Depletion of Kindlin-2 induces cardiac dysfunction in mice

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Kindlin-2, a member of the Kindlin family focal adhesion proteins, plays an important role in cardiac development. It is known that defects in the Z-disc proteins lead to hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). Our previous investigation showed that Kindlin-2 is mainly localized at the Z-disc and depletion of Kindlin-2 disrupts the structure of the Z-Disc. Here, we reported that depletion of Kindlin-2 leads to the disordered myocardial fibers, fractured and vacuolar degeneration in myocardial fibers. Interestingly, depletion of Kindlin-2 in mice induced cardiac myocyte hypertrophy and increased the heart weight. Furthermore, decreased expression of Kindlin-2 led to cardiac dysfunction and also markedly impairs systolic function. Our data indicated that Kindlin-2 not only maintains the cardiac structure but also is required for cardiac function.

Kindlin-2, depletion, myocardial hypertrophy, cardiac dysfunction, mouse

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INTRODUCTION
Cardiomyopathy is one of the major causes for sudden death and progressive heart failure (Marsiglia and Pereira, 2014). Based on the morphological and functional changes, cardiomyopathy is mainly classified into hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). HCM is characterized by left ventricular hypertrophy accompanied by myofibrillar disarray and manifests as diastolic dysfunction of the cardiac ventricles. Whereas, DCM is characterized by a dilated ventricular cavity with systolic dysfunction (Ahmad et al., 2005; Grunig et al., 1998). Recently, components of the sarcomere such as the Z-Disc related proteins have been suggested to be involved in pathologic hypertrophy process of HCM. Mutations in the genes for sarcomeric proteins, such as MYH7 (cardiac β-myosin heavy chain), TNNT2 (cardiac troponin T) and ACTC (cardiac alpha-actin) have been revealed to be involved in the pathogenesis of HCM. The Z-disc plays important roles in sarcomeric organization and force transmission (Arad et al., 2002; Ervasti, 2003; Marian and Roberts, 2001). Defects in the Z-disc proteins lead to HCM or DCM (Vatta et al., 2003). Recent genetic investigations have revealed that mutations of Titin, muscle LIM protein MLP and ACTN2 cause HCM (Frank et al., 2006, 2007).

Kindlin-2, as one of integrin-interacting protein, mediates cell-cell and cell-matrix adhesions. There are three subfamily members, Kindlin-1, Kindlin-2 and Kindlin-3 in

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the Kindlin family. Compared to a restricted expression in epithelial cells for Kindlin-1 and in hematopoietic cells for Kindlin-3, Kindlin-2 is ubiquitously expressed (Meves et al., 2009; Zhan et al., 2014, 2015). Dowling et al. found that Kindlin-2 mainly expressed at the intercalated disc and costamere of the heart. Knockout of Kindlin-2 resulted in embryonic lethality at or before E7.5, suggesting that Kindlin-2 may play an important role in the regulation of heart development in mice (Dowling et al., 2008). However, the role of Kindlin-2 in cardiac muscle of postnatal mice remains unclear. In our previous investigation, we found that Kindlin-2 interacts with α-actinin-2, an important structural component of the Z-disc (Qi et al., 2015). Kindlin-2 is required for maintaining the integrity of the Z-disc by forming a complex with α-actinin-2 and integrin β1. Depletion of Kindlin-2 causes disruption of the Z-disc (Qi et al., 2015). Given that mutations of the Z-disc proteins have been implicated in HCM, Kindlin-2 may be involved in the pathogenesis of hypertrophy. To test this idea, we depleted the endogenous Kindlin-2 in mice by injecting chemically modified small interference RNA (siRNA) of murine Kindlin-2 via tail vein. We found that depletion of Kindlin-2 in mice leads to the disordered myocardial fibers, fractured and vacuolar degeneration in myocardial fibers, features indicating that Kindlin-2 is an important molecule in maintaining the normal structure and function of the heart.

RESULTS

Depletion of Kindlin-2 in the heart affects the structure of cardiac myocytes but not other cell types

To knockdown the endogenous Kindlin-2 in mice we used small interference RNA (siRNA) specific to murine Kindlin-2, which was chemically modified and lasts long in mice. Two to four weeks after tail vein injection of Kindlin-2 siRNA or the control siRNA, Western blot analysis was performed. Result showed that Kindlin-2 siRNA effectively suppressed the expression of endogenous Kindlin-2 (Figure 1A). Further, we observed a serial change of cardiac structure with or without Kindlin-2 siRNA treatment at different time points by immunohistochemical staining using α-actinin-2 antibody. Two weeks after Kindlin-2 knockdown, the structure of the Z-disc is normal. However, at weeks 3–4, the structure of the Z-disc is disrupted (Figure 1B and C). Since Kindlin-2 expression is not just restricted to cardiac myocytes, we set to determine the expression of Kindlin-2 in other cardiac cell types including endothelial cells, fibroblasts and smooth muscle cells. Similar to cardiomyocytes, the expression of Kindlin-2 were also decreased in smooth muscle cells, fibroblasts and vascular endothelial cell (Figure 2 left). However, depletion of Kindlin-2 did not lead to the obvious structure changes of these cells (Figure 2 middle and right). These data indicates that Kindlin-2 may mainly impact the structure and function of cardiomyocytes in the heart.

Figure 1 Injection of modified Kindlin-2 siRNA via tail vein leads to Kindlin-2 depletion in the mouse heart. Kindlin-2 siRNA was injected into the tail vein of ICR mice. Hearts were dissected at different time points. A, The depletion efficacy of Kindlin-2 siRNA was detected in cardiac muscle by Western blot analysis using indicated antibodies. B, Kindlin-2 associated protein α-actinin-2 was examined by immunohistochemical staining in cardiac sections (2–4 weeks) of mouse heart with Kindlin-2 depletion by siRNA. Bars: 20 μm. C, Higher magnification of the boxed areas from B.
Figure 2  Depletion of Kindlin-2 in the heart affects the structure of cardiac myocytes but not other cell types. Immunofluorescent staining was carried out to show smooth muscle cells (α-SMA) (A), fibroblasts (Vimentin) (B) and vascular endothelial cells (CD31) (C) in sections of cardiac muscle treated with control siRNA and Kindlin-2 siRNA in mice. Left line showed image of Kindlin-2 staining, the middle line showed low-magnification image of α-SMA, Vimentin and CD31 staining respectively. The right line showed higher magnification of the boxes area from B.

Depletion of Kindlin-2 induces cardiac myocyte hypertrophy

It was known that defects of the Z-disc proteins lead to hypertrophy, and we have determined that Kindlin-2 is one of structure proteins of the Z-disc. Thereby we wanted to investigate whether knockdown of Kindlin-2 may cause hypertrophy phenotype in mice. Hematoxylin and Eosin (H&E) staining was performed and result showed that Kindlin-2 knockdown resulted in myocardial fibers disordered arrangement, vacuolar degeneration and distributed unevenly (Figure 3A). Interestingly, compared with myocardium of control siRNA injected mice, cross section of
myocardium of Kindlin-2 siRNA injected mice showed that myofibers are thicker (Figure 3B), and the number of myoccardial cells per unit area was counted and result showed that the cardiomyocyte density was decreased in Kindlin-2 siRNA-treated cardiac muscle (Figure 3C). To examine the changes of cardiomyocyte size, DIO perchlorate was used to label the cell membrane of cardiomyocytes (Figure 3D) and the size of the cells was measured by quantifying the average diameter of cardiomyocytes. As showed in Figure 3E, the average diameter of cardiomyocytes of Kindlin-2 siRNA-treated mice is larger than that of the control siRNA-treated mice. These results indicated that depletion of Kindlin-2 may induce cardiac myocyte hypertrophy.

Depletion of Kindlin-2 increases the weight of the heart in mice

Given that hypertrophic cardiac myocytes were induced by Kindlin-2 depletion, we then wanted to examine whether the body weight under Kindlin-2 depletion is changed in mice. We found that there was no obvious difference for the whole body weight between the two groups of mice with or without Kindlin-2 depletion (Figure 4A). However, when we detected the ratio of heart weight to body weight (mg g⁻¹) and also the ratio of heart weight to the tibia length (mg mm⁻¹) we found that the ratios are higher in Kindlin-2 depleted group than the control group (Figure 4B and C). These results demonstrated that depletion of Kindlin-2 increased the heart weight in mice. The increased ratio of heart weight/body weight might be caused by concentric hypertrophy with accompanying cellular hypertrophy or by the dilation.

Depletion of Kindlin-2 causes cardiac dysfunction

Given that Kindlin-2 depletion caused myocardial fibers disordered arrangement and hypertrophy, it is possible that Kindlin-2 depletion may affect cardiac function. To this end, echocardiography analysis was performed in control siRNA or Kindlin-2 siRNA-treated mice. As showed in Figure 5A, while Kindlin-2 siRNA treating time was prolonged, diastole of left ventricular posterior wall (diastole, LVPWd) increased significantly. We thus continued to examine the ejection fraction (%EF) and ventricular shorting fraction (%FS) as indicators of ventricular contractility. Results showed that both %EF and %FS were reduced over time (Figure 5B and C), suggesting that Kindlin-2 depletion markedly weaken cardiac systolic function.

Since Kindlin-2 levels were also decreased in smooth muscle cells and vascular endothelial cells upon Kindlin-2 siRNA treatment, we detected the depth of aorta anterior and posterior wall and diameter of aorta root by echocardiography analysis in the control or Kindlin-2 siRNA-treated mice. As showed in Table 1, there was no obvious difference between the two groups of mice. In addition, we as-
Figure 4  Depletion of Kindlin-2 increases the weight of heart. A, The body weight (g) was determined in control or Kindlin-2 siRNA-injected mice. B, The ratio of heart weight to body weight (mg g$^{-1}$) was determined in control or Kindlin-2 siRNA-injected mice. C, The ratio of heart weight to tibia length (mg mm$^{-1}$) was determined in control or Kindlin-2 siRNA-injected mice. Data are shown as mean±SD. **, *P*<0.01 by Student’s *t* test.

Figure 5  Depletion of Kindlin-2 impairs heart function. Control or Kindlin-2 siRNA were injected every other day and the echocardiographic parameters were measured at days 7, 14, 21 and 28. A, LVPPWd, left ventricular posterior wall (diastole); B, EF, left ventricular ejection fraction. C, FS, left ventricular shorting fraction; Values are means±SD. from 9 animals. *, *P*<0.05, by Student’s *t* test.

Table 1  Echocardiographic parameters of aorta in different group

| Group                  | ARd (mm) | AAWd (mm) | APWd (mm) |
|------------------------|----------|-----------|-----------|
| Control siRNA (n=9)    | 1.4±0.02 | 0.16±0.02 | 0.18±0.04 |
| Kindlin-2 siRNA (n=9)  | 1.37±0.03| 0.17±0.01 | 0.19±0.03 |

Table 2  Values of bleeding time (BT) and prothrombin time (PT) in different group

| Group                  | BT(s)      | PT(s)      |
|------------------------|------------|------------|
| Control siRNA (n=9)    | 42.02±1.204| 12.91±0.32 |
| Kindlin-2 siRNA (n=9)  | 46.30±1.906*| 13.98±0.141*|

DISCUSSION

Our previous studies have demonstrated that Kindlin-2 expressed in the Z-disc and knockdown of Kindlin-2 disrupted the structure of the Z-Disc (Qi et al., 2015). Due to the early embryonic lethality of Kindlin-2 KO mice, in this study, we used chemically modified siRNA to deplete the endogenous Kindlin-2 in adult mice. The modified siRNA could enhance the stability of siRNA and prolongs the silencing effects in vivo. We found that the protein level of Kindlin-2 is obviously decreased in the heart after tail vein injection of Kindlin-2 siRNA within two weeks. It may be an aid that intravenous injection transports siRNA to the heart first, therefore the depletion efficiency of Kindlin-2 in the heart is higher than other organs (Figure S1 in Supporting Information). Since Kindlin-2 expression is not only restricted to cardiac myocytes, we also detected the expression of Kindlin-2 in other cell types in the heart, including endothelial cells, fibroblasts and smooth muscle cells. Similar to cardiomyocytes, Kindlin-2 levels were also decreased in those cells. However, downregulation of Kindlin-2 did not lead to obvious structural changes of these cells. Multiple lines of evidences pointed out that Kindlin-2 plays a crucial role in vascular endothelial cells. For example, knockdown assessment of hemostatic function of Kindlin-2 siRNA-treated mice, and result showed that bleeding time (BT) and prothrombin time (PT) were both slightly increased (approximately 10% and 8%) in Kindlin-2 treated mice (Table 2). Based on these observations we believed that the cardiac dysfunction caused by Kindlin-2 depletion is mainly due to the direct Kindlin-2 effect on cardiomyocytes.
of Kindlin-2 in endothelial cells led to a defect in cell adhesion, spreading, and migration mediated by β3 integrin in vitro (Ma et al., 2008). Kindlin-2 knockout mice showed significantly reduced angiogenesis in growing tumors. Aortic endothelial cells (ECs) isolated from the Kindlin-2+/-mice showed reduced activation of β3 integrin. VEGF and PLGF did not induce intracellular signaling in Kindlin-2+/-ECs and VEGF did not rescue aberrant angiogenesis (Pluskota et al., 2011). Pluskota et al. also found that Kindlin-2 regulates homeostasis by controlling endothelial cell-surface expression of ADP/AMP catabolic enzymes. Bleeding time was prolonged in Kindlin-2+/-mice (Pluskota et al., 2013). In our study, we found that the level of Kindlin-2 is decreased in vascular endothelial cells, but aorta function detected by echocardiography analysis showed normal. In addition, we also assessed hemostatic function of Kindlin-2 siRNA-treated mice, and found that bleeding time (BT) and prothrombin time (PT) were increased upon Kindlin-2 knockdown. However, due to the fact that myocardium is highly perfused by a range of smaller caliber vessels that can be affected by the Kindlin-2 reduction. If these vessels are affected, the oxygen supply to the cardiac muscle will be altered and this could greatly affect the end phenotype. Therefore, to unequivocally establish the connection between Kindlin-2 and cardiac function, myocyte-specific knockout mice for Kindlin-2 will be a good model system. We are currently working on the establishment of cardiac myocyte-specific knockout for Kindlin-2 in mice. We will thereby confirm what we found in the present investigation.

Dowling et al. found that knockdown of Kindlin-2 in cardiac muscle results in the failure of myofibrils attachment to the cellular adhesive complexes (Dowling et al., 2008). A similar function has been demonstrated for Unc-112, the homolog of Kindlin-2 in C. elegans. Migfilin is an interacting protein of Kindlin-2 that expresses in cardiomyocytes and is able to associate with actin by binding with filamin and vasodilator-stimulated phosphoprotein (VASP). Thus Kindlin-2 may associate with actin by interacting with migfilin and VASP (Zhang et al., 2006). The Z-Disc is a highly organized three-dimensional structure containing several proteins assembled in multi-protein complexes. For example, α-actinin-2 is a major structural protein of the Z-Disc, plays an essential role in maintaining the structure and function of the Z-Disc (Yang and Xu, 2012). In our previously study, we demonstrated that Kindlin-2 interacts with α-actinin-2 by its FERM domain (Qi et al., 2015). All of these studies suggest that Kindlin-2 has an important role in maintaining the structures of the sarcomere and the Z-Disc.

It has been known that many genes encoding the Z-Disc proteins and mutations of these genes, for example, mutations of the α-actinin-2 encoding gene ACTN2 could leads to cardiomyopathies (Bagnall et al., 2014). In the Z-Disc, ACTN2 binds to a variety of proteins including ALP-encoded actinin-associated LIM protein and CAPZ-encoded actin capping protein (Bagnall et al., 2014). ALP and CAPZ are excellent candidates accounting for HCM. Interestingly, disruption of VASP also causes dilated cardiomyopathy in mice. Moreover, mutations in ILK were recently uncovered in patients with dilated cardiomyopathy (Traister et al., 2013).

Although Kindlin-2 has not been reported to be involved in cardiomyopathy, Kindlin-2 interacting proteins were shown clearly participating in the pathogenesis of cardiomyopathy. Therefore, it is possible that Kindlin-2 may involve in cardiomyopathy. We tested this hypothesis by depletion of Kindlin-2 to investigate the function of Kindlin-2 in mice. Our data showed that depletion of Kindlin-2 results in cardiac muscle hypertrophy and influences cardiac systolic function.

Although the evidence demonstrated that genes encoding the Z-Disc proteins are involved in the pathogenesis of cardiomyopathy (Mu et al., 2014; Wang et al., 2010), the molecular mechanisms underlying this phenotype are still unclear. In response to chronic pressure the α-actinin-binding muscle LIM protein (MLP) translocates from the cytoplasm to the nucleus. MLP in the nucleus stimulates myogenic differentiation through binding to the muscle-specific transcription factor MyoD, which enhances transcriptional activity of the downstream targets (Wang et al., 2014; Yu et al., 2013). In our previous study, Kindlin-2 translocates into nucleus to form a tripartite complex with active β-catenin and TCF4 and regulates myogenic regulatory factor myogenin via a canonical Wnt/β-catenin signaling (Wu et al., 2015; Yu et al., 2013). Future investigations warrant to elucidate the molecular mechanisms and signaling events that are associated with cardiac muscle development and cardiomyopathy.

In conclusion, our study demonstrated that Kindlin-2 not only functions as a structural protein at the Z-disc but also involves in the pathological process of cardiac dysfunction.

MATERIALS AND METHODS

Animal model

All experiments were approved by Peking University Ethics Committee for Animal Experiments. Six to eight-week-old male ICR mice (weighting 20–25 g) were housed at Peking University Animal Facility. Mice were divided into two groups and were injected with control siRNA or Kindlin-2 siRNA respectively. Injection of Kindlin-2 siRNA or control siRNA into mice was performed as described previously (Wei et al., 2013). Briefly, synthetic Kindlin-2 or control siRNA (5 nmol L⁻¹) was rapidly injected (within 10 s) into the mouse tail vein (twice a week). Mice were euthanized after 1 to 4 weeks of Kindlin-2 or control siRNA injection. Control siRNA or Kindlin-2 siRNA was designed and synthesized by Ribobio Co. (Guangzhou). Sequences of RNA
interference (RNAi) oligonucleotides were as follows: Control siRNA, sense: 5′-UUCUCCGAACGUGUCAGUTT-3′; Kindlin-2 siRNA, sense: 5′-AAGUUGUGGAAAACUG- CGAUGTT-3′.

**Histology**

Hearts were fixed with 4% paraformaldehyde overnight at 4°C and embedded in Optimal Cutting Temperature compound (O.C.T). Serial sections (6 μm) were stained in Hematoxylin and Eosin (H&E) and Iron Hematoxylin for morphometric analysis. To determine the cell size, the sections were stained with 3,3′-Dioctadecyloxacar- boyamine perchlorate (DIO) (Beyotime Co, Beijing) at the concentration of 2 μmol L⁻¹ for 5 min at room temperature and then incubated with DAPI for 2 min for the detection of nuclei. The cell membrane was detected as green fluorescence by confocal microscopy (Leica, Germany). The average diameter of cardiomyocyte was analyzed with image analysis software. All measurements were averaged from three slices. All data were expressed as mean±SD. Statistical analysis was performed using Student t test. A probability of P<0.05 was considered to be statistically significant.

**Immunofluorescent staining and confocal microscopy**

Hearts were fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Serial sections (6 μm) were stained then incubated with 1:100 dilution of anti-CD31(Santa Cruz), or 1:200 dilution of anti-vimentin and 1:50 anti-α-smooth muscle actin (Epitomics) antibodies overnight at 4°C and then with a 1:100 dilution of Alexa Fluor 488 or 568-conjugated IgG (Invitrogen) for 1 h at room temperature. The images were captured with a TCS SP5 confocal microscope (Leica, Germany).

**Echocardiographic analysis**

Echocardiography images were obtained by use of a Vevo 770TM Imaging System (Visual Sonics Inc, Canada) (Huang et al., 2013). Briefly, mice were anesthetized with 1.5%–2.0% isoflurane (Baxter Healthcare Corp, USA) and maintained in a heating pad to keep the body temperature within a narrow range (37.0°C±0.5°C). Caution was taken not to apply excessive pressure over the chest as this will distort the signal. Two dimensional parasternal short-axis imaging at the level of the papillary muscle was used as a guide to obtain a left-ventricular M-mode tracing. Left ventricular posterior wall (diastole, LVPWd), left ventricular shorting fraction (%FS) and left ventricular ejection fraction (%EF) were calculated. All measurements were averaged from three consecutive cardiac cycles. All data were analyzed off-line at the end of the study with software resident on the ultrasound system. All parameters were expressed as mean±SD. Statistical analysis was performed using t test. A probability of P<0.05 was considered to be statistically significant.

**Bleeding time (BT) and prothrombin time (PT)**

Bleeding time (BT), a small incision was made in the middle of the tail with blade. The time from the moment blood to emerge from the wound until cessation of blood flow was recorded.

Prothrombin time (PT), Firstly, hold the mouse neck skin with left hand, gently pressed mice on the experimental platform to keep the mouse in a lateral decubitus position. Then, try to make the eye proptosis and quickly remove one of eye ball with the tweezers. Blood was collected into a 1.5 mL Eppendorf tube. After 2 h at room temperature, the blood was centrifuged at 1,000 rpm for 15 min. The supernatant was obtained as plasma. PT assay was performed according to the instruction from assay kit (Ji Ning, Shanghai).

**Compliance and ethics** The author(s) declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Figure S1 Depletion of Kindlin-2 in various organs in mouse with tail vein injection of siRNA.

The supporting information is available online at life.scichina.com and link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.