Targeted Focal Adhesion Kinase Activation in Cardiomyocytes Protects the Heart From Ischemia/Reperfusion Injury


Objective—We previously reported that cardiac-restricted deletion of focal adhesion kinase (FAK) exacerbated myocyte death following ischemia/reperfusion (I/R). Here, we interrogated whether targeted elevation of myocardial FAK activity could protect the heart from I/R injury.

Methods and Results—Transgenic mice were generated with myocyte-specific expression of a FAK variant (termed SuperFAK) that conferred elevated allosteric activation. FAK activity in unstressed transgenic hearts was modestly elevated, but this had no discernible effect on anabolic heart growth or cardiac function. Importantly, SuperFAK hearts exhibited a dramatic increase in FAK activity and a reduction in myocyte apoptosis and infarct size 24 to 72 hours following I/R. Moreover, serial echocardiography revealed that the transgenic mice were protected from cardiac decompensation for up to 8 weeks following surgery. Mechanistic studies revealed that elevated FAK activity protected cardiomyocytes from I/R-induced apoptosis by enhancing nuclear factor-κB (NF-κB)-dependent survival signaling during the early period of reperfusion (30 and 60 minutes). Moreover, adenoviral-mediated expression of SuperFAK in cultured cardiomyocytes attenuated H2O2 or hypoxia/reoxygenation-induced apoptosis, whereas blockade of the NF-κB pathway using a pharmacological inhibitor or small interfering RNAs completely abolished the beneficial effect of SuperFAK.

Conclusion—Enhancing cardiac FAK activity attenuates I/R-induced myocyte apoptosis through activation of the prosurvival NF-κB pathway and may represent a novel therapeutic strategy for ischemic heart diseases. (Arterioscler Thromb Vasc Biol. 2012;32:924-933.)

Key Words: apoptosis ■ extracellular matrix ■ hypoxia ■ ischemic heart disease ■ reactive oxygen species

Cardiovascular diseases are the number 1 cause of death globally, and current estimates indicate that as many as 1 of 6 deaths per year in the United States can be attributed to coronary disease and associated myocardial ischemia.1 Although rapid reperfusion is necessary to reduce ischemia-dependent myocyte necrosis, it can also result in intracellular calcium overload and oxidative stress, which can initiate apoptosis.2 Importantly, although apoptosis can account for up to 60% of myocyte death within infarcted tissue,3 this programmed cell death cascade (unlike necrosis) can be reversed by activation of prosurvival signals.4 5 Therefore, defining the mechanisms that govern the transition between cardiomyocyte apoptosis and survival will undoubtedly have a significant impact on the generation of promising treatments to reduce ischemia-induced myocardial dysfunction.

Attachment of cells to the extracellular matrix results in the clustering of integrin receptors and initiates the recruitment of numerous structural and catalytically active signaling proteins to the adhesion complexes that are necessary for maintaining tissue integrity, for conveying tensile strength, and for the transduction of growth and survival signals.6 Several recent studies indicate that integrin signaling may play a pivotal role in preserving the myocardium from pathological stressors. For example, myocardial-restricted deletion of β1 integrin in mice leads to myocardial dysfunction in postpartum females and in male mice subjected to hemodynamic overload.7 8 As well, mice with myocyte-restricted deletion of β1 integrin exhibit elevated cardiomyocyte apoptosis and concomitant heart failure following isoproterenol infusion.9 Moreover, the finding that human ischemic cardiomyopathy is associated with downregulation of the muscle selective β1-D-integrin and the integrin-activated kinase focal adhesion kinase (FAK) supports the possibility that targeting this pathway may be beneficial in the prevention of ischemic heart failure.10

Nearly two thirds of the known integrin heterodimers, including all β1-, β3-, or βα-containing integrins, couple to the nonreceptor protein tyrosine kinase FAK,11 12 as do several growth factor receptors, including those activated by vascular endothelial growth factors and fibroblast growth...
FAK activation proceeds by a 2-step process that involves dimerization and autophosphorylation of Tyr397, which creates a high-affinity Src homology 2 binding site for the tyrosine kinase Src. Once bound, Src phosphorylates FAK on 2 additional sites within the activation loop (Tyr576 and Tyr577), leading to further enhancement of FAK catalytic activity. FAK binding partners/substrates include the adapter proteins paxillin, p130 CAS, and GRB2, which can activate the extracellular signal–regulated kinase (ERK) and c-Jun N-terminal kinase growth promoting mitogen-activated protein kinases. FAK also coordinates signaling to the prosurvival nuclear factor-κB (NF-κB) and Akt pathways through interactions with receptor-interacting protein and PI3 kinase, respectively. Although FAK activity is necessary for myocyte proliferation and midgestational heart growth, we recently showed that myocyte-specific deletion of FAK in the adult myocardium did not affect anabolic growth or basal contractility. However, on challenge, FAK-depleted hearts progressed to profound cardiac degradation following pressure overload and exhibited markedly increased myocyte apoptosis and infarct size relative to wild-type mice following ischemia/reperfusion (I/R). Because our mechanistic studies supported a critical role for myocardial FAK in promoting NF-κB-induced survival signaling following I/R, we predicted that enhanced myocardial FAK activity might provide cardioprotection from an ischemic insult.

To date, attempts to enhance prosurvival signaling in the myocardium by expression of upstream kinases have met with limited success, likely because of strategies that often lead to supraphysiological expression of constitutively active kinases. In these cases, overexpression alone can lead to striking changes in myocardial remodeling that culminate in phenotypes ranging from hypertrophy to heart failure and sudden death as has been observed in several transgenic mouse models with targeted expression of active Akt variants. Because we hypothesized that transient elevation of FAK-dependent survival signals would be cardioprotective, whereas uncontrolled activation could lead to detrimental remodeling, we sought to express a variant of FAK that requires signal-dependent activation but exhibits enhanced catalytic activity. Thus we took advantage of known mutations within the FAK activation loop (Lys578Glu/Lys581Glu) that mimic the charge transfer (and enhanced catalytic activity) induced by Src phosphorylation. Herein, we found that transgenic mice with myocyte-restricted expression of this superactivatable FAK variant (termed SuperFAK [SF]) exhibited remarkable protection from I/R-dependent cell death. These studies provide the first evidence that FAK could be a tractable target for gene therapy and provide proof-of-concept for a methodological approach to exploit posttranslational modifications to enable spatial and temporal control of kinase activation in vivo.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Generation of SF Transgenic Mice
The SF cDNA variant was kindly provided by Dr Michael Schaller. Cardiac-specific transgenic mice with SF overexpression was achieved with a 3.4-kb piece of the cardiac β-myosin heavy chain promoter that was modified to prevent downregulation at birth. All animals were housed in a university animal care facility accredited by the American Association for Accreditation of Laboratory Animal Care, and all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Statistical Analysis
All values are presented as means±SEM. Comparisons were performed by using unpaired Student t test or 1-way analysis of variance with the Tukey’s post hoc test as appropriate. All tests were 2-tailed, and significance was accepted at P<0.05.

Results
Generation of Transgenic Mice That Confer Enhanced Allosteric FAK Activity in the Myocardium
We previously demonstrated that cardiac-restricted deletion of FAK exacerbates I/R-induced apoptosis and leads to enhanced cardiac decompensation following I/R or prolonged pressure overload. Because the toggling between prosurvival and proapoptotic signals remains central to preventing irreversible damage to the heart, we strove to determine whether enhanced FAK activity could salvage at-risk myocytes in the ischemic heart. To address this critical issue, we generated transgenic mice that expressed a superactivatable variant of FAK (SF) in cardiomyocytes. SF contains glutamic acid substitutions for 2 lysine residues in the activation loop of FAK (Lys578Glu, Lys581Glu) that render the protein primed for allosteric activation (Figure IA in the online-only Data Supplement), SF has substantially increased catalytic activity in comparison to wild-type FAK when expressed at comparable levels (Figure IB in the online-only Data Supplement). Nonetheless, SF is not constitutively active. Indeed, cells transfected with SF for 48 hours and maintained on tissue culture plastic exhibited levels of FAK activity comparable to those of nontransfected or LacZ-transfected cells (Figure IA, left). However, when replated on a fibronectin-coated surface for 30 minutes, the SF transfected cells exhibited much more pronounced FAK activity (Figure IA, right), indicating that SF confers enhanced allosteric activation.

Two novel mouse lines were generated in which a flag epitope tagged SF transgene was expressed under the control of a truncated β-myosin heavy chain promoter that was modified to drive cardiomyocyte-specific expression into adulthood. The so-named SF1 and SF2 mice exhibited a persistent and myocardial-secreted increase (2- to 6-fold respectively) in basal cardiac FAK activity relative to age-matched nontransgenic (NTG) littermate control hearts (Figure 1B–1D). Both SF1 and SF2 mice were born in the expected Mendelian frequency, and no noticeable morbidity or mortality was observed up to 18 months of age.

Enhanced Cardiomyocyte-Specific FAK Activity Does Not Influence Cardiac Growth or Basal Cardiac Function
We carefully assessed the consequence of elevated myocyte-restricted FAK activity on postnatal myocardial growth in SF1 and SF2 mice. No significant differences were observed in myocardial shape or size (Figure IIA in the online-only Data Supplement), heart weight/body weight ratios (Figure IIB in the online-only Data Supplement), or cardiomyocyte...
cross-sectional area (Figures IIC, IID, and III in the online-only Data Supplement) when SF mice 8 weeks to 12 months of age were compared with age-matched littermate controls. To determine whether elevated myocyte-specific FAK activity affected cardiac performance, we first measured left ventricular function by conscious echocardiography in young adult male SF2 and NTG control mice. No significant differences in either ejection fraction or fractional shortening were observed between these lines (Figure IIE and IIF in the online-only Data Supplement). As well, serial echocardiography in a second cohort of anesthetized mice revealed comparable left ventricular wall thickness, chamber diameter, mass, and function between NTG and SF2 mice from 1 to 12 months of age (Figure IV in the online-only Data Supplement). Hemodynamic measurements by cardiac catheterization also revealed no significant differences in intrinsic cardiac contractility or diastolic function between 12-month-old NTG and SF2 mice (Figure V in the online-only Data Supplement). However, postnatal SF2 and NTG hearts exhibited comparable levels of cardiac Akt and ERK activity, signals that regulate anabolic and pathological hypertrophic growth of the postnatal heart (Figure IIH in the online-only Data Supplement).

Elevated FAK Activity Ameliorates Adverse Cardiac Remodeling and Dysfunction Following I/R

Because we previously showed that cardiac-restricted deletion of FAK exacerbated I/R-induced myocardial infarction,22 we hypothesized that elevated FAK activity would confer cardioprotection in this setting. To this end, we subjected NTG and SF2 mice to 30 minutes of ischemia (induced by transient ligation of the left anterior descending coronary artery) followed by reperfusion for 24 hours to 8 weeks and assessed infarct size and myocardial function. Evans blue/2,3,5-triphenyltetrazolium chloride staining showed that the relative area at risk (nonblue/total left ventricular area) was comparable between the 2 groups, indicating that a similar level of ischemia was induced following surgical ligation (data not shown). However, 24 hours following reperfusion, the SF2 mice exhibited a significantly decreased relative infarct size (white/nonblue area) compared with control mice (control, 50.5 ± 6.0%, vs SF2, 34.6 ± 3.2%; \( P < 0.05; n = 6 \) for control and \( n = 7 \) for SF2; Figure 2A, top, and 2B). A much greater difference was observed between the 2 groups after 72 hours of reperfusion (control, 79.4 ± 4.1%, vs SF2, 34.7 ± 5.6%; \( P < 0.001; n = 5 \) for control and \( n = 4 \) for SF2; Figure 2A, bottom, and 2B). Notably, in the NTG controls the relative infarct size increased dramatically from 24 to 72 hours following I/R, whereas the SF2 mice exhibited no significant increase in infarct size during this critical time period. To determine whether SF expression promoted long-term cardioprotection, cardiac function was monitored by conscious echocardiography at 2, 4, and 8 weeks post-I/R in
a third cohort of NTG and SF2 mice. Both interventricular septal and posterior wall thickness were much better preserved in the SF2 hearts (Figure VII in the online-only Data Supplement). Concomitantly, although both groups exhibited a decline in ejection fraction and fractional shortening following I/R, the SF2 hearts maintained significantly greater function than the NTG controls (Figure 2C and 2D). Ultimately, I/R-induced chamber dilatation was observed in the NTG but not SF2 hearts at the 4- and 8-week time points (Figure VII in the online-only Data Supplement). Collectively, these data indicate that enhanced myocardial FAK activity protects the myocardium from I/R-dependent cell death and promotes functional recovery following an ischemic insult.

Elevated FAK Activation Attenuates Cardiomyocyte Apoptosis Following I/R

I/R-induced myocyte death results from irreversible (necrotic) and reversible (apoptotic) signals. Because our previous studies indicated that FAK depletion renders cardiomyocytes more susceptible to ischemia-induced apoptosis, we reasoned that enhanced FAK activity might limit cardiomyocyte apoptosis following I/R. We first sought to determine the extent to which SF is activated in ischemic myocytes. To aid in the demarcation of the ischemic zone, I/R-treated mice were injected with hypoxyprobe-1 (pimonidazole hydrochloride), which forms protein adducts in cells with a pO₂ of 10 mmHg or less that can be detected by immunostaining.34 As shown in Figure 3A, sham SF2 hearts contained little immunoreactivity for hypoxyprobe-1, whereas those subjected to I/R for 24 hours revealed intense focal reactivity, typical of hypoxia. Consistent with previous studies from us and others indicating that wild-type FAK was activated following I/R22 or ischemic preconditioning,35 costaining with the phospho-FAK(Tyr397) antibody revealed that FAK activity was relatively low in sham SF2 hearts but was markedly induced within the hypoxic myocytes in I/R treated SF2 hearts (Figure 3A). As expected, the magnitude of ischemia-induced FAK activation was much higher in SF2 hearts than control hearts subjected to I/R despite a similar extent of hypoxyprobe staining (Figure 3B and data not shown), indicating that SF is primed for robust activation in response to myocyte hypoxia. We next asked whether ischemic myocytes with elevated FAK activity were more resistant to I/R-induced apoptosis. As shown in Figure 3B and 3C, the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive myocytes 24 hours following I/R was significantly lower in the SF2 hearts than in NTG controls in both the ischemic border zone and in remote regions. Moreover, SF2 hearts exhibited a stark reduction in I/R induced cleaved caspase 3 levels as assessed by immunohistochemistry (Figure VIII in the online-only Data Supplement). Taken together with our results that infarcts in SF2 hearts did not increase between 24 and 72 hours following I/R (time points at which apoptosis contributes significantly to myocyte death), these studies provide strong evidence that enhanced FAK activity can protect myocytes from ischemia-induced apoptosis. Importantly, capillary and coronary vascular density was comparable between control and SF2 hearts (Figure IX in the online-only Data Supplement), indicating that the beneficial effect of elevated FAK activity was likely myocyte autonomous and was not due to enhanced collateral blood flow.

Elevated FAK Activity Protects Cultured Cardiomyocytes From Oxidative Stress–Induced Apoptosis

We developed an in vitro assay to further test whether elevated FAK activity confers protection from ischemia-
induced programmed cell death in a cell-autonomous fashion. To this end, we infected primary neonatal rat cardiomyocytes with adenoviruses expressing green fluorescent protein (GFP) or SF and subjected these cells to oxidative stress by treatment with $10^5$ mol/L H$_2$O$_2$ or by incubation in low O$_2$ as previously described. Oxidative stress is known to play a critical role in I/R-induced cardiomyocyte apoptosis, and we found that treatment of cultured cardiomyocytes with $10^5$ mol/L H$_2$O$_2$ for 5 hours induced marked apoptosis in noninfected or GFP-infected cardiomyocytes (as assessed by TUNEL), whereas significantly fewer TUNEL-positive cells were found in SF-infected cultures (Figure 4A; Figure X in the online-only Data Supplement). SF also decreased TUNEL in neonatal rat cardiomyocytes with adenoviruses expressing green fluorescent protein (GFP) or SF and subjected these cells to oxidative stress by treatment with $10^5$ μmol/L H$_2$O$_2$ or by incubation in low O$_2$ as previously described. Oxidative stress is known to play a critical role in I/R-induced cardiomyocyte apoptosis, and we found that treatment of cultured cardiomyocytes with $10^5$ μmol/L H$_2$O$_2$ for 5 hours induced marked apoptosis in noninfected or GFP-infected cardiomyocytes (as assessed by TUNEL), whereas significantly fewer TUNEL-positive cells were found in SF-infected cultures (Figure 4A; Figure X in the online-only Data Supplement). SF also decreased TUNEL in neonatal rat cardiomyocytes subjected to hypoxia (1% O$_2$) for 2 hours followed by reoxygenation for 1 hour, which closely mimics the hypoxia observed following I/R in vivo (Figure 4B). As a secondary measure of apoptosis, we evaluated cleavage of caspase 3 in cell lysates by Western analysis. As shown in Figure X in the online-only Data Supplement, H$_2$O$_2$ treatment led to cleavage of caspase 3 in GFP-infected but not SF-infected neonatal rat cardiomyocytes. Taken together, these data indicate that FAK acts in a cell-autonomous fashion to protect myocytes from oxidative stress–induced apoptosis.

**SF Enhanced Nuclear Translocation and Transcriptional Activity of NF-κB in Cardiac Myocytes**

Because we recently showed that FAK was required for I/R-mediated activation of NF-κB, and some studies indicate that NF-κB protects cardiomyocytes from I/R-induced apoptosis, we interrogated the activity of this pathway in NTG and SF2 hearts. As shown in Figure 5, SF2 hearts subjected to 30 minutes of ischemia followed by 30 or 60 minutes of reperfusion exhibited increased levels of phosphorylated Ser32/36 IκBα relative to control hearts, with a concomitant decrease in IκBα levels, and enhanced phosphorylation of p65 (Ser536) that returned to baseline by 24 hours of reperfusion (Figure 5A and 5B; Figure XI in the online-only Data Supplement). As well, SF2 hearts exhibited enhanced p65 NF-κB nuclear localization, enhanced DNA binding activity at 60 minutes post-I/R and higher levels of the NF-κB-transcriptional targets X-linked inhibitor of apoptosis.
To confirm that regulation of NF-κB was attributable to a primary change in myocyte FAK activity, we next examined activation of this survival pathway in cultured cardiomyocytes. As expected, SF expressing cardiomyocytes that were plated on FN to induce FAK activity exhibited a decrease in IkBα and a concomitant increase in NF-κB activity (Figure XIII in the online-only Data Supplement) and expression of 2,3,5-triphenyltetrazolium chloride, bcl-2 and bcl-xl (Figure 6A) compared with noninfected or GFP-infected cells. To determine the functional importance of this survival pathway with respect to SF-dependent protection from oxidative stress, we next explored whether inhibition of NF-κB signaling would restore myocyte apoptosis in SF-expressing cardiomyocytes. As shown in Figure 6A, treatment with JSH-23, a cell-permeable inhibitor of NF-κB p65 nuclear translocation, suppressed the capacity of SF to induce 2,3,5-triphenyltetrazolium chloride, bcl-2 and bcl-xl expression. Moreover, although treatment of JSH-23 had little effect on H2O2- or hypoxia/reoxygenation-induced apoptosis in GFP-expressing cells, it completely abolished the protection conferred by SF overexpression (Figure 6B and 6C). Indeed, in the vehicle control group, the number of TUNEL-positive SF expressing cardiomyocytes was significantly lower than GFP-expressing cardiomyocytes exposed to oxidative stress, whereas the numbers of TUNEL-positive SF- and GFP-expressing cardiomyocytes were comparable in cultures pretreated with JSH-23. We next used small interfering RNAs targeted toward the p65 NF-κB transcript to confirm a role for this pathway in SF-mediated myocyte survival. As shown in Figure 6D to 6F, treatment of cultured cardiomyocytes with NF-κB small interfering RNAs led to an approximate 90% reduction of p65 levels as assessed by Western blotting and nearly completely reversed the beneficial effect of SF expression when cells were exposed to H2O2 or hypoxia/reoxygenation. In combination, these results indicate that induction of the NF-κB pathway is a major mechanism by which elevated FAK activity confers cardioprotection.

**Discussion**

Ischemic heart disease is a significant cause of morbidity and mortality worldwide, and thus an enormous interest exists for the development of cardioprotective gene therapies. One potentially promising avenue involves attempts to enhance the activity of intrinsic prosurvival signals that are hard-wired in postmitotic cardiomyocytes to impart relatively high levels of resistance to oxidative stress. Under physiological conditions, most signaling molecules exhibit transient waves of activation to achieve the appropriate biological response, and 1 of the current challenges of this therapeutic approach is how to achieve such spatial and temporal control of prosurvival signals, because prolonged activation can lead to maladaptive cardiac remodeling.25–27 We previously reported that FAK plays an important role in the induction of intrinsic survival pathways in cardiomyocytes following I/R and sought to explore whether targeted FAK activation might ameliorate ischemia-induced apoptosis. To this end, we used the knowledge that mutations in the activation loop of FAK enhanced catalytic activity but also required signal-dependent activation for maximal induction. We found that transgenic mice that expressed this superactivatable variant of FAK (SF) in the myocardium exhibited elevated FAK activity in unstressed hearts without consequence on cardiac growth or
function. Importantly, FAK activity was profoundly increased in SF expressing hearts following I/R, and these hearts displayed remarkable cardioprotection. Indeed, hearts with transiently elevated FAK activity exhibited a dramatic reduction in myocyte apoptosis and infarct size and were protected from myocardial infarction-induced cardiac decompensation. We reason that the attenuation of myocyte apoptosis and initial infarct size by FAK activation leads to a long-standing benefit in contractile performance.

It is important to note that our studies reflect a beneficial effect of FAK when activated solely in cardiomyocytes. Indeed, hearts with transiently elevated FAK activity exhibited a dramatic reduction in myocyte apoptosis and infarct size and were protected from myocardial infarction-induced cardiac decompensation. We reason that the attenuation of myocyte apoptosis and initial infarct size by FAK activation leads to a long-standing benefit in contractile performance.

Figure 5. Focal adhesion kinase (FAK) enhanced nuclear translocation and transcriptional activation of nuclear factor-κB (NF-κB) in cardiomyocytes. A to D, Nontransgenic (NTG) littermate control mice and SuperFAK 2 (SF2) mice were subjected to 30 minutes of ischemia (I30) followed by 30 or 60 minutes of reperfusion (R30 and R60, respectively). The border zone of the infarcted myocardium was collected for subcellular fractionation or immunohistochemistry. A, Representative immunoblots of whole cell lysates 30 and 60 minutes post–ischemia/reperfusion (I/R), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. B, Quantification of protein expression in whole cell lysate at 60 minutes post-I/R showed a significant increase of phospho-NF-κB p65(Ser536) (p-p65(S536)) and phospho-IκBα(S32/36) (pI-κBα(S32/36)) and a decrease of total IκBα in the SF2 group compared with control hearts. NTG, n=3; SF2, n=4. *P<0.05 vs NTG. C, Representative immunoblots of nuclear and cytosolic fractions 30 and 60 minutes post-I/R. Histone H3 served as a nuclear loading control and GAPDH as a cytosolic loading control. See Figure XII in the online-only Data Supplement for quantification. D, Confocal images of NTG and SF2 hearts 60 minutes post-I/R immunolabeled for NF-κB p65 (green), cardiac troponin T (cTnT; red), and 4’,6-diamidino-2-phenylindole (DAPI) (blue). SF2 hearts exhibited increased NF-κB p65 signal in the nuclei of cardiac myocytes (arrows). Scale bar=20 μm. E, Protein levels of 2,3,5-triphenyltetrazolium chloride, bcl-2, and bcl-xl were significantly higher in the SF2 heart than in controls at 24 hours post-I/R. See Figure XII in the online-only Data Supplement for Western images. NTG, n=3; SF2, n=4. *P<0.05 vs NTG, **P<0.01 vs NTG. Data are mean±SEM.
posed to shear stress. FAK has been shown to induce NF-κB activation by different mechanisms in cytokine versus shear stress-induced signaling. These authors found that following mechanical signals, FAK acts in a direct fashion to promote NF-κB phosphorylation and activation. Although we did find elevated activity of the IκB kinase and decreased levels of IκB in the SF2 hearts, our data do not rule out the possibility that I/R-dependent activation of FAK does not play a role in cardioprotection.

Cardiomyocytes are known to be particularly sensitive to the levels of NF-κB-dependent survival genes. Indeed studies in cultured myocytes indicated that NF-κB suppressed mitochondrial defects and apoptosis through transcriptional upregulation of survival genes (including bcl-2, bcl-xl, and X-linked inhibitor of apoptosis protein) or by silencing proapoptotic genes, such as bim. However, the function of NF-κB in the intact heart is controversial, as it has been reported to induce both prosurvival and proapoptotic signals. More recent studies indicate that timing of activation may be causal for this disparity. Our studies are consistent with a FAK-dependent cardioprotective effect of transient NF-κB activation.
activation, as we found elevated NF-κB activity in the SF2 hearts 30 to 60 minutes following I/R and a return to baseline by the 24-hour time point. To determine which cell type was responsible for elevated NF-κB activity in SF2 hearts, immunohistochemistry was performed with an NF-κB p65-specific antibody. Our results showed that enhanced nuclear translocation of NF-κB p65 was present in cardiac myocytes of SF2 hearts compared with NTG hearts (Figure 5D), although we cannot exclude the possibility that NF-κB is activated in additional resident heart cells, such as the endothelium, in which I/R has been reported to lead to activation of NF-κB.49 Moreover, although we did not observe enhanced activation of other major survival signals, such as ERK1/2 or Akt, in the SF2 hearts, it is formally possible that other FAK-dependent survival pathways contribute to the cardioprotective effects of FAK. Nonetheless, our finding that blockade of NF-κB-dependent signals reverses the protection induced by SF expression in cardiomyocytes exposed to oxidative stress indicates that intrinsic NF-κB activation does play a causative role in FAK-mediated myocyte survival.

We and others have previously identified a significant role for FAK in mediating the robust growth responses necessary for both midgestational cardiac development and the promotion of pressure-overload induced myocyte hypertrophy21,33,50 but not for anabolic cardiac growth. Subsequent mechanistic studies indicated that basal FAK activity in embryonic hearts was necessary to repress p38 kinase activity and to promote cardiomyocyte cell cycle progression through G1/M.51 However, in terminally differentiated cardiomyocytes, we found that FAK activity was necessary for ERK-dependent promotion of pathological hypertrophic growth.32 Although postnatal SF2 hearts exhibited elevated levels of FAK activity, no change in cardiac ERK activity was observed, consistent with previous findings that SF did not enhance adhesion- or serum-stimulated ERK activation in cultured cells.28 Concomitantly, adult SF2 hearts exhibited no signs of pathological hypertrophic remodeling. Indeed, SF2 and littermate control NTG mice had comparable heart weight/body weight ratios, cardiomyocyte cross-sectional area, atrial natriuretic factor expression, and cardiac function. These results confirm and extend previous studies indicating that overexpression of wild-type FAK in neonatal rat cardiomyocytes did not increase stretch-induced atrial natriuretic factor expression.51 Taken together, these data indicate that FAK is necessary but not sufficient to promote ERK activation and pathological hypertrophic growth.

In sum, there are 2 major findings from the studies presented herein: (1) they support the feasibility of exploiting mutations in the activation domain of kinases to augment the successes of gene therapy approaches, and (2) they indicate that methodologies to enhance FAK activity (including administration of upstream agonists,52,53 or possibly via ischemic pre- and postconditioning54,55 or heat shock56) may represent a novel therapeutic strategy for ischemic heart diseases. Because several recently developed chemotherapeutic agents that target receptor tyrosine kinases upstream of FAK, including sunitinib and imatinib, induce myocyte apoptosis and cardiomyopathy,57,58 it will be of future importance to evaluate whether enhancing FAK activation might also be an effective strategy to preserve myocardial function in this setting.

Acknowledgments
The authors thank C. Robert Bagnall, Jr., and Steven J. Ray (Microscopy Services Laboratory, University of North Carolina, Chapel Hill); Jackie Kylander and Tayler Kopple (Mouse Cardiovascular Models Core Laboratory, University of North Carolina, Chapel Hill); Morgan V. Cameron; and Devin Bailey for excellent technical assistance, as well as Dr Thomas J. O’Neill, Zhigang Zhou, and Kaitlin Lenhart for helpful discussions and critical comments throughout the duration of these studies.

Sources of Funding
This work was supported by grants from the National Heart, Lung, and Blood Institute, National Institutes of Health (HL-081844 and HL-071054 to J.M.T.) and the American Heart Association (AHA.0355776U to J.M.T.). Dr Cheng was supported by an American Heart Association Postdoctoral Fellowship (#11POST7600008).

Disclosures
None.

References


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Zhaokang Cheng, Laura A. DiMichele, Zeenat S. Hakim, Mauricio Rojas, Christopher P. Mack and Joan M. Taylor

Arterioscler Thromb Vasc Biol. 2012;32:924-933; originally published online March 1, 2012; doi: 10.1161/ATVBAHA.112.245134
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/4/924

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Supplemental Information includes an expanded Methods section and additional data including: thirteen figures with legends, and reference citations.
Detailed Methods

Generation of SuperFAK transgenic mice

Cardiac-specific transgenic mice with SuperFAK overexpression was achieved with a 3.4 kb piece of the cardiac βMHC promoter that was modified to prevent down-regulation at birth\(^1\). All animals were housed in a University Animal Care Facility accredited by the American Association for Accreditation of Laboratory Animal Care.

Myocardial ischemia/reperfusion

Ischemia/reperfusion injury was induced in male SF2 or littermate control mice (Age 8-12 weeks) as previously described\(^2\). In brief, the left anterior descending (LAD) coronary artery was ligated with a 6–0 suture for 30 minutes followed by reperfusion for indicated times. Sham operations were performed by passing a suture around the LAD without ligation.

Evans blue/TTC staining

The infarct region and area at risk (AAR) were determined by Evans blue and triphenyltretrazolium chloride (TTC) staining as previously described\(^2\). Briefly, the LAD was re-occluded at 24 h or 72h after reperfusion and 1ml of 1% Evans Blue (in PBS) was injected via the right carotid artery. The hearts were excised, sliced transversely into 1 mm thick slices, and incubated with 1% TTC (in PBS) at 37°C for 30 min. Evans Blue unstained area was defined as AAR and TTC unstained area was considered as the infarct region. Relative infarct size was calculated by dividing the infarct region by the AAR.

Echocardiography

Heart function was measured by transthoracic echocardiography using the Visualsonic Ultrasound System (Vevo 660) equipped with a 30 MHz variable frequency pediatric probe as described previously\(^3\).
Standard long axis and short axis M-mode views were recorded. Echocardiographic measurements were obtained from gray scale M-mode images and averaged from three consecutive contractions using Visual Sonics software.

**Neonatal rat cardiomyocyte cell culture and treatment**

Primary neonatal rat cardiomyocytes (NRCM) were isolated by using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, NJ) according to the vendor’s instructions. In brief, P0 hearts were minced to 1 mm³ pieces and digested with 50 μg/ml trypsin at 4°C overnight followed by 100 U/ml collagenase at 37°C for 45 min. Isolated cardiomyocytes were plated on 10 μg/ml fibronectin-coated chamber slides with 100 μM BrdU to prevent fibroblast contamination. Cells were infected with adenoviruses overexpressing GFP or SuperFAK at an MOI of 50 in serum-free M199 media for 48 hours prior to exposure to hydrogen peroxide (H₂O₂, 10 μM, Fisher BioReagents) for 5 hours.

In vitro hypoxia/re-oxygenation was performed as described earlier². In brief, cells were cultured in glucose- and serum-free media. Hypoxia was achieved by using a hypoxia chamber maintained at 37 °C, 1% O₂, 5% CO₂, and 95% N₂ for 2 hours. For re-oxygenation, the media was changed to maintenance medium under a water-saturated atmosphere of 5% CO₂ and 95% air for 1 h.

To block NF-κB activation, cells were pre-treated for 2 hrs with the NF-κB inhibitor 4-Methyl-N¹-(3-phenylpropyl)benzene-1,2-diamine (JSH-23, 25 μM, Calbiochem, 481408), a cell-permeable diamino compound that selectively blocks nuclear translocation and transcriptional activity of NF-κB p65.

**Small interfering RNA (siRNA) transfection**

NRCM were transfected with siRNA (25 nM) by using HiPerfect transfection reagent (Qiagen) as previously described⁴. Briefly, siRNA and HiPerfect were diluted in 100 μl serum-free M199 medium. After incubation for 5-10 minutes, transfection complexes were added to the cells for indicated times. The rat
NF-κB p65 siRNA was a pool of 3 target-specific 19-25 nt siRNAs obtained from Santa Cruz Biotechnology (sc-61876); The control siRNA was obtained from Invitrogen.

Subcellular fractionation

Subcellular fractionation was performed as previously described\(^4\). Briefly, heart tissues were homogenized in isolation buffer (70mM sucrose, 190mM D-Mannitol, 20mM Hepes, 0.2mM EDTA) by using a Teflon-glass dounce homogenizer. Nuclear fractions were separated by centrifugation at 600g for 10min followed by discontinuous sucrose density centrifugation. Cytosolic fractions were separated by centrifugation at 20,000g for 60min. Samples were resuspended in RIPA buffer and 20μg protein was used for Western blotting. Purity of preparations was confirmed by immunoblotting for the presence or absence of cytoplasmic GAPDH or nuclear histone H3.

NF-κB DNA binding assay

NF-κB DNA-binding activity was measured in nuclear extracts using the TransAM\textsuperscript{TM} NF-κB p65 Transcription Factor Assay kit (Active Motif, 40096). In brief, nuclear extracts (10μg/well) were added to Elisa plates pre-immobilized with an oligonucleotide containing the NF-κB consensus site (5’-GGGACTTTCC-3’). Following a 1 hour incubation at room temperature, wells were washed and sequentially probed with an anti-NF-κB p65 antibody and an HRP-conjugated secondary IgG. NF-κB binding activity was quantified by spectrophotometry (450 nm absorbance). Nuclear extracts from Jurkat cells stimulated with TPA and calcium ionophore served as a positive control (data not shown).

Western blotting

Western blotting was performed as described previously\(^2\). In brief, cell and tissue lysates were subjected to SDS-PAGE and separated proteins were transferred onto wither nitrocellulose or polyvinylidene
fluoride (PVDF) membranes, blocked with 5% skim milk for 1 h at room temperature, and incubated with specific primary antibodies including: rabbit anti-pFAK (Y397) (44624G, Invitrogen, 1:1000), mouse anti-FAK (clone 4.47, Millipore, 1:1000), rabbit anti-pNF-κB p65 (S536) (#3033, Cell signaling, 1:500), rabbit anti-NF-κB p65 (#3987, Cell signaling, 1:500), mouse anti-pIκBα (S32/36) (#9246, Cell signaling, 1:500), IκBα (#4814, Cell signaling, 1:1000), rabbit anti-Bcl-xl (#2764, Cell signaling, 1:1000), rabbit anti-pAkt(S473) (#9271, Cell signaling, 1:1000), rabbit anti-Akt (#9272, Cell signaling, 1:1000), rabbit anti-pERK1/2(Thr202/Tyr204) (#9101, Cell signaling, 1:1000), rabbit anti-ERK1/2 (#4695, Cell signaling, 1:1000), rabbit anti-Histone H3 (ab1791, Abcam, 1:1000) and rabbit anti-GAPDH (sc-25778, Santa Cruz Biotechnology, 1:1000). Blots were next incubated with either horseradish peroxidase (HRP, Sigma) or alkaline phosphatase (AP, Jackson ImmunoResearch, West Grove, PA)-conjugated secondary antibodies. Blots were visualized after incubation with SuperSignal West Pico Chemiluminescent Substrate or Lumi-Phos WB Chemiluminescent Substrate (Thermo Scientific).

**Immunohistochemistry and confocal microscopy**

Paraffin-embedded heart sections were deparaffinized, rehydrated and subjected to antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) or 0.1% trypsin solution. Slides were blocked with 3%H2O2 in PBS for 20min, and 10% horse serum for 1 hour. Tissue sections were then incubated with rabbit anti-pFAK (Y397) (44624G, Invitrogen, 1:50), rabbit anti-NF-κB p65 (#3987, Cell signaling, 1:50), rat anti-mouse CD31 (550274, BD, 1:50), mouse anti-cardiac Troponin T (MS-295-P, Thermo scientific, 1:100) at 4°C overnight. Sections were stained with Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen 1:100), HRP-conjugated donkey anti-rabbit IgG (Sigma, 1:200) or donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1.5h followed by development using the Tyramide Signal Amplification (TSA) system (Perkin Elmer). Nuclei were labeled with DAPI (1:200). For active caspase 3
staining, sections were incubated with rabbit anti-cleaved caspase 3 (#9664, Cell signaling, 1:50) followed by VectaStain ABC Kit (PK-4001, Vector labs). Signals were visualized by development using ImmPACT DAB Peroxidase Substrate (SK-4105, Vector labs).

Neonatal rat cardiomyocytes (NRCM) were permeabilized with 0.1% Triton-X100 for 5min, blocked with 10% horse serum for 1h, and incubated with mouse anti-cardiac Troponin T (MS-295-P, Thermo scientific, 1:100) at 4°C overnight. The next day, slides were incubated with Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen, 1:100) for 1.5 h at room temperature. Nuclei were stained with DAPI (Invitrogen, 1:200). Confocal images were acquired by using a Zeiss LSM 710 confocal laser-scanning microscope (Zeiss, Germany).

**Tissue hypoxia assessment**

Tissue hypoxia was assessed by using the Hypoxyprobe™-1 Kit (Hypoxyprobe, Inc. Burlington, MA) according to the manufacturer’s instructions. Briefly, mice were injected i.p. with 60 mg/kg hypoxyprobe-1 60 min prior to sacrifice. Hearts were fixed in 4% PFA, embedded in paraffin, sectioned at 8-μm thickness, and stained with the mouse anti-Hypoxyprobe-1 monoclonal antibody (1:50), which binds to protein adducts of hypoxyprobe-1 in hypoxic cells. Sections were then incubated with HRP-conjugated swine anti-mouse secondary antibody (Sigma, 1:200) and visualized by using the Tyramide Signal Amplification (TSA) system (Perkin Elmer).

**TUNEL staining**

TUNEL staining was performed with the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science) as per the manufacturer’s instructions. Briefly, heart sections were deparaffinized, rehydrated and antigen-retrieved in 10 mmol/L citrate buffer (pH 6.0). NRCM were permeabilized with fresh permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS). Slides were then incubated
with TUNEL reagent for 1 hour at 37°C and examined by using a Zeiss LSM 710 confocal microscope (Zeiss, Germany).
Supplemental Figure I. Mutations within the activation loop of FAK lead to enhanced allosteric FAK activity. (A) Schematic diagram of FAK and the K578E, K581E FAK variant (SuperFAK). (B) Non-transfected (NT), wild-type FAK (wtFAK) or SuperFAK (SF) transfected COS7 cells were cultured at low density in serum-containing media. Cell lysates were blotted for indicated antibodies and FAK activity was determined by phosphorylation of the autophosphorylation site Y397.
Supplemental Figure II. Mice with cardiac-restricted SuperFAK expression exhibited normal cardiac performance and morphology. (A) Cross sections of 8-12-week-old non-transgenic (NTG) and SF2 hearts stained with Masson’s Trichrome. (B) Heart weight to body weight ratio over time revealed no significant difference between NTG and SF2 mice. n=3-5 for each group per time point. (C, D) Cardiomyocyte cross-sectional area measured from laminin stained sections was comparable between 8-12-week-old NTG and SF2 mice. NTG, n=3; SF2, n=4 (200-300 cells/heart) (E,F) Conscious echocardiography revealed similar ejection fraction (EF) and fractional shortening (FS) between 8-12-week-old NTG and SF2 mice. NTG, n=4; SF2, n=6. (G) RNA was isolated from ventricular tissues from NTG and SF2 mice and qRT-PCR was performed for ANF, the canonical pathological hypertrophy marker. Data represent mean ± SEM of values obtained from 3-5 mice/genotype. (H) Western blot for pERK and pAkt levels in NTG and SF2 hearts during development (note these are the identical lysates used for Figure 1D).
Supplemental Figure III. SF1 mice did not exhibit signs of cardiac hypertrophy. (A) Representative images of non-transgenic (NTG) littermate control and SF1 heart sections stained with wheat germ agglutinin (WGA, red). Scale bar = 20μm. (B) Cardiomyocyte cross-sectional area measured from WGA stained sections was comparable between NTG and SF1 mice. Data represent 200-300 cells/heart from NTG, n=5 and SF2, n=8 and are expressed as mean ± SEM.
Supplemental Figure IV. Serial echocardiographic measurements of NTG and SF2 hearts revealed no significant difference in cardiac growth or function. Non-transgenic (NTG) littermate control mice and SF2 mice were anesthetized with inhaled isoflurane, and echocardiographic parameters were assessed at 1, 2, 5 and 12 months of age. (A) LVPWT, left ventricular posterior wall thickness; s, end systole; d, end diastole; (B) IVST, interventricular septal thickness; s, end systole; d, end diastole; (C) LVD, left ventricular diameter; s, end systole; d, end diastole; (D) LVM, left ventricular mass; (E) EF, ejection fraction; (F) FS, fractional shortening. Data represent mean ± SEM of 4-6 mice at each time point. All parameters are comparable between NTG mice and SF2 mice.
Supplemental Figure V. Hemodynamic assessment of 12-month-old NTG and SF2 hearts revealed no significant difference in myocardial contractility or function. (A) Representative pressure-volume loops of non-transgenic (NTG) mice and SF2 mice at 12 months of age. (B) LVESP, left ventricular end-systolic pressure; (C) LVEDP, left ventricular end-diastolic pressure; (D) $+\!dP/dt_{max}$, maximal rate of pressure increase; (E) $-\!dP/dt_{max}$, maximal rate of pressure decrease; (F) LVESV, left ventricular end-systolic volume; (G) LVEDV, left ventricular end-diastolic volume; (H) Stroke volume; (I) EF, ejection fraction; (J) Cardiac output. Data represent mean ± SEM of 5 mice in each group. All parameters are comparable between NTG mice and SF2 mice.
Supplemental Figure VI. Cardiac-specific FAK activation does not induce fibrosis. (A) Representative Masson’s trichrome staining of 8-12-week-old NTG and SF2 mice hearts at 20X magnification. Muscles appear red and collagen fibers appear blue. (B) Quantification of cardiac fibrosis was performed as previously described with modifications. Fibrosis score: 0, none; 1, little; 2, mild; 3, moderate; 4, substantial; 5, severe. NTG, n=7; SF2, n=6.
Supplemental Figure VII. Echocardiographic analysis of NTG and SF2 hearts following I/R. Non-transgenic (NTG) littermate control mice and SF2 mice were monitored before, 2, 4, and 8 weeks after I/R. (A) Representative M-mode tracings of NTG (left) and SF2 (right) hearts before (top) and 4 weeks post I/R (bottom); (B) IVSTd, interventricular septal thickness at end diastole; (C) IVSTs, interventricular septal thickness at end systole (IVSTs); (D) PWTd, left ventricular posterior wall thickness at end diastole; (E) PWTs, left ventricular posterior wall thickness at end systole; (F) LVDd, left ventricular diameter at end diastole; (G) LVDs, left ventricular diameter at end systole. NTG, n=6; SF2, n=5. * $p < 0.05$ vs. NTG; ** $p < 0.01$ vs. NTG; *** $p < 0.001$ vs. NTG. Data are mean ± SEM.
Supplemental Figure VIII. SF2 hearts exhibited decreased active caspase 3 compared with NTG hearts. Immunostaining of cleaved caspase-3 in the ischemic area of heart sections in NTG and SF2 mice subjected to I/R for 24h (data are representative of n=3 hearts/condition).
Supplemental Figure IX.  Cardiac-specific FAK activation does not affect basal coronary or capillary density.  (A) Representative confocal images of 8-12-week-old non-transgenic (NTG) and SF2 hearts immunolabeled for α-smooth muscle actin (α-SMA, green), cardiac troponin T (cTnT, red) and DAPI (blue). Scale bar = 40μm. (B) Number of α-SMA-positive blood vessels was similar between NTG and SF2 hearts. Data represent mean ± SEM of 4 mice in each group. (C) Representative confocal images of 8-12-week-old NTG and SF2 hearts immunolabeled for the endothelial marker PECAM (green). Scale bar = 20μm. (D) Number of capillaries was similar between NTG and SF2 hearts. Data represent mean ± SEM of 4 mice in each group.
Supplemental Figure X. Overexpression of SuperFAK protected cultured cardiomyocytes from H$_2$O$_2$-induced apoptosis. (A) Non-infected, GFP- or SuperFAK-infected neonatal rat cardiomyocytes (NRCM) were treated with 10μM H$_2$O$_2$ for 5h and then stained with TUNEL (red), DAPI (blue) and cardiac troponin T (cTnT, white). GFP fluorescence was shown as green. Scale bar = 20μm. (B) Expression of SuperFAK in NRCM prevented caspase-3 cleavage in response to H$_2$O$_2$ treatment. Images are representative of 4 independent experiments.
Supplemental Figure XI.  Assessment of pro-survival pathways in SF2 mice at 60min and 24h after ischemia/reperfusion. (A) NF-κB activity was similar between SF2 and non-transgenic (NTG) mice at 24h post-I/R; (B) Akt and ERK1/2 were equally activated in NTG and SF2 hearts at 60min post-I/R; (C) Akt and ERK1/2 activity were not significantly increased in SF2 heart when compared with NTG heart at 24h post-I/R.
Supplemental Figure XII. SF2 hearts exhibited enhanced NF-κB activation following ischemia/reperfusion. (A) Quantification of protein expression in nuclear fractions at 60min post I/R showed a significant increase of phospho-NF-κB p65 (S536) and total NF-κB p65 in the SF2 hearts. NTG, n=3; SF2, n=4. * p < 0.05 vs. NTG. (B) Quantification of protein expression in cytosolic fractions at 60min post I/R showed a significant decrease of total NF-κB p65 in the SF2 hearts. NTG, n=3; SF2, n=4. * p < 0.05 vs. NTG. (C) Nuclear NF-κB p65 DNA binding activity 60min post I/R was measured by using the TransAM™ NF-κB p65 Transcription Factor Assay kit (Active Motif). NTG, n=3; SF2, n=4. * p < 0.05 vs. NTG. (D) Immunoblot of NTG and SF2 heart lysates probed for flag tag and the pro-survival NF-κB targets XIAP, Bcl-2 and Bcl-xl at 24h post I/R, with GAPDH as a loading control.
Supplemental Figure XIII. SuperFAK promotes NF-κB activity in cultured cardiomyocytes.

Non-infected (N), GFP or SuperFAK (SF) infected neonatal rat cardiomyocytes (NRCM) were plated on fibronectin-coated dishes for 30 or 60min. Western blotting was performed with indicated antibodies. Elevated FAK activity induced IκBα degradation and NF-κB activation. GAPDH served as loading control.
Supplemental References


