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J Mol Cell Cardiol. Author manuscript; available in PMC 2015 August 13.

Published in final edited form as:

J Mol Cell Cardiol. 2009 February ; 46(2): 241–248. doi:10.1016/j.yjmcc.2008.10.017.

FAK regulates cardiomyocyte survival following ischemia/reperfusion

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Abstract

Myocyte apoptosis is central to myocardial dysfunction following ischemia/reperfusion (I/R) and during the transition from hypertrophy to heart failure. Focal adhesion kinase (FAK), a non-receptor tyrosine kinase regulates adhesion-dependent survival signals and unopposed FAK activation has been linked to tumor development. We previously showed that conditional myocyte-specific deletion of FAK (MFKO) in the adult heart did not affect basal cardiomyocyte survival or cardiac function but led to dilated cardiomyopathy and heart failure following pressure overload. In the present study, we sought to determine if FAK functions to limit stress-induced cardiomyocyte apoptosis. We reasoned that (I/R), which stimulates robust apoptotic cell death, might uncover an important cardioprotective function for FAK. We found that depletion of FAK markedly exacerbates hypoxia/re-oxygenation-induced cardiomyocyte cell death *in vitro*. Moreover, deletion of FAK in the adult myocardium resulted in significant increases in I/R-induced infarct size and cardiomyocyte apoptosis with a concomitant reduction in left ventricular function. Finally, our results suggest that NF-κB signaling may play a key role in modulating FAK-dependent cardioprotection, since FAK inactivation blunted activation of the NF-κB survival signaling pathway and reduced levels of the NF-κB target genes, Bcl2 and Bcl-xl. Since the toggling between pro-survival and pro-apoptotic signals remains central to preventing irreversible damage to the heart, we conclude that targeted FAK activation may be beneficial for protecting stress-dependent cardiac remodeling.

Keywords

FAK; NF-κB; Ischemia/reperfusion; Cardiomyocytes; Apoptosis

1. Introduction

Obstruction of blood flow to the heart limits myocyte oxygen and nutrient supply and induces programmed (apoptotic) and un-programmed (necrotic) cell death, both important causal components of myocardial infarction and heart failure [1–6]. Apoptosis can be

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initiated by extrinsic (receptor-mediated) and intrinsic pathways (due to mitochondrial dysfunction), both of which ultimately lead to activation of the caspase family of proteases, which cleave DNA and multiple contractile and cytoskeletal proteins. Although the initiation of apoptosis was originally thought to be irreversible, studies now indicate that apoptotic cells (unlike necrotic cells) can actually be salvaged by activation of pro-survival signaling cascades [2,6–9]. This is likely because of the fact that apoptosis is characterized by maintenance of plasma membrane integrity until late in the process unlike necrosis which leads to early irreversible disintegration of the plasma membrane. Therefore, understanding the precise signaling pathways involved in the regulation of cardiomyocyte survival will be important for future development of agents that could be used to treat ischemia-induced myocardial dysfunction.

Integrins are transmembrane receptors that mediate cell attachment to extracellular matrix (ECM), a critical event for numerous cell responses including cell migration, differentiation, proliferation, and protection from apoptosis [10]. In addition to acting as contact points for cell attachment, integrin engagement leads to induction of intracellular signals that serve to integrate diverse extrinsic cues from growth factor and cytokines receptors. The finding that deletion of β_1 integrin in adult hearts leads to dilated cardiomyopathy and concomitant heart failure [11,12] underscores the possibility that defects in integrin signaling may play a direct role in regulating cardiomyocyte survival.

FAK is a non-receptor protein tyrosine kinase, which is strongly and rapidly activated by all β_1, β_3 or β_5 containing integrins, and its activation is considered central to integrin-dependent signal transduction [13]. In some, but not all cell types, FAK stimulates activation of the pro-survival kinases, ERK and AKT, and in these cells, FAK inactivation leads to apoptosis [14–17]. We recently reported that ventricular myocyte-specific deletion of FAK in the adult heart did not affect basal cardiomyocyte survival or cardiac function [18]. However, prolonged transverse aortic constriction (TAC) in mice with cardiac-restricted FAK deletion led to dilated cardiomyopathy and heart failure. Several studies indicate that the switch from hypertrophy to cardiac failure may involve activation of apoptotic pathways. For instance, aortic banding of mice with heart-restricted overexpression of $G\alpha_q$, result in dilated cardiomyopathy accompanied by cellular apoptosis, and either overexpression of the anti-apoptotic protein, Nix or administration of caspase inhibitors significantly reversed myocardial dysfunction in these mice [19–21]. Although we did not detect significant cardiomyocyte apoptosis in the banded FAK null hearts [18], we could not rule out a cardioprotective effect of FAK in this setting since apoptosis induced by chronic TAC is spatially diffuse and occurs over a prolonged time scale.

In the present study, we explored the possibility that the stress induced by I/R, which results in robust localized apoptotic and necrotic cell death [1,22] might uncover an important function for FAK as a cardioprotective signaling molecule. Herein, we found that oxidative stress induced more pronounced apoptotic cell death in FAK null cardiomyocytes than in the FAK-containing cardiomyocytes using both *in vitro* and *in vivo* model systems. Subsequent mechanistic studies revealed that FAK was necessary for NF-κB-induced survival signaling following I/R injury. We conclude that targeted FAK activation may be beneficial for protecting I/R-induced myocardial dysfunction.

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2. Materials and methods

2.1. Antibodies and chemicals

The FAK antibody was purchased from Upstate Biotechnology, Inc, the antibodies directed towards phosphorylated ^{ser536} NF-κB (p65), phosphorylated $^{ser32/36}$ IκBα IκBα and Bcl-xl were purchased from Cell Signaling Technology, the phosphorylated $^{Tyr\ 397}$ FAK antibody was purchased from BioSource, the α-tubulin antibody was purchased from Sigma, the Bcl2 antibody was purchased from Upstate and the NF-κB and troponin T antibodies were a generous gift from Dr. Craig Selzman (CCBC, UNC-CH) and Nadia Malouf (Department of Pathology and Laboratory Medicine, UNC-CH) respectively.

2.2. In vitro hypoxia/re-oxygenation (Hypox/Reox)

FAK null and FAK containing ventricular cardiomyocytes expressing GFP under the control of the β-MHC promoter were isolated from *fak*$^{flox/flox}$*nkx*2-5$^{Cre/wt}$β*MHC*^{gfp} (FAK$^{-/-}$/βMHC^{gfp}) and littermate control *fak*$^{flox/flox}$*nkx*2-5$^{wt/wt}$β*MHC*^{gfp} (FAK$^{+/+}$/βMHC^{gfp}) hearts respectively, and cultured as described previously [23]. Cardiac cells were plated at cell density of 10,000 cells on fibronectin (FN)-coated 96-well plates and cultured for 12 h in serum medium. Viable attached cardiomyocytes (identified by nuclear GFP labeling and verified by Trypan Blue dye exclusion) were counted in 4 fields at 10× magnification. Prior to experimentation, serum-containing media was replaced with glucose- and serum-free medium in the hypoxic group, which were then placed in a hypoxia chamber set to confer an atmosphere of 1% O_2, 5% CO_2, and 95% N_2. Cells were incubated for 2 h prior to re-oxygenation for an hour. Cells were rinsed briefly with PBS, fixed with 4% PFA for 20 min at 25 °C and counted as described above. Cell death was expressed as a ratio of the number of GFP positive cells counted after Hypox/Reox to that before Hypox/Reox. Finally, cardiomyocyte apoptosis in response to Hypox/Reox was determined by calculating the percentage of viable cardiomyocytes (spread GFP positive cells that excluded Trypan Blue) in Hypox/Reox compared to the viable cardiomyocytes in the normoxia group.

For biochemical analysis of FAK phosphorylation following Hypox/Reox, neonatal (P0) rat cardiomyocytes were isolated using a commercially available kit from Worthington according to the vendor's instructions. Isolated cardiomyocytes were plated on 0.1% gelatin-coated 60 mm dishes with 100 μM BrDu to prevent fibroblast contamination. The cells were cultured in heart medium and then exposed to normoxia (2.5 h) or Hypox/Reox (2 h and 15 min or 2 h and 30 min). Cell lysates were prepared and subjected to western analysis as described below.

2.3. Mice

fak$^{flox/flox}$ or *fak*$^{flox/wt}$ (genetic control) mice with FAK-containing cardiomyocytes, *fak*$^{flox/flox}$/*mlc2v*$^{Cre/wt}$ (MFKO) mice with FAK-deficient cardiomyocytes, FAK$^{+/+}$/βMHC^{gfp} mice with GFP-positive, FAK-containing cardiomyocytes and FAK$^{-/-}$/βMHC^{gfp} mice with GFP-positive, FAK-deficient cardiomyocytes were generated and genotyped as described previously [16,18,23]. All mice used for the I/R experiments were backcrossed to the C57black6 background at least 8 generations prior to subsequent breeding.

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All animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AALACI) according to the Institutional Animal Care and Use Committee (IACUC)-approved guidelines. All surgical procedures were approved by the AALACI-accredited IACUC at the University of North Carolina at Chapel Hill, Chapel Hill, NC.

2.4. Coronary artery ligation

Briefly, aged matched genetic control and MFKO male and female mice were anesthetized to perform a left thoracotomy under mechanical ventilation. Mice were anesthetized using isoflurane from an automated and calibrated device. The body temperatures of the mice were maintained by a heated surgical platform and body temperature was monitored using a rectal sensor during the surgical procedure. ECG electrodes were attached to the mouse (in the supine position) and a frontal plane 3-lead ECG was also recorded during the surgical procedure. The left anterior descending (LAD) coronary artery was occluded near its origin with a 6–0 suture for 30 min to produce transient ischemia. Consistent elevation of the ST segment was observed in lead II tracings following occlusion of the LAD coronary vessel and in each case removal of the suture was followed by a concomitant depression of this segment. After 24 h of reperfusion, the LAD was re-occluded and the right carotid artery was cannulated to allow injection of 1 ml of KCL (40 mEq/L) followed by 1 ml of 1% Evan's Blue dye (in PBS) for identification of the area at risk (AR). The hearts were excised and rinsed briefly in PBS. The atria were removed and ventricles were sliced transversely into 6×1 mm thick slices using an acrylic heart matrix. Starting from the base of the heart, slice 1 was used to obtain DNA for FAK recombination PCR, slice 2 was used for RNA analysis, slice 3 was used for protein analysis, and slices 4 and 5 were utilized for infarct size measurements. Briefly, slices 4 and 5 were incubated with 1% triphenyltretrazolium chloride (TTC, pH 7.4 in phosphate buffer) at 37°C for 30 min. After immersion in TTC, the slices were fixed in 4% PFA and infarct size was quantified as described below.

2.5. Quantification myocardial infarction

Slices 4 and 5 stained with Evan's Blue dye and TTC were photographed and the images were used to quantify I/R-induced myocardial infarction using the software, NIH Image J. The Evan's Blue stained area defined the perfused area whereas; the Evan's Blue unstained area defined the area at risk (AR). The area lacking the red TTC staining within the AR was considered as the infarct area (I). Relative AR (RAR) was considered as the AR expressed as percentage of the total slice area (T), and the relative infarct area (RI) was calculated by dividing I by the RAR. Both the surgeon and the two independent evaluators of infarct size were blinded to mouse genotypes.

2.6. Heart function

Echocardiography was performed prior to I/R injury as described previously [18] to determine the baseline heart function and ventricular dimensions in the experimental groups. We performed hemodynamic measurements after 24 h of reperfusion using a 1.0 F catheter tip micromanometer (Millar PVR-1035). Following calibration, the catheter (connected to a Powerlab 40/3 data acquisition box; ADInstruments) was inserted through the right carotid artery and into the left ventricular cavity of anesthetized mice. We analyzed the first

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derivative of left ventricular pressure (dP/dT) using PVAN 3.6 software (Millar) following administration of a saline bolus of 2 μl of 15% NaCl via the left subclavian vein.

2.7. Apoptosis

In some mice, slices 4 and 5 (procured as described above) were fixed in 4% PFA, embedded in paraffin, sectioned, and processed to detect DNA strand breaks by the TUNEL-reaction using a commercially available kit (Roche, 12156792910) according to the manufacturer's instructions. IHC was performed on the same slides for troponin T to label cardiomyocytes as described previously [18]. TUNEL positive cardiomyocyte nuclei were counted within the left ventricle juxtaposed to the necrotic area (delineated by regions devoid of troponin T immunoreactivity).

2.8. Western blot analysis

Cell and tissue lysates (procured as described above) were prepared as previously described [23]. Antibodies for phosphorylated $^{Tyr\ 397}$FAK, FAK, phosphorylated $^{Ser\ 536}$ NF-κB (p65), NF-κB (p65), phosphorylated $^{Ser32/36}$ IκBα, IκBα Bcl2, Bcl-xl and α-tubulin were used at a 1:1000 dilution for immunoblot analysis as previously described [23].

2.9. Semi-quantitative reverse transcription-PCR (RT-PCR)

Heart slice 2 (procured as described above) from the experimental mice were snap-frozen in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was obtained by reverse transcription using a commercially available kit (iScript; Bio-Rad). Semi-quantitative RT-PCR analysis of the cDNA was carried out using 1 μl of diluted cDNA (1:2) as a template, PCR reaction mix Jumpstart (Sigma) and previously published primers and cycling conditions for Bcl2 [23] and Bcl-xl [24]. The primers used for 18S were forward, AGAAACGGCTACCACATCCA and reverse, CTCGAAAGAGTCCTGTATTGT.

2.10. Statistical analysis

All replicate data are expressed as mean and standard error of mean (SEM). Statistical significance was tested using the two-tailed, unpaired Students' *t* test or one-way ANOVA followed by post-hoc Tuckey test using commercial software.

3. Results

3.1. Hypoxia-reperfusion induced cell death is enhanced in FAK null cardiomyocytes

The enhanced systolic dysfunction and rapid progression to cardiac failure observed following persistent pressure overload in hearts with myocardial-restricted deletion of FAK indicated the possibility that FAK might play a cardioprotective role in this setting. Since previous reports indicated that hypoxia induced transient activation of FAK in both cultured myocytes and neurons [25,26] we sought to determine whether FAK might function to promote cell survival under ischemic conditions. We first employed an *in vitro* model of I/R (i.e. hypoxia/serum and glucose deprivation followed by re-oxygenation; Hypox/Reox) in FAK containing and FAK null cardiomyocytes to determine whether FAK plays a critical role in protecting cardiomyocytes from apoptosis associated with this process. To this end,

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cardiomyocytes were isolated from E17.5 $FAK^{-/-}/\beta MHC^{gfp}$ and littermate control $FAK^{+/+}/\beta MHC^{gfp}$ mice, in which myocytes can be identified by nuclear GFP expression. As previously reported, both $FAK^{+/+}/\beta MHC^{gfp}$ and $FAK^{-/-}/\beta MHC^{gfp}$ cardiomyocytes exhibit comparable well-defined sarcomeric actin organization when plated on fibronectin matrix (Fig. 1A. Left) [23]. We next subjected these cultured cells to low (1% O_2) for 2 h followed by re-oxygenation in serum- and glucose-containing medium for an hour to stimulate Hypox/Reox-dependent apoptosis [27]. Approximately 25% of the $FAK^{+/+}/\beta MHC^{gfp}$ cardiomyocytes died following this treatment (Fig. 1A. middle and right panels, Fig. 1B). However, Hypox/Reox-induced cell death was significantly greater (approximately 60%) in comparably treated $FAK^{-/-}/\beta MHC^{gfp}$ cardiomyocytes. Importantly, we found that Hypox/Reox induced rapid activation of FAK in cultured cardiomyocytes (Fig. 1C), confirming and extending previous reports that hypoxia alone induced transient activation of FAK in both cultured myocytes and neurons [26]. Since FAK activity is not necessary for survival of cardiomyocytes under basal conditions [18,23], we speculate that hypoxia and Hypox/Reox-induced activation of FAK may reflect an adaptive response to stress, and that FAK may play a critical role in promoting cardiomyocyte survival under these conditions.

3.2. Cardiac-restricted deletion of FAK exacerbates I/R-induced myocardial infarction in vivo

We next examined whether FAK also functions as a cardioprotective signal transducer *in vivo*. To this end, we bred $fak^{flox/flox}$ mice [16] to $mlc2v^{Cre/wt}$ mice [28] to generate the MFKO ($fak^{flox/flox}/mlc2v^{Cre/wt}$) mice that were deficient in ventricular FAK from 13 weeks post-natal onwards [18]. Corroborating our previous findings that global cardiac structure and function is preserved in MFKO hearts [18], the cohort of MFKO and $fak^{flox/flox}/mlc2v^{wt/wt}$ (genetic control) mice used for the present studies exhibited no significant differences at baseline in heart rate, ventricular chamber and wall dimensions, ejection fraction, or fractional shortening as assessed by echocardiography (Table 1).

We next subjected the genetic control and MFKO mice to 30 min of ischemia followed by 24 h of reperfusion (induced by transient ligation of the LAD coronary artery) and assessed infarct size and myocardial function. Although, the non-perfused region or relative area at risk (RAR; non-blue/total area in Fig. 2A) was comparable between the two groups (MFKO; 32.6±2.9% and genetic controls; 33.5±5.8%, Fig. 2B), the MFKO mice exhibited a significantly increased relative infarct size (RI; ratio of the non-viable white area to the RAR; Fig. 2A) compared to the genetic control mice (36.1±7.3% vs. 14.4±3.1%; $p<0.05$; $n=6$ for each genotype, Fig. 2C). Importantly, we observed comparable infarcts in our two groups of control mice [genetic control and $fak^{wt/wt}/mlc2v^{Cre/wt}$ (Cre control)], indicating that neither expression of the Cre recombinase, nor haplo-insufficiency of Mlc2v influenced this injury response (Fig. 2C). Mice were also analyzed for myocardial function by cardiac catheterization. As shown in Table 2, both LVESP and contractility were significantly reduced in the I/R MFKO group when compared to either the I/R genetic controls or the sham MFKO groups, in strong support of the thesis that FAK plays a critical role in mediating cardioprotection following an ischemic insult.

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3.3. FAK deficiency enhances I/R-induced apoptosis

We next examined whether the increased infarct size in the MFKO hearts was accompanied by enhanced apoptosis within the AR. Indeed, although baseline levels of apoptosis were low and not significantly different between the two experimental groups (data not shown), we observed a significantly higher number of TUNEL positive cardiomyocyte nuclei in the region juxtaposed to the infarct zone in the left ventricle of the MFKO mice compared to the genetic control mice following I/R (Figs. 3A–C).

3.4. Activation of the NF-κB survival pathway is attenuated in MFKO hearts

FAK has been recently shown to modulate activation of pro-survival signals induced by NF-κB [29–31]. Since cardiac NF-κB levels progressively increase following I/R and some studies indicate that NF-κB protects cardiomyocytes from I/R-induced apoptosis [32–34], we sought to examine the activity of this pathway in the MFKO hearts. We first examined the level of phosphorylated inhibitor κB (IκB), since FAK has previously been shown to be necessary for receptor-dependent activation of the IκB kinase (IKK) As shown in Fig. 4A, the level of phosphorylated Ser32/36 IκBα in the MFKO hearts was markedly reduced in comparison to the genetic controls. Accordingly, a concomitant increase in IκB protein and decrease in NF-κB activity (as assessed by phosphorylation of Ser536) were also detected in the post I/R MFKO hearts in comparison to the post I/R control hearts (Figs. 4A, B).

To confirm and extend the observation that NF-κB-dependent signaling is defective in MFKO hearts, we next explored the expression levels of the NF-κB target genes, Bcl2 and Bcl-xl. As shown in Figs. 4C–E, we found that both the message and protein levels of the anti-apoptotic genes, Bcl2 and Bcl-xl, which can be induced by NF-κB-dependent signaling [29,35,36], were significantly reduced in post I/R MFKO in comparison to the genetic controls. Collectively, these studies support the possibility that FAK may promote cell survival in ischemic hearts, at least in part, by enhancing NF-κB-dependent survival signaling.

4. Discussion

In the present study, we investigated whether FAK has a cardioprotective role in response to ischemia/reperfusion, a stimulus known to induce robust myocardial apoptosis and necrosis. We found that FAK activation is induced by Hypox/Reox and that depletion of FAK markedly exacerbates Hypox/Reox-induced cardiomyocyte cell death *in vitro*. Moreover, deletion of FAK in the adult myocardium resulted in significant increases in I/R-induced infarct size and cardiomyocyte apoptosis and a concomitant reduction in left ventricular function. Finally, our results suggest that NF-κB signaling may play a role in modulating FAK-dependent cardioprotection, since FAK inactivation blunted activation of NF-κB and reduced levels of the NF-κB target survival-genes, Bcl2 and Bcl-xl.

Although post-mitotic cells, like cardiomyocytes and neurons, are notoriously resistant to apoptosis, it is evident that apoptotic cardiomyocyte cell death plays a critical role in ischemia-induced myocardial damage. Notably, loss of function mutations in the so-called death receptor signaling components including Fas, Bid, or caspase 9, each lead to a 50–60% reduction in infarct size [37–39]. Thus, the importance of defining pro-apoptotic and

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pro-survival signals in cardiomyocytes is clear. Results from the current study confirm that FAK activity is important for survival of cultured neonatal rat cardiomyocytes exposed to ischemia [40] and our report is the first to show that FAK activation protects cardiomyocytes from I/R-induced apoptosis *in vivo*. The mechanism/s by which I/R stimulates FAK activation is not known. Integrin ligation is the main inducer of FAK phosphorylation, however the activity of FAK is also regulated by a number of G protein coupled receptors and growth factors (such as VEGF). Since many of these circulating factors are increased following I/R, they are likely to induce FAK activation in this setting [25,41]. Our demonstration that FAK inactivation resulted in enhanced cardiomyocyte apoptosis under stressed conditions, is consistent with previous studies implicating FAK in the survival of normal and cancerous cells [42]. Interestingly, FAK expression is up-regulated in several solid tumors and FAK activity has been linked to tumor progression [13,42]. In light of our findings, it is tempting to speculate that enhanced FAK levels might function to protect tumor cells from their typically ischemic environment.

Our data suggest that FAK may regulate cardiomyocyte survival following I/R via activation of the NF-κB signaling cascade. Indeed, we observed decreased IκB phosphorylation, increased IκB protein levels, decreased NF-κB phosphorylation and lower levels of the NF-κB-pro-survival target Bcl2 in the post I/R MFKO hearts in comparison to the genetic controls. Previous studies indicate that inactivation of FAK in either mouse embryo fibroblasts or kidney epithelial cells promotes TNFα-stimulated apoptosis by impairing RIP/TRAF2 complex formation and blocking induction of NF-κB-dependent survival signals [29,30,43,44]. It is likely that a similar mechanism resulted in diminished I/R-dependent NF-κB activation in the MFKO hearts. It is important to note, however, that the function of NF-κB in the heart is controversial as it has been reported to induce both pro-survival and pro-apoptotic signals [32–34,45–50]. Thus, further experimentation is required to determine whether dampened NF-κB activation plays a causative role in the exacerbation of I/R-induced apoptosis in MFKO hearts, or whether FAK may exert cardioprotective effects by additional mechanisms, such as, stimulation of PI3Kinase/AKT, Ras/ERK, and Cas/JNK signaling pathways, which promote survival in many cell types including cardiomyocytes [42,51,52].

An effect of FAK on cardiomyocyte apoptosis may have important implications on the role of FAK during heart failure. Although, apoptosis was originally thought to play a minor role in heart failure more recent evidence suggests that activators or inhibitors of this process influence this pathogenic process [53–55,19,20]. Our findings herein strongly support a critical cardioprotective role for FAK in response to I/R, however whether FAK plays a similar role in the transition from hypertrophy to heart failure is currently unclear. Although we previously showed that FAK inactivation leads to dilated cardiomyopathy after prolonged TAC, we found comparable (but limited) apoptosis following TAC in the genetic control and MFKO hearts (less than 0.03% TUNEL positive cells/condition) [18]. As quantifying differences at these low levels is technically challenging, it is possible that we missed a slight but significant difference in apoptosis in our previous studies. Importantly, recent data suggest that even very low rates of cardiac myocyte apoptosis (2–6 fold over control) are sufficient over time to cause a lethal dilated cardiomyopathy [56]. In light of our

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new findings, it will be of future interest to determine whether anti-apoptotic treatments will halt the rapid progression to dilated cardiomyopathy in banded MFKO hearts.

In conclusion, using loss of function experiments, we have demonstrated that FAK is not necessary for myocyte survival or function in the adult myocardium. However, the absence of FAK in the myocardium exacerbates I/R-dependent apoptosis. Since the toggling between pro-survival and pro-apoptotic signals remains central to preventing irreversible damage to the heart [9], it will be of future interest to determine whether spatial-temporal activation of FAK can salvage “at risk” myocytes in the ischemic heart.

Acknowledgments

We thank Dr. Nancy Moss (UNC-CH) and Dr. Ruhang Tang (CCBC, UNC-CH) for helpful advice and discussions during the course of the present study and Jackie Kylander (CCBC, UNC-CH) for technical assistance with echocardiography. This work was supported by grants from NIH-NHLBI (HL-081844 and HL-071054) and the American Heart Association (AHA. 0355776U) to J.M.T.

Abbreviations

AKT	RAC-alpha serine/threonine-protein kinase
ERK1/2	extracellular signal-regulated kinase 1/2
FN	fibronectin
GFP	green fluorescent protein
IκBα	NF-kappa-B inhibitor alpha
MHC	myosin heavy chain
MLC2V	myosin light chain-2V
NF-κB	nuclear factor-kappa B
PFA	paraformaldehyde

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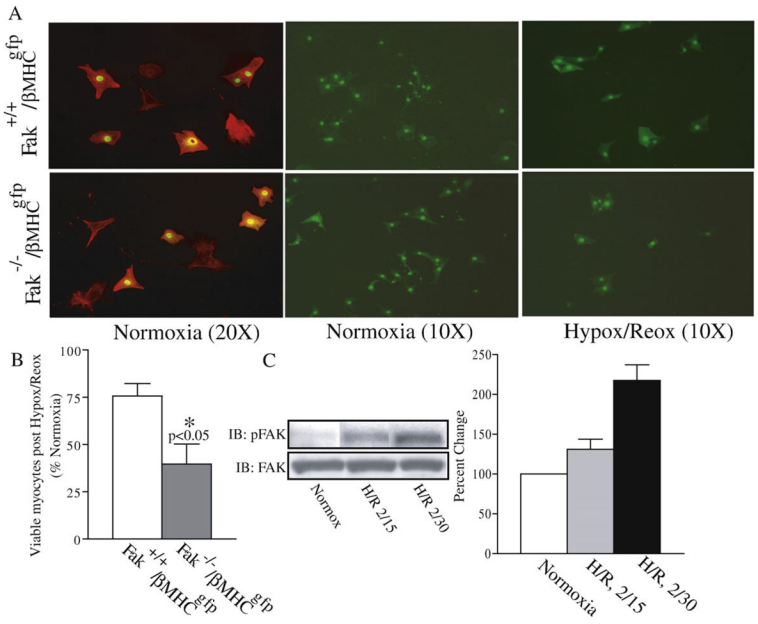

Fig. 1.

Hypoxia/reperfusion-induced cell death is enhanced in FAK null cardiomyocytes. (A) left panel: cultured ventricular cardiomyocytes obtained from embryonic (E17.5) $FAK^{-/-}$/βMHC^{gfp} and littermate control $FAK^{+/+}$/βMHC^{gfp} mice exhibit comparable sarcomeric actin (red) organization (20× power). Middle and right panels: representative images of viable $FAK^{+/+}$/βMHC^{gfp} and $FAK^{-/-}$/βMHC^{gfp} cardiomyocytes after exposure to normoxia or hypoxia/re-oxygenation (Hypox/Reox) as described in the Material and methods section. (10× power). (B) Viable cardiomyocytes following Hypox/Reox were expressed as a percentage of total viable cells in parallel normoxia cultures (n= 6 experiments in which 50–300 cells were counted per experiment per condition). (C) Cultured rat neonatal (P0) cardiomyocytes were exposed to normoxia for 2.5 h or Hypox/Reox for 2 h and 15 min or 2 h and 30 min and FAK activity was assessed by immunoblotting with an anti pY397-FAK antibody. Total FAK is shown as a loading control. (Right panel) Densitometric quantification is shown and data are expressed as mean ± SEM (n=3–4).

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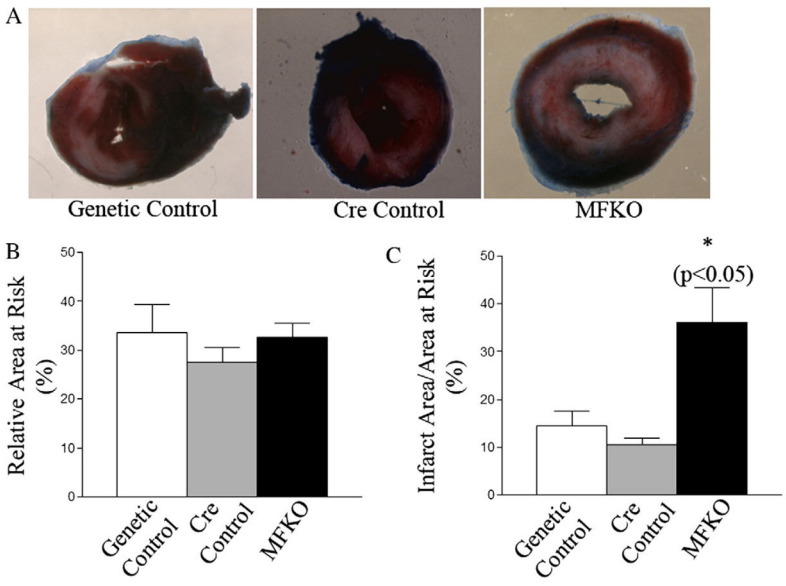

Fig. 2.

Inactivation of FAK in the adult myocardium exacerbates I/R-induced myocardial infarction. MFKO and genetic control mice were subjected to I/R and injected with Evan's blue dye to delineate the perfused regions and area at risk (AR) as described in the Materials and methods section. 24 h post I/R the myocardial tissue was harvested and stained with TTC. (A) The red area indicates the viable cardiac tissue while the white indicates the infarct in these representative sections. (B, C) Quantification of relative area at risk and the ratios of the infarct size to area at risk. Data are expressed as means±SEM (n=4–6).

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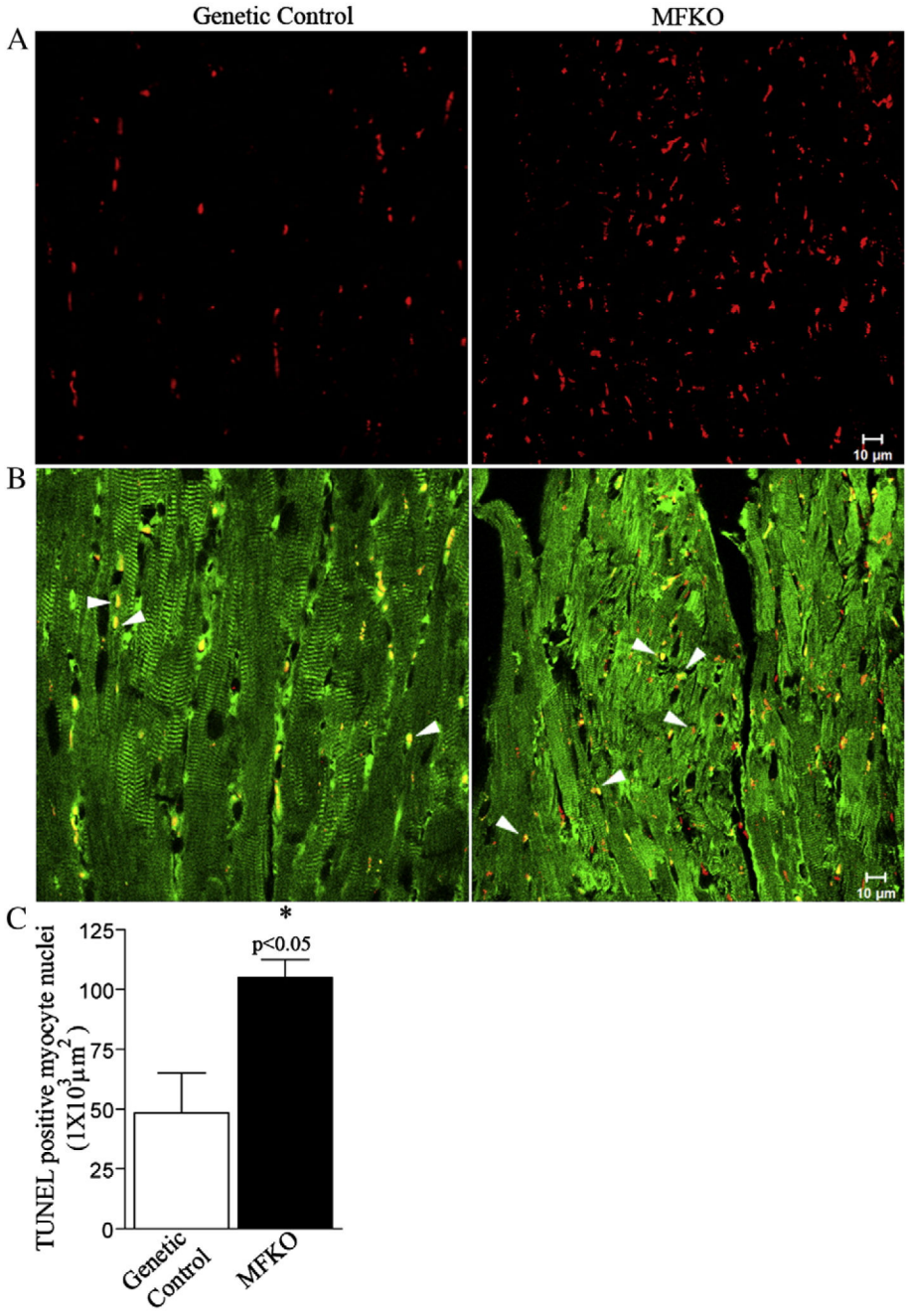

Fig. 3.

I/R-induced apoptosis is enhanced in MFKO hearts. Genetic control and MFKO mice were subjected to I/R as described above. Hearts were sectioned and processed for TUNEL labeling (red) and troponin T IHC (green) to detect DNA strand breaks in apoptotic cardiomyocytes. (A, B) Representative images from the region of the left ventricle juxtaposed to the infarct area in the control and MFKO hearts. (C) Quantification of apoptotic cardiomyocyte nuclei counted in the defined left ventricular area. Data are expressed as means±SEM (*n*=4).

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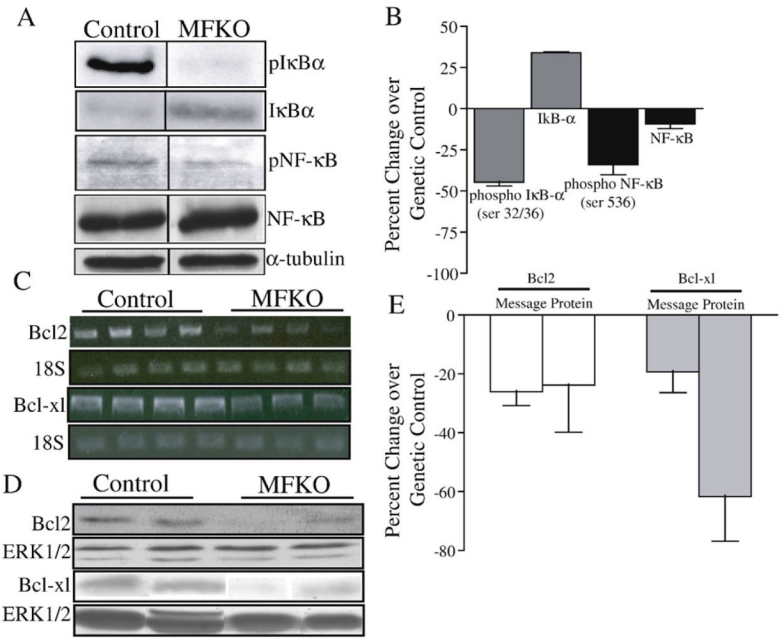

Fig. 4.

NF-κB activation is impaired in MFKO hearts in response to I/R. (A) Cardiac lysates procured from control and MFKO heart sections 24 h following I/R were analyzed by Western analysis using the indicated antibodies. (B) Densitometric quantification (*n*=3–4) is shown and data are expressed as mean percent change from control values±SEM. (C) Isolated RNAs from control and MFKO heart sections 24 h following I/R were subjected to semi-quantitative RT-PCR for Bcl2 and Bcl-xl. Message levels were normalized to 18S. (D) Cardiac tissue lysates from control and MFKO heart sections 24 h following I/R injury were immunoblotted for Bcl2 and Bcl-xl. ERK1/2 is shown as a loading control. (E) Densitometric quantification (*n*=3–4) is shown and data are expressed as mean percent change from control values±SEM.

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Table 1

Baseline echocardiographic analysis of control and MFKO mice

Parameters	Genetic control	MFKO
LVEdD (mm)	4.0±0.3	3.9±0.2
LVEsD (mm)	2.8±0.2	2.6±0.4
LVPWTd (mm)	0.9±0.1	0.8±0.1
LVPWTs (mm)	1.2±0.04	1.1±0.1
IVSd (mm)	1.3±0.1	1.1±0.2
IVSs (mm)	1.6±0.1	1.6±0.1
FS (%)	30.2±3.4	34.3±6.6
EF (%)	57.7±5.3	63±9.0
HR (bpm)	543±6	556±16

EF, ejection fraction; FS, fractional shortening; HR, heart rate; IVSd, interventricular septal thickness during diastole; IVSs, interventricular septal thickness during systole; LVEdD, left ventricular end diastolic diameter; LVEsD, left ventricular end systolic diameter; LVPWTd, left ventricular posterior wall thickness during diastole; and LVPWTS, left ventricular posterior wall thickness during systole.

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Table 2

Hemodynamic analysis of sham and I/R treated MFKO and control mice

Group	HR (bpm)	LVESP (mm)	LVEDP (mm)	Ejection fraction (%)	dP/dT max (mmHg/sec)	dP/dT min (mmHg/sec)	*n*
Genetic control, sham	491±10.5	86.7±4.7	5.0±0.8	64±4	8213±1030	8517±745	5
MFKO, sham	555±39.6	90.0±4.3	7.2±1.6	62±6	8819±748	8063±1132	5
Genetic control, MI	555±14.7	77.5±4.8	4.9±0.6	48±6	7267±391	7550±866	6
MFKO, MI	515±13.5	64.6±6.5*,**	6.8±2.2	46±6	4942±427*,**	4905±600*	5

EF, ejection fraction; HR, heart rate; LVEDP, left ventricular end diastolic pressure; LVESP, left ventricular end systolic pressure; dP/dT max, the derivative of intraventricular pressure during contraction; and dP/dT min, the derivative of intraventricular pressure during relaxation. Data are expressed as means±SEM.

* indicates $p<0.05$ when compared to genetic control, sham.

** indicates $p<0.05$ when compared to MFKO, sham.