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Krüppel-like factor 4 regulates pressure-induced cardiac hypertrophy

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Abstract

Krüppel-like factors (KLF) are a subfamily of the zinc-finger class of transcriptional regulators that play important roles in diverse cellular processes. While a number of KLFs are expressed in cardiomyocytes, little is known about their specific roles in the heart *in vivo*. Here, we demonstrate that KLF4 is induced by hypertrophic stimuli in cultured cardiomyocytes and in the mouse heart. Overexpression of KLF4 in neonatal rat ventricular myocytes inhibits three cardinal features of cardiomyocyte hypertrophy: fetal gene expression, protein synthesis, and cell enlargement. Conversely, mice with cardiomyocyte-specific deletion of KLF4 (CM-K4KO) are highly sensitized to transverse aortic constriction (TAC) and exhibit high rates of mortality. CM-K4KO mice that survive TAC display severe pathologic cardiac hypertrophy characterized by increased cardiac mass, depressed LV systolic function, pulmonary congestion, cavity dilation and attenuated LV wall thickening when compared to control genotypes. In addition, CM-K4KO mice develop increased myocardial fibrosis and apoptotic cell death after TAC. Collectively, these studies implicate KLF4 as a novel transcriptional regulator that is indispensable for the hearts response to stress *in vivo*.

Keywords

Krüppel; hypertrophy; myocyte; transcription; pressure overload

1. Introduction

Cardiac hypertrophy is a common adaptive response of the heart to hemodynamic and neurohormonal stress, in which individual cardiomyocytes grow in length and/or width as a means of maintaining circulatory function. However, in the long term, myocardial hypertrophy predisposes to heart failure, arrhythmia and sudden death [1]. At the cellular level,

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cardiomyocyte hypertrophy is characterized by cellular enlargement, increased protein content, and re-expression of fetal genes (e.g. ANF, BNP, β -MHC) [1,2]. Cardiac hypertrophy and its attendant consequences remain a source of considerable morbidity and mortality despite currently available therapies [1]. Thus, a more precise understanding of the molecular mechanisms regulating cardiac hypertrophy and failure is of considerable scientific and therapeutic interest.

Divergent signals that cause cardiac hypertrophy ultimately converge upon the nucleus and lead to alterations in gene expression. A number of nuclear factors, including myocyte enhancer factor-2 (MEF2), GATA4, Nuclear factor κ B (NF κ B), and nuclear factor of activated T cells (NFATs) have been implicated as key mediators of the hypertrophic transcriptional program [1]. Despite the increasing body of knowledge describing transcriptional activators of cardiac hypertrophy, there is much less information regarding transcriptional repressors of hypertrophy.

Krüppel-like factors (KLF) are a subfamily of the zinc-finger class of transcriptional regulators. Members of this gene family have been shown to play important roles in cell growth and differentiation across multiple tissues [3,4]. A recent study has shown that 9 of the 17 known mammalian KLFs are expressed in neonatal rat ventricular myocytes (NRVM) and differentially regulated by hypertrophic stimuli (e.g. ET-1) [5]. Of these 9 factors, only the roles of KLF15, KLF10 and KLF5 have been evaluated *in vivo* [6–8]. Our previous work has highlighted KLF15 as a negative regulator of cardiac hypertrophy [7,8]. KLF15 deficient mice develop severe cardiac hypertrophy and accelerated heart failure after pressure overload or Angiotensin II (Ang II) infusion [7,8]. In contrast, KLF5 was shown to be a mediator of Ang II-induced cardiac hypertrophy and fibrosis [6]. Spelsberg et al also reported KLF10 deficiency results in spontaneous pathological cardiac hypertrophy in male mice at the age of 16 months [9]. While previous studies from our group and others have demonstrated critical roles for KLF4 in endothelial cells and smooth muscle cells [10–12], the role of KLF4 in the cardiac muscle is not known. Here, using cardiac-restricted gene targeting in mice, we provide evidence that KLF4 is a novel negative regulator of cardiac hypertrophy *in vivo*.

2. Materials and methods

2.1 Primary cardiomyocyte cultures

Neonatal rat ventricle myocytes (NRVM) were isolated and cultured as described previously [8]. Pharmacologic stimulation was performed with 50 μ M phenylephrine (PE) (P6126, Sigma, St. Louis, MO), 1 μ M Ang II (A9526, Sigma), or 50 nM endothelin-1 (ET-1) (E7764, Sigma).

2.2 Adenoviral infection and transient transfection studies

NRVMs were infected with adenovirus (Welgen, Inc. Worcester, MA) carrying GFP (EV), or KLF4 cDNA at 10 MOI, 48 h after plating for a period of 48 h (>90% infection efficiency as revealed by GFP signal). For transient transfections, NRVMs were transfected with FuGENE 6 (Roche Applied Science, Indianapolis, IN) following manufacturer's protocol using luciferase based reporters and assayed on a luminometer.

2.3 Protein synthesis measurements

Protein synthetic rate in NRVMs was determined by [3 H]-leucine incorporation as described [8].

2.4 Immunocytochemistry analysis of NRVM

For immunocytochemical analysis, NRVMs were placed on laminin-coated coverslips, infected with KLF4 or EV adenovirus and followed by 48 h of PE stimulation. The cells were

then washed with PBS, fixed with formalin, blocked in 1% BSA, and incubated with monoclonal antibody against sarcomeric α -actinin (A7732, Sigma) at a dilution of 1:800. Immunostaining was visualized by Alexa Fluor® 594-conjugated anti-mouse secondary antibody (A21135, Invitrogen, Carlsbad, CA) under a fluorescent microscope.

2.5 Gene expression analysis using quantitative RT-PCR (qPCR)

Total RNA was isolated from cultured cells or tissue samples using TRIzol reagent following the manufacturer's protocol (15596-026, Invitrogen). First-strand cDNA was synthesized and subjected to qPCR with either Sybr green (4309155, Applied Biosystems, Foster City, CA) or Roche universal probe TaqMan reagent (Universal ProbeLibrary, Roche Applied Science) on a StepOnePlus real-time PCR system (Applied Biosystems). Gene expression was normalized to GAPDH using the $\Delta\Delta CT$ method. Primer efficiencies were within the range of 90%–110%.

2.6 Animal models

All mice were maintained in the animal resource center and protocols are approved by IACUC. The α -MHC^{cre/+} mice were generous gift from Dr. Michael D. Schneider (British Heart Foundation Centre of Research Excellence, National Heart and Lung Institute, Imperial College London, UK) [13]. The derivation of the KLF4^{flox/flox} mice has been described previously [14]. All mice were bred on C57BL/6J background. To generate cardiomyocyte-specific deficient mice for KLF4, KLF4-floxed mice were bred with α -MHC^{cre/+} mice to get α -MHC^{cre/+}; KLF4^{flox/flox} (designated CM-K4KO). KLF4^{flox/flox} (designated K4FL) and α -MHC^{cre/+} (designated MHC-cre) mice were used as controls.

Transverse aortic constriction (TAC) with 27-gauge needle and Ang II infusion with osmotic pump was performed in male mice (age 10–12 weeks) as described [7,8].

2.7 Histology

Mice heart samples were fixed in 10% formalin, embedded in paraffin and sectioned following standard histological protocol. Fibrosis was determined using Gomori Trichrome Stain Kit (23-900-662, Thermo Scientific, Waltham, MA). Apoptosis was accessed by TUNEL method with an ApopTag® Plus Peroxidase In Situ Apoptosis Kit (S7101, Millipore, Billerica, MA).

2.8 Statistics

All statistical analysis was performed using one way ANOVA followed by Bonferroni's multiple-comparison test for multiple comparisons, and Logrank test for survival curves.

3. Results and discussion

3.1 KLF4 inhibits hypertrophy in cultured cardiomyocytes

We first profiled the expression pattern of KLF4 in NRVMs in response to various hypertrophic stimuli, including PE, ET-1 and Ang II. Consistent with prior observations [5], KLF4 mRNA was transiently induced by all three stimuli (Fig 1A). To assess whether KLF4 induction also occurred *in vivo*, we assessed KLF4 mRNA levels in two murine models of cardiac hypertrophy, chronic Ang II infusion and transverse aortic constriction (TAC). Fourteen-day Ang II infusion resulted in a 1.5-fold increase in KLF4 mRNA (Fig 1B). TAC induced sustained levels of KLF4 mRNA expression at 6 hour, 2-week, and 4-week time points (Fig 1C). These data demonstrate that KLF4 is expressed in the heart and induced in the setting of neurohormonal and pressure-overload mediated cardiac hypertrophy.

Given these expression patterns, we next assessed whether KLF4 overexpression could affect cardiomyocyte hypertrophy in cultured NRVM. Adenoviral over-expression KLF4 in NRVM

attenuated the induction of ANF expression in response to multiple pro-hypertrophic agonists (Fig 1D). Furthermore, transient transfection of KLF4 in NRVM markedly inhibited the PE-induced activity of the ANF (−638) and BNP (−112) promoters (Fig 1E). Lastly, KLF4 overexpression significantly attenuated PE-induced protein synthesis as assessed by [³H]-leucine incorporation (Fig 1G) and cell enlargement as assessed by immunocytochemistry for sarcomeric α -actinin (Fig 1H). These data demonstrate that KLF4 overexpression inhibits three cardinal features of cardiomyocyte hypertrophy: fetal gene expression, protein synthesis, and cellular growth.

3.2 Cardiomyocyte-specific deficiency KLF4 sensitizes mice to pressure overload-induced cardiac hypertrophy and heart failure

As KLF4 negatively regulated cardiomyocyte hypertrophy *in vitro*, we next assessed the biologic role of KLF4 *in vivo* using a tissue-restricted loss-of-function strategy. We employed a Cre-loxP approach to target KLF4 in a cardiomyocyte-specific fashion (CM-K4KO) using the α -MHC-cre transgene as previously described [13,14]. CM-K4KO mice were born in Mendelian ratios, and viable into adulthood. At baseline, CM-K4KO mice had mildly increased cardiac mass and elevated levels of ANF mRNA (Fig 1A). We next subjected CM-K4KO and control mice to pressure-overload hypertrophy using the transverse aortic constriction model (TAC) [8]. CM-K4KO mice exhibited significant intolerance to pressure overload with 50% mortality after one week and 75% mortality after 2 weeks postoperatively, which is significantly higher than controls (Fig 2A). The surviving CM-K4KO mice developed severe pathologic hypertrophy with eccentric cardiac remodeling and heart failure after TAC (Fig 2C–D). When compared to control mice, CM-K4KO mice had increased cardiac and lung mass (Fig 2C), impaired LV systolic function, cavity dilation, and attenuated wall thickening (Fig 2D). The echocardiographic abnormalities seen in survived CM-K4KO mice were apparent as early as 2-weeks post TAC, persisted at 4-week post-TAC, and were significantly different than control genotypes (Fig 2D). In the CM-K4KO mice, there was no observed difference in echocardiographic parameters between the 2-week and 4-week time points. As the majority of CM-K4KO mice die by the 4-week time point, this result may be influenced by survival bias. The hypertrophic marker genes ANF, BNP were induced to similar degree 4 weeks post-TAC, likely reflecting a saturation of this readout in late-stage pathologic hypertrophy (Fig 2E). However, β -MHC expression was significantly higher in CM-K4KO heart at 4 weeks post-TAC (Fig 2E). As further evidence of severe pathologic hypertrophy, we detected severe fibrosis (Fig 2F–G) and increased apoptotic cell death (Fig 2H) in CM-K4KO mouse hearts at 4-weeks post-TAC.

In summary, we show that KLF4 is induced by hypertrophic stimuli and is necessary for the compensated response to pressure overload *in vivo*. While previous studies have demonstrated that KLF4 can affect hypertrophic signal transduction in cultured cardiomyocytes [15], the current work utilizes cardiomyocyte-specific gene targeting to confirm the role of KLF4 as a critical negative regulator of cardiac hypertrophy *in vivo*. As the majority of CM-K4KO mice cannot tolerate prolonged pressure overload, it is likely that the phenotypic data described in Figure 2 significantly underestimate the true severity of the KLF4-deficient phenotype. Further studies using milder forms of stress (e.g. low-intensity pressure overload or chronic agonist infusion) may be revealing and allow for further mechanistic dissection of KLF4 function in the myocardium. However, our current work provides cogent evidence implicating KLF4 as a hypertrophy-inducible transcription factor that is indispensable for the heart's response to stress *in vivo*. These observations contribute to the increasing appreciation that KLFs are key molecular regulators of cardiac biology.

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Fig 1. KLF4 negatively regulate cardiomyocyte hypertrophy *in vitro*

(A) Regulation of KLF4 in NRVMs by hypertrophic stimuli. * $p < 0.05$ vs 0 h. (B) Expression of KLF4 in 2-week Ang II infused mouse heart. Control (normal saline): $n = 5$; Ang II: $n = 9$. * $p < 0.05$ vs Control. (C) Expression of KLF4 mouse heart at 6h, 2-week and 4-week after TAC. Sham: $n = 4$ or 5; TAC: $n = 5$ or 6. * $p < 0.05$ vs corresponding sham. (D) ANF mRNA level in KLF4 or EV adenovirus infected NRVMs after 24h treatment with hypertrophic stimuli. * $p < 0.05$ vs EV control. # $p < 0.05$ vs EV+treatment in its own treatment group. (E,F) Effect of KLF4 co-transfection on the transcription of a luciferase construct driven by ANF promoter (E) and BNP promoter (F). * $p < 0.05$ vs untreated control of luc+pcDNA3.1 plasmid (vector). # $p < 0.05$ vs PE treated group of luc+vector. (G) PE-induced protein syntheses in KLF4

or EV adenovirus infected NRVMs as revealed by [^3H]-leucine incorporation. * $p < 0.05$ vs EV control. # $p < 0.05$ vs EV+PE. (H) PE-induced cell size enlargement of KLF4 or EV adenovirus infected NRVMs as revealed by sarcomeric α -actinin immunostaining. Sarcomeric α -actinin was stained as red by Alexa Fluor® 594. Nuclei were stained as blue by DAPI. Pictures were taken at 400 $\times$. Gene expression was determined by qPCR using GAPDH as control. Gene expression level was expressed as fold change over control sample (set as 1). Error bar indicates s.e.m.

Fig 2. Cardiomyocyte-specific deficiency of KLF4 exacerbate pressure overload-induced cardiac hypertrophy and heart failure *in vivo*

(A) CM-K4KO mice developed heart hypertrophy at the age of 4-month as indicated by heart weight (left panel) and hypertrophic marker gene ANF expression (right panel). (B) Postoperative survival curve of MHC-cre, K4FL and CM-K4KO mice. MHC-cre: n=10; K4FL: n=11; CM-K4KO: n=19. p: Logrank test (CM-K4KO vs MHC-cre or K4FL). (C) Echocardiography parameter at 2-week and 4-week after TAC. Left panel: fractional shortening. Middle panel: left ventricular internal dimensions in diastole. Right panel: interventricular septal thickness at end-diastole. *p<0.05 vs MHC-cre at 2-week after TAC. #p<0.05 vs MHC-cre at 4-week after TAC. (D) Heart weight and lung weight after 4-week

TAC. Heart or lung weight (mg) was normalized to body weight (g) to control body weight difference. Data were expressed as the ratio of heart or lung weight/body weight (mg/g). * $p < 0.05$ vs MHC-cre. (E) Hypertrophic marker genes expression analyzed by qPCR. GAPDH served as control. Gene expression level was expressed as fold change over MHC-cre sham samples (set as 1). Error bar indicates s.e.m. * $p < 0.05$ vs MHC-cre sham. # $p < 0.05$ vs MHC-cre TAC. (F) Fibrosis staining of heart sections. Pictures were taken at 400 $\times$ in bright field. Red: cells. Blue: collagen. (G) Calculated fibrosis deposition. Pictures were taken at 400 $\times$ from random field (>10 fields per section) and blue staining was calculated as area percentage versus whole image area using ImagePro software. * $p < 0.05$ vs MHC-cre sham. # $p < 0.05$ vs MHC-cre TAC. (H) Apoptosis in TAC heart by TUNEL assay. Pictures were taken at 200 $\times$ from random field (>20 fields per section). Methyl green labeled nuclei and DAB positively stained nuclei were counted. Data expressed as percentage of DAB positive counts (apoptotic cells) versus methyl green positive counts (total cells). * $p < 0.05$ vs MHC-cre.