Supplementary Figure 1

(a) LVEDd and LVEDs over time. (b) Fibrosis area (%) over time. (c) BrdU-positive cells over time.

Supplementary Figure 2

(a) HW/BW (mg/g). (b) LVPWd (mm). (c) CSA of cardiomyocytes. (d) Representative images of vessel cardiomyocytes.
Supplementary Figure 6

a) Hypoxia

- control
- 2
- 3
- 5
- 7 (days)

Hif-1
p53
Vegf
Actin

b) Hypoxia

- control
- Ac5z
- Ac5p53

Hif-1
p53
Vegf
Actin

Supplementary Figure 7

Supplementary Figure 8
Supplementary Figure 9

Supplementary Figure 10

Supplementary Figure 11
Supplementary Figure legends

Figure 1  Analyses of echocardiography

a, Echocardiographic analysis of mice during 28 days of TAC.  LVD, left ventricular
dimension; LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic
dimension.  b, Cardiac fibrosis was evaluated by staining with Masson’s trichrome.
The graph depicts the extent of cardiac fibrosis.  c, BrdU (50mg/kg) was administered
by intraperitoneal injection everyday.  Mice were sacrificed at the indicated time
points after TAC, and the number of BrdU-positive cells was counted as described in
Methods.  Error bars indicate s.e.m.  n=5 for a and b; n=4 for c.  *P<0.05, **P<0.001 vs. day 0; †P<0.005 vs. day 3; #P<0.05, ##P<0.001 vs. day 7; §P<0.01 vs. day 14.

Figure 2  Effects of TNP-470 on Ang II-induced hypertrophy

a, HW/BW of mice that were subjected to 4 weeks of infusion of angiotensin II (Ang II)
or vehicle and treated with (+) or without TNP-470 (–).  Error bars indicate s.e.m.  n=3.  b, Echocardiographic analysis of mice that were subjected to 4 weeks of infusion of
angiotension II (Ang II) or vehicle and treated with (+) or without TNP-470 (–).  Error
bars indicate s.e.m.  n=3.  c, Histological analysis of the hearts from mice that were
subjected to 4 weeks of infusion of angiotension II (Ang II) or vehicle and treated with
(+ ) or without TNP-470 (–).  CSA of vehicle-treated mice without TNP-470 was
designated as 1.  Error bars indicate s.e.m.  n=3.  All P values in (a)–(c) are
determined by ANOVA.  *P<0.05, **P<0.001 vs. vehicle without TNP-470; †P<0.05 vs.
vehicle with TNP-470; #P<0.05, ##P<0.005, ###P<0.001 vs. Ang II without
TNP-470.  d, Double-immunostaining for dystrophin (brown) and PECAM (black) of
the hearts from mice that were subjected to 4 weeks of infusion of angiotension II (Ang
II) or vehicle and treated with or without TNP-470. Scale bar, 20 µm.

Figure 3  Expression of Hif-2α, p53, and anti-angiogenic factors during 28 days of TAC

a, Expression of Hif-2α was examined by Western blot analysis. b, The levels of p53 mRNA were examined by Northern blot analysis. c, Expression of thrombospondin (Tsp)-1 during 28 days of TAC. d, Expression of plasminogen activator inhibitor (Pai)-1 during 28 days of TAC. Error bars in (a)–(d) indicate s.e.m. n= 3–4. There were no significant changes of the expression during 28 days of TAC.

Figure 4  Quantitative analyses of Hif-1 activity, Vegf, p53 and Anf expression during 28 days of TAC

Relative values of Hif-1 activity and expression of Vegf, p53 and Anf were plotted in the graphs. The values on day 0 are designated as 1. Error bars indicate s.e.m. n= 5. *P<0.005, **P<0.001 vs. day 0; †P<0.005, ††P<0.001 vs. day 7; #P<0.05, ##P<0.01 vs. day 14.

Figure 5  Cardiomyocyte specific ablation of Hif-1α

a, Genomic PCR analysis. The 441 bp products represent the floxed allele, and the 355 bp products represent the deleted allele. b, Northern blot analysis of Hif-1α using tissue samples from the mutant mice 10 days after the treatment with tamoxifen (+) or vehicle (−).
Figure 6 Accumulation of p53 inhibits induction of angiogenic factors

a, Primary cultured cardiomyocytes were exposed to hypoxia. Nuclear extracts (Hif-1α) and whole cell lysates (p53, Vegf and Actin) were prepared at the indicated time points (days). Hif-1 activity was examined by gel mobility shift assay. Expression of p53 and Vegf was examined by Western blot analysis. Control samples (control) extracted from cardiomyocytes cultured under normoxia were also prepared.

b, Primary cultured cardiomyocytes were infected with an adenoviral vector encoding p53 (Adp53) or mock (AdLacZ). Infected cells were exposed to hypoxia 24 hours later. Cell lysates were prepared at 24 hours after treatment and examined for expression of p53 and Vegf by Western blot analysis. Hif-1 activity was examined by gel mobility shift assay. Control samples (control) extracted from cardiomyocytes cultured under normoxia were also prepared.

c, COS-7 cells were transfected with the Hif-1α-expressing vector plus the expression vector encoding p53 (+) or the empty vector (−). Transfected cells were treated with (+) or without MG132 (−). Cell lysates were prepared and subjected to Western blot analysis for Hif-1α and p53 (middle panels) or immunoprecipitation (IP) with anti-p53 antibody followed by immunoblotting (IB) with anti-Hif-1α antibody (upper panel). Actin served as a loading control (lower panel).

d, COS-7 cells were transfected with the luciferase reporter gene plasmid containing the Hif-1-binding sequence with (+) or without the expression vector encoding p53 (−). Transfected cells were then cultured under hypoxia (H) or normoxia (N) and the luciferase activity was measured 48 hours after transfection. The luciferase activity in control cells cultured under normoxia (p53(−)/N) is designated as 1. Error bars indicate s.e.m. n= 6. *P<0.005, **P<0.001 vs. p53(−)/N; †P<0.001 vs. p53 (−)/H.

e, COS-7 cells were transfected with the luciferase reporter gene plasmid containing the Hif-1-binding sequence with (+) or without the expression vector encoding p53 (−). Transfected cells were then
treated with (+) or without CoCl$_2$ (−). The luciferase activity was measured 24 hours after transfection. The luciferase activity in control cells without CoCl$_2$ treatment (p53 (−)/CoCl$_2$ (−)) is designated as 1. Error bars indicate s.e.m. n = 6. *$P<0.001$ vs. p53 (−)/CoCl$_2$ (−); †$P<0.001$ vs. p53 (−)/CoCl$_2$ (+).

**Figure 7** Expression of Vegf and Anf in the heart of p53-deficient mice

Western blot analysis for expression of Vegf and Anf in the heart of wild-type (Wild) and p53-deficient mice (KO) 4 weeks after TAC (TAC) or sham-operation (sham). The values of sham-operated wild-type mice were designated as 1. Error bars indicate s.e.m. n = 5. *$P<0.001$ vs. wild-type sham; †$P<0.05$, ††$P<0.001$ vs. KO sham; #*$P<0.001$ vs. wild-type TAC.

**Figure 8** Role of p53 in the MI heart

a, Cardiac expression of p53 was examined at the indicated time points after MI by Western blot analysis. b, Echocardiography of wild-type (Wild) and p53-deficient mice (KO) 14 days after myocardial infarction (MI) or sham-operation (sham). Error bars indicate s.e.m. n = 5. LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension. c, Histological analysis of the heart of wild-type (Wild) and p53-deficient mice (KO) was performed 14 days after myocardial infarction as described in Methods. Error bars indicate s.e.m. n = 5. All $P$ values in (b) and (c) are determined by ANOVA. *$P<0.001$ vs. wild-type sham; †$P<0.001$ vs. KO sham; #*$P<0.05$, ##$P<0.01$, ###$P<0.005$ vs. wild-type MI.
Figure 9  Expression of p53, Anf, and Vegf in the heart after quinacrine treatment

Wild-type mice were treated with quinacrine (+) or vehicle (−) and subjected to 2 weeks of TAC or sham-operation (sham). Whole cell lysates were prepared and analyzed for expression of p53, Vegf, and Anf by Western blot analysis. The graphs depict the relative expression of p53, Vegf, and Anf. The values of sham-operated mice treated with vehicle were designated as 1. Error bars indicate s.e.m. n= 5. *P<0.05, **P<0.001 vs. vehicle sham; †P<0.001 vs. quinacrine sham; #P<0.005, ##P<0.001 vs. vehicle TAC.

Figure 10  Effects of quinacrine on cardiac function and angiogenesis in p53-deficient mice

a, b, Wild-type (Wild) or p53-deficient mice (KO) were treated with (+) or without (−) quinacrine and subjected to TAC, and echocardiography (a) and histological analysis (b) were performed. Error bars indicate s.e.m. n= 5. *P<0.01, **P<0.001 vs. wild-type mice without quinacrine treatment.

Figure 11  Role of p53 in cardiomyocyte death

a, TUNEL analysis in the heart of wild-type (Wild) and p53-deficient mice (KO) 4 weeks after TAC or sham-operation (sham). The number of TUNEL-positive cardiomyocytes is plotted in the graph. Error bars indicate s.e.m. n= 4. All P values are determined by ANOVA. *P<0.01 vs. wild-type sham; #P<0.005 vs. wild-type TAC. b, TUNEL analysis in the hearts from mice that were subjected to TAC or sham-operation (sham) and treated with quinacrine (+) or vehicle (−) for 2
weeks. The number of TUNEL-positive cells is plotted in the graphs. Error bars indicate s.e.m. n = 4. All P values are determined by ANOVA. *P<0.001 vs. vehicle sham; †P<0.001 vs. quinacrine sham; #P<0.005 vs. vehicle TAC. c, Expression of p53 and Bax was examined in the hearts of mice prepared in Figure 11b. Error bars indicate s.e.m. n = 5. *P<0.05, **P<0.005, ***P<0.001 vs. vehicle sham; †P<0.001 vs. quinacrine sham; #P<0.05, ##P<0.01, ###P<0.001 vs. vehicle TAC.

Supplementary Methods

Animal models

All protocols were approved by our institutional review board. TAC was performed as described previously on 8-week-old male C57BL/6 mice (SLC, Shizuoka, Japan). As an alternative cardiac hypertrophy model, angiotensin II (200 ng/kg/min) was continuously administered by osmotic minipump (DURECT, Cupertino, California) implanted subcutaneously into 8-week-old male C57BL/6 mice. MI was produced by ligation of the left anterior descending artery and analyzed as described previously. To inhibit cardiac angiogenesis, TNP-470 (30 mg/kg; Takeda Pharmaceutical Company Limited, Osaka, Japan), known as an angiogenic inhibitor, was administered by subcutaneous injection once every other day. p53-deficient mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). For p53 activation mice were treated with quinacrine (5 mg/kg/day, Sigma, St. Louis, Missouri). The treatment of wild-type mice with quinacrine did not affect their health status including food intake and body weight gain during the experiment periods (data not shown).
Physiological analysis and histological analysis

Echocardiography was performed as described previously. Frozen sagittal-sections of the heart samples were stained with hematoxylin and eosin. Frozen cross-sections of the heart samples were immunohistochemically double-stained by use of antibodies against platelet and endothelial cell adhesion molecule (PECAM) (PharMingen, San Jose, California) and dystrophin (Novocastra Laboratories, Newcastle, UK). For measurement of cross-sectional area (CSA) of cardiomyocytes, 50 randomly selected cardiomyocytes in the left ventricle of the cross-sectional heart samples were measured by tracing an immunostaining of dystrophin with NIH Image-J software. Using the same sections, the number of PECAM-positive vessels was counted, and the number of microvessels per cardiomyocyte was calculated. Frozen cross-sections of the heart samples were also stained by using an antibody against p53 (Santa Cruz Biotechnology, Santa Cruz, California) or Bax (Santa Cruz Biotechnology) in combination with immunostainings for appropriate cell markers. Tissue hypoxia was estimated with the Hypoxyprobe (Chemicon, Temecula, California) according to the manufacturer’s instructions. TUNEL labeling was performed according to the manufacturer’s protocol (In Situ Apoptosis Detection kit; Takara, Shiga, Japan) in combination with immunostainings for appropriate cell markers. For in vivo cell proliferation assay, BrdU (50mg/kg, PharMingen) was administered by intraperitoneal injection everyday after TAC until sacrifice. Frozen cross-sections of the heart samples were then stained by use of an antibody against BrdU (Becton Dickinson Immunocytometry Systems, San Jose, California) in combination with immunostainings for appropriate cell markers. Digital photographs were taken at ×400 magnification, and 25 random high-power fields (HPF) from each heart sample were chosen and blindly quantified. We examined vascularization by measuring the number of capillary endothelial cells in light-microscopic sections taken from the border zone of the hearts 2 weeks after MI.
Capillary endothelial cells were identified by immunohistochemical staining with anti-PECAM antibody. The extent of fibrosis was measured in 3 sections from each heart and the value was expressed as the ratio of Masson’s trichrome stained area to total LV free wall.

**Conditional ablation of the Hif-1α gene in cardiomyocytes of adult murine heart**

We prepared transgenic mice in which a transgene encoding Cre recombinase fused to the mutated estrogen receptor domains (MerCreMer) was driven by the cardiomyocyte specific α-myosin heavy chain (MHC) promoter. We then crossed the MHC-MerCreMer mice with mice that carried floxed Hif-1α alleles (Hif-1α\textsuperscript{floxed/floxed}) and produced the MHC-MerCreMer;Hif-1α\textsuperscript{floxed/floxed} mutant mice. Since the MerCreMer protein is sensitive to tamoxifen but not to estrogen, treatment of the MHC-MerCreMer;Hif-1α\textsuperscript{floxed/floxed} mutant mice with tamoxifen induced Cre-mediated recombination of floxed Hif-1α alleles in cardiomyocytes. The MHC-MerCreMer transgenic mice and the floxed Hif-1α mice (Hif-1α\textsuperscript{floxed/floxed}) were genotyped as previously described. To induce Cre-mediated recombination, the MHC-MerCreMer;Hif-1α\textsuperscript{floxed/floxed} mutant mice were treated with 20 mg/kg of tamoxifen (Sigma) by intraperitoneal injection once a day for five consecutive days. Ten days after the treatment, the mutant mice underwent TAC as described above. We utilized two types of littermate controls: transgenic, floxed mice without tamoxifen treatment; and non-transgenic, floxed mice with tamoxifen treatment. There were no significant differences in left ventricular posterior wall thickness (LVPWTD), fractional shortening (FS), CSA of cardiomyocytes and the number of microvessels per cardiomyocyte between the Hif-1α mutant mice with tamoxifen treatment and two types of littermate controls at the basal condition.
**In vivo gene transfer**

Soluble Flt-1 is known to bind to vascular endothelial growth factor (VEGF), thereby acting as an inhibitor for VEGF⁸. We injected an adenoviral vector encoding the murine soluble Flt-1 gene (10⁹ pfu; InvivoGen, San Diego, California) into the thigh muscles of mice twice a week during TAC. To promote angiogenesis, an adenoviral vector encoding the Vegf gene (10⁹ pfu)⁹ and an adenoviral vector encoding the angiopoietin-1 (Ang-1) gene (10⁹ pfu)¹⁰ were injected into left ventricular cavity under temporary clamping of ascending aorta and pulmonary artery 2 days prior to the TAC procedure. For the control mice, an adenoviral vector encoding LacZ was injected into left ventricular cavity 2 days before TAC.

**RNA analysis**

Total RNA (10 µg) was isolated from the left ventricle of mice by using the RNAZol-B (Molecular Research Center, Cincinnati, Ohio) and subjected to the ribonuclease protection assay (BD RiboQuant™, PharMingen, San Jose, California). Expression of Vegf and Ang-1 was detected with mAngio-1 Multi-Probe Template Set (PharMingen). For Northern blot analysis, total RNA (30 µg) was separated on a formaldehyde denaturing gel and transferred to a nylon membrane (Amersham, Buckinghamshire, UK). The blot was then hybridized with radiolabelled p53, Hif1-α, Pai-1 cDNA probes using the Quickhyb hybridization solution (Stratagene, Tokyo, Japan) according to the manufacturer’s instructions. Pai-1 cDNA fragment was a gift from Dr. K Maemura (University of Tokyo, Tokyo, Japan).
Western blot analysis

Whole-cell lysates (30–50 µg) or nuclear extracts (10–20 µg) were resolved by SDS polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Massachusetts) and incubated with the first antibody followed by an anti-rabbit immunoglobulin G-horseradish peroxidase antibody or anti-mouse immunoglobulin G-horseradish peroxidase antibody (Jackson, West Grove, Pennsylvania). Specific proteins were detected using enhanced chemiluminescence (Amersham). The first antibodies used for Western blotting are as follows: anti-Vegf antibody (Santa Cruz Biotechnology, Santa Cruz, California), anti-actin antibody (Sigma), anti-Hif-1α antibody (Novus Biologicals, Littleton, Colorado), anti-p53 antibody (Santa Cruz Biotechnology), anti-phosphorylated p53 antibody (Santa Cruz Biotechnology), anti-Bax antibody (Santa Cruz Biotechnology), anti-Chk2 antibody (Upstate Cell Signaling, Charlottesville, Virginia), anti-Anf antibody (Santa Cruz Biotechnology), anti-TFIIB antibody (Santa Cruz Biotechnology), and anti-thrombospondin-1 antibody (Ab-11, Lab Vision, Fremont, California). Immunoprecipitation was performed as described previously.11

Cell culture

Cardiomyocytes were prepared from ventricles of one-day-old Wistar rats, seeded onto 100-mm plastic culture dishes at a density of 1×10^5 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a mixture of 95% air and 5% CO₂. Two days after seeding, cardiomyocytes were exposed to 250 µM cobalt chloride (CoCl₂) or hypoxia (<1% O₂, PO₂ 18~20 mmHg) and harvested at the indicated time points. In some experiments, cardiomyocytes were
infected with an adenoviral vector encoding p53 or LacZ (20 multiplicity of infection). Twenty-four hours after infections cardiomyocytes were exposed to CoCl$_2$ or hypoxia. Cells were harvested 24 hours after exposure, and nuclear extracts and whole cell lysates were prepared. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a mixture of 95% air and 5% CO$_2$. Transfection was performed by using fugene 6 (Roche, Indianapolis, Indiana) according to the manufacturer’s instructions. The expression vector encoding p53 (ref $^{12}$) and the vector encoding Hif-1α$^{13}$ were gifts from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, Maryland) and Dr. L.E. Huang (Harvard Medical School, Boston, Massachusetts), respectively.

**Luciferase assays**

The reporter gene plasmid (1 µg) was transfected into COS7 cells with or without the p53 expression vector. The control vector encoding *Renilla* luciferase (0.1 µg) was cotransfected as an internal control. Transfected cells were then exposed to CoCl$_2$ or hypoxia. Luciferase assay was carried out 24 hours later using dual-luciferase reporter assay system (Promega, Madison, Wyoming) according to the manufacturer’s instructions. The luciferase reporter gene containing the hypoxia response elements was a gift from Dr. M Nangaku (University of Tokyo, Tokyo, Japan).

**Electrophoretic mobility shift assay (EMSA)**

Tissue nuclear extracts were prepared as described previously$^{14}$ with modifications. Left ventricles were minced and homogenized with Dounce homogenizer (Wheaton, Millville, New Jersey) in buffer A with 0.5% NP-40. After incubation on ice for 15
minutes, the samples were centrifuged and the pellets were resuspended in buffer A containing 1% NP-40. The samples were filtered with 90 µm-pore size filter in spin-column (MoBiTec, Goettingen, Germany). Then nuclear pellets were resuspended in buffer C and mildly vortexed for 30 minutes at 4°C. The samples were centrifuged and the supernatants were used as nuclear extracts. The oligonucleotide probe used in EMSA for Hif-1 consisted of the VEGF promoter sequence, 5'-gcggatccgggCCACAGTGCATACGTGGGCTCCAACAGGTCCTCTTagatcttc-3'. Nuclear extracts were incubated with 32P-labeled oligonucleotide in binding buffer containing 10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.05% NP-40, and 50 µg/ml poly(dI-dC) for 20 minutes at room temperature. Competition experiments were performed with 40~300-fold molar excess of unlabeled oligonucleotides relative to the labeled probe. The reaction mixtures were subjected to electrophoresis using 5% nondenaturing polyacrylamide gels.

**Statistical analysis**

Data were shown as mean±s.e.m. Multiple group comparison was performed by one-way ANOVA followed by the Bonferroni procedure for comparison of means. Comparisons between two groups were analyzed by two-way ANOVA. Values of $P<0.05$ were considered statistically significant.

**Supplementary References**


