mice

	NTG (n=7)	TG (n=7)	p-value
Age (days)	108.3±5.4	111.6±4.5	0.64
LVESD (cm)	0.129±0.028	0.332±0.033	<0.0001
LVEDD (cm)	0.307±0.028	0.437±0.042	<0.0001
IVSD	0.104±0.011	0.093±0.014	0.13
LVPWd	0.094±0.02	0.077±0.01	0.076
FS%	58.086±6.368	24.014±5.134	<0.0001
%EF	0.785±0.005	0.424±0.04	0.003
HW/BW ratio (mg/g)	4.186±0.73	5.671±0.622	0.0015

Echocardiographic analyses of human AT1 receptor transgenic mice (TG) and the non-transgenic control mice (NTG) at 3 months of age. LVESD: left ventricular end systolic dimensions; LVEDD: left ventricular end diastole dimensions; IVSD: Intraventricular septal width; LVPWd: left ventricular posterior wall (diastole); FS%: percentage of fraction shortening; %EF: percentage of ejection fraction; HW: heart weight, and BW: body weight.

Supplementary Table 3. Expression of 33 U-STAT3 targeted genes activated by Ang II/AT1R in mouse and HEK-AT1R

Matched genes with Y705FSTAT3-MEF and -hTERT-HME1Cells	Gene Symbol	AT1R-TG vs. NTG	HEK293 Ang II (+) vs. (-)
		Log_2 (Fold change)	Log_2 (Fold change)
* Actin, alpha 1, skeletal muscle	Acta1	2.07	NC
* Connective tissue growth factor	Ctgf	1.59	32
* Inactive X specific transcripts	Xist	1.73	NC
* Lectin, galactoside-binding, soluble, 3 binding protein	Lgal3bp	1.99	NC
* Regulator of G-protein signaling 2	Rgs2	-1.78	2.46
* Secreted phosphoprotein 1	Spp1	1.51	NC
CAP, adenylate cyclase-associated protein, 2 (yeast)	CAP2	NC	-2.14
Chloride intracellular channel 2	CLIC2	NC	-3.25
Chromosome 1 open reading frame 63	C1orf63	NC	-2.3
Cystathionase (cystathionine gamma-lyase)	CTH	NC	2
Discs, large (Drosophila) homolog-associated protein 4	DLGAP4	NC	2.83

Early growth response 1	EGR1	1.4	3.25
Elongation factor, RNA polymerase II, 2	ELL2	NC	2.3
Enolase 2 (gamma, neuronal)	ENO2	NC	3.73
FOS-like antigen 1	FOSL1	1.0	18.38
Growth arrest and DNA-damage-inducible, alpha	GADD45A	NC	2.46
GTP binding protein overexpressed in skeletal muscle	GEM	NC	19.7
Guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	NC	-2.46
Heterogeneous nuclear ribonucleoprotein D-like	HNRPDL	NC	-2.14
Limb bud and heart development homolog (mouse)	LBH	1.2	3.03
Matrix metallopeptidase 1 (interstitial collagenase)	MMP1	1.26	2.46
Neural precursor cell expressed, developmentally down-regulated 4-like	NEDD4L	1.0	2
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2	NC	5.28
Serine peptidase inhibitor, Kazal type 1	SPINK1	1.09	39.4

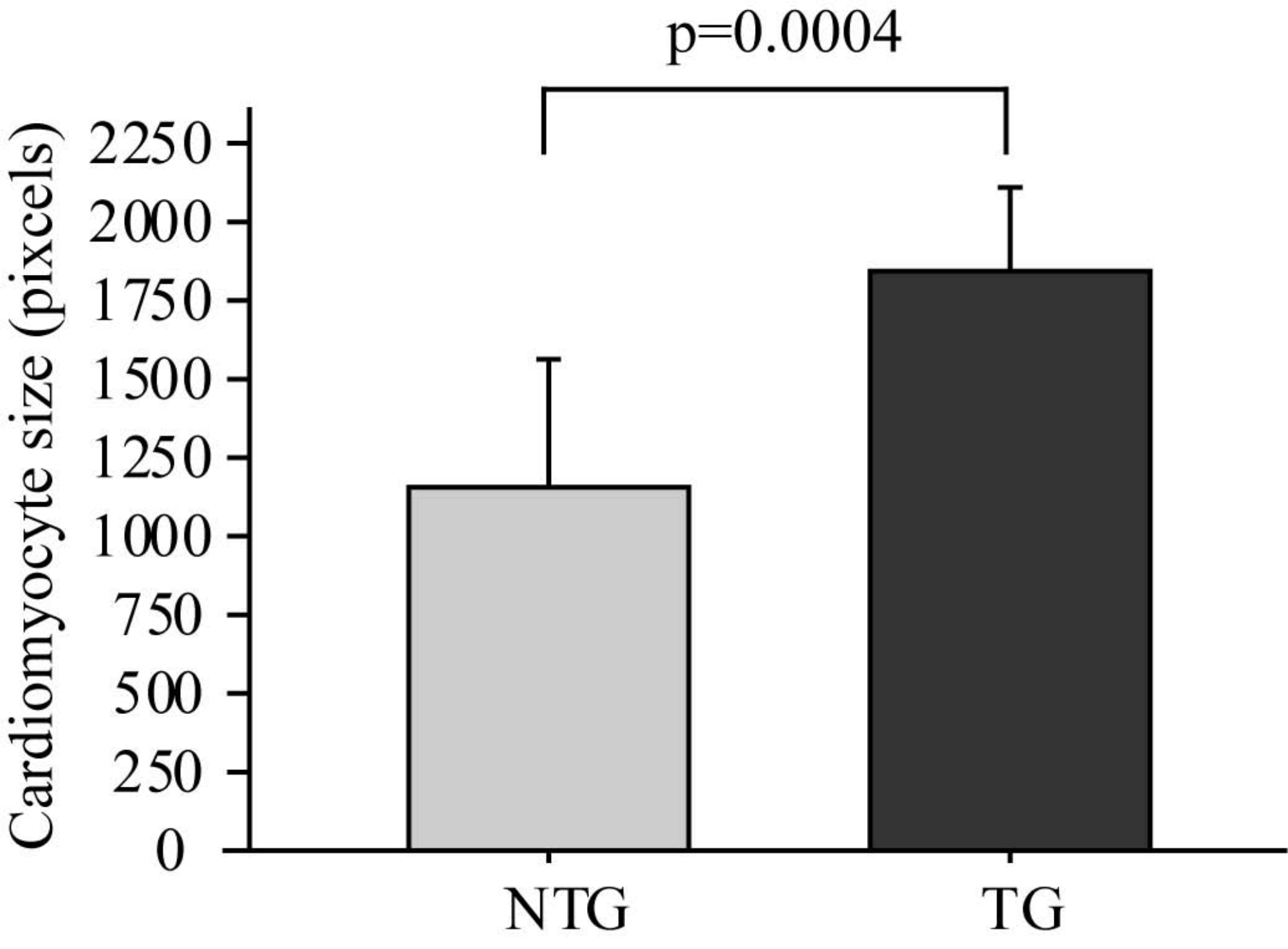
Solute carrier family 2, member 3	SLC2A3	1.0	4.59
Thioredoxin interacting protein	TXNIP	NC	-3.03
Tryptophanyl-tRNA synthetase	WARS	2.42	2.46
Tumor necrosis factor receptor superfamily, member 12A	TNFRSF12A	NC	10.56
V-maf musculoaponeurotic fibrosarcoma oncogene homolog F	MAFF	NC	17.15
Zinc finger protein 185 (LIM domain)	ZNF185	NC	-2.3

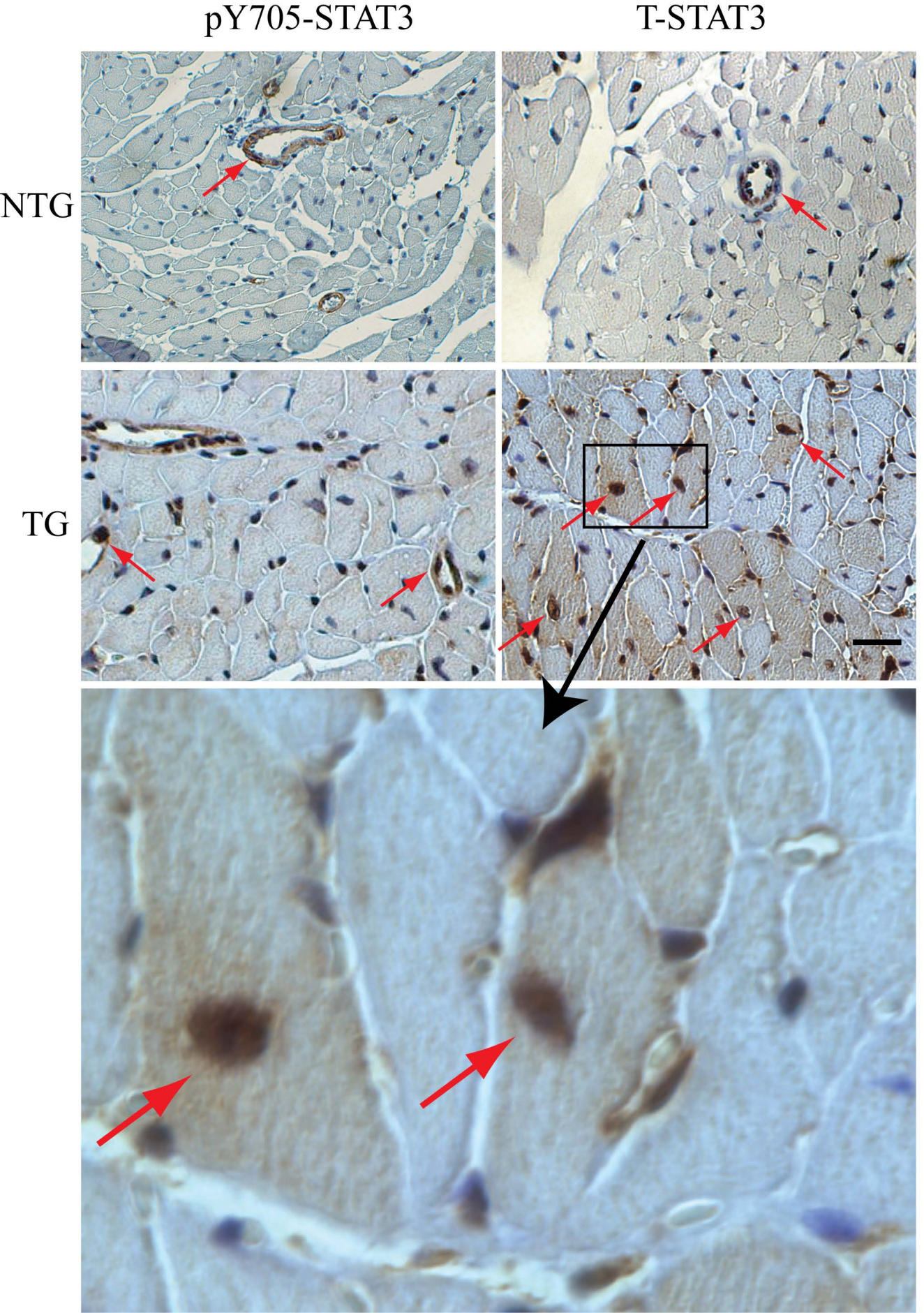
Genes that matched with microarray analysis from STAT3-null mouse embryonic fibroblasts overexpressing Y705F mouse STAT3 mutant to wild-type cells, and with primary human mammary epithelial cells (immortalized by the hTERT subunit of telomerase, have relatively low levels of STAT3 comparable with the levels in normal human fibroblasts) overexpressing Y705F mouse STAT3 mutant to non transfected cells [17]. * indicated genes found in mouse. Only the absolute value of $\log_2$ [fold change] ≥ 1 was considered as significant change, otherwise was considered as no change (NC).

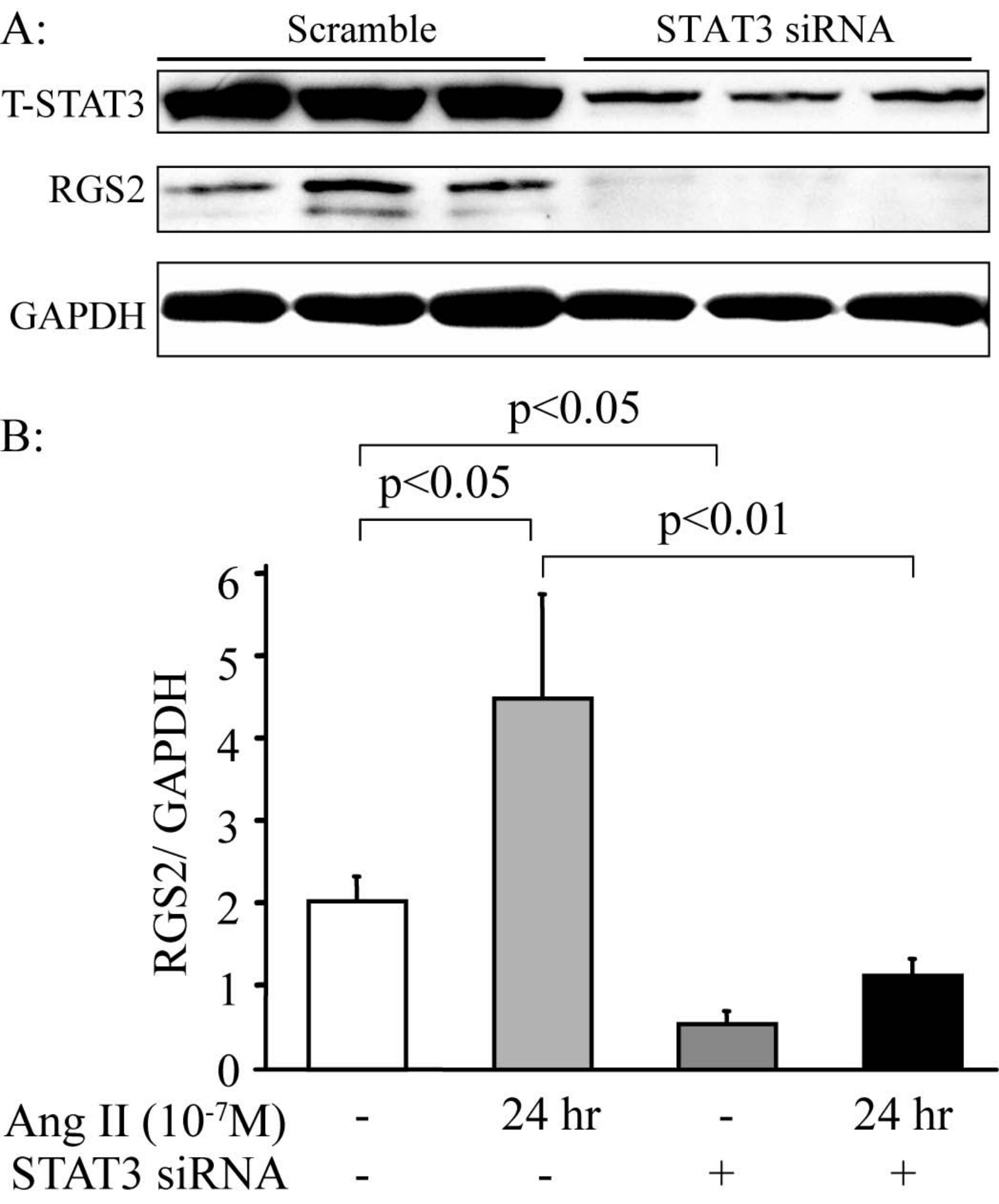
Supplementary Table 4: Primers designed based on the 8000 bp upstream sequence of human OPN (AF052124). Pare 22 amplified the immunoprecipitated promoter fragments as shown in Figure 5.

Primer name	Sequence
hOPNPRU20	GCA AGG GAG GCT GAG AAA TGT AGT
hOPNPRL20	CAG TGT GGG AGA GTG TCA GTA TGT C
hOIPNPRU21	CTC TCT TCG GTA TCA TTG GAA CTT
hOPNPRL21	TGT ATA TTT ACA GGG CCA GTA GGG
hOPNPRU22	GGG CAA ACT GAT TCT GGA TGA CTC
hOPNPRL22	CCA GAG TAG GGG ATT GAG GAG AAA C
hOPNPRU23	CCA ATC TCT TCA GTC AGT TTC TCC
hOPNPRL23	ACA ACA AGA GGA GTA AAC AGA TGG T
hOPNPRU24	AAG GCA ACA GAA AAT AAT CAG CAA
hOPNPRL24	AGA GGG AGA TAG AAC AAG GTG GAA
hOPNPRU25	GCT TTT CCA CCT TGT TCT ATC TCC
hOPNPRL25	TAT CAC AGG GTA GCA AGC AAA GGT
hOPNPRU26	GCA CCC TCT TAT CAT CCA CCT TTG
hOPNPRL26	GAA TGT GTC TGG GAT GCT TTG GAG
hOPNPRU27	TTG GGA CTA GGA ACT TTT TGA ATG
hOPNPRL27	TTG TCA ATT TAG TGG AGG GAA GTC
hOPNPRU28	CCT GTA AAG GGT CGT ATG GTT CA

hOPNPRL28	TCT TCA TTT GCT TTC TCA GTT GTG
hOPNPRU29	ACA ACT GAG AAA GCA AAT GAA GAT
hOPNPRL29	ACC ACT CTT GCC TGT ATG ATT GTA
hOPNPRU30	GCA GCC CTC TCA AGC AGT CAT CCT
hOPNPRL30	GCT GCA GAC ATC CTC CAC CAA CAC


p=0.0004
Cardiomyocyte size (pixcels)
2250
2000
1750
1500
1250
1000
750
500
250
0
NTG
TG


pY705-STAT3
T-STAT3
NTG
TG


A:
Scramble
STAT3 siRNA
T-STAT3
RGS2
GAPDH
B:
p<0.05
p<0.05
p<0.01
RGS2/ GAPDH
0
1
2
3
4
5
6
Ang II (10-7M)
-
24 hr
-
24 hr
STAT3 siRNA
-
-
+
+

Supplementary Figure legends

Supplementary Figure 1. Analysis of cardiomyocyte hypertrophy in AT1R TG mice based on the immunohistochemical stained images (as shown in Supplementary Figure 2), from TG and NTG mice (n=3). Single cells were selected using the Quick selection tool in Adobe Photoshop CS4 software, and then the pixels in the histogram were used for statistical analysis. The sizes of 15 randomly selected cardiomyocytes were calculated from 3 different mice in each group.

Supplementary Figure 2. Accumulation of U-STAT3 in the nuclei of TG mice heart cells. Immunohistochemical staining of pY705-STAT3 and T-STAT3 in heart sections showed a differential staining pattern. Brown color indicates positive staining, blue indicates nuclear staining.positive. The pY705-STAT3 staining was predominantly localized in vascular cells, but positive T-STAT3 staining was in the cardiac myocytes and localized mainly in the nucleus in TG mice. pY705-STAT3: phosphorylated STAT3; T-STAT3: total STAT3; TG: hAT1R transgenic mice; NTG: non-transgenic control mice. Scale bar, 30 μm

Supplementary Figure 3. U-STAT3 regulates RGS2 expression. A, HEK-AT1R cells were transfected with siRNA targeting human STAT3 or scrambled siRNA, and expression of STAT3 and RGS2 were analyzed by immunoblotting assay. GAPDH was blotted as a loading control. Cells transfected with STAT3 siRNA or scrambled siRNA were treated with (+) or without (-) Ang II for 24 hrs, and RGS2 (B) mRNA expression was assessed by real-time RT-PCR assay (n=4). The expression of STAT3 was shown in Figure 5C (left).