Gene 33/RALT Is Induced by Hypoxia in Cardiomyocytes, Where It Promotes Cell Death by Suppressing Phosphatidylinositol 3-Kinase and Extracellular Signal-Regulated Kinase Survival Signaling

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Ischemia in the heart deprives cardiomyocytes of oxygen, triggering cell death (myocardial infarction). Ischemia and its cell culture model, hypoxia, elicit a stress response program that contributes to cardiomyocyte death; however, the molecular components required to promote this process remain nebulous. Gene 33 is a 50-kDa cytosolic adapter protein that suppresses signaling from receptor Tyr kinases of the epidermal growth factor receptor/ErbB family. Here we show that adenoviral expression of Gene 33 swiftly stimulates cardiomyocyte death coincident with reduced Akt and extracellular signal-regulated kinase (ERK) signaling. Subjecting cardiomyocytes to hypoxia and then reoxygenation induces *gene 33* mRNA and Gene 33 protein. RNA interference experiments indicate that endogenous Gene 33 reduces Akt and ERK signaling and is required for maximal hypoxia-induced cardiomyocyte death. Gene 33 levels are also strikingly increased in myocardial ischemic injury and infarction. Our results identify a new role for Gene 33 as a component in the molecular pathophysiology of ischemic injury.

In myocardial infarction, occlusion of coronary arteries by atherosclerotic thrombus functions to deprive the myocardium of oxygen (ischemia). A consequence of this is the death of cardiomyocytes normally supplied by the occluded vessel (4, 17, 36). Both necrotic and apoptotic cardiomyocyte death occur in myocardial infarction, with fibrotic tissue replacing the lost myocardium. Cardiomyocytes can be provoked to die in vivo and in tissue culture in response to numerous stresses (6). Ischemia-reperfusion injury in the heart is best recapitulated in cultured cardiomyocytes by hypoxia, followed by reoxygenation (hypoxia/reoxygenation), which, in addition to depriving the cell of critical nutrients, can foster the formation of toxic free radicals. These stresses precipitate cardiomyocyte death (5, 6, 15). The hypoxia model provides a convenient system with which the molecular components involved in promoting cardiomyocyte death can be identified.

Cardiomyocyte death triggered in response to hypoxia, glucose deprivation, or redox stress is complex and can resemble both apoptosis and necrosis. Typically, chronic, low-grade insults trigger a death response that includes recruitment of proapoptotic elements of the mitochondrial cytochrome *c* pathway (2, 6, 12, 21, 30, 33).

Signals that promote cardiomyocyte death are countered by cytoprotective signaling. In the heart, members of the epidermal growth factor (EGF)/ErbB family of receptor Tyr kinases, especially ErbB2 and ErbB4 (the receptor for neuregulin-1 [NRG]), are required for cardiomyocyte survival during redox stress and pressure overload (11, 20, 25). Of particular importance to the resistance to cell death—especially with regard to

the mitochondrial pathway—are the Akt family kinases and to a lesser extent, the extracellular signal-regulated kinase (ERK) group of mitogen-activated protein kinases (MAPKs) (7, 16).

In situ, it is the relative and cumulative influences of these pro- and antisurvival mechanisms that determines whether a cell will survive exposure to chronic stress. The exposure of the heart to a chronic stress, such as hypoxia, would be expected to promote the accumulation of proapoptotic elements to a point where they would tip the balance to cell death. The identification of such proapoptotic signaling intermediates, especially those which might be induced at the transcriptional level in response to chronic stress, remains poorly characterized.

gene 33 (also called receptor-associated late transducer [*ralt*], mitogen-inducible gene 6 [*mig6*], and ErbB receptor feedback inhibitor 1 [*erfi1*]) encodes a 50-kDa cytosolic adapter protein that is rapidly induced by a wide variety of mitogenic, endocrine, and stress signals (26, 37). Gene 33 associates in situ with all members of the ErbB family and inhibits signaling from these receptors (1, 18, 37). Accordingly, Gene 33 suppresses the transformed phenotype of Erb (especially ErbB2)-dependent tumor cells and reduces the abilities of mitogens in the Erb family to trigger cell proliferation in cultured cells and in vivo (1, 3, 18). In addition, glucocorticoids, which are known to suppress cell proliferation, induce *gene 33* which, in turn blunts EGF-dependent ERK and Akt activation, as well as cell cycle entry (37). Consistent with an antiproliferative role for Gene 33, disruption of murine *gene 33* leads to excessive osteophyte formation due to the hyperproliferation and chondrocytic differentiation of mesenchymal progenitor cells. This causes a form of premature joint degeneration that closely resembles osteoarthritis (38).

Given the observations that Gene 33 can suppress EGF/ErbB family mitogen activation of Akt and ERK and that ErbB family receptor Tyr kinases are essential to cardiomyocyte sur-

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vival during redox stress, we wondered whether cardiomyocyte Gene 33 was induced in response to hypoxia and whether it could foster cardiomyocyte death in part by downregulating ERK and Akt signaling. We observe that Gene 33 is induced in myocardial infarction and in cardiomyocytes subjected to hypoxia. Our findings also highlight a previously unidentified requirement for Gene 33 in the promotion of hypoxia-induced cardiomyocyte death.

MATERIALS AND METHODS

Antibodies and adenoviruses. A polyclonal antibody against Gene 33 was generated as described previously (37). Anti-ErbB-2 polyclonal antibody was from Santa Cruz Biotechnology. Polyclonal antibodies to cleaved caspase 3, cleaved caspase 9, and phospho-forkhead (phospho-FKHR) were purchased from Cell Signaling Technology. Polyclonal antibody against sarcomeric α -actinin was purchased from Sigma. Cy3- and rhodamine-conjugated anti-rabbit secondary antibodies were purchased from Jackson Immunogicals. Other antibodies used have been described elsewhere (37). Recombinant adenoviral constructs expressing Gene 33, β -galactosidase, myristoylated (Myr) Akt, and constitutively active MAPK/ERK kinase 1 (MEK1) (with S218D S222D [MEK-DD]) were constructed as described previously (10, 22, 37).

Cell culture, adenoviral infection, cell survival determination, and RNA interference (RNAi). Rat neonatal cardiomyocytes were prepared from 1- to 2-day-old animals and cultured in F10 medium supplemented with 10% donor horse serum and 5% fetal bovine serum as described previously (10, 31). Cells were treated with agonist (EGF, 50 ng/ml; platelet-derived growth factor [PDGF], 10 ng/ml; NRG, 50 ng/ml; insulin-like growth factor 1 [IGF-1], 10 nM; insulin, 10 nM; endothelin [ET-1], 100 nM) as described in the figure legends. As indicated in the figures, cells were treated for 1 h with signaling inhibitors (the phosphatidylinositol [PI] 3-kinase inhibitor LY294002 [20 μ M] or wortmannin [100 nM], the MEK inhibitor U0126 [10 μ M], the EGF receptor (EGFR) family inhibitor AG1478 [10 μ M] and its inactive analogue, AG9 [10 μ M]) prior to the addition of mitogens. Adenoviral infection of cardiomyocytes was performed under serum-free conditions at a multiplicity of infection (MOI) of 50 or as indicated. The cell survival rate was calculated as the ratio of the number of attached cells in control and experimental cell groups. Assays were performed in triplicate, and the data were subjected to statistical analysis (unpaired Student's *t* test, with a *P* of <0.05 indicating statistical significance).

Small interfering RNA (siRNA)-mediated RNAi of rat *gene 33* was performed as described previously (37). The control oligonucleotide was based on human *gene 33*, which differs from the rat sequence by 2 nucleotides (37).

Western and Northern analyses. Cells were harvested and lysed in RIPA buffer with a cocktail of protease inhibitors after the indicated treatments (37). Western blotting was performed with the indicated antibodies according to standard protocols. Immunoblots were quantitated with ImageJ software (<http://rsb.info.nih.gov/ij/>) on a Windows XP platform. Total cellular RNA was isolated with Ultraspec total RNA isolation kit from Biotecx. Northern blotting was performed on 10 μ g total RNA/sample with 32 P-labeled DNA probe of Gene 33 as described previously (26).

Immunohistochemistry and immunocytochemistry. Immunohistochemistry was performed on frozen sections of mouse hearts subsequent to myocardial infarction or sham operation as described below. Frozen tissue sections on glass slides were fixed in cold acetone (-20°C) for 15 min followed by air drying and rehydration in phosphate-buffered saline (PBS). The sections were then treated for 20 min at room temperature with PBS containing 5% fetal bovine serum (blocking buffer), followed by incubation with primary antibodies in blocking buffer for 1 h at room temperature. Consecutive sections on the same corresponding slides were incubated with blocking buffer only. The sections were washed with PBS and then incubated with fluorescein-conjugated secondary antibodies for 30 min at room temperature. The sections were then washed with PBS, mounted with VectorShield mounting medium (Vector Lab) containing 4',6'-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope. For immunocytochemistry, cells were cultured on coverslips. After the indicated treatments, cells on coverslips were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton in PBS, stained, and observed as described above for immunohistochemistry.

TUNEL staining. Terminal dUDP-nicked end labeling (TUNEL) staining was performed on cells grown on coverslips using the ApopTag fluorescein in situ apoptosis detection kit from Chemicon International, according to the manufacturer's protocol. TUNEL staining was followed by standard immunostaining with

anti-sarcomeric α -actinin antibody and Cy3-conjugated secondary antibody. For quantification of TUNEL staining, five fields from each slide were photographed, and the number of TUNEL-positive spots (with matching DAPI-stained nuclei) and number of nuclei in the field were counted. The ratio of the two values was calculated as a percentage of TUNEL-positive cells. The data were subjected to statistical analysis (unpaired Student's *t* test, with a *P* of <0.05 indicating statistical significance).

Hypoxia/reoxygenation treatments. Cells were cultured for 1 to 2 days or for 2 days after siRNA oligonucleotide transfection before being subjected to hypoxia. Culture medium, flushed with 5% CO_2 and 95% N_2 at 37°C for at least 6 h, was then added to the cell culture to substitute for the normoxic culture medium. Hypoxia was achieved by placing cells in a hypoxia chamber filled with 5% CO_2 and 95% N_2 at 37°C . Cells were then kept under hypoxic conditions for the indicated time periods. At the end of the hypoxic treatment, cells were either harvested immediately or reoxygenated by placing the cells in normoxic culture medium for the indicated time periods. For control normoxic treatment, cells were kept in fresh normoxic medium for the duration of the treatments used for hypoxia.

Mouse models for ischemia and myocardial infarction. The mouse models for ischemic injury (brief ischemia or ischemia/reperfusion without myocardial infarction) and myocardial infarction used in this study have been described in detail previously (8, 31). As indicated, after each treatment, left ventricular tissue (ischemic, nonischemic, infarct zone, and noninfarct zones) were separated and snap frozen. Later, samples were lysed as described previously (8, 31) and used for Western blotting to detect Gene 33 expression. In parallel, 72 h after myocardial infarction, transverse sections of the left ventricle were prepared for immunohistochemistry as described previously (8, 31) and used for immunohistochemical detection of Gene 33.

RESULTS

Adenoviral vector-driven expression of Gene 33 in cardiomyocytes suppresses ERK and Akt activation by multiple stimuli. The observation that EGFR/ErbB agonists, whose effects are reversed by Gene 33, were important to cardiomyocyte survival (1, 11, 18, 20, 25, 37) prompted us to examine the effects, in primary cultures of neonatal rat cardiomyocytes, of ectopic Gene 33 expression. For these studies, we used an adenoviral expression construct of FLAG-tagged rat Gene 33 with which we can attain >90% transduction efficiency.

EGF, PDGF, and NRG all strongly activate cardiomyocyte Akt and ERK (Fig. 1A). Expression of recombinant Gene 33 substantially blunts ERK and Akt activation incurred by EGF and NRG, but not that by PDGF or insulin (Fig. 1A and B). This result is consistent with those described by us and others indicating that Gene 33 strongly inhibits signaling from EGF family receptors (1, 3, 18, 37). Interestingly, expression of Gene 33 also modestly reduces the activation of cardiomyocyte ERK and Akt stimulated by IGF-1 and ET-1 (Fig. 1B). Inhibition of IGF-1 signaling by Gene 33 has not been reported previously and may represent a broader function for cardiomyocyte Gene 33. ET-1 may transactivate the EGFR (32), thus accounting for the ability of Gene 33 to disrupt ET-1 signaling.

Adenoviral expression of Gene 33 triggers cardiomyocyte apoptosis: reversal by Akt, MEK, and polypeptide mitogens. The ERK and PI 3-kinase/Akt pathways have been shown to protect cells, including cardiomyocytes, from death signaling (7, 16, 27). The finding that expression of Gene 33 reduced cardiomyocyte ERK and Akt signaling suggested to us that Gene 33 might foster cardiomyocyte death. To test this, we expressed Gene 33 in cardiomyocytes and assayed for the detachment of cells from the substratum and the appearance of cleaved caspase 3, a marker for cell death (23). Expression of Gene 33 in cardiomyocytes was sufficient to produce massive cardiomyocyte death comparable to that triggered by UV ra-

FIG. 1. Effects of ectopically expressed Gene 33 on cardiomyocyte signaling pathways. (A) Gene 33 suppresses signaling by the ErbB family agonists EGF and NRG. Cardiomyocytes were infected with either adenovirus expressing Gene 33 or control adenovirus expressing β -galactosidase (β -Gal). After 24 h, cells were treated with the indicated agonists for 5 min. Extracts were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with phospho-specific (P-) or conventional antibodies (indicated on the right of each blot). The bar graphs on the right are quantitations of the results for ERK and Akt phosphorylation in the gels on the left. (B) Same as panel A except that insulin, IGF-1, and endothelin (ET-1) signaling were also examined. The bar graphs on the right are quantitations of the results for ERK and Akt phosphorylation in the left panel. C, control; P-38, p38.

diation (Fig. 2A and B). Thus, infection with adenoviral Gene 33 at a multiplicity of infection of 20 to 50—which resulted in >90% of the cells expressing Gene 33—was sufficient to kill 70 to 80% of the cardiomyocytes within 72 h.

The cleavage of procaspase 3 to mature caspase 3 is a hall-

mark of apoptotic cell death (6, 23). Expression of Gene 33 produced a striking appearance of cleaved caspase 3 in the infected cardiomyocytes, as assessed by immunocytochemistry (Fig. 2C) and immunoblotting (Fig. 2D). Thus, Gene 33-induced cardiomyocyte death has at least one feature of apop-

tosis. As is discussed below (see Fig. 5), Gene 33 is necessary for stress-induced internucleosomal DNA cleavage, another feature of apoptosis (6).

The established cytoprotective role of Akt, coupled with the observation that Gene 33 could suppress activation of Akt prompted us to investigate whether contemporaneous expression of active Akt could reduce the cardiomyocyte death incurred by coexpressed Gene 33. Accordingly, cardiomyocytes were infected with adenoviral Gene 33 either alone or together with adenoviral active (myristoylated) Akt. From Fig. 2E, it is clear that expression of Gene 33 alone is sufficient to kill >70% of the cardiomyocytes within 48 h. Coexpression of Akt reduces this rate of cell death by more than half. Coincident with this cytoprotective effect, coexpression of Akt reduces dramatically the ability of Gene 33 to trigger cleavage of procaspase 3 (Fig. 2F).

The ERK pathway has also been shown to protect cells from apoptosis, primarily through recruitment of the protein kinase Rsk which, among other things, can, like Akt, phosphorylate and inhibit the proapoptotic protein Bad (7). Coexpression from an adenoviral vector of a constitutively active form of the ERK-specific MAPK kinase MEK1 (S218D S222D [MEK-DD]) rescues cardiomyocytes from Gene 33-triggered cell death to a degree commensurate with that incurred by Akt. MEK-DD and Akt coinfect together with adenovirus expressing Gene 33 provides an additional modest degree of cell survival (Fig. 2G, top panel). Coincident with provoking cardiomyocyte cell death, expression of Gene 33 strikingly reduces basal ERK activity. Expression of MEK-DD alone enhances ERK activation, and expression of MEK-DD with Gene 33 can partly restore ERK activation blunted by Gene 33 expression, but not to levels seen in cells not expressing ectopic Gene 33. Of note, MEK-DD produces the same approximately twofold ERK activation in the presence or absence of coexpressed Gene 33; however, the basal ERK activity in the pres-

ence of Gene 33 is ~10% of that in the absence of Gene 33 (Fig. 2G, bottom panel, and H).

Our results (Fig. 1) and those of others (1, 18, 37) indicate that while EGFR/ErbB signaling is strongly inhibited by Gene 33, PDGF is resistant to Gene 33, while Gene 33 only modestly inhibits IGF-1 signaling. We wondered whether PDGF or IGF-1 could counter the cell death signaling mediated by Gene 33. Thus, cardiomyocytes were serum starved and infected with either control adenovirus or adenovirus expressing Gene 33. Cells were then treated with purified PDGF, IGF-1, or EGF. From Fig. 2I, it is evident that Gene 33 kills >70% of infected cardiomyocytes. Both PDGF and IGF-1 can very modestly (albeit statistically significantly) reverse Gene 33-induced cardiomyocyte death. Interestingly, EGF, whose signaling is suppressed more completely by Gene 33, cannot rescue cardiomyocytes from Gene 33-induced death (Fig. 2I).

Induction of endogenous cardiomyocyte Gene 33 by hypoxia.

We next examined the regulation and function of endogenous Gene 33. Previous work has shown that the *gene 33* transcript is strongly induced by a number of stress, endocrine, and mitogenic signals (26, 37). Consistent with this, we find that endogenous cardiomyocyte Gene 33 protein (Fig. 3A) and *gene 33* mRNA (Fig. 3B) are induced upon prolonged (≥ 1 -h) stimulation with PDGF and ET-1. Of note, although Gene 33 can suppress EGF and NRG signaling, neither of these factors can stimulate induction of *gene 33* to a degree commensurate with that stimulated by either PDGF or ET-1 (Fig. 3A and B). We also observe that hypoxia strongly induces Gene 33 protein (Fig. 3C) and *gene 33* mRNA (Fig. 3D). The induction of Gene 33 by PDGF requires both the PI 3-kinase and ERK pathways and can be prevented by the specific pharmacologic PI 3-kinase inhibitor wortmannin and by U0126, a specific inhibitor of MEK1, the immediate upstream activator of ERK (14) (Fig. 3E). Hypoxia activates cardiomyocyte Akt but produces at most a very modest activation of ERK (Fig. 3F; also see Fig.

FIG. 2. Ectopically expressed Gene 33 rapidly kills cardiomyocytes in a manner reversed by ectopically expressed Akt. (A) Gene 33 triggers cardiomyocyte death to a degree commensurate with that incurred by UV radiation (UV). Cardiomyocytes were infected with either adenovirus expressing Gene 33 (Adeno-Gene 33) or control adenovirus expressing β -galactosidase (Adeno- β -Gal). In parallel, uninfected cells were subjected to UV irradiation. After 48 h, cells were examined by phase-contrast microscopy. (B) Quantitation of cardiomyocyte survival as a function of the MOI of adenovirus expressing Gene 33 (or control β -galactosidase [β -Gal]). Cardiomyocytes were counted, plated, and infected with the indicated adenovirus constructs at the indicated MOI. After 72 h, the remaining live cells (attached to the dish) were counted. Data are expressed as a ratio (in percentage) of the number of attached cells in virus-infected groups and that of a control group without viral infection. Values shown are the means $\pm$ standard errors of the means (SEM) (error bars) for triplicate determinations. (C) Ectopic expression of Gene 33 in cardiomyocytes leads to elevated levels of cleaved caspase 3. Cardiomyocytes were infected with Gene 33-expressing adenovirus, and after 48 h, the cells were stained as indicated with cleaved caspase 3 antibody or an antibody against green fluorescent protein (GFP) (indication of infection with the recombinant adenovirus). Note that the cells expressing GFP (infected cells) also manifest elevated levels of cleaved caspase 3. (D) Same as panel C except that parallel cultures of cardiomyocytes were infected with control (β -Gal) adenovirus or Gene 33 (G33) adenovirus as indicated. The levels of Gene 33 and cleaved caspase 3 (casp 3) or, as a loading control, ERK2 were determined as indicated by immunoblotting. (E) Coexpression of constitutively active Akt1 alleviates cardiomyocyte killing incurred by Gene 33. Cardiomyocytes were infected (+) with adenovirus expressing β -galactosidase, Gene 33, and/or constitutively active myristoylated Akt1 (Myr-AKT). Cell killing was assayed as described above for panel B. The values shown are means $\pm$ SEM (error bars) for triplicate determinations. (F) Same as panel E except that cell extracts were immunoblotted with the indicated antibodies. Note that coexpression with Akt1 suppresses the level of caspase 3 cleavage triggered by Gene 33 expression. (G) Constitutively active MEK1 partially alleviates cardiomyocyte killing incurred by Gene 33. Cardiomyocytes were infected as indicated with β -galactosidase, Gene 33 and/or constitutively active MEK1 (MEK-DD) or myristoylated Akt1. In the bar graph at the top, cell killing was measured as described above for panel E (means $\pm$ SEM for triplicate samples). NS indicates a statistically nonsignificant difference in the indicated measurements (unpaired Student's *t* test, $P > 0.05$). *P* values are shown for the indicated statistically significant assay results. For the gels at the bottom, parallel extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with the indicated antibodies. (H) Quantitation of the ERK phosphorylation attained in the samples from the gels at the bottom of panel G. (I) Mitogens partially rescue cardiomyocyte killing incurred by Gene 33. Cells were infected with the indicated adenoviral constructs, serum starved, and treated with the mitogens indicated. Cell survival was measured as described above for panel B. The values shown are the means $\pm$ SEM for triplicate determinations. C, control.

FIG. 3. Endogenous cardiomyocyte Gene 33 protein and *gene 33* mRNA are induced by mitogens, ET-1, and hypoxia. (A) Induction of Gene 33 protein by mitogens and ET-1. Neonatal rat cardiomyocytes were treated with the indicated mitogens for 4 h. Extracts were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with the indicated antibodies (p-ERK, phosphorylated ERK). C, control. (B) *gene 33* mRNA is induced by the same mitogens that trigger Gene 33 protein expression. Cardiomyocytes were treated as described above for panel A for 2 h, and total cellular RNA was extracted and subjected to Northern blot analysis. The 28S/18S ethidium bromide staining served as a loading control. (C) Hypoxia induces cardiomyocyte Gene 33 protein. Cells were subjected to hypoxic treatment (Hypo) (4 h; Materials and Methods), and then cell extracts were prepared and subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Control cells (C) were retained under normoxic conditions. (D) Hypoxia induces cardiomyocyte *gene 33* mRNA. Cells were subjected to hypoxic treatment (Materials and Methods). Total cellular RNA was extracted and subjected to Northern blot analysis. The 28S/18S ethidium bromide staining served as a loading control. (E) Mitogen induction of cardiomyocyte Gene 33 protein requires PI 3-kinase and ERK. Cardiomyocytes were treated with PDGF as indicated (+), either in the presence (+) or absence of U0126 (MEK1 inhibitor) or wortmannin (Wort) (a PI 3-kinase inhibitor). Induction of Gene 33 protein and efficacy of the pharmacological inhibitors was analyzed as described above for panel A. (F) Hypoxia induction of Gene 33 protein requires ERK and PI 3-kinase. Cells were treated with the indicated inhibitors (LY294002 [LY], a PI 3-kinase inhibitor) and then subjected to 6 h of hypoxia treatment. Gene 33 induction and phosphorylation of Akt and ERK (positive controls for the inhibitor drugs) were determined by immunoblotting. Total p38 MAPK immunoblotting served as a gel loading control.

5D). Nevertheless, induction of Gene 33 by hypoxia also requires PI 3-kinase and at least basal ERK activity inasmuch as LY294002 (a second, specific PI 3-kinase inhibitor (14) and U0126 both blunt hypoxia induction of Gene 33 protein (Fig. 3F).

Hypoxia treatment of cardiomyocytes triggers activation of the EGF receptor, and hypoxia or hypoxia/reoxygenation of ERK and Akt requires in part EGFR family receptors. The antagonistic relationship between Gene 33 and EGFR/ErbB signaling combined with the requirement for PI 3-kinase and ERK for hypoxia induction of endogenous Gene 33 led us to ask whether Akt and ERK were recruited by hypoxia through a mechanism that required in part receptor Tyr kinases of the EGFR family. From Fig. 4A, it is clear that hypoxia triggers EGFR Tyr phosphorylation.

AG1478 is a small-molecule inhibitor specific for receptor Tyr kinases of the EGFR family. AG9 is an inactive analogue of AG1478. Use of these compounds reveals a complex and divergent regulation of ERK and Akt by hypoxia and hypoxia/reoxygenation—an in vitro condition mimicking ischemia/reperfusion. Thus, modest activation of ERK, but not the stronger activation of Akt by hypoxia, is partially suppressed by AG1478, but not AG9, indicating a role for EGFR family receptors in hypoxia recruitment of ERK, but not Akt. By

contrast, the maintenance of Akt, but not ERK, activation during reoxygenation (subsequent to hypoxia) is reduced but not eliminated by AG1478 (but not AG9), indicating a role for EGFR family receptors in continued Akt activity, but not ERK activity subsequent to hypoxia, during reoxygenation (Fig. 4B). It is known that hypoxia can lead to the production of reactive oxygen species, and reoxygenation can trigger further production of reactive oxygen species as well as the paracrine release of proinflammatory cytokines. These additional elements may, independently of EGFR family receptors, contribute to ERK and Akt activation. Figure 4C shows that AG1478 and AG9 produce the expected effects on EGF and NRG recruitment of ERK.

Endogenous Gene 33 functions to suppress cardiomyocyte ERK and Akt activation and is required for optimal hypoxia- or hypoxia/reoxygenation-induced cardiomyocyte death. Our results suggest the following. (i) Gene 33 suppresses cardiomyocyte EGFR/ErbB signaling and kills cardiomyocytes. (ii) Gene 33 is induced by hypoxia in an Akt/ERK-dependent manner. (iii) Hypoxia signals in part through EGFR-based mechanisms to recruit Akt and ERK. We wished next to extend the above results and examine the functional role of endogenous Gene 33 induction. Specifically, we were interested in whether Gene 33 was required for optimal cardiomyo-

FIG. 4. Hypoxia triggers activation of EGFR signaling, and hypoxia activation of ERK requires in part EGFR signaling. (A) Hypoxia stimulates Tyr phosphorylation of EGFR. Cardiomyocytes were subjected to hypoxic treatment for the indicated times (10 min [10'] and 30 min [30']). EGFR was immunoprecipitated, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting with the indicated antibodies (P-EGFR, phosphorylated EGFR). C, control. (B) EGFR inhibitors differentially affect hypoxia and hypoxia/reoxygenation activation of cardiomyocyte ERK and Akt. Cells were pretreated with either AG1478 or the inactive analogue AG9 and then subjected to treatment with hypoxia (Hypo) or hypoxia/reoxygenation (Hypo + Rep) as indicated. Extracts were prepared and subjected to SDS-PAGE and immunoblotting with the indicated antibodies. The bar graphs are quantitations of ERK and Akt phosphorylation for the immunoblots shown. (C) Efficacy of AG1478 and AG9. Cardiomyocytes were pretreated with AG1478 or AG9 as indicated and then treated with EGF or NRG as indicated. Extracts were subjected to SDS-PAGE and immunoblotting with the indicated antibodies.

cyte killing triggered by hypoxia. To that end, we exploited small interfering RNA-mediated RNA interference to silence endogenous *gene 33* and characterize the effects of loss of Gene 33 function. A rat-specific *gene 33* siRNA, but not an analogous siRNA based on the human *gene 33* sequence, could effectively silence neonatal rat cardiomyocyte Gene 33 protein induced by hypoxia (Fig. 5A).

To examine directly the role of endogenous Gene 33 in promoting hypoxia-induced cardiomyocyte death, we exploited TUNEL staining, a measure of cell death-induced internucleosomal cleavage. Thus, cells were treated with hypoxia alone or hypoxia followed by reoxygenation. Hypoxia alone triggers significant TUNEL staining which is sustained during reoxygenation. Silencing of rat cardiomyocyte *gene 33* strikingly reduces TUNEL staining (Fig. 5B and C)—a finding consistent with the ability of *gene 33* siRNA to blunt hypoxia/reoxygenation-induced procaspase 9 and 3 cleavage (below).

Consistent with the observations that ERK and Akt can promote cell survival and that Gene 33 blunts recruitment of ERK and Akt by EGFR family receptors (which participate in hypoxia/reoxygenation-induced activation of ERK and Akt [Fig. 4]), we find that silencing of *gene 33* substantially enhances hypoxia- and hypoxia/reoxygenation-induced activation of cardiomyocyte ERK and Akt coincident with protecting cardiomyocytes from cell death (Fig. 5D).

The FOXO subgroup of forkhead transcription factors includes FKHR/FOXO1. FKHR promotes apoptosis by transactivating the genes for several proapoptotic proteins, including FasL, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and tumor necrosis factor receptor-associated death domain (TRADD) (6, 16, 23, 35). Akt phosphorylates FKHR, triggering the binding of 14-3-3 proteins which, in turn, leads to dissociation from DNA and nuclear exit of FKHR (6, 16, 23, 35). Silencing of *gene 33* elevates hypoxia- and hypoxia/reoxy-

FIG. 5. (A) Silencing of endogenous *gene 33* mRNA by siRNA-mediated RNAi. Neonatal rat cardiomyocytes were treated with rat-specific *gene 33* siRNA or, as a control, human-specific *gene 33* siRNA. Cells were then treated with hypoxia (Hypo) or hypoxia/reoxygenation (Hypo+rep) as indicated. C, control. (B) Hypoxia and hypoxia/reoxygenation-induced cardiomyocyte cell death is suppressed by silencing *gene 33*. TUNEL staining of cells subjected to hypoxia or hypoxia/reoxygenation was performed. Rat cardiomyocytes were treated with control (human-specific) or specific (rat-specific) *gene 33* siRNA. Cells were then subjected to hypoxia (12 h) or hypoxia followed by 24 h of reoxygenation as indicated. Cells were then fixed and stained by using the TUNEL kit or anti-sarcomeric α -actinin as indicated. oligo, oligonucleotide. (C) Quantification of results from panel A. TUNEL-positive cells from five different fields were quantitated as a percentage of the total number of DAPI-stained nuclei. The values shown are means $\pm$ standard errors of the means (SEM) (error bars) ($n = 5$). The asterisks indicate that silencing *gene 33* results in a statistically significant reduction in hypoxia and hypoxia/reoxygenation-induced cell death (P values are shown in the figure and were generated using an unpaired Student's t test on the values indicated by the brackets). (D) Silencing of *gene 33* increases prosurvival signaling and reduces cell death signaling recruited by hypoxic stress. RNAi was performed as described above for panel A. As indicated, cells were subjected to hypoxia (12 h) or hypoxia/reoxygenation (12 h and 1 h, respectively) as described for Fig. 4C. Cell extracts were subjected to immunoblotting with the indicated antibodies (P-ERK, phosphorylated ERK; casp 9, caspase 9). H-Ras immunoblotting served as a gel loading control. The bar graphs on the right are quantitations of ERK and Akt phosphorylation for the immunoblots shown on the left panel. C, control. (E) Maximal hypoxia/reoxygenation-induced cleavage of cardiomyocyte caspase 3 requires Gene 33. Cardiomyocyte Gene 33 was silenced as described in the legend to Fig. 5. Cells were subjected to hypoxia or hypoxia/reoxygenation treatments described in the legend to Fig. 5B. Reoxygenation (rep) was for the indicated times. Cell extracts were prepared and subjected to immunoblotting with the indicated antibodies (casp3, caspase 3). The total ERK2 blot served as a gel loading control.

genation-induced inhibitory, Akt-catalyzed FKHR phosphorylation (Fig. 5D). Thus, the component of Akt's survival signaling program involving inhibition of FKHR is suppressed in part by Gene 33 induced during hypoxia. This coincides with a requirement for Gene 33 for maximal hypoxia/reoxygenation-induced cardiomyocyte death.

Activation of procaspase 9 involves proteolytic cleavage consequent to association with the apoptosome (9, 13, 23). The ability of hypoxia to trigger procaspase 9 cleavage (as visualized on immunoblots with cleavage-specific antibodies) is also reduced upon silencing of *gene 33* (Fig. 5D). A consequence of procaspase 9 activation is procaspase 9-dependent cleavage and activation of procaspase 3. Activation of procaspase 3 is critical to the progress of cell death (6, 23). We find that hypoxia, followed by reoxygenation, triggers procaspase 3 cleavage with kinetics that are slower than those of procaspase 9 activation (compare Fig. 5D and E). Thus, procaspase 9 cleavage is first apparent after hypoxia, while procaspase 3 cleavage (as also visualized on immunoblots with cleavage-specific antibodies) is first seen after reoxygenation—consistent with the idea that procaspase 9 activation is a prerequisite for procaspase 3 activation in the mitochondrial apoptosis pathway (6, 23) (Fig. 5D and E). Gene 33 polypeptide appears during hypoxia—prior to the activation of either procaspase 9 or procaspase 3. Silencing of *gene 33* substantially reduces hypoxia/reoxygenation-stimulated procaspase 9 and procaspase 3 cleavage (Fig. 5E). Thus, optimal activation of four markers of hypoxia/reoxygenation-triggered cardiomyocyte cell death, internucleosomal cleavage, FKHR phosphorylation, and cleavage of procaspases 9 and 3, requires Gene 33 and can be substantially reduced upon silencing of endogenous *gene 33* (Fig. 5). Moreover, ectopic expression of recombinant Gene 33 is sufficient to trigger cardiomyocyte death (Fig. 2).

Endogenous Gene 33 is induced in ischemic injury and myocardial infarction. In order to determine whether Gene 33 was induced by ischemia and ischemia/reperfusion, we subjected mouse hearts to ischemic injury (ligation of the left anterior descending coronary artery) transiently (60 min, followed by sacrifice or reperfusion for 30 min). We monitored Gene 33 protein levels following ischemia or ischemia/reperfusion by immunoblotting samples taken from the ischemic region and the unaffected region (Fig. 6A). We find that at 1 h postischemia, the ischemic region shows elevated Gene 33 immunoreactivity—both in heart treated with ischemia or with ischemia/reperfusion. Of note, control hearts manifested tonic ERK activity which declined substantially upon ischemic injury or ischemic/reperfusion injury. This decline was coincident with elevated levels of Gene 33—a result consistent with the idea that Gene 33 suppresses ERK activation (Fig. 6A). For studies of myocardial infarction, ligation of the left anterior descending coronary artery was maintained until myocardial infarction (8, 31). Samples from the noninfarct, infarct, and border zones were prepared and subjected to immunoblotting with anti-Gene 33 antibodies. In contrast to ischemia, or ischemia/reperfusion injury, myocardial infarction triggers enhanced expression of Gene 33 protein in all three zones 6 hours after myocardial infarction (Fig. 6B). However, by 24 h after myocardial infarction, Gene 33 levels in the noninfarct and border zones return to normal, while Gene 33 levels in the infarct zone remain elevated. We also prepared thin sections

for immunohistochemical analysis from hearts 72 h after myocardial infarction. After 72 h, significant endogenous Gene 33 immunoreactivity remains present in the infarct area (Fig. 6C and D). Hearts subjected to a sham operation show little or no detectable Gene 33 immunoreactivity (compare Fig. 6E to C and D). The results from Fig. 6 suggest that Gene 33 is induced by ischemia or ischemia/reperfusion coincident with a suppression of ERK activation. In this instance, Gene 33 induction is restricted to the affected region. By contrast, in actual myocardial infarction—essentially an extreme form of ischemic injury—Gene 33 is upregulated in noninfarct regions as well as the infarct zone but returns to basal levels in the noninfarct regions, while remaining elevated in the infarct zone for up to 72 h postinfarction. Thus, myocardial infarction leads to a sustained induction of Gene 33 within the myocardial infarct zone.

DISCUSSION

Our results suggest a model wherein Gene 33 functions in the cardiomyocyte, at least in part, as a stress-inducible polypeptide that functions to suppress prosurvival signaling emanating from ErbB receptors (or in the case of ET-1, possibly via transactivation of EGFR)—especially that mediated by Akt or ERK. By this process, Gene 33 promotes cell death. ErbB ligands and IGF-1 have been linked to cardiomyocyte stress resistance and survival (11, 20, 25), and Akt and ERK activation are particularly important in the promotion of cardiomyocyte survival following ischemic injury (6, 27, 29, 34).

gene 33 is induced by a variety of stimuli and likely is involved in multiple functions. We find that in cardiomyocytes, *gene 33* is induced by hypoxia and by mitogens. Our previous findings from fibroblasts indicate that glucocorticoids trigger *gene 33* expression, which is required not for cell death but for glucocorticoid-mediated prevention of EGF-induced cell cycle entry (37). In ErbB2-dependent tumor cells, Gene 33 is cytostatic, but not apoptogenic (1, 3, 18). Thus, in some systems, Gene 33 functions to suppress proliferative signaling (notably EGF family mitogens)—perhaps in a negative-feedback manner. Accordingly, depending on the cell background and the spectrum of signals to which a cell is exposed coincident with Gene 33 induction, Gene 33 may have either cytostatic or cell death functions.

Consistent with this, transgenic mouse studies support a role for Gene 33 in the suppression of cell proliferation. Thus, mice engineered to overexpress *gene 33* in skin manifest a phenotype (a wavy coat, eyes open at birth, and curly whiskers) resembling that incurred by hypomorphic or antimorphic alleles of *egfr*, the gene encoding the EGF receptor (3). Likewise, disruption of *gene 33* in mice leads to early onset joint degeneration that strikingly resembles osteoarthritis. In *gene 33*^{-/-} joints, there is an apparent hyperplasia of mesenchyme-like cells leading to the development of bony outgrowths and osteophyte formation in the affected joints (38). This observation also fits with the idea that Gene 33 can suppress cell proliferation.

In response to chronic, or even severe, acute stress stimuli, however, Gene 33 may be induced in conjunction with both proliferative and death signals and may function, in conjunction with other concerted stress signaling mechanisms, to tip

FIG. 6. Upregulation of Gene 33 protein expression in ischemia, ischemia/reperfusion, and myocardial infarction. Ischemic induction of Gene 33 coincides with a decrease in tonic ERK activation. To trigger ischemic injury, ischemia/reperfusion injury, or myocardial infarction, mice were subjected to banding of the left anterior descending coronary artery either transiently (ischemia or ischemia/reperfusion) or for a sustained period (so as to induce myocardial infarction). Control animals were subjected to a sham operation. (A) For ischemia and ischemia/reperfusion injury, the coronary artery was banded for 60 min (60'). Reperfusion was for 30 min as indicated. Tissue samples from the ischemic zone (affected region [AR]) and nonischemic zone (NI) were isolated and subjected to immunoblotting with the indicated antibodies (P-ERK, phosphorylated ERK). C, control. (B) Expression of Gene 33 in myocardial infarction. Mice were subjected to left anterior descending coronary artery banding until myocardial infarction. Samples from the noninfarct (NI), infarct (I), and infarct border (B) zones were taken at 6 and 24 h after infarction as indicated. Samples were subjected to immunoblotting with the indicated antibodies. (C) Expression of Gene 33 in myocardial infarct zones. Thin frozen sections were prepared from two infarct zones taken from a single representative animal (indicated by the large numbers 1 and 2) 72 h after myocardial infarction. Sections were stained with rabbit anti-Gene 33 antibody (Gene33Ab) plus fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (2nd Ab) plus DAPI or second antibody only, plus DAPI (negative control) as indicated. Sections incubated without the primary antibody showed no background staining. (D) Same as panel C but the sections were from a different animal also subjected to myocardial infarction. The leftmost panel is a low-magnification image, the area highlighted with the red border was examined at a higher magnification (middle panels), with staining with anti-Gene 33 antibody and FITC-conjugated anti-rabbit secondary antibody. The region highlighted with the red border in the middle panel was examined at a higher magnification in the rightmost panel. (E) Sham-operated control. Staining with anti-Gene 33 antibody and FITC-conjugated anti-rabbit secondary antibody was performed as described above for panels C and D.

the balance to the promotion of cell death. This idea fits with our observations that Gene 33 protein levels are elevated in myocardial infarct zones for up to 72 h, that hypoxia recruitment of ERK and Akt is partly dependent upon EGFRs, and that upregulation of *gene 33* is necessary for optimal hypoxia-induced cardiomyocyte death.

Still, in pathophysiologic circumstances, induction of Gene 33 may not automatically lead to widespread cell death. In this regard, it is noteworthy that endogenous *gene 33* is also strongly induced in the progression to diabetic nephropathy. Here, induction does not coincide with cell death but does

track with disease progression. In the diabetic animal, renal *gene 33* is rapidly induced with the onset of hyperglycemia and continues to rise steadily until complete renal failure. This contrasts with *c-fos* and *c-jun*, which are induced in the diabetic kidney much more transiently (26). The steady increase in renal *gene 33* induction during diabetic nephropathy resembles that of *p8*, which encodes a small basic helix-loop-helix transcriptional regulatory protein required for renal mesangial cell hypertrophy (19). This suggests that *p8* and *gene 33* may be part of a suite of genes that are stably induced in chronic stress-related diseases that contribute to development of the patho-

physiologic phenotype. The functions of Gene 33 and p8 are, however, clearly different. We find no role for endogenous Gene 33 in hypertrophy or fibrosis. Similarly, p8 has no discernible function in cardiomyocyte death (S. Goruppi and J. M. Kyriakis, unpublished observations).

The biochemical mechanisms by which Gene 33 functions remain somewhat nebulous. We and others have found that Gene 33 binds directly to Erb family Tyr kinases and reduces their autophosphorylating activity (1, 18, 37). This likely accounts for the Gene 33-dependent suppression of ErbB family receptors, ERK and Akt activation, inasmuch as the recruitment of these pathways depends on the binding of Src homology 2 domain-containing proteins to autophosphorylated receptor Tyr kinases. Given that ErbB signaling, especially to Akt and ERK, fosters cardiomyocyte survival during stress (6, 20, 25, 27, 29, 34), one consequence of inhibition of cardiac ErbB family signaling could be cardiomyocyte death. Consistent with this, we find that expression of recombinant Gene 33 suppresses EGF and NRG activation of Akt and ERK coincident with promoting cardiomyocyte death in an Akt- and MEK-DD-reversible manner. Interestingly, trastuzumab/Herceptin, a novel anticancer therapeutic monoclonal antibody that acts to block signaling through ErbB receptors has also been shown to produce elevated cardiotoxicity in clinical trials (24), and this cardiotoxicity may be due in part to suppression of Akt activation (20).

We see recruitment of cardiomyocyte EGFRs by hypoxia, partial EGFR-dependent ERK and Akt activation by hypoxia, as well as Gene 33-dependent hypoxia-induced cell death coincident with suppression of ERK and Akt activation. From these observations, it is reasonable to hypothesize that hypoxia recruitment of ErbB family receptors is in part a survival mechanism that serves to counter death signaling, with the eventual outcome, death or survival, dependent upon the relative intensity and kinetics of survival signals (Akt/ERK) versus death signals (Gene 33 and others).

Gene 33 has a complex structure that suggests an adapter protein with multiple functions (26, 28). The carboxyl-terminal region of Gene 33 is remarkably homologous to the noncatalytic region of the Ack1 nonreceptor Tyr kinase (26, 28). It is solely this domain that is necessary for ErbB receptor family binding and inhibition of ERK and Akt activation (1, 18, 37). Gene 33 also possesses an amino-terminal Cdc42/Rac interaction and binding domain that binds the Rho GTPase Cdc42 in a GTP-dependent manner. In addition, Gene 33 binds 14-3-3 proteins tightly, and upon overexpression, Gene 33 activates the Jun N-terminal protein kinase pathway in some, but not all, cell lines (26, 37). It will be important to determine the functions of these different portions of the Gene 33 polypeptide, especially with regard to cell death.

Our results indicate that the induction of Gene 33 in stressed heart contributes to cardiomyocyte death. These findings provide a framework for future studies that will establish the molecular mechanisms by which Gene 33 affects heart function. In addition, the appearance of Gene 33 could serve as a marker useful in the development of new therapies designed to prevent cardiomyocyte death in the ischemic or failing heart.

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