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E4BP4 is a cardiac survival factor and essential for embryonic heart development

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Abstract The bZIP transcription factor E4BP4, has been demonstrated to be a survival factor in pro-B lymphocytes. GATA factors play important roles in transducing the IL-3 survival signal and transactivating the downstream survival gene, E4BP4. In heart, GATA sites are essential for proper transcription of several cardiac genes, and GATA-4 is a mediator of cardiomyocyte survival. However, the role E4BP4 plays in heart is still poorly understood. In this study, Dot-blot hybridization assays using Dig-labeled RNA probes revealed that the E4BP4 gene was expressed in cardiac tissue from several species including, monkey, dog, rabbit, and human. Western blot analysis showed that the E4BP4 protein was consistently present in all of these four species. Furthermore, immunohistochemistry revealed that the E4BP4 protein was overexpressed in diseased heart tissue in comparison with normal heart tissue. In addition, the overexpression of E4BP4 in vitro activated cell survival signaling pathway of cardiomyocytes. At last, siRNA-mediated knock down of E4BP4 in zebrafish resulted in malformed looping of the embryonic heart tube and decreased heart beating. Based on these results, we conclude that E4BP4 plays as a survival factor in heart and E4BP4 is essential for proper embryonic heart development.

Keywords E4BP4 · Heart development · Cardiac survival factor

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Introduction

E4BP4 (adenovirus E4 promoter-binding protein) is a PAR (proline and acidic rich)-related bZIP factor that has been shown to play essential roles in regulating mammalian circadian oscillatory mechanism, anti-inflammatory response, and cell survival effect [1]. E4BP4 was originally isolated in mammalian systems as a transcription factor recognizing the adenovirus E4 promoter with repressor activity [2]. E4BP4, also known as NFIL3, was later identified as an activator of the IL-3-induced survival signaling pathway in T cells [3] and Hematopoietic cells [4–6]. However, in *Caenorhabditis elegans*, cell death selector (CES-2), an E4BP4 homologue, induces apoptosis by repressing the downstream survival factor *ces-1* [1, 7]. E4BP4 has also been shown to be a repressor of various promoters in HeLa cells [2] and in HepG2 and HuH-7 cell lines [8].

Human E4BP4 shares greater than 90% sequence identity with mouse [4], rat [9], and chicken [10] E4BP4 and, therefore, their gene products might share common features in function. E4BP4 protein in mammals and its homologues CES-2 in *C. elegans* [7] and vrille protein in *Drosophila* [11] commonly lack a PAR region but contain a PAR-like basic domain. The characteristic feature that allows E4BP4 to exert transcriptional repression activity is a repression domain lying between residues 299 and 363 that interacts with the TBP binding repressor protein Dr1 [12, 13].

E4BP4 is highly expressed in liver, moderately expressed in heart, lung, brain, spleen, and skeletal muscle of rat [9]. However, it is unknown what role E4BP4 plays in heart function. It has been shown in vivo that GATA-1 activates the transcription of E4BP4 by binding to the *E4bp4* promoter and that it is also involved in the anti-apoptotic signaling of IL-3 in the Ba/F3 hematopoietic cell line [5]. Phosphorylation of GATA-1 by ERK mediates the induction of the cell survival molecule Bcl-X_L [6]. Therefore, GATA-1 appears to mediate survival responses and anti-apoptotic mechanisms. GATA sites have also been shown to be important in the regulation of several cardiac genes. GATA-4 was reported to function as a mediator of cardiomyocyte differentiation, proliferation, and survival [14]. Therefore, we speculated that E4BP4 might also as a survival mediator in heart. The aim of this study was to investigate the role E4BP4 plays in heart, to determine whether E4BP4 acts as a survival factor and to evaluate whether E4BP4 plays a role in heart function.

Materials and methods

Dot blotting

Two plasmid templates were used to make DIG-labeled antisense RNA probes: the linearized pT7 RNA 18S (Ambion, Astin, TX, USA) containing an 80 bp insert (nucleotides 715–794) of a highly conserved region of the human 18S-rRNA gene, and pBluescript II SK containing a 494-bp insert of highly conserved sequences from the human E4BP4 gene. The latter plasmid was cloned in *Escherichia coli*, purified with the Wizard plus Minipreps DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's protocol, extracted with phenol–chloroform, and linearized [15]. Linearized plasmids were transcribed with T7 RNA polymerase and DIG-UTP using the DIG/Genius 4 RNA labeling kit SP6/T7 (Boehringer-Mannheim, Indianapolis, IN, USA). DIG-labeled antisense transcripts were precipitated, washed, dried, and dissolved in 0.1% DEPC–H_2O, according to the manufacturer's protocol. These antisense RNA probes were then synthesized and the sizes were verified by electrophoresis on 1.2% non-formaldehyde agarose gels. The loading buffer contained 80% formamide, 1 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol. Probe specificity was verified by Northern blotting done under the same hybridization conditions, including temperature and probe concentration, as used for dot blotting as described below [16].

Hearts were obtained from dog, rabbit, monkey, and human. Obtainments of all the hearts were approved by ethical committee. Total RNA was isolated from 100 to 150 mg whole heart tissue using the Ultraspec reagent (Biotecx, Houston, TX, USA) according to the manufacturer's protocol, denatured in formamide–formaldehyde–10× MOPS buffer for 10 min at 65°C and diluted to 80 ng/μl with the same buffer [16].

Total RNA was spotted on a positively charged Nylon membrane (no. 1209299, Boehringer-Mannheim) using a Bio-Dot apparatus (Bio-Rad, Hercules, CA) under a vacuum. The membrane was dried at 80°C and the RNA was fixed to the membrane by UV cross-linking for 5 min. A DIG-labeled antisense RNA probe was denatured in a 65°C water bath for 10 min and then kept at room temperature until used. The membrane was pre-hybridized for 70 min at 80°C. The pre-hybridization solution was then poured off, and the denatured DIG-labeled RNA probe/hybridization buffer mixture was added to the membrane, and incubated overnight at 68°C with gentle agitation. The hybridized membrane was washed twice with 2× washing buffer (2× SSC, 0.1% SDS) for 7.5 min, and washed with 0.5× washing buffer (0.5× SSC, 0.1% SDS) for 20 min, and then washed with 0.1× washing buffer (0.1× SSC, 0.1% SDS) for 20 min, and finally rinsed for 1 min in washing buffer (0.1 M Maleic acid, 0.15 M NaCl, 0.3% Tween 20). The membrane was then incubated with gentle shaking for 1 h in blocking solution, followed by incubation for 30 min in antibody solution, anti-DIG-AP conjugate (Roche, Mannheim, Germany) diluted 1:10000 in blocking buffer, washed

twice for 15 min in washing buffer, equilibrated for 2–5 min in detection buffer (0.1 M Tris–HCl, 0.1 M NaCl, 50 mM $MgCl_2$), and then incubated in CDP-Star (Roche) solution (1:100 diluted in detection buffer) for 5 min and detected the chemiluminescent signal [16].

Immunohistochemistry

A human cardiovascular tissue array slide comprising normal heart sections ($n = 10$), acute infarction ($n = 20$), and granulation tissue ($n = 8$) was purchased from GroPep (Thebarton, SA, AU). The tissues were obtained from both female and male, age range from 25 to 84 years old. The infarct locations were including anterior, posterior, and septal parts. The normal tissues were including both right and left ventricles.

Slides were placed on a rack and dried at 58°C overnight. The slides were dewaxed in xylene for 40 min and rehydrated in graduated ethanol solutions for 30 min, and finally placed in ddH_2O for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 13 min. Slides were then rinsed with tap water for 15 min, placed in pre-warmed citrate buffer (0.01 M citric acid, pH 6.0) heated in a microwave for 15 min, and then allowed to cool down to room temperature for 30 min. The slides were then rinsed with PBS buffer for 10 min. Nonspecific binding was blocked with 5% FBS for 10 min. A 1:50 dilution of polyclonal E4BP4 antibody (Santa Cruz, CA, USA) was then added at 37°C for 1 h. Then, the slides were washed with PBS for 10 min and followed by application of secondary antibody (dilution of 1:100; Santa Cruz, CA, USA) for 30 min and then washed by PBS buffer for 10 min. Immunoreactivity was visualized with DAB chromogen (3,3′-diaminobenzidine) provided in the DAB substrate kit (Roche). Tissues were counterstained with hematoxylin (blue, nuclei) and eosin (red, cytoplasm) and mounted in aqueous mountant.

Primary cell culture and transfection

Primary cardiomyocyte cells were grown in NS medium (Cellutron life technologies, Highland park, NJ, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), incubated at 37°C in 5% CO_2 incubator. E4BP4 plasmids were transfected into the cells using GeneJuice® transfection reagent (Novagen, WI, USA) according to the manufacturer's guidelines. After 6 h, the cells were fed with fresh medium, and 18 h thereafter the cells were harvested.

RT-PCR

An aliquot of total RNA (0.5 μg) was reverse transcribed using 0.5 μM oligo dT primers in a reaction solution (50 μl) containing 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM DTT, 10U RNase inhibitor (Promega, Madison, WI, USA), 0.8 mM total dNTPs, and 200U of Moloney murine leukemia virus (MMLV) reverse transcriptase (promega). The sample was incubated at 42°C for 1 h and at 99°C for 5 min before chilling on ice for 10 min. The RT product (2 μl) was diluted with the PCR buffer (50 mM KCl, 10 mM Tris–HCl, 2 mM $MgCl_2$) to a final volume of 50 μl, containing 0.5 μM dNTPs (final concentration, 0.8 mM) and 0.5 U of Taq DNA polymerase. Following the hot start (5 min at 95°C); the samples were subjected to 30 cycles of 1 min at 95°C, 30 s at annealing temperature, and 1 min at 72°C. After 30 cycles, the final cycle was extended for 10 min at 72°C and held at 4°C. The IGF-1 forward primer: *TGG ATG CTC TTC AGT TCG TG*; reverse primer: *CAA CAC TCA TCC ACA ATG CC*; IGF-1R forward primer: *CCT AGA CAA CCA GAA CTT GC*; reverse primer: *GTC TAC ATC CAC CAT GTT CC*; Bcl-2 forward primer: *GCG AAG TGC TAT TGG TAC CTG*; reverse primer: *ATA TTT GTT TGG GGC AGG TCT* (Mission Biotech, Taipei, Taiwan). The PCR products were analyzed by 1.2% agarose gel electrophoresis, and imaged using the Kodak Scientific ID Imaging System (Eastman Kodak Company, CT, USA).

Western blot

The tissue and cell lysate proteins were separated using 12% SDS-PAGE and transferred to PVDF membranes. Residual protein sites were blocked in Tween/Tris-buffer saline (TBS) containing 5% skin milk. The filters were incubated with 1:1000 diluted primary antibody E4BP4, caspase 3 (Santa Cruz, CA, USA), p-IGF-1R, IGF-1R (Abcam, Cambridge, UK), Bcl-2 (BD, NJ, USA) in TBS plus 2.5% skim milk at the recommended concentrations at 4°C overnight and incubated with secondary antibodies for 1 h at room temperature. Antibody reaction was visualized with enhanced chemiluminescence (ECL) reagent.

Zebrafish SiRNA and microinjection

The siRNA designed against zebrafish E4BP4 was produced using the Silencer® Express kit (Ambion, USA). We designed two template oligonucleotide sequences: Sense (CAA [CTA CAC AAA] TTG ATG ACA GAG ATG CAA CCG GTG TTT CGT CCT TTC CAC AAG); antisense (CGG CGA AGC TTT TTC CAA AAA GT TGC ATC TCT GTC ATC AA[C TAC ACA AA]T TGA) using web software offered by Ambion. According to the manufacturer's protocol, the sense template and promoter primer provided in the kit were used in the first PCR amplification run to anneal the promoter region and sense sequence. The first-run PCR products were then added to a mixture

containing the promoter primer and antisense template and subjected to a second PCR resulting in promoter–sense–loop–antisense sequences. The second-run PCR products were added to a mixture containing the promoter and terminator primer (offered in the kit) and subjected to a third PCR to produce a functional siRNA construct. The siRNA (1.5 ng/embryo) were microinjected into the embryo yolk at the 1- to 2-cell stage. After 48 or 72 h, heart morphology was observed using a dissection microscope and pictures of the embryonic heart were taken. The number of heart beats were accounted from in vivo cardiac imaging and the heart rate was counted.

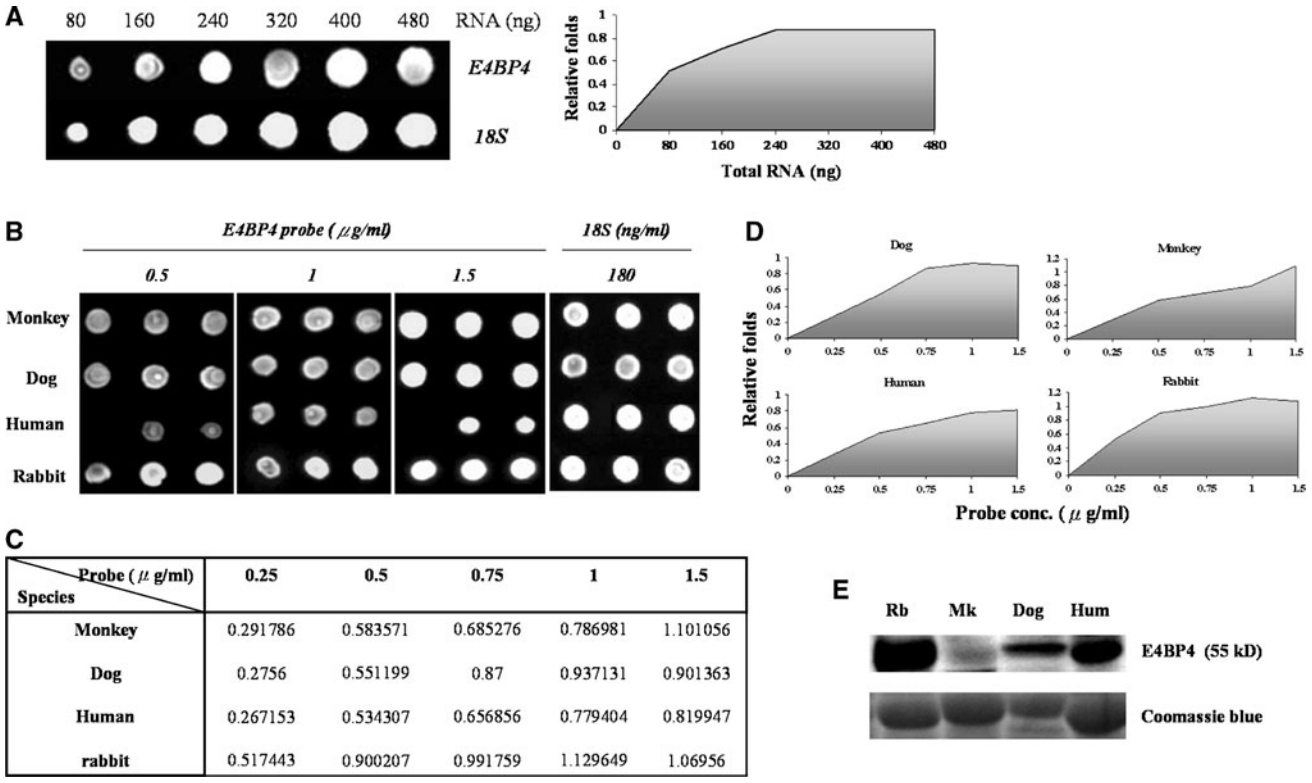
Statistical analysis

Each experiment was repeated thrice at least, and results were presented as the mean ± SEM, and statistical comparisons were made using the Student's *t*-test. Significance was defined at the $P < 0.05$ level.

Results

E4BP4 expressed in hearts of different mammalian species

The Dot blot assay described here is more rapid and sensitive than Northern blotting. E4BP4 mRNA was reliably detected up to 240 ng total RNA with hybridization mixture (0.6 μg probe/ml) (Fig. 1a). Total RNA (240 ng) were extracted from different species of mammalian hearts to quantitatively detect the expression pattern of E4BP4 with Dot-blot assay. E4BP4 mRNA was indeed expressed in heart tissue from dog, rabbit, monkey and human (Fig. 1b–d). Western blot assay confirmed that E4BP4 protein was expressed in all four of these species (Fig. 1e). These results suggest that E4BP4 is actually expressed in the hearts of many mammalian species. However, what role E4BP4 play in heart is still unknown.



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Species \ Probe (μ g/ml)	0.25	0.5	0.75	1	1.5
Monkey	0.291786	0.583571	0.685276	0.786981	1.101056
Dog	0.2756	0.551199	0.87	0.937131	0.901363
Human	0.267153	0.534307	0.656856	0.779404	0.819947
rabbit	0.517443	0.900207	0.991759	1.129649	1.06956

Fig. 1 E4BP4 expressed in different species' hearts. Rapidly detection of RNA in dot-blot assay. **a** To determine the linear range of detection of E4BP4 mRNA, total heart RNA was spotted at 80–480 ng/dot, applied the amount of E4BP4 probe (0.6 μg/ml), 18S probe (180 ng/ml). Relative densities of the dots are plotted versus the amount of total RNA. **b** To detect E4BP4 mRNA expression in different mammalian species including monkey, dog, human, and rabbit, 240 ng total heart RNA was spotted per dot and hybridized with 0.5–1.5 μg DIG-labeled antisense E4BP4 mRNA probe/ml hybridization mixture. **c** Relative density of the dots is expressed as the number of dots of E4BP4 divided by 18S. **d** Relative curve of density fold. **e** Western blot showed E4BP4 protein present in hearts of rabbit, dog, monkey, and human. Coomassie blue stain showed the protein quantity as a loading control

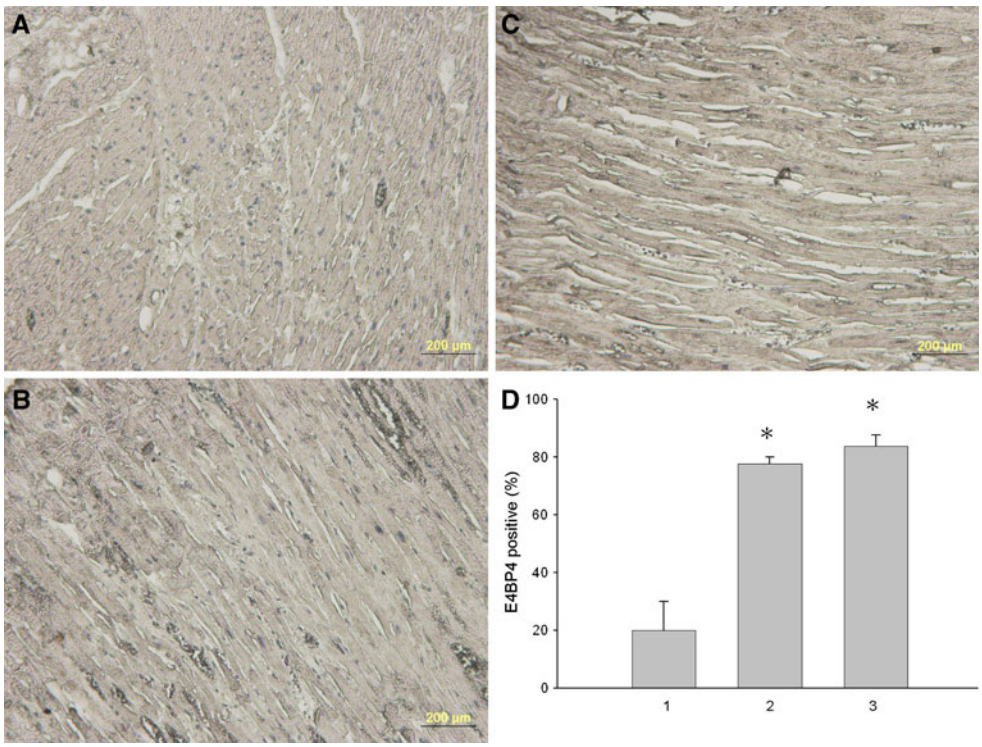

Fig. 2 Comparison of E4BP4 protein expression between normal and infarct human hearts. E4BP4 protein expression was assayed by IHC and hematoxylin counter stain then visualized *brown* color. The protein expression increased with disease progression. **a** Normal ($n = 10$), **b** acute infarction ($n = 20$), **c** granulation tissue ($n = 8$). **d** The histogram of E4BP4 IHC positive results. *Bar 1*: normal hearts, *bar 2*: acute infarction, *bar 3*: granulation tissue. The experiment was repeated thrice. Statistical represent as the mean value ± SEM, * $P < 0.05$ significant difference (Color figure online)

E4BP4 protein expression is elevated in diseased heart and promoted the cardiac survival signalings

To understand the pattern of E4BP4 protein expression in diseased hearts, we analyzed tissues representing different stages of heart disease by immunohistochemistry. The results revealed that the level of E4BP4 protein expression was higher in acute infarct tissue (77.5%) and granulation tissue (83.75%) than in normal heart tissue (20%) (Fig. 2). To further explore why E4BP4 overexpressed in disease heart, we transiently transfected E4BP4 plasmids in primary cardiomyocytes of neonatal rats to overexpress E4BP4. The RT-PCR results showed the overexpression of E4BP4 up-regulated survival related factors, such as IGF-1, IGF-1R (survival signals), and Bcl-2 (anti-apoptotic protein) (Fig. 3a). Western blot results also showed the activation of survival signaling pathway (p-IGF-1R, p-Akt), and increase of Bcl-2 when E4BP4 overexpressed (Fig. 3b). Additionally, the pro-apoptotic protein, caspase 3 showed down-regulation of protein cleavage while E4BP4 overexpressed. When E4BP4 was presented, that improved survival signalings, and inhibited pro-apoptotic protein caspase 3 activation (Fig. 3). These results suggest that E4BP4 might activate cell survival signalings in diseased hearts to maintain heart function and prevent heart from damage.

Faulty heart development in zebrafish after E4BP4 knocked down by siRNA treatment

The zebrafish is a well-established animal model for studying vertebrate development and gene function. Therefore, we adopted the zebrafish model to investigate the role that E4BP4 plays in the development of heart. We used the sequence aligning software "DNA star" to compare the homology of vertebrates and mammals. The E4BP4 protein sequence of zebrafish was 49.47% homologous to the corresponding human sequence, 50.11% homologous to that of the mouse sequence, and 50.32% homologous to that of the rat sequence. The functional binding domain of the basic region leucine zipper (BRLZ), located at amino acids 60–122, was highly conserved in all four species (Fig. 4A). siRNA-mediated knock down of E4BP4 in zebrafish resulted in malformed looping of the embryonic heart tube which occluded blood flow (Fig. 4Bc, d) and a decrease in heart rate (Fig. 4C). Knockdown of E4BP4 also retarded cardiac cell growth.

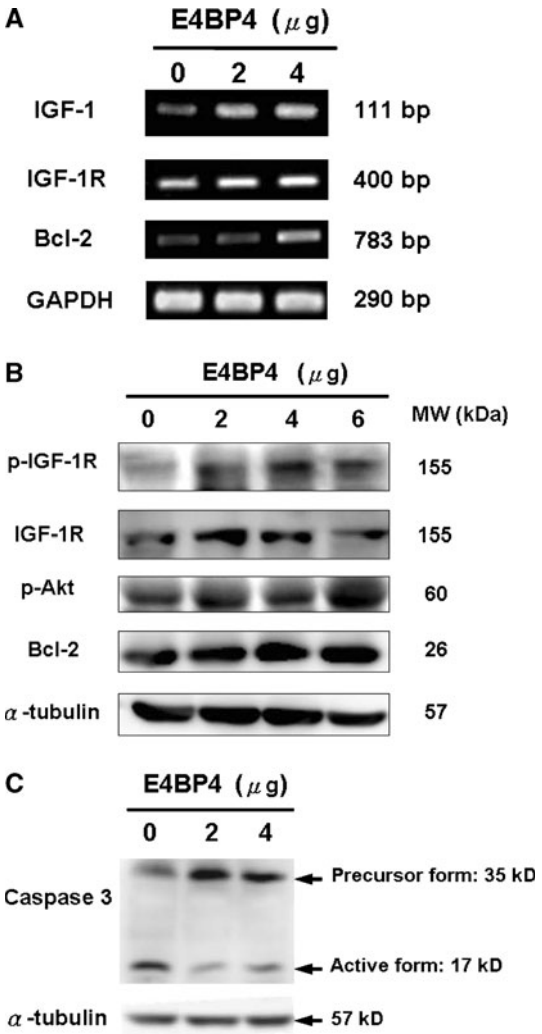

Fig. 3 Up-regulation of cardiac survival signalings and blockade of caspase 3 activation in E4BP4 overexpressed- rat primary cardiomyocytes. Transiently transfected E4BP4 with 0, 2, 4 or 6 μg in cardiomyocytes, and cell lysate was analyzed by **a** RT-PCR and **b**, **c** Western blot. *IGF-1* insulin-like growth factor-1, *IGF-1R* insulin-like growth factor-1 receptor. GAPDH and α-tubulin were used as internal control. All experiments were repeated thrice

These data suggest that E4BP4 plays a prominent role in heart development and in maintaining heartbeat.

Discussion

In this study, we found that E4BP4 proteins are expressed in cardiac tissue from many species. We also observed that E4BP4 proteins were overexpressed in diseased hearts. Moreover, the E4BP4 overexpression activated the cardiac survival signalings and inhibited the apoptotic proteins in cardiomyocyte. At last, siRNA-mediated knock down of E4BP4 revealed that E4BP4 plays as a key role in embryonic heart development.

The zebrafish (*Danio rerio*) model was originally used to study genetics and developmental biology; however, this model is now used in pharmacology, toxicology, and in the study of human disease [17–22]. Using the siRNA knock down technique, we found that E4BP4 deficiency led to malformations of the atrium and ventricle as well as a decrease in heartbeat. In normal zebrafish, the straight heart tube bent slightly in the first 36 h [23]. In E4BP4-deficient zebrafish, the heart tube was still straight at 72 h. It means that E4BP4 deficiency retards cardiogenesis during the pharyngula to early larval period (36–72 h). The initial rationale for the beat of the embryonic heart is to aid in subtle but significant aspects of cardiac growth, shaping, maturation, and to facilitate maturation of cardiac angiogenesis [24]. In this study, we showed that E4BP4-deficient zebrafish had a significantly lower-than-normal heart rate. E4BP4 deficiency led to occlusion of blood flow; therefore, we speculate that E4BP4 deficiency affects the developing circulatory system. E4BP4 acts like an embryonic gene in the heart, such as insulin-like growth factor II (IGF-II), and plays an essential role in embryonic heart development [25, 26]. Both of them have the characteristic that elevated protein level when hearts suffered from stress. We also found that the level of hematopoiesis decreased (data not showed), a finding similar to that of GATA-1 deficiency [27, 28].

More than 80% of acute myocardial infarctions are due to coronary atherosclerosis with superimposed luminal thrombi. Myocardial ischemia shares features with myocyte necrosis, such as that caused by inflammation [29]. Necrosed myocardium is replaced by fibroblasts and type III collagen; the resulting increased left ventricular (LV) chamber stiffness leads to diastolic dysfunction [30–32]. We propose that E4BP4 is overexpressed in myocardial infarct lesions to blockade inflammatory effect and blockade the disease aggravation. The development of heart disease comprises several complicated events, including myocyte hypertrophy; cell apoptosis and necrosis; excessive accumulation of interstitial matrix; chamber dilatation; fibrosis and scar formation, and continued deterioration of cardiac function [33]. E4BP4 and insulin-like growth factor-I (IGF-I) are overexpressed in disease heart [34]. We proposed that E4BP4 represents anti-apoptotic or anti-inflammatory effectors in heart disease progression. E4BP4 might play a role as a rescuer in heart disease progression. There were many reasons caused E4BP4 overexpression, IHC results revealed E4BP4 overexpression might related with aging and disease. The data we showed in Fig. 1 explained that E4BP4 expressed widely in many mammalian hearts, this hinted somewhat important in hearts. The protein level should not be compare because of the individual different.

NFIL3/E4BP4, which is downstream of the IL-3 receptor, functions to block programmed cell death [4].

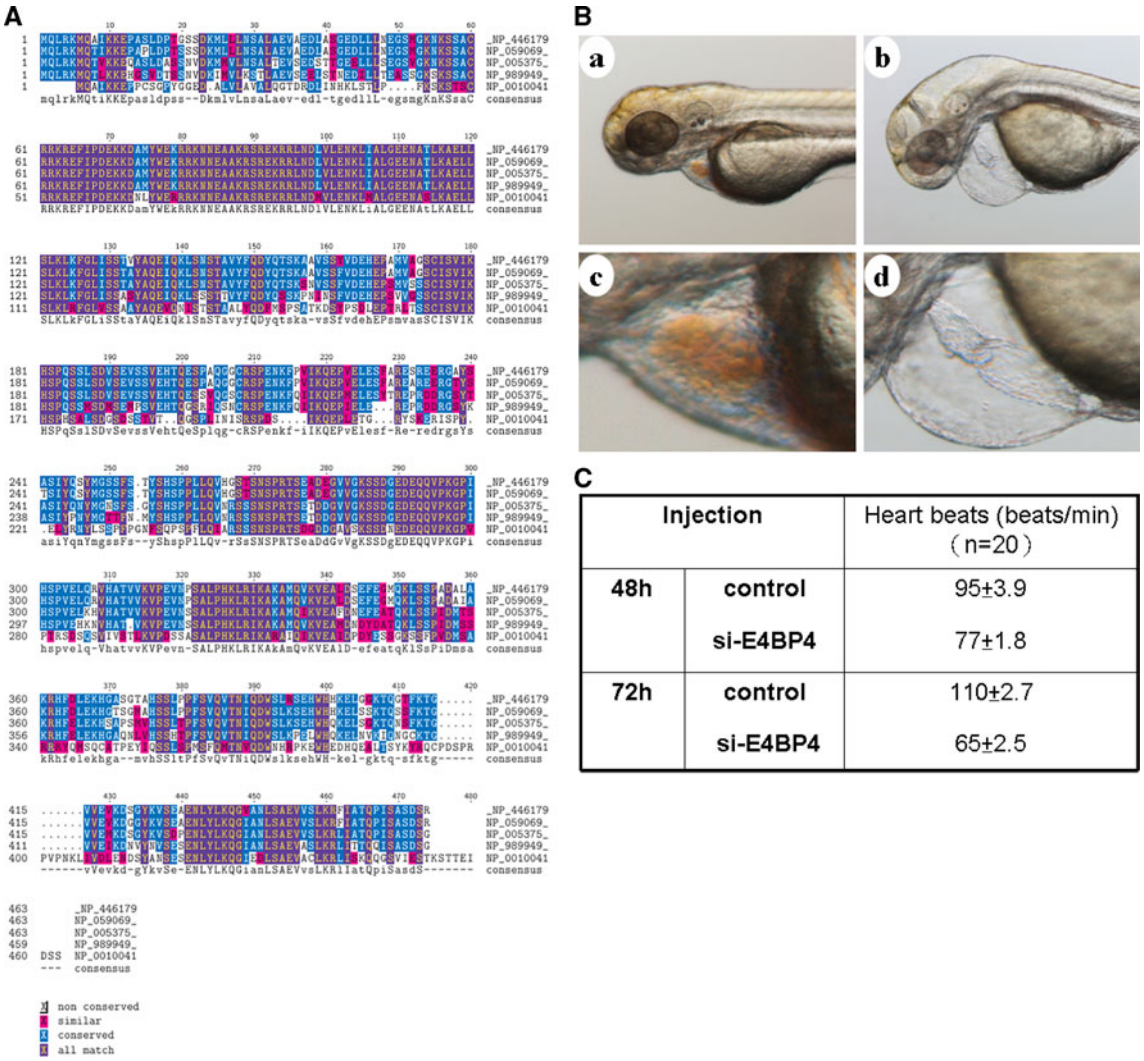

Fig. 4 Changes of heart beats and heart looping after siRNA-mediated knock down of E4BP4 in zebrafish. **A** Comparison of the E4BP4 amino acid sequence homology between chicken (NP_989949), human (NP_005375), mouse (NP_059069), Rat (NP_446179), and zebrafish (NP_001004120). Zebrafish E4BP4 contains several highly conserved domains: 60–122 amino acids were defined as bZIP region; 144–452 amino acids were defined as IL3 binding site region. **B** Knockdown of E4BP4 by microinjection (1.5 ng/embryo) into zebrafish in two-cell stage. *a* Injection of short DNA fragment as negative control. *b* 72 h after E4BP4 siRNA injection, showed malformed heart looping (straight heart tube) leading to incomplete heart function. *c*, *d* are heart area enlarged figure of *a*, *b*. **C** Changes of the zebrafish heart beats in 48–72 h after E4BP4 siRNA injection. Knocking down of E4BP4 led to a slower heart rate ($n = 20$). * $P < 0.05$, ** $P < 0.01$ significant difference

Constitutive activation of NFIL3 can block apoptosis in murine IL-3-dependent pro-B lymphocytes [35, 36]. In a cytokine-mediated anti-apoptotic response, GATA-1 transactivates the *E4bp4* gene, *Bcl-*X_L expression and improves cell survival [5, 6]. E4BP4 has also been shown to be a novel intrinsic regulator of motor neuron growth and survival, and shown to strongly enhance neuronal cell size and axonal growth [37]. There is abundant evidence that E4BP4 is an anti-inflammatory and anti-apoptotic factor [1, 3–6, 35–37]. The pathways regulated by E4BP4 are of potential interest both for understanding heart development and for promoting heart survival in pathological situations.

Conclusions

We conclude that E4BP4 is a cardiac survival factor and essential for embryonic heart development.

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