Hepatocyte Growth Factor Induces GATA-4 Phosphorylation and Cell Survival in Cardiac Muscle Cells*

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Hepatocyte growth factor (HGF) is released in response to myocardial infarction and may play a role in regulating cardiac remodeling. Recently, HGF was found to inhibit the apoptosis of cardiac muscle cells. Because GATA-4 can induce cell survival, the effects of HGF on GATA-4 activity were investigated. Treatment of HL-1 cells or primary adult rat cardiac myocytes with HGF, at concentrations that can be detected in the human serum after myocardial infarction, rapidly enhances GATA-4 DNA-binding activity. The enhanced DNA-binding activity is associated with the phosphorylation of GATA-4. HGF-induced phosphorylation and activation of GATA-4 is abolished by MEK inhibitors or the mutation of the ERK phosphorylation site (S105A), suggesting that HGF activates GATA-4 via MEK-ERK pathway-dependent phosphorylation. HGF enhances the expression of anti-apoptotic Bcl-xL, and this is blocked by dominant negative mutants of MEK or GATA-4. Forced expression of wild-type GATA-4, but not the GATA-4 mutant (S105A) increases the expression of Bcl-xL. Furthermore, expression of the GATA-4 mutant (S105A) suppresses HGF-mediated protection of cells against daunorubicin-induced apoptosis. These results demonstrate that HGF protects cardiac muscle cells against apoptosis via a signaling pathway involving MEK/ERK-dependent phosphorylation of GATA-4.

The hepatocyte growth factor (HGF) is a heterodimeric protein composed of a 69-kDa α-chain and a 34-kDa β-chain (1). The biologic activity of HGF as a potent mitogen of hepatocytes was first demonstrated in the sera of normal and partially hepatectomized rats (2, 3). HGF has been purified, cloned, and sequenced (1, 4). The HGF receptor was identified as the c-met proto-oncogene that is translated to a protein product, c-Met (5, 6). Signal transduction pathways for HGF in these cells involve tyrosine phosphorylation of c-Met and subsequent activation of p21ras (7), ERK (8), and phosphatidylinositol 3-kinase (8).

Earlier findings that HGF expression is increased after liver injury induced by CCl4 (9) suggest that HGF may be an oxidative stress-inducible factor. Similarly, in a rat model of myocardial ischemia and reperfusion, HGF expression was found to be enhanced (10). Furthermore, human studies have shown that serum HGF levels are elevated 25–50-fold after acute myocardial infarction (11, 12). HGF has been postulated to serve as an endogenously produced cardioprotective factor (13). Recent studies supported this hypothesis by demonstrating that gene transfection (14) or injection (15) of HGF attenuated myocardial ischemia-reperfusion injury in rats. The mechanism of HGF-mediated protection is at least, in part, because of its actions on cardiac myocytes, as HGF has been shown to attenuate oxidative stress-induced death of neonatal rat ventricular myocytes (15), adult rat ventricular myocytes (16, 17), and HL-1 adult mouse cardiac muscle cells (16). Furthermore, HGF was found to augment the expression of antiapoptotic Bcl-xL both in vivo and in vitro (15).

GATA-4 is a member of the GATA family of zinc finger transcription factors, which plays important roles in transducing nuclear events that modulate cell lineage differentiation during development. Six GATA family members have been identified and shown to alter transcription of target genes via binding to the consensus 5′-GATAAR-3′ sequence. Three members of this family, GATA-4/5/6, are expressed in the heart. Functionally relevant GATA-binding sites have been identified in numerous cardiac transcriptional regulatory regions (18, 19).

In addition to regulating differentiation, there is increasing evidence that GATA factors also control cell survival. Weiss and Orkin (20) reported that the GATA-1-deficient erythroid precursors undergo apoptosis. In erythroleukemia cells, the induction of apoptosis by estrogen was dependent on the inhibition of GATA-1 (21, 22). The bcl-x gene has two GATA consensus motifs in the 5′ promoter region (23), and GATA-1 induces the expression of the antiapoptotic protein Bcl-xL (24). GATA-1 also regulates the expression of Bcl-2 (25). GATA elements are found in the promoters of other genes involved in antiapoptotic activities such as nitric-oxide synthases (26, 27) and antioxidant enzymes (28). GATA-4 may play a role in promoting cell survival, as the apoptosis of ovarian cells was found to be associated with a decrease in the expression of GATA-4 (29). Furthermore, a lack of GATA-4 is also associated with activation of apoptosis in the presumptive foregut (30). We...
recently found that the apoptosis of cardiac myocytes induced by anthracycline is associated with decreased GATA-4 expression and that the forced expression of GATA-4 or -6 attenuates apoptosis, indicating that GATA-4 is involved in cell survival signaling in cardiac myocytes (31).

To gain insights to the mechanism of HGF-mediated survival signaling in cardiac myocytes, the present study explored the effects of HGF on GATA-4. Results show that HGF activates GATA-4 in HL-1 cells and in the primary culture of adult rat cardiac myocytes via MEK/ERK-dependent phosphorylation, and this signaling pathway is involved in HGF-mediated anti-apoptotic responses.

**EXPERIMENTAL PROCEDURES**

**Culture of Cardiac Muscle Cells**—HL-1 cardiac muscle cells (32) were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, LA). Cells were maintained in EXCELL 320 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Invitrogen), 10 μg/ml insulin (Invitrogen), 20 μg/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 1 μM retinoic acid (Sigma), 100 μM norepinephrine (Sigma), 1% nonessential amino acid supplements (Invitrogen), 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma) in plastic dishes, coated with 12.5 μg/ml fibronectin and 0.02% gelatin, in a 5% CO₂ atmosphere at 37 °C. Cells were replenished with fresh media every 2–3 days. For treatment, cells were starved in EXCELL 320 medium supplemented only with nonessential amino acids, penicillin, streptomycin, and amphotericin B for 18 h and treated with human recombinant HGF (Sigma).

Cardiac myocytes were isolated from ventricles of adult male Lewis rats (3–6 months old) using an enzymatic isolation technique previously described (33). Viable myocytes were purified via a series of gravity sedimentations in 0.2, 0.5, and 1 mM Ca²⁺, and then placed in Dulbecco's modified Eagle's medium/F-12 medium. Ca²⁺-tolerant myocytes purified through these procedures were >90% viable.

**Adenovirus-mediated Gene Transfer**—Adenovirus-directed gene transfer was implemented by adding 30 plaque forming units of recombinant adenovirus. The culture medium was aspirated from the cell culture growing in a 35-mm dish, and 0.5 ml of the fetal bovine serum-free medium containing the recombinant adenovirus was added. 1.5 ml
of growth medium was added following 2 h of culture and maintained for 24–48 h before performing experiments. Adenovirus constructs expressing GATA-4 (wild-type and mutants) and dominant negative MEK1 were kindly provided by Dr. J. Molkentin (University of Cincinnati).

Nuclear Extraction—To prepare nuclear extracts, cells were washed in phosphate-buffered saline and incubated in 10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 1 mM tetrasodium pyrophosphate for 15 min at 4 °C. Nonidet P-40 was then added at a final concentration of 0.6% (v/v). Samples were mixed vigorously, and centrifuged. Pelleted nuclei were resuspended in 50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, and 1% (v/v) glycerol, then mixed for 20 min and centrifuged for 5 min. The supernatants were harvested, protein concentrations determined, and supernatants were stored at −80 °C.

Electrophoretic Mobility Shift Assay (EMSA)—To perform EMSA, binding reaction mixtures containing 2 µg of protein of nuclear extract, 1 µg of poly(dI-dC)poly(dI-dC), and 32P-labeled double stranded oligonucleotide probe containing consensus GATA sequence (5'-CACTTGATAACAGAAAGTGATAACTCTTTCACT-3') in 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, and 20 mM Tris-HCl (pH 7.5) were incubated for 20 min at 25 °C. Electrophoresis of samples through a native 6% polyacrylamide gel was followed by autoradiography.

Western Blot Analysis—SDS-PAGE gels (10%) used to separate phosphorylated from nonphosphorylated GATA-4 contained acrylamide/bis at a ratio of 30:0.165. Nuclear extracts (20 µg of protein) were electrophoresed and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked and incubated with rabbit polyclonal IgG for GATA-4 (H-112) (Santa Cruz Biotechnology) at a concentration of 1 µg/ml. Levels of proteins were detected with horseradish peroxidase-linked secondary antibodies and ECL (enhanced chemiluminescence) system (Amersham Biosciences). To determine the effects of α-protein phosphatase (APPase), nuclear extracts were isolated without the use of phosphatase inhibitors (sodium fluoride, sodium orthovanadate, and tetrasodium pyrophosphate). Nuclear extracts were incubated with 2,500 units/ml APPase (New England Biolabs) at 30 °C for 30 min before subjecting to SDS-PAGE.

Comet Assay—The neutral comet assay was employed to measure double stranded DNA breaks as an indication of cardiomyocyte apoptosis (16). Treated cells were embedded in situ in 1% agarose, then placed in lysis solution (2.5 mM NaCl, 1% Na-lauryl sarcosinate, 100 mM EDTA, 1% sodium orthovannadate, and 1 mM tetrasodium pyrophosphate) for 15 min at 4 °C. Nonidet P-40 was then added at a final concentration of 0.6% (v/v). Samples were mixed vigorously, and centrifuged. Pelleted nuclei were resuspended in 50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, and 1% (v/v) glycerol, then mixed for 20 min and centrifuged for 5 min. The supernatants were harvested, protein concentrations determined, and supernatants were stored at −80 °C.

RESULTS

HGF Induces GATA-4 Activation—HGF has been shown to protect cardiac myocytes against apoptotic death (15, 16). Because our recent study (31) has shown that GATA-4 serves as a cell survival factor of cardiac myocytes, we examined the effects of HGF on GATA-4. We found that HGF increases the GATA DNA-binding activity. As shown in Fig. 1A, nuclear extracts from cultured HL-1 cardiac muscle cells had some constitutive DNA-binding activity toward a double stranded oligonucleotide containing the consensus GATA sequence as determined by EMSA. This binding activity was further increased by the treatment of cells with HGF (25 ng/ml). The stimulation of GATA DNA-binding activity occurred within 3 min of HGF treatment, and the densitometry analysis determined that the intensity of the band increased 4-fold after 10 min of HGF treatment (Fig. 1A, bar graph). Both constitutive and activated GATA bands were effectively eliminated by cold double stranded oligonucleotide competitors containing the GATA consensus sequence in a concentration-dependent fashion (Fig. 1B). As shown in the line graph in Fig. 1B, the densitometry analysis revealed that, at a given amount of cold competitor, the GATA-binding activity in HGF-treated cells (closed squares) was higher than that in untreated cells (open circle).

To determine the exact transcription factor species in HL-1 cells that binds to the oligonucleotide containing the GATA consensus sequence and is activated in response to HGF treatment, we performed supershift experiments using GATA-4 and GATA-6 antibodies. As shown in Fig. 1C, both the constitutive and activated GATA complexes were completely supershifted with the GATA-4 antibody whereas the GATA-6 antibody had no effects.

Similarly, HGF-activated GATA DNA-binding activity in primary cultures of adult rat cardiac myocytes (Fig. 1D) in which GATA-4 is the major GATA-binding protein (31). In the same nuclear extract samples, we observed that the DNA-binding activity of NFAT (a known mediator of GATA-4 activation) was not affected by HGF (Fig. 1D, lower panel). These results indicate that HGF activates the DNA-binding activity of GATA-4 in adult cardiac myocytes. In contrast, other known cell survival factors such as NF-κB (Fig. 1E) and Akt (data not shown) are not activated by HGF.

HGF Induces GATA-4 Phosphorylation—We also noticed that the complex between DNA and the HGF-activated GATA-4 migrated slightly slower through the native gel compared with the complex with the unstimulated GATA-4. Western blot analysis detected two GATA-4 bands at −50 kDa in
untreated HL-1 cells (Fig. 2A). The lower band was slightly denser than the higher band (see densitometry analysis shown in the bar graph of Fig. 2A). Treatment of cells with HGF resulted in a time-dependent upward shift of the lower band. By 10 min of treatment with HGF, all of the GATA-4 protein molecules existed as a single species with a reduced mobility, and this was sustained for at least 30 min.

To test the hypothesis that GATA-4 may be phosphorylated and thus give a species with reduced mobility, we incubated nuclear extracts from untreated and HGF-treated HL-1 cells with H9261 PPase in vitro. For these experiments, we isolated nuclear extracts without the use of phosphatase inhibitors. As shown in the Western blot results in Fig. 2B, the lower GATA-4 band shifted to a higher band in response to treatment with HGF (first and second lanes). This upward shift was slightly attenuated by incubating the nuclear extracts at 30°C (third and fourth lanes), presumably because of actions of endogenous protein phosphatases. The treatment of nuclear extract samples from untreated and HGF-treated HL-1 cells with λPPase at 30°C completely abolished the higher band (fifth and sixth lanes). Similarly, λPPase treatment completely abolished the upward shift of the GATA-4 band induced by HGF in EMSA experiments (Fig. 2C). These results suggest that HGF induced the phosphorylation of GATA-4 in HL-1 cells.

**Role of MEK in HGF-induced GATA-4 Phosphorylation**—Towatari et al. (35) reported that interleukin-3 activated GATA-2 through the ERK pathway in hematopoietic progenitor cells. The GATA-4 molecule contains putative ERK phosphorylation sites (36), and recent studies showed that hypertrophic stimuli such as 1-adrenergic agonist (37) and endothelin-1 (38) induced the phosphorylation of GATA-4 in cardiac myocytes. We, therefore, examined whether HGF phosphorylated GATA-4 through the MEK-ERK pathway. EMSA experiments showed that pretreatment of cells with a specific inhibitor of MEK, PD98059 (39), effectively blocked the upward shift (phosphorylation) of the GATA-4 band induced by HGF (Fig. 3A). Furthermore, these results revealed that HGF-mediated enhancement of the GATA-4 DNA-binding activity was also blocked by the MEK inhibitor (Fig. 3A, bar graph). PD98059 was dissolved in Me2SO and an equal amount of Me2SO (0.2% final concentration) alone had no effect (data not shown). Similarly, U0126 (another inhibitor of MEK) blocked the phosphorylation and activation of GATA-4 induced by HGF (data not shown). Western blot experiments confirmed our
GATA-4 DNA-binding activity was monitored by EMSA. With alanine (S105A) for 48 h, then treated with HGF for 10 min. Wild-type (WT) GATA-4 or GATA-4 mutant with serine 105 replaced with alanine (S105A) for 48 h, then treated with HGF for 10 min. GATA-4 DNA-binding activity was monitored by EMSA.

To confirm the effects of these MEK inhibitors, we used adenovirus expressing the dominant negative MEK described by Liang et al. (40). As shown in Fig. 3C, HGF-induced activation of ERK was blocked by dominant negative MEK expression without altering the ERK protein levels. Expression of this dominant negative mutant abolished the phosphorylation and activation of GATA-4 induced by HGF (Fig. 3D).

Liang et al. (40) reported that GATA-4 is phosphorylated at serine residue 105, an ERK phosphorylation site, in response to phenylephrine treatment of neonatal ventricular myocytes. Consistently, the mutant GATA-4 with serine 105 replaced with alanine was not phosphorylated or activated by HGF in HL-1 cells (Fig. 4A) nor in primary culture of adult rat cardiac myocytes (Fig. 4B), whereas endogenous (Fig. 4A) or exogenous (Fig. 4B) wild-type GATA-4 was activated by HGF. Taken together, these results demonstrate that HGF induces phosphorylation and activation of GATA-4 via MEK/ERK phosphorylation of serine 105.

Role of MEK in HGF-induced expression of Bcl-x<sub>L</sub>. Cells were infected with adenovirus expressing dominant negative MEK (AdDN-MEK; 30 plaque-forming units/cell) for 48 h, then treated with HGF (25 ng/ml) for 24 h. Cell lysates were prepared and subjected to Western blot analysis using the Bcl-x<sub>L</sub> antibody. The bar graph indicates the intensities of Bcl-x<sub>L</sub> bands as determined by densitometry. Values represent mean ± S.E. (n = 4). An asterisk (*) denotes the value that is significantly different from the untreated control value at p < 0.05.

Role of MEK/ERK-dependent Phosphorylation of GATA-4 in HGF-mediated Cell Survival—HGF has been reported to stimulate antiapoptotic Bcl-x<sub>L</sub> protein expression in a human epithelial cell line (41) and cardiomyocytes (15). Similarly, HGF up-regulated the expression of Bcl-x<sub>L</sub> in HL-1 cells (Fig. 5). To examine the role of the MEK/ERK pathway in the up-regulation of Bcl-x<sub>L</sub>, the adenovirus expressing a dominant negative mutant of MEK was employed. As shown in Fig. 5, the dominant negative mutant of MEK blocked the HGF-induced Bcl-x<sub>L</sub> expression. To determine the role of GATA-4 in HGF-induced Bcl-x<sub>L</sub> expression, a dominant mutant of GATA-4 as described by Liang et al. (42) was used. EMSA experiments demonstrate that the expression of this dominant negative mutant GATA-4 indeed inhibited that GATA-4 DNA-binding activity in HL-1 cells (Fig. 6A). Reverse transcriptase-PCR analysis was used to monitor levels of bcl-x mRNA. The data were quantified by calculating the ratio of bcl-x to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. As shown in Fig. 6B, a dominant negative mutant GATA-4 increased the basal level of bcl-x, but inhibited the HGF-induced enhancement of bcl-x mRNA expression. Furthermore, forced expression of wild-type GATA-4 induced the expression of Bcl-x<sub>L</sub> (Fig. 6C), supporting the experiments with dominant negative mutants demonstrating that GATA-4 regulates the bcl-x expression.

To determine the role of serine 105 phosphorylation of GATA-4 in cell survival, adenovirus expressing the S105A mutant of GATA-4 was used. Unlike wild-type GATA-4, the S105A mutant did not cause enhancement of Bcl-x<sub>L</sub> expression (Fig. 7A). Interestingly, we found that this mutant serves as a dominant negative mutant. As demonstrated in Fig. 7B, HGF inhibited the apoptotic cell death caused by an anthracycline antibiotic, daunorubicin, but S105A mutant expression blocked the HGF-induced protection of cells against daunorubicin. These results suggest that HGF-mediated protection of cardiac muscle cells is, at least partly, elicited by the up-regulation of antiapoptotic Bcl-x<sub>L</sub> mediated by phosphorylation of GATA-4 at serine 105.
DISCUSSION

HGF has been shown to be released in response to myocardial ischemia-reperfusion injury (10) and acute myocardial infarction (11, 12). Human serum HGF has been reported to increase from 0.3 to 0.4 ng/ml to a level as high as 37 ng/ml after acute myocardial infarction (11). Studies using rat models have supported a protective role by demonstrating that gene transfection (14) or injection (15) of HGF attenuated ischemia-reperfusion injury in the heart. Therefore, HGF appears to play an important role as an endogenous cardioprotective factor. Furthermore, HGF may be useful as a therapeutic agent to protect the heart against various oxidative stress stimuli. Thus, it is important to understand the molecular mechanism of HGF action in the heart.

Some of the cardioprotective effects of HGF may be attrib-

FIG. 6. Role of GATA-4 in HGF-induced expression of Bcl-xL. A, HL-1 cells were infected with control adenovirus (AdCont) without functional insert or adenovirus expressing a dominant negative GATA-4 mutant (AdDN-GATA4) for 48 h. Nuclear extracts were prepared and the GATA DNA-binding activity was monitored by EMSA. B, HL-1 cells were infected with control adenovirus or adenovirus expressing a dominant negative GATA-4 mutant for 48 h, then treated with HGF (25 ng/ml) for the durations as indicated. Total RNA was isolated and subjected to reverse transcriptase-PCR analysis for bcl-x and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNAs. The line graph shows 5-fold increase in the bcl-x:glyceraldehyde-3-phosphate dehydrogenase ratio in response to HGF treatment as determined by densitometry (open circle, AdCont; closed square, AdDN-GATA4). C, HL-1 cells were infected with control adenovirus or adenovirus expressing wild-type GATA-4 for 48 h. Cell lysates were prepared and subjected to Western blot analysis using the Bcl-xL antibody. The membrane was re-blotted with the antibody for GATA-4 (lower panel).

FIG. 7. Role of serine 105 of GATA-4 in HGF-induced expression of Bcl-xL. A, HL-1 cells were infected with control adenovirus (AdCont) without functional insert or adenovirus expressing wild-type GATA-4 (AdGATA4 wt) or GATA mutant (S105A) for 48 h. Cell lysates were prepared and subjected to Western blot analysis using the Bcl-xL antibody. The bar graph indicates the intensity of Bcl-xL bands as determined by densitometry. Values represent mean ± S.E. An asterisk (*) denotes the value that is significantly different from the value in AdCont expressing cells at p < 0.05. B, HL-1 cells were infected with control adenovirus or adenovirus expressing a GATA-4 mutant (S105A) for 24 h, pretreated with HGF (25 ng/ml) for 2 h, then treated with daunorubicin (DNR; 1 μM) for 24 h. Apoptotic cells were identified using the neutral comet assay. Values represent mean ± S.E. (n = 4). An asterisk (*) denotes the value that is significantly different from the value in DNR-treated, control adenovirus-infected cells at p < 0.05.

FIG. 8. A proposed model for the mechanism of HGF-induced cardiac myocyte survival. The signal from HGF (via c-Met tyrosine kinase receptor) activates MEK-ERK pathway which, in turn, phosphorylates GATA-4. The phosphorylated GATA-4 up-regulates the expression of anti-apoptotic proteins such as Bcl-xL, and prevents oxidative stress-induced cardiac myocyte apoptosis.
uted to its ability to induce angiogenesis by acting on vascular endothelial cells (43). However, HGF may also directly affect cardiac myocytes by attenuating the oxidative stress damage. In support of this hypothesis, we have recently demonstrated that HGF directly protected adult cardiac myocytes against oxidative stress stimuli such as serum deprivation, H$_2$O$_2$, and daunorubicin (16). These results suggest that the mechanism of HGF action to protect the heart may, in part, involve its direct action on cardiac muscle cells to prevent the occurrence of cell death.

HGF has also been shown to inhibit the apoptosis in noncardiac tissues. Revollo et al. (44) reported that C28 mouse embryonic hepatocytic cells require exogenous HGF to survive and proliferate in serum-free medium. Apoptosis of hepatocytes induced by interferon-γ was inhibited by HGF (45). In human endometrial epithelial cells, HGF suppressed apoptosis induced by Fas antigen (46). A protective role for HGF in acute renal failure is suggested because HGF protected renal epithelial cells against apoptosis induced by serum starvation (47) or renal failure is suggested because HGF protected renal epithelial cells against apoptosis induced by serum starvation (47). A protective role for HGF in acute endometrial epithelial cells, HGF suppressed apoptosis induced by Fas antigen (46). A protective role for HGF in acute renal failure is suggested because HGF protected renal epithelial cells against apoptosis induced by serum starvation (47) or renal failure is suggested because HGF protected renal epithelial cells against apoptosis induced by serum starvation (47).

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HGF failed to activate this transcription factor. Thus, HGF action to protect the heart may, in part, involve its direct action on cardiac muscle cells to prevent the occurrence of cell death.

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