

Cardiac-specific Activation of Signal Transducer and Activator of Transcription 3 Promotes Vascular Formation in the Heart*

Received for publication, August 27, 2001, and in revised form, December 13, 2001
Published, JBC Papers in Press, December 14, 2001, DOI 10.1074/jbc.M108246200

Tomoaki Osugi, Yuichi Oshima, Yasushi Fujio‡, Masanobu Funamoto, Atsuko Yamashita§, Shinji Negoro, Keita Kunisada, Masahiro Izumi, Yoshikazu Nakaoka, Hisao Hirota, Masaru Okabe§, Keiko Yamauchi-Takahara, Ichiro Kawase, and Tadamitsu Kishimoto

From the Department of Molecular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka Suita City, Osaka 565-0871, Japan and the §Genome Information Research Center, Osaka University, 3-1 Yamada-oka Suita City, Osaka 565-0871, Japan

Signal transducer and activator of transcription 3 (STAT3) functions in cell proliferation, differentiation, and cell survival. Previously, we have demonstrated that the activation of STAT3 is required for glycoprotein 130-mediated induction of VEGF in cardiac myocytes, but the functional importance of STAT3 as an angiogenic mediator remains to be determined. To address this issue, we first generated the adenoviral vector expressing constitutively active STAT3 (caSTAT3). Adenoviral gene transfer of caSTAT3 induced an increase in the expression of VEGF in cultured cardiomyocytes. The conditioned medium from caSTAT3-transfected cardiomyocyte culture promoted endothelial tubule formation, which was inhibited by anti-VEGF antibody. Next, we generated the transgenic (TG) mice with cardiac-specific overexpression of caSTAT3 and demonstrated that caSTAT3 TG mice showed evidence of VEGF induction in the hearts. The caSTAT3 TG hearts also demonstrated increased capillary density accompanied by an increase in the expression of VE-cadherin, an endothelial-specific component. These data indicate that caSTAT3 TG hearts exhibit an enriched vascular structure compared with non-transgenic hearts. The study presented here provides the first evidence that activation of STAT3 controls vessel growth *in vivo* and suggests that STAT3 contributes to cardiac adaptation by regulating vascular function under the conditions of stress.

Signal transducer and activator of transcription 3 (STAT3)¹ was originally identified as a molecule responsible for cytokine signaling in the interleukin-6 family system (1). Stimulation of GP130, a common subunit of the interleukin-6 family cytokine

receptor, leads to activation of Janus kinase, which phosphorylates STAT3 on the tyrosine residue. Phosphorylated STAT3 translocates from the cytoplasm to the nucleus and activates the transcription of target genes. STAT3 is involved in proliferation (2), differentiation (3, 4), and cell survival (5, 6). It is also demonstrated that STAT3 plays a crucial role in the regeneration or remodeling of tissues *in vivo* (7, 8).

In cardiac myocytes, the GP130/STAT3-signaling pathway is activated by extracellular stresses including catecholamine stress (9) and mechanical stretch (10). The stimulation of GP130 activates Janus kinase/STAT, mitogen activated protein kinase, and phosphatidylinositol 3-kinase/Akt pathways (11, 12). The signals through GP130 transduce cell survival signals as well as hypertrophic signals (13–16). Mice with *GP130* gene knocked out in a cardiac-specific manner showed the decompensation in response to pressure overload (17), suggesting that the GP130/Janus kinase/STAT pathway is critical for adaptation in response to extracellular stresses.

When cardiac hypertrophy occurs, angiogenesis has been demonstrated to be essential for adaptation (18). Recently, we have demonstrated that STAT3 is required for GP130-mediated induction of VEGF, a potent angiogenic factor, in cardiomyocytes (19). Interestingly, a recent study (20) demonstrated that cardiac-specific ablation of the *VEGF* gene resulted in failure to maintain cardiac function. Based on these findings, it is hypothesized that the activation of STAT3 in cardiac myocytes plays an important role in vascular formation in the heart through the paracrine system and contributes to the maintenance of cardiac function.

In this study, we aimed to clarify the biological significance of STAT3 in cardiac myocyte-vascular endothelial cell interaction. For this purpose, we generated the adenovirus vector expressing the constitutively active form of STAT3 (caSTAT3) (6). Adenoviral transfection of caSTAT3 in cardiomyocytes leads to an increase in VEGF mRNA expression. The conditioned medium from caSTAT3-transfected cardiac myocytes promoted endothelial tubule formation, which was inhibited by anti-VEGF antibody. Next, we generated cardiac myocyte-specific transgenic mice (TG) expressing caSTAT3 under the control of *α-myosin heavy chain (α-MHC)* gene promoter and analyzed vascular formation in the hearts of these mice that showed the up-regulation of VEGF both in mRNA and protein level accompanied by an increase in capillary density and enhanced expression of VE-cadherin compared with those in non-transgenic littermates. These findings indicate that constitutive activation of STAT3 in cardiomyocytes promotes vascular formation in the heart, suggesting a novel function of STAT3 as a regulator of vascularization in the heart.

* This work is supported in part by grants from the Study Group of Molecular Cardiology, the Kanae Foundation, the Japan Heart Foundation Research, the Takeda Science Foundation, and the Osaka Foundation for Promotion of Clinical Immunology (to Y. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 81-6-6879-3835; Fax: 81-6-6879-3839; E-mail: yfujio@imed3.med.osaka-u.ac.jp.

¹ The abbreviations used are: STAT3, signal transducer and activator of transcription 3; GP130, glycoprotein 130; VEGF, vascular endothelial growth factor; caSTAT3, constitutively active STAT3; mRNA, messenger RNA; PIPES, 1,4-piperazinediethanesulfonic acid; dnSTAT3, dominant negative STAT3; *α-MHC*, myosin heavy chain; adeno-caSTAT3, adenovirus vector expressing caSTAT3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; moi, multiplicity of infection; HUVEC, human umbilical vein endothelial cell; SSC, standard saline citrate; TG, transgenic.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of rat neonatal cardiac myocytes were prepared as described previously (13). The ventricles of the hearts were isolated from neonatal rats and treated with trypsin and collagenase. Isolated cells were collected by centrifugation and resuspended in M-199 containing 10% calf serum. Cultures were then enriched with myocytes by preplating for 60 min to deplete non-myocyte population, and non-attached cells were cultured in M-199 with 10% calf serum. Immunofluorescent examination with anti-sarcomeric α -actinin antibody revealed that >90% cultured cells consisted of cardiac myocytes (data not shown).

Construction of Adenoviral Vector Expressing caSTAT3—caSTAT3 cDNA was kindly provided by Drs. J. F. Bromberg and J. E. Darnell, Jr. (The Rockefeller University, New York) (6). The adenovirus vector expressing caSTAT3 (adeno-caSTAT3) was generated according to the protocol described elsewhere (21). caSTAT3 cDNA was subcloned into the multicloning site of the pACCMVpLpA vector, and pACCMVpLpA-caSTAT3 plasmid was co-transfected with JM17 plasmid into 293 cells to allow for homologous recombination. The adeno-caSTAT3 construct was cloned with the plaque formation method and amplified in 293 cells. Finally, the adenovirus vector was purified by ultracentrifugation in the presence of CsCl.

Northern Blot Analyses—Northern blot analyses were performed as described previously (19). The probe for GAPDH was kindly donated by Dr. K. R. Chien (University of California, San Diego, CA), and the VEGF probe was generated as described previously (19). Total RNA was prepared with the acid guanidinium-phenol-chloroform method (22). 10 μ g of total RNA was size-fractionated by 1.5% formaldehyde-agarose gel electrophoresis and blotted onto a nylon membrane (Hybond N+, Amersham Biosciences, Inc.) in 20 \times SSC. After prehybridization in a hybridization buffer (650 mM sodium chloride, 100 mM PIPES, pH 6.8, 5 \times Denhardt's solution, 0.1% SDS, 50% formamide, 10 μ g/ml of denatured salmon sperm DNA), the membranes were hybridized with the 32 P-labeled probes at 42 $^{\circ}$ C for 15 h. Membranes were washed twice with 2 \times SSC containing 0.1% SDS at room temperature and twice with 0.1 \times SSC containing 0.1% SDS at 60 $^{\circ}$ C. Autoradiography was performed at -80° C.

Western Blot Analyses—Isolated hearts were washed with phosphate-buffered saline and homogenized in lysis buffer (50 mM HEPES, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride) in a Polytron homogenizer. Protein concentration was measured with BCA protein assay kit (Pierce). Proteins (30 μ g) were separated in 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking in TBS-T (150 mM NaCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4) containing 2% skim milk, membranes were probed with anti-VE-cadherin, anti-Cdk4, anti-STAT3 (Santa Cruz Biotechnology) or anti-FLAG (Upstate Biotechnology) antibody. ECL system (Amersham Biosciences, Inc.) was used for the detection.

Immunofluorometric Assays of VEGF—VEGF contents in the hearts were measured as described previously (19). The hearts from TG or non-TG mice were homogenated in lysis buffer ($n = 4$ each). Crude extracts were then prepared by centrifugation at 15,000 rpm for 10 min, and VEGF concentrations were measured by using a VEGF enzyme-linked immunosorbent assay kit (Quantikine M mouse VEGF, R&D Systems). VEGF concentrations were normalized with protein concentration assessed with a BCA protein assay kit (Pierce).

Measurement of Angiogenic Activities—Angiogenic activities of the conditioned medium were measured with an angiogenesis kit (KURABO) according to the protocol by the manufacturer (23). Cardiac myocytes (5×10^6 cells/well) were incubated with adenoviral vector-expressing β -galactosidase or caSTAT3 at a moi 20 in M-199 with 2% fetal calf serum for 12 h. After transfection, cells were washed three times and cultured in 1 ml of serum-depleted M-199 for 24 h. An equal amount of the conditioned medium from the cardiac myocyte culture was added to the basal medium of the human umbilical vein endothelial cell (HUVEC)/fibroblast co-culture. The medium was changed every 3 days. HUVECs were cultured in the presence or absence of 1 μ g/ml of anti-mouse VEGF antibody (Genzyme Techne) or non-immune IgG (Caltag Laboratories) for 9 days and stained with anti-CD31 antibody according to the protocol by the manufacturer. Capillary density was estimated with the Chalkley count method (24).

Generation of Transgenic Mice Expressing STAT3 Mutants—Transgenic mice with cardiac-specific overexpression of the caSTAT3 gene, caSTAT3 TG, were generated as described previously (15). caSTAT3 cDNA was subcloned into *SalI* site of pBSIISK(+)- α -MHC plasmid, a kind gift from Dr. J. Robbins (Children's Hospital, Cincinnati, OH) (25).

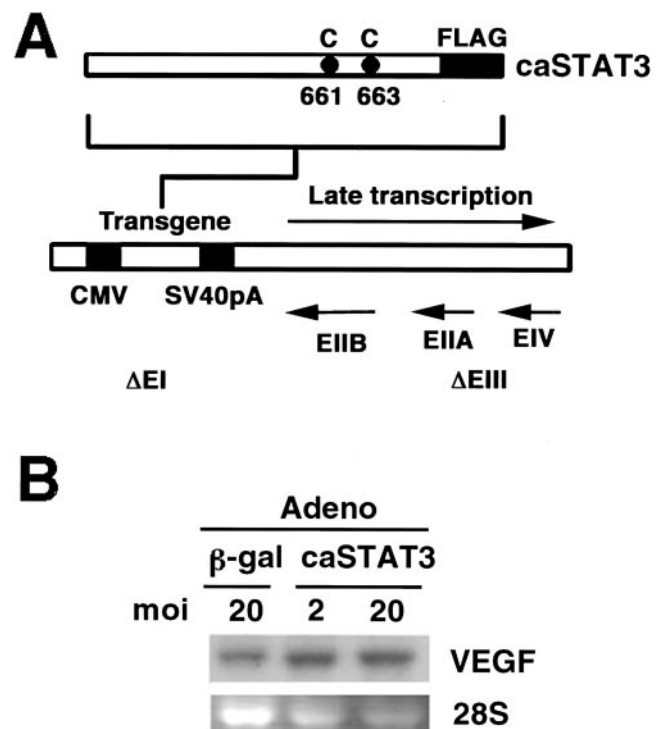


FIG. 1. Adenoviral transfer of constitutively active STAT3 results in increased VEGF mRNA expression. A, structure of adenovirus vector expressing constitutively active STAT3 (caSTAT3). Substitution of Ala-661 and Asn-663 to cysteine renders the molecule capable of dimerizing without extracellular stimuli. The transgene is driven under the control of the CMV promoter. B, cardiomyocytes were transfected with adenovirus vector expressing caSTAT3 or β -galactosidase (β -gal) at the indicated by moi. Cells were cultured for 8 h in serum-depleted medium, and total RNA was prepared and Northern blotted for VEGF mRNA.

The plasmid was digested with *NotI* to generate an 8.4-kbp DNA fragments. Transgenic mice lines were generated using BDF-1 xBDF-1 fertilized eggs as described elsewhere (26).

The generation of transgenic mice with cardiac-specific overexpression of the dominant negative form of STAT3 (dnSTAT3) was described previously (19). The care of all animals was in compliance with Osaka University animal care guidelines.

Electrophoretic mobility shift assay—Electrophoretic mobility shift assay was performed as described previously (6) with some modification. Whole extracts (5 μ g of proteins) from non-TG or TG heart ventricles were incubated with 32 P-labeled *m67* (5'-dGATTTCCCGTAAATCAT-3') probe (0.5 ng) in 20 μ l of reaction buffer (14 mM HEPES, pH 7.8, 52 mM KCl, 4.2 mM MgCl₂, 0.9 mM EDTA, 12.5% glycerol). The DNA-protein complex was analyzed by polyacrylamide gel electrophoresis.

Immunohistochemical Analyses—Immunohistochemical analyses were performed with Vecstatin ABC kit (Vector Laboratories) according to the protocol by the manufacturer. Four transgenic mice and four of their non-transgenic littermates (10–12-weeks-old) were sacrificed, and their hearts were isolated immediately. The hearts were frozen, and 10- μ m sections were prepared with a Microm HM500MS (Zeiss) and fixed with ice-cold acetone. After blocking with 2% rabbit serum in phosphate-buffered saline, sections were incubated first with anti-CD31 antibody (PharMingen) and then with biotinylated secondary antibody and finally with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. Sections were developed in peroxidase substrate solution.

RESULTS

Adenoviral Gene Transfer of caSTAT3 Induced VEGF mRNA in Cultured Cardiac Myocytes—To achieve a high frequency of the transfection in cardiac myocytes (27), adenovirus vector was generated as described previously (21). The structure of the adeno-caSTAT3 is shown in Fig. 1A. Mutations at positions 661 and 663 induce the conformational changes in the STAT3 molecule, leading to the formation of activated STAT3 (6).

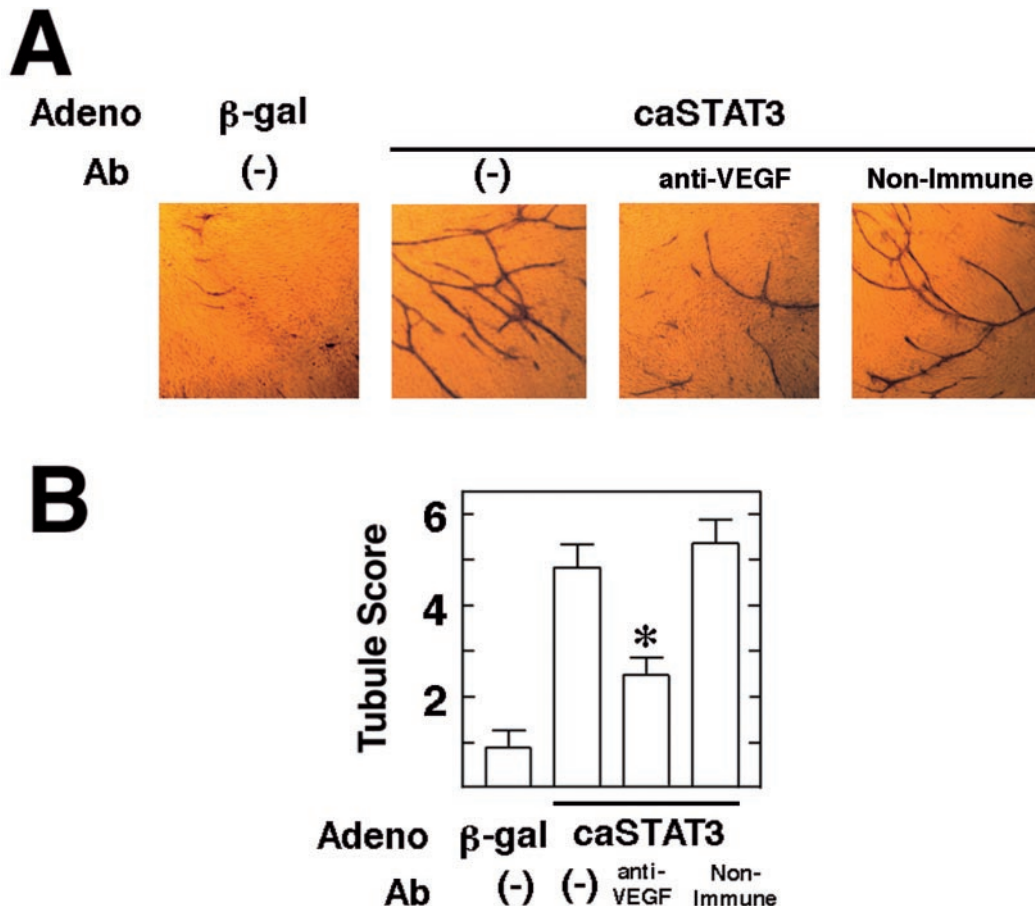


FIG. 2. Conditioned medium from cardiomyocytes transfected with constitutively active STAT3 promotes endothelial tubule formation. Rat cardiomyocyte cultures were transfected with the adenovirus vector expressing caSTAT3 or β -galactosidase (β -gal) at moi of 20 as described under "Experimental Procedures." Equal amounts of the conditioned medium from the cardiac myocyte cultures were added to the basal culture medium in the HUVEC/fibroblast co-culture system either in the presence or absence of anti-mouse VEGF or non-immune IgG. Cells were cultured for 9 days and stained with anti-CD31 antibody. *A*, representative photographs of tubule formation. *B*, tubule formation was quantified with the Chalkley count methods, and the tubule score was estimated in 10 fields. Data are shown as the means \pm S.D. *, $p < 0.05$ versus the medium from caSTAT3-transfected cells with non-immune IgG.

Adenovirus vector expressing β -galactosidase was used as a control. Cells were incubated with adenovirus vectors for 12 h and cultured in serum-depleted medium for 8 h. Total RNA was prepared and Northern blotted for VEGF mRNA. As shown in Fig. 1*B*, transfection of caSTAT3 resulted in an increase in VEGF mRNA expression. A higher level of VEGF mRNA expression was also detected in caSTAT3-transfected cardiomyocytes even at moi 2 but not in β -galactosidase-transfected cells at moi 20, indicating that VEGF mRNA up-regulation is unlikely to result from the artificial effects of adenovirus infection.

The Conditioned Medium from caSTAT3-transfected Cardiomyocyte Culture Showed Angiogenic Activities—To examine the functional relevance of up-regulation of VEGF in caSTAT3-transfected cells, we examined the angiogenic activities in the culture medium from the caSTAT3-transfected cardiomyocytes. The conditioned medium was prepared from cardiomyocyte culture transfected with adenoviral vector-expressing β -galactosidase or adeno-caSTAT3 and added to the basal medium of HUVEC/fibroblast co-culture. Tubule formation was examined by staining with anti-CD31 antibody. As shown in Fig. 2, the conditioned medium from caSTAT3-transfected cardiomyocytes promoted tubule formation, which was inhibited by two-thirds by anti-mouse VEGF antibody but not by non-immune IgG.

Generation of Cardiac-specific Transgenic Mice with Overexpression of caSTAT3—We generated transgenic mice with car-

diac-specific overexpression of caSTAT3 (Fig. 3). Three transgenic lines were obtained that showed an approximate 10-fold increase in STAT3 protein expression compared with endogenous STAT3 in non-TG littermates. All the transgenic lines showed a similar phenotype.

Using an oligonucleotide probe containing the STAT3 binding motif, electrophoretic mobility shift assay demonstrated that the protein extract from caSTAT3 TG hearts showed enhanced DNA binding activity in the absence of extracellular stimuli such as leukemia inhibitory factor (11). Anti-STAT3 antibody inhibited DNA-protein complex formation, and the DNA-protein complex was supershifted by anti-FLAG antibody (Fig. 3*C*). These results show that caSTAT3 transgene product functions *in vivo* as a constitutively active STAT3 in cardiomyocytes without extracellular stimuli.

VEGF Expression Was Up-regulated in caSTAT3 TG Hearts—To analyze the expression of VEGF mRNA in caSTAT3 TG hearts, total RNA from the hearts of TG mice ($n = 3$) and from those of non-TG littermates ($n = 3$) (5-weeks-old) were Northern blotted for VEGF. As shown in Fig. 4*B*, VEGF mRNA expression in caSTAT3 TG increased by $49 \pm 13\%$ ($p < 0.05$) compared with that in non-TG hearts.

We next measured VEGF protein in the hearts from transgenic mice. Crude protein extracts were prepared from TG mice ($n = 4$) and from non-TG mice ($n = 4$), and VEGF concentrations were measured with the enzyme-linked immunosorbent assay system as described under "Experimental Procedures."

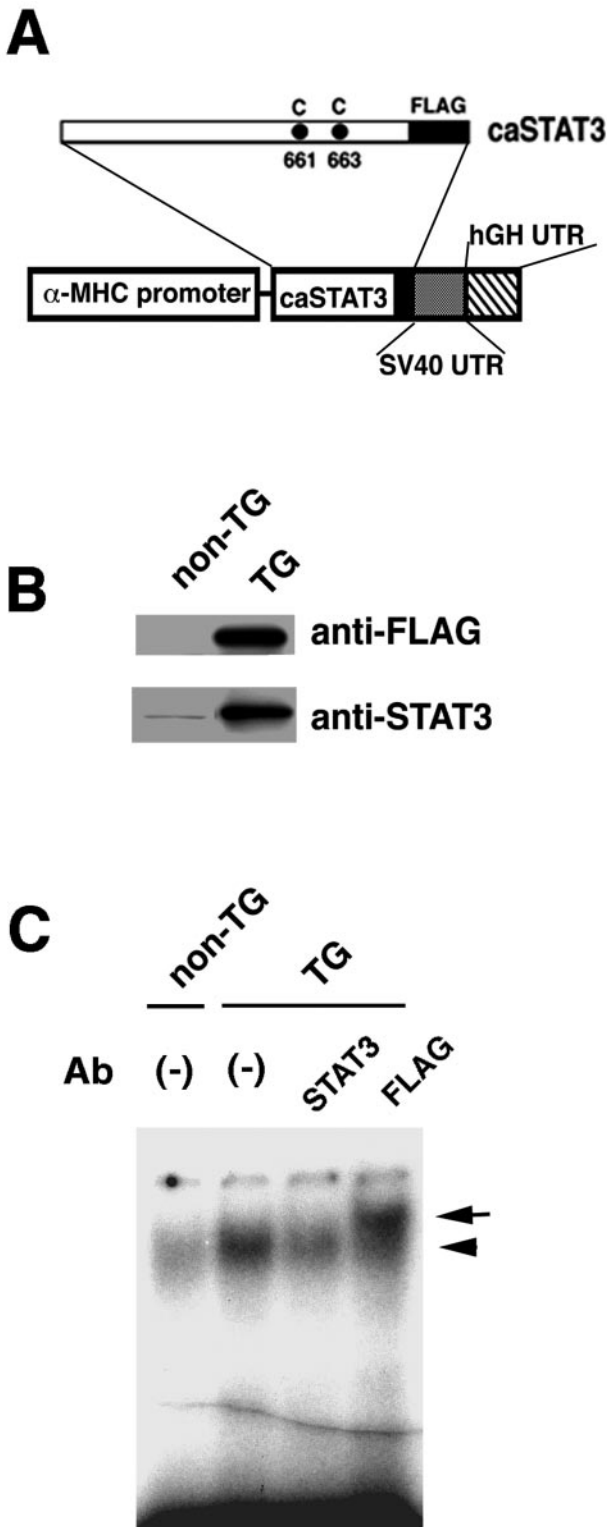


FIG. 3. Generation of the transgenic mice with cardiac-specific overexpression of constitutively active STAT3. *A*, structure of the transgenic mice expressing constitutively active STAT3. *B*, 30 μ g of proteins from caSTAT3 TG and non-transgenic (*non-TG*) littermate hearts was immunoblotted with anti-FLAG (*upper panel*) or anti-STAT3 (*lower panel*) antibody. *C*, electrophoretic mobility shift assay of the whole extracts from caSTAT3 TG or non-TG hearts. Heart extracts from TG or non-TG mice were incubated with the 32 P-labeled DNA probe containing the STAT3 binding motif either in the presence or absence of anti-STAT3 or anti-FLAG antibody. The DNA-protein complex was analyzed by polyacrylamide gel electrophoresis. Note that the extract from the TG heart formed the DNA-protein complex (*arrowhead*), which was disrupted by anti-STAT3 antibody and supershifted by anti-FLAG antibody (*arrow*).

As shown in Fig. 4C, VEGF protein was also up-regulated in caSTAT3 TG hearts in comparison with that in non-TG hearts.

Capillary Density Increased in the Hearts from caSTAT3 TG Mice—To examine the biological activities of STAT3 as a regulator of vessel formation *in vivo*, we examined the capillary density of hearts from TG and non-TG littermates by immunostaining with anti-CD31 antibody. To avoid inaccurate estimation because of angling of the sections, the sections were cut perpendicularly to the major axis, and the number of CD31-positive vasculatures in the endocardium were counted at the papillary muscle level. As shown in Fig. 5, capillary density was increased by 17% in caSTAT3 TG hearts ($n = 4$) compared with non-TG hearts ($n = 4$) (3928 ± 415 and 3360 ± 97 , respectively, $p < 0.05$).

Expression of VE-Cadherin Was Enhanced in the caSTAT3 TG Hearts—For quantitative evaluation of the vasculature in the hearts, immunoblot analyses of VE-cadherin, an endothelial-specific component, and of Cdk4, an internal control, were performed. VE-cadherin expression was enhanced in caSTAT3 TG hearts but not in dnSTAT3 TG (Fig. 6A). Using densitometer, we quantified the relative intensity of the bands for VE-cadherin in caSTAT3 TG heart extracts in comparison with that in non-TG heart extracts. As shown in Fig. 6B, caSTAT3 TG hearts ($n = 3$) showed an increase in VE-cadherin protein expression by $47 \pm 7\%$ compared with non-TG hearts ($n = 3$) ($p < 0.01$).

DISCUSSION

In this study, we provided evidence that cardiac myocyte-specific activation of STAT3 mediates vascular formation in the heart. In detail, adenoviral gene transfer of caSTAT3 induced VEGF mRNA. The conditioned medium from caSTAT3-transfected cardiomyocytes showed angiogenic activities, which were inhibited by anti-VEGF antibody. We generated cardiac-specific transgenic mice expressing caSTAT3 and demonstrated that VEGF mRNA is induced in caSTAT3 TG hearts and that VE-cadherin expression is increased in caSTAT3 TG hearts, which is consistent with the increase in capillary density.

Previously, we demonstrated that STAT3 regulates VEGF production in cardiomyocytes (19). Consistently, in the current study, cardiomyocytes transfected with caSTAT3 showed enhanced expression of VEGF mRNA. To clarify the biological significance of STAT3 as a regulator of vascularization, we generated an adenoviral construct expressing caSTAT3 and analyzed the angiogenic activities in the conditioned medium from caSTAT3-transfected cardiomyocytes by means of endothelial tubule formation assay. The conditioned medium from caSTAT3-transfected cardiomyocytes was found to promote endothelial tubule formation, whereas anti-VEGF antibody inhibited caSTAT3-mediated angiogenic activity significantly. Although it remains unclear why anti-VEGF antibody could not produce the complete inhibition, these data indicated that activation of STAT3 in cardiac myocytes stimulated angiogenic paracrine system and that VEGF is one of the important angiogenic paracrine factors from cardiomyocytes.

To examine the effects on caSTAT3 on vascular formation *in vivo*, we generated cardiac-specific transgenic mice expressing caSTAT3 that showed an increase in VEGF mRNA expression in the hearts by $49 \pm 13\%$ at the age of 5-weeks-old, similar to the results from the *in vitro* assay. Later at 20-weeks-old, the expression of VEGF mRNA in the caSTAT3 TG hearts was reduced to the same level as that in non-TG hearts (data not shown). As α -MHC promoter starts to be activated remarkably in the ventricles at the age of 2 weeks (28), VEGF mRNA is induced transiently and might be negatively regulated, possibly by endogenous negative regulators such as protein inhibitor

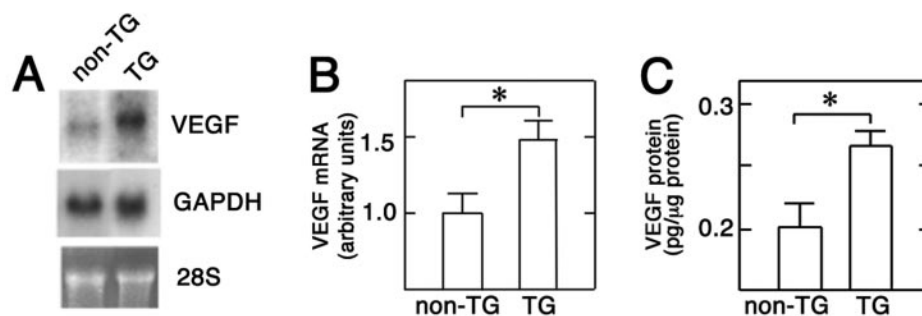


FIG. 4. VEGF expression was up-regulated in constitutively active STAT3 transgenic hearts. *A*, increase in VEGF mRNA expression in constitutively active STAT3 transgenic hearts. Total RNA was prepared from the hearts of constitutively active STAT3 TG mice or their non-transgenic (*non-TG*) littermates (5-weeks-old). VEGF mRNA was estimated by Northern blot analysis, and GAPDH mRNA was blotted as an internal control. Ribosomal 28 S RNA was used to demonstrate equal loading. *B*, the relative intensity of VEGF mRNA was assessed as the ratio to the intensity of the band for GAPDH. Values are shown as the means \pm S.D. *, $p < 0.05$. *C*, enhanced expression of VEGF protein in constitutively active STAT3 TG hearts. The protein extracts were prepared from the STAT3 TG and non-TG hearts. VEGF concentrations were measured as described under "Experimental Procedures." VEGF concentrations were normalized with protein concentration and expressed as the ratio of VEGF content over total protein. Values are shown as the means \pm S.D. * $p < 0.05$.

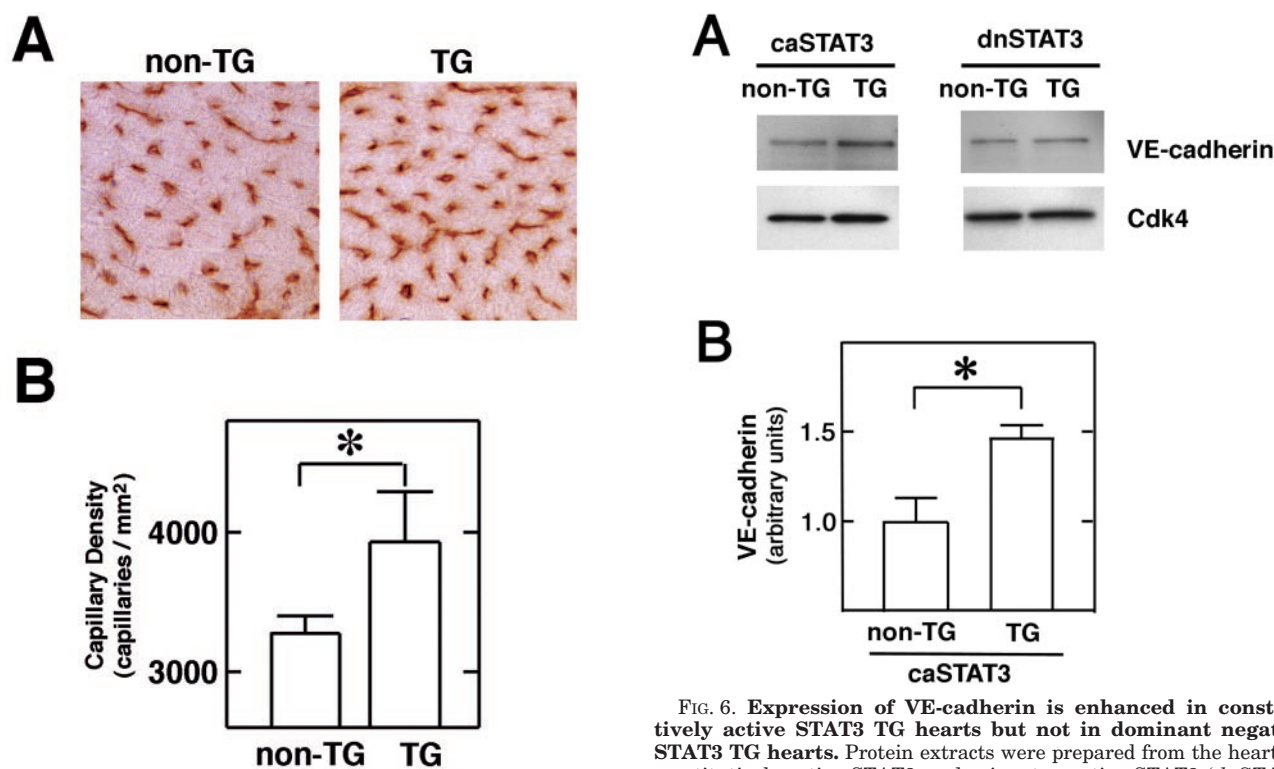


FIG. 5. Capillary density increased in the hearts from constitutively active STAT3 transgenic mice. The hearts were isolated from constitutively active STAT3 transgenic mice or their non-transgenic (*non-TG*) littermates (10–12-weeks-old). Frozen sections, cut perpendicularly to the major axis of the heart, were fixed and immunostained with anti-CD31 antibody. *A*, representative photographs of the endocardium. *B*, CD31-positive vasculatures in the endothelium were counted. The number of capillaries/mm² indicates capillary density. Values are shown as the means \pm S.D. *, $p < 0.05$.

of activated STAT (29). Immunohistochemical analyses with anti-CD31 antibody revealed that capillary density increased in caSTAT3 TG hearts, demonstrating that vessel growth is promoted in caSTAT3 TG hearts. Because capillary density estimation might be influenced by hypercontraction during sample preparation, we estimated vessel formation by quantifying the expression of VE-cadherin, which is an endothelial-specific component and is essential for endothelium maintenance (30). VE-cadherin was up-regulated in caSTAT3 TG hearts by 47% in comparison with non-TG hearts, providing the corroborative evidence that vasculature is enriched in

FIG. 6. Expression of VE-cadherin is enhanced in constitutively active STAT3 TG hearts but not in dominant negative STAT3 TG hearts. Protein extracts were prepared from the hearts of constitutively active STAT3 or dominant negative STAT3 (*dnSTAT3*) transgenic mice and from those of their non-transgenic (*non-TG*) littermates (10–12-weeks-old). 30 μ g of proteins was separated on SDS-PAGE and immunoblotted with anti-VE-cadherin (*upper panel*), and Cdk4 was immunoblotted as a loading control (*lower panel*). Experiments were performed three times with similar results. *B*, the relative intensities of the bands for VE-cadherin were quantified with densitometry. Values are shown as means \pm S.D. *, $p < 0.05$.

caSTAT3 TG hearts. Moreover, VE-cadherin induction is specific for the activation of STAT3, because VE-cadherin expression did not increase in the dnSTAT3 TG hearts. These findings indicate that STAT3 is a positive modulator of vascular formation in the hearts.

Our study has demonstrated that the promotion of vascular formation in caSTAT3 TG hearts is accompanied by VEGF induction. Theoretically, causality could be proved by generating VEGF gene-ablated mice overexpressing caSTAT3. However, at the present time, the feasibility of such an experimental procedure is severely limited, because VEGF gene-ablated mice are characterized by the embryonic lethality (31), and because cardiac-specific ablation of the VEGF gene itself leads

to cardiac abnormality (20). For these reasons, we cannot completely exclude the possibility that STAT3 could regulate vessel growth through systems other than the VEGF paracrine pathway.

Coronary vessel growth is a critical event in the adaptation to left ventricular hypertrophy (18, 32, 33). Among various kinds of angiogenic growth factors, VEGF is thought to play an important role in the regulation of vessel formation in the heart, because VEGF paracrine pathway of cardiac myocytes is needed to maintain cardiac function (20). A recent study (19) has shown that the activation of STAT3 is required for GP130-mediated induction of VEGF. Interestingly, cardiac-specific ablation of *GP130* gene results in heart failure in conjunction with inactivation of STAT3 during pressure overload-induced hypertrophy (17). Based on recent reports (34, 35) that insufficient vascular function leads to cardiac dysfunction and results in heart failure, the promotion of vascular formation through the STAT3 pathway could contribute to the adaptation of cardiac function, especially in response to extracellular stresses.

The results presented here are also reminiscent of oncogenic activities of STAT3, because tumor growth is accompanied by tumor angiogenesis (36). STAT3 is required for cellular transformation by *v-src* (37), whereas constitutive activation of STAT3 is sufficient for tumorigenesis (6). Interestingly, the suppressor of cytokine signaling-1, a negative regulator of the Janus kinase/STAT pathway, is silenced by methylation in human hepatocellular carcinoma, indicating that STAT3 activation is responsible for oncogenesis in some cases of human hepatoma (38). The totality of findings presented here suggests that activated STAT3 could well transduce angiogenic signals as well as cell-proliferative and -cytoprotective signals, thus leading to the formation of feeding arteries in growing tumors.

In conclusion, we have demonstrated that cardiac-specific activation of STAT3 promotes vascular formation in the hearts. Therefore, we suggest a novel function of STAT3 namely as a positive modulator of vessel growth.

Acknowledgment—We thank J. Hironaka for secretarial assistance.

REFERENCES

- Kishimoto, T., Taga, T., and Akira, S. (1994) *Cell* **76**, 253–262
- Shirogane, T., Fukada, T., Muller, J. M. M., Shima, D. T., Hibi, M., and Hirano, T. (1999) *Immunity* **11**, 709–719
- Minami, M., Inoue, M., Wei, S., Takeda, K., Matsumoto, M., Kishimoto, T., and Akira, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3963–3966
- Nakajima, K., Yamanaka, Y., Nakae, K., Kojima, H., Ichiba, M., Kiuchi, N., Kitaoka, T., Fukada, T., Hibi, M., and Hirano, T. (1996) *EMBO J.* **15**, 3651–3658
- Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Fernandez-Luna, J. L., Nunez, G., Dalton, W. S., and Jove, R. (1999) *Immunity* **10**, 105–115
- Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. (1999) *Cell* **98**, 295–303
- Sano, S., Itami, S., Takeda, K., Tarutani, M., Yamaguchi, Y., Miura, H., Yoshikawa, K., Akira, S., and Takeda, J. (1999) *EMBO J.* **18**, 4657–4668
- Cressman, D. E., Greenbaum, L. E., DeAngelis, R. A., Ciliberto, G., Furth, E. E., Poli, V., and Taub, R. (1996) *Science* **274**, 1379–1383
- Funamoto, F., Hishinuma, S., Fujio, Y., Matsuda, Y., Kunisada, K., Oh, H., Negoro, S., Tone, E., Kishimoto, T., and Yamauchi-Takahara, K. (2000) *J. Mol. Cell. Cardiol.* **32**, 1275–1284
- Pan, J., Fukuda, K., Saito, M., Matsuzaki, J., Kodama, H., Sano, M., Takahashi, T., Kato, T., and Ogawa, S. (1999) *Circ. Res.* **84**, 1127–1136
- Kunisada, K., Hirota, H., Fujio, Y., Matsui, H., Tani, K., Yamauchi-Takahara, K., and Kishimoto, T. (1996) *Circulation* **94**, 2626–2632
- Oh, H., Fujio, Y., Kunisada, K., Hirota, H., Matsui, H., Kishimoto, T., and Yamauchi-Takahara, K. (1998) *J. Biol. Chem.* **273**, 9703–9710
- Fujio, Y., Kunisada, K., Hirota, H., Yamauchi-Takahara, K., and Kishimoto, T. (1997) *J. Clin. Invest.* **99**, 2898–2905
- Kunisada, K., Tone, E., Fujio, Y., Matsui, H., Yamauchi-Takahara, K., and Kishimoto, T. (1998) *Circulation* **98**, 346–352
- Kunisada, K., Negoro, S., Tone, E., Funamoto, M., Osugi, T., Yamada, S., Okabe, M., Kishimoto, T., and Yamauchi-Takahara, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 315–319
- Negoro, S., Oh, H., Tone, E., Kunisada, K., Fujio, Y., Walsh, K., Kishimoto, T., and Yamauchi-Takahara, K. (2001) *Circulation* **103**, 555–561
- Hirota, H., Chen, J., Betz, U. A. K., Rajewsky, K., Gu, Y., Ross, J. J., Muller, W., and Chien, K. R. (1999) *Cell* **97**, 189–198
- Tomaneck, R. J. (1990) *J. Am. Coll. Cardiol.* **15**, 528–533
- Funamoto, M., Fujio, Y., Kunisada, K., Negoro, S., Tone, E., Osugi, T., Hirota, H., Izumi, M., Yoshizaki, K., Walsh, K., Kishimoto, T., and Yamauchi-Takahara, K. (2000) *J. Biol. Chem.* **275**, 10561–10566
- Giordano, F. J., Gerber, H.-P., Williams, S.-P., VanBruggen, N., Bunting, S., Ruiz-Lozano, P., Gu, Y., Nath, A. K., Huang, Y., Hickey, R., Dalton, N., Peterson, K. L., Ross, J. J., Chien, K. R., and Ferrara, N. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5780–5785
- Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D., and Newgard, C. B. (1994) *Methods Cell Biol.* **43**, 161–189
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Bishop, E. T., Bell, G. T., Bloor, S., Broom, I. J., Hendry, N. F. K., and Wheatley, D. N. (1999) *Angiogenesis* **3**, 335–344
- Fox, S. B., Leek, R. D., Weekes, M. P., Whitehouse, R. M., Gatter, K. C., and Harris, A. L. (1995) *J. Pathol.* **177**, 275–283
- Gulick, J., Subramaniam, A., Neumann, J., and Robbins, J. (1991) *J. Biol. Chem.* **266**, 9180–9185
- Ikawa, M., Yamada, S., Nakanishi, T., and Okabe, M. (1998) *FEBS Lett.* **430**, 83–87
- Kirshenbaum, L. A., MacLellan, W. R., Mazur, W., French, B. A., and Schneider, M. D. (1993) *J. Clin. Invest.* **92**, 381–387
- Palermo, J., Gulick, J., Colbert, M., Fewell, J., and Robbins, J. (1995) *Circ. Res.* **78**, 504–509
- Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997) *Science* **278**, 1803–1805
- Carmeliet, P., Lampugnani, M.-G., Moons, L., Breviario, F., Compernelle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuysse, B., Dewerchin, M., Zanetti, A., Angellilo, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., Ruitter, M. C., Groot, A. G., Poelmann, R., Lupu, F., Herbert, J.-M., Collen, D., and Dejana, E. (1999) *Cell* **98**, 147–157
- Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996) *Nature* **380**, 435–439
- Tomaneck, R. J., Schalk, K. A., Marcus, M. L., and Harrison, D. G. (1989) *Circ. Res.* **65**, 352–359
- Rakusan, K., Flanagan, M. F., Geva, T., Southern, J., and Praagh, R. V. (1992) *Circulation* **86**, 38–46
- Carmeliet, P., Ng, Y.-S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V. V., Stalmans, I., Mattot, V., Perriard, J.-C., Dewerchin, M., Flameng, W., Nagy, A., Lupu, F., Moons, L., Collen, D., D'Amore, P. A., and Shima, D. T. (1999) *Nat. Med.* **5**, 495–502
- Coral-Vazquez, R., Cohn, R. D., Moore, S. A., Hill, J. A., Weiss, R. M., Davison, R. L., Straub, V., Barresi, R., Bansal, D., Hrstka, R. F., Williamson, R., and Campbell, K. P. (1999) *Cell* **98**, 465–474
- Folkman, J. (1995) *N. Engl. J. Med.* **333**, 1757–1763
- Bromberg, J. F., Horvath, C. M., Besser, D., Lathem, W. W., and Darnell, J. E. (1998) *Mol. Cell. Biol.* **18**, 2553–2558
- Yoshikawa, H., Matsubara, K., Qian, G.-S., Jackson, P., Groopman, J. D., Manning, J. E., Harris, C. C., and Herman, J. G. (2001) *Nat. Genet.* **28**, 29–35