A mutation in NFkB interacting protein 1 results in cardiomyopathy and abnormal skin development in wa3 mice

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We have identified waved 3 (wa3), a novel recessive mutation that causes abnormalities of the heart and skin. The cardiac defect results in a severe and rapidly progressive dilated cardiomyopathy. We identified the gene mutated in these mice, which we call NFkB interacting protein1 (Nkip1), using positional cloning. Nkip1 is expressed in skin, heart and vascular endothelium and shares homology with a small family of proteins that play a role in the regulation of transcription factors. A C-terminal fragment of this protein was previously identified as the RelA associated inhibitor (RAI). We show that the full-length protein is larger than previously described, and we confirm that it interacts with NFkB in vivo. Expression analysis of genes known to be regulated by NFkB revealed that Intercellular adhesion molecule 1 (Icam1) expression is consistently elevated in mutant mice. This result suggests that wa3 mutant mice represent a potentially important model for the analysis of the role of inflammatory processes in heart disease.

INTRODUCTION

In recent years, tremendous progress has been made in identifying the genetic basis of familial hypertrophic and dilated cardiomyopathies (1). This has been complemented by studies of cardiac gene expression in model systems. One conclusion from these discoveries is that, although the phenotype of these respective disorders may be consistent, they can be caused by mutations that affect a wide spectrum of cellular functions including contractility, cytoskeletal integrity, signal transduction and apoptosis. Of note is that the contribution of defects in these genes to non-familial cardiovascular disease is not clear.

Recent studies in human populations have implicated inflammatory processes in the development of cardiac and peripheral vascular disease. It would be of tremendous utility if this potential disease pathway could be analyzed in a model system. Here, we report our discovery of a mutant mouse with a phenotype that includes a rapidly progressive cardiomyopathy. Positional cloning identified the affected gene as Nkip1. This is a putative repressor of NFkB, which is thought to mediate inflammatory pathways that contribute to cardiac disease. This phenotype-driven discovery has uncovered an entirely novel mechanism for the causation of cardiovascular disease.

RESULTS

We identified mice with a spontaneous mutation resulting in open eyelids at birth and abnormal coat (Fig. 1). On the basis of the similarity of the phenotype to waved 1 (wal) and waved 2 (wa2) mice, which both have open eyelids at birth and wavy hair (2,3), and the evidence from mapping data that this was a novel locus (discussed subsequently), we named our mutation waved 3 (wa3). Unlike wal mice,
homozygous \textit{wa3} mice on an inbred C57BL/6J (B6) background have reduced postnatal viability. Affected mice were born in Mendelian ratios and were of normal size, but most \textit{wa3/wa3} mice failed to thrive. Two-week-old \textit{wa3/wa3} mice were often only 60\% the weight of normal littermates (Fig. 1B) and fewer than 10\% of \textit{wa3/wa3} mice survived beyond 4 weeks (data not shown). Homozygous \textit{wa3} mice bred in a mixed genetic background had a greater survival rate and were fertile; however, affected dams often died suddenly during the post-partum period.

Although the open eyelid and curly hair phenotype in \textit{wa3/wa3} mice was fully penetrant, only subtle histological abnormalities were found in skin at birth. By postnatal day 8 (P8), differences in anagen follicle shape and position in the dermis were noted (Fig. 1C). Hair shafts from \textit{wa3/wa3} mice were consistently thinner than that of normal controls (Fig. 1D). This is most evident in awl hairs that were reduced in width, but not in length. No abnormalities in hair cuticles were found.

Necropsies of \textit{wa3/wa3} mice revealed hearts of abnormal shape and size (Fig. 2). The first visible abnormalities were small white patches on the ventricles of \textit{wa3/wa3} mice, which were evident within the first 2 weeks of life (Fig. 2C). Histologically, these lesions consisted of focal areas of myofiber degeneration, necrosis and mineralization. Usually this lesion first appeared on the anterior aspect of the right ventricle near the outflow tract and extended into the anterior aspect of the adjacent septum (Fig. 2B, D and F). The lesion was evident at birth (P0) in some of these mice and became progressively larger with age. It was accompanied by a maturing inflammatory response that consisted of macrophage infiltration, fibrosis and neovascularization. Thromboses of coronary arteries proximal to cardiomyocyte degeneration were occasionally noted. By P14, dilation was evident in the right ventricle, which is likely to be a secondary defect due to the extensive necrosis of the anterior free wall and septum (Fig. 2F). As the disease progressed, the size, number and distribution of affected regions increased. Although the appearance of the lesion initially on the anterior of the heart was consistent, the histological pattern of affected regions in more severely affected animals was quite variable, consistent with what

**Figure 1.** Phenotype of \textit{wa3} mice. (A) Mutant mice can be identified by their precocious open eyelids at birth. (B) Affected mice develop a waved coat by day 10 and are significantly smaller than normal mice by 2 weeks. (C) Skin histology shows abnormalities in the shape and orientation (arrow) of hair follicles in skin from \textit{wa3/wa3} mice. Hair shafts from \textit{wa3/wa3} mice are thinner than normal hair (D) Note the Auchene (AU) and Awl (AW) hairs are two cell diameters wide in mutant mice compared with three cell widths in controls. G, guard hair; Z, zigzag.
might be expected for a multivessel inflammatory coronary disease process.

Echocardiography of 8-week-old \(wa3/wa3\) mice showed a severe dilated cardiomyopathy characterized by dilatation of left ventricular chambers, left atrial enlargement and marked reduction in contractile (systolic) function (Table 1). Fractional shortening was \(24 \pm 14\%\) for mutant mice when compared with \(53 \pm 6\%\) for wild-type \((P < 0.001)\). Studies

Figure 2. Heart abnormalities in \(wa3\) mice. (A, C, E) Hearts from homozygous mutant mice develop pale, focal lesions that first appear on the right ventricle adjacent to the ventricular septum. (B, D, F) Transverse sections of ventricles reveal focal lesions of cardiomyocyte necrosis that appear initially in the right ventricular free wall and progress into the adjacent septum at later stages. Thromboses of coronary vessels were occasionally identified proximally to these lesions (B). Inflammation, mineralization and dilation accompany disease progression (F). (G) Eight-month-old \(wa3/wa3\) hearts show extensive necrosis, mineralization and dilation. (H) Masson trichrome staining of a right ventricular free wall showing fibrosis and a mature mural thrombus.
Table 1. Echocardiographic characteristics of wild-type, wa3/+ heterozygote and wa3/wa3 homozygote mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/+ (n = 3)</th>
<th>wa3/+ (n = 3)</th>
<th>wa3/wa3 (2-month-old) (n = 4)</th>
<th>wa3/wa3 (8-month-old) (n = 4)</th>
</tr>
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<tbody>
<tr>
<td>Age (weeks)</td>
<td>12 ± 4</td>
<td>10 ± 1</td>
<td>8 ± 2</td>
<td>38 ± 3</td>
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<tr>
<td>HR (bpm)</td>
<td>520 ± 9</td>
<td>562 ± 127</td>
<td>516 ± 25</td>
<td>489 ± 67</td>
</tr>
<tr>
<td>LVWT (mm)</td>
<td>0.77 ± 0.01</td>
<td>0.86 ± 0.05</td>
<td>0.87 ± 0.07</td>
<td>0.87 ± 0.06</td>
</tr>
<tr>
<td>LVDD (mm)</td>
<td>2.15 ± 0.05</td>
<td>2.78 ± 0.17</td>
<td>3.50 ± 0.59†</td>
<td>3.63 ± 0.24†</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.90 ± 0.13</td>
<td>1.16 ± 0.24</td>
<td>2.72 ± 0.90†</td>
<td>3.10 ± 0.25†</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>55 ± 6</td>
<td>58 ± 7</td>
<td>24 ± 14†</td>
<td>15 ± 4†</td>
</tr>
<tr>
<td>LA (mm)</td>
<td>1.50 ± 0.13</td>
<td>1.53 ± 0.02</td>
<td>1.77 ± 0.19†</td>
<td>1.80 ± 0.07†</td>
</tr>
</tbody>
</table>

LVWT, LV wall thickness; LVDD, LV end-diastolic diameter; LVFS, LV fractional shortening; LVPW, LV posterior wall thickness; LVSD, LV systolic diameter; LA, left atrium; HR, heart rate.

†Significant difference (P < 0.001) compared with +/+ mice.

Significant difference (P < 0.01) between wa3/+ and wa3/wa3 mice.

LVWT, LV wall thickness; LVDD, LV end-diastolic diameter; LVFS, LV fractional shortening; LVPW, LV posterior wall thickness; LVSD, LV systolic diameter; LA, left atrium; HR, heart rate.

Significant difference (P < 0.01) between wa3/+ and wa3/wa3 mice.

in older wa3/wa3 mice indicated a gradual progression of disease, with increased chamber dilation and further deterioration in contractile function, and without left ventricular hypertrophy.

The wa3 mutation was localized to proximal mouse chromosome 7 using interval haplotype analysis (4). We genotyped 20 affected F2 progeny from an intercross of F1 D2/C3H wa3/+ mice using 40 markers, and significant linkage was obtained with the marker D7Mit57 (X² = 45, P < 0.001). We then constructed a high-resolution genetic map by genotyping analysis of 86 affected mice from the same cross. Two recombinant haplotypes localized wa3 between D7Mit57 and D7Mit191, a region of the mouse genome that shares conserved synteny with human Chr. 19q13.2 (5). Informative SSLPs from the muscle creatine kinase gene (D7Nds6), dystrophia myotonica kinase (DM135) and a novel marker within Ercc2 genomic sequence (GenBank accession no. L47235) were used to refine the recombinant interval to <200 Kb (Fig. 3), representing in a single bacterial artificial chromosome that contains the flanking markers (GenBank accession no. AC073787).

We analyzed 21 mouse cDNAs localized to this region by hybridization to northern blots and found that expression of RelA associated inhibitor (RAI) (6) was reduced in hearts and skin from wa3/wa3 mice (Fig. 3E). Amplification of RAI cDNAs revealed that transcripts from mutant mice were larger than that of controls (Fig. 3). We sequenced transcripts and found that mutant cDNA was 92 bp larger than that of wild-type because of retained intronic sequence between exons eight and nine. Comparison of genomic DNA sequence reveals a 14 bp deletion in wa3 mice. This includes the splice donor site of exon 8, which accounts for the addition of intron sequence to the mutant transcript (Fig. 3).

Our analysis of the cDNA for the gene mutated in wa3 indicated that the reported transcript for RAI (GenBank accession No. AF078037), which encodes a 351 amino acid peptide, contains a frame-shifted region in its 5’ end and excludes a substantial N-terminal portion of the open reading frame (1). We used RT–PCR and 5’ RACE to characterize the full-length cDNA for the wa3 gene, revealing a transcript of ~3.2 kb that contains an 824 amino acid open reading frame (GenBank accession no. AY869711; Fig. 4). The insertion of intron sequence in the wa3 transcript introduces a premature stop codon that truncates the protein product (Fig. 4).

Comparison of the predicted full-length protein sequence to the non-redundant database using BLASTP revealed mammalian sequences with significant similarity, including p53 binding protein 2 (53BP2) and ASPP1 (7,8). These family members share high sequence similarity only at their C-termini, which include ankyrin repeats, and a C-terminal Src Homology 3 (SH3) domain. However, analysis of domain structure using the Simple Modular Architecture Research Tool (9) identified multiple additional protein motifs that are conserved along the length of these proteins, including alpha helices and several proline-rich regions (Fig. 4). In vitro studies of these proteins have identified interactions with p53, Bcl-2, protein phosphatase 1 and NFKB (7,10–12). As discussed subsequently, we have confirmed that the wa3 protein interacts with NFKB in vivo and have therefore named this gene NFKB-interacting protein 1 (Nkip1).

We confirmed the predicted protein product by generating polyclonal antibodies to an N-terminal peptide of Nkip1 that includes its proline-rich domains and to a C-terminal peptide that contains the ankyrin repeats and SH3 domain. We tested protein extracts by western blot analysis and found that both antibodies identify a 98 kD band in normal hearts, which is not detected in wa3/wa3 hearts (Fig. 6A). This demonstrates that the protein is the size predicted by our analysis and that the wa3 mutation results in the loss of its expression.

We analyzed Nkip1 expression by northern hybridization and found it is most abundant in skin, followed by testis, heart and stomach (Fig. 5). Nkip1 can be found in most epithelial cell types by RNA in situ analysis (Fig. 5B–D). Cardiovascular expression can be detected in adult and embryonic hearts (Fig. 5E). Atria of e15.5 embryos have a higher expression level than that of ventricles. High levels of expression can be detected in the endothelial cell layer of major arteries. This is most obvious in the aorta of adult mice (Fig. 5F) and is also evident in smaller vessels (see arrows in Fig. 5E). Skin expression is highest in anagen hair follicles and is restricted to epithelial cells (Fig. 5B). Expression was detected in all simple and stratified epithelia (Fig. 5B–D).

RAI was originally identified as an NFKB-interaction factor by yeast two-hybrid analysis using RelA and by immunoprecipitation of epitope-tagged constructs of RAI and RelA expressed in cultured cells (6). We sought to determine whether the full-length protein also interacted with p65 in vivo. Anti-Nkip1 antibodies were used to immunoprecipitate the wa3 gene product from skin lysates. Western analysis revealed that the immunoprecipitate also contained p65, proving that this gene interacts with NFKB in vivo (Fig. 6B). We also tested wa3/wa3 skin and found p65 was not co-immunoprecipitated using anti-Nkip1 antibodies (Fig. 6B).

We performed quantitative PCR on heart RNA from P0 mice to investigate whether the absence of Nkip1 activity in wa3/wa3 hearts altered the expression of genes whose transcription is normally regulated by NFKB. Icam1 expression is significantly higher (P < 0.0001) in wa3/wa3 hearts when
compared with normal hearts (Fig. 6C). This difference was not seen in all NFkB-regulated genes, including, for example, Anf or Vcam, suggesting that Nkip1 regulates a subset of these targets.

**DISCUSSION**

We report here our characterization of wa3, a spontaneous mutation that affects skin and heart development. These mutant mice have open eyes at birth and hair abnormalities similar to those found in wa1 and wa2 mice (which carry mutations in Tgfa and Egfr, respectively). However, wa3 mice also develop a reproducible pattern of focal cardiac necrosis at early ages, which progresses to a global and ultimately fatal dilated cardiomyopathy.

We used a positional cloning strategy and found that the wa3 mutation is due to a protein-truncating 14 bp deletion in a novel gene we call Nkip1, for NFkB interacting protein 1. This deletion is not present in the parental strain in which the wa3 mutation arose. As discussed subsequently, the 351 amino acid C-terminal portion of this gene was previously identified as the protein RAI. Our analysis of the Nkip1 transcript reveals an open reading frame of 824 amino acids with a predicted molecular weight of 98 kDa. As shown in Figure 4, this longer protein has a domain structure that is conserved among its mammalian paralogs and across species.

Affinity-purified antibodies generated against bacterially expressed peptides derived from either N- or C-terminal of the predicted protein identified the same 98 kDa band in extracts from skin and heart tissue (Fig. 5), supporting our conclusion that the full-length protein derived from this locus is much larger than the previously reported RAI peptide. As our northern and sequencing analysis would predict, this band is not detectable in extracts derived from wa3 mutant tissue (Fig. 6).
**Nkip1** is the third member of a gene family in mammals that includes tumor protein p53 binding protein 2 (TP53BP2) and protein phosphatase 1, regulatory (inhibitor) subunit 13B (PPP1R13B) (also called ASPP2 and ASPP1, respectively) (7,8). The function of these proteins is not well understood and they have only been evaluated previously in tissue culture. As noted previously, the C-terminal of *Nkip1* corresponds to RAI, which was originally found as an NFkB-interacting factor by yeast two-hybrid analysis (6). Our analysis confirms that this function is retained by full-length *Nkip1* and occurs in vivo, as NFkB can be co-immunoprecipitated from wild-type skin tissue using anti-*Nkip1* antibodies. The absence of *Nkip1* protein in *wa3/wa3* tissues results in a loss of co-immunoprecipitation of RelA, suggesting that these tissues may have defects in NFkB activity.

*Nkip1* family members are uniquely identified by the presence of three consecutive tryptophans in their SH3 domains; these have been proposed to convey binding specificity to these proteins (13). SH3 domains facilitate protein–protein interactions with binding sites that contain a core PXXP binding motif. The proline-rich region of *Nkip1* contains 28 PXXP motifs that may serve as intermolecular or intramolecular interaction sites. Family members also share ankyrin repeats. The second ankyrin repeat of 53BP2 has been shown to interact specifically with the DNA binding domain of p53 (14). Other NFkB repressors bind RelA via ankyrin repeats (15), raising the possibility that *Nkip1* may interact with RelA in a similar fashion.

Recently, Bergamaschi *et al.* (16) have suggested that RAI, which they call iASPP, inhibits the pro-apoptotic activity of 53BP2 through a direct interaction with p53. The significance of this result is unclear, as our data indicates that the full-length protein encoded by this locus is much larger than the RAI/iASPP peptide they studied. In addition, we cannot detect co-immunoprecipitation of p53 using antibodies against RAI/iASPP.
directed against Nkip1. Although this negative result is not definitive evidence that a direct interaction does not occur, it is consistent with previous results. An alternative explanation for the observation by Bergamaschi et al., (17) that antisense RNA treatment of human cells induces p53-dependent apoptosis, is that these actions are mediated through NFkB activity, which is also known to mediate the apoptotic activity of p53.

The epidermal phenotype of wa3/wa3 mice affects highly proliferative compartments of the skin including the advancing margins of the eyelid and the anagen hair follicle. Previous work has shown that NFkB negatively regulates proliferation and promotes differentiation of the epidermis and its appendages (18). Nkip1 may act in this process by fine tuning the affects of NFkB in the skin when additional proliferative capacity is required.

The ectodermal dysplasias also have abnormalities in epidermal appendage development (OMIM 305100 and 129500). Recent cloning of the X-linked and autosomal recessive forms of this disorder has implicated abnormalities in NFkB signaling (19,20). NFkB activity in humans has been linked to hypohidrotic ectodermal dysplasia, as mutations have been found in the TNF superfamily member ectodysplasin, its receptor EDAR and adapter molecule EDARADD, which require NFkB to function (21). As Nkip1 is a putative repressor of NFkB activity, it should affect similar tissues in the opposite fashion.

Figure 5. Expression analysis of Nkip1. (A) Multiple tissue northern blots show highest levels of expression in tissues affected in mutant mice. RNA *in situ* reveals that Nkip1 expression is restricted to simple and stratified epithelia (white staining on black background) including epidermis and hair follicles (B) the fore stomach and esophagus (C) and bladder epithelium (D). Heart expression is also detected in atria and ventricles (E) and in the endothelia of arteries but not veins (arrows). Analysis of postnatal day 9 (F) aorta shows that this expression is restricted to the endothelia.
The most striking aspect of the wa3 phenotype is the profound cardiomyopathy. Although heart development in these mice appears normal, the progressive postnatal deterioration of heart muscle is pronounced and ultimately lethal. Two human syndromes with skin and cardiac abnormalities have been described. Naxos disease (OMIM 601214) is due to mutations in plakoglobin (22), and dilated cardiomyopathy with woolly hair and keratoderma (OMIM 605676) can be caused by mutations in desmoplakin (23). Both of these proteins are principal components of desmosomes, which are particularly prominent in the epidermis and cardiac tissue and are important for cellular rigidity and strength. No defects in the expression of these genes have been detected in wa3/wa3 mice and desmosomes appear normal in wa3/wa3 hearts (data not shown) but abnormalities in other proteins associated with desmosomes cannot be excluded at this time.

The focal nature of the cardiac necrosis suggests that these mice have defects in cardiovascular perfusion. This idea is supported by our in situ studies that show high levels of Nkip1 RNA in vascular endothelia (Fig. 5). A potential explanation for the wa3 heart phenotype is that Nkip1 participates in the modulation of NFkB-specific responses to stress in endothelial cells. Loss of Nkip1 may result in an inappropriate activation of NFkB, as suggested by the evidence that Icam1 is markedly elevated in wa3/wa3 heart tissue, resulting in intravascular thrombosis and ischemic necrosis. This result is consistent with the observation that activation of NFkB in endothelia by oxidative stress or microbial infection will result in conversion from a quiescent, non-thrombogenic phenotype to an activated state that promotes inflammation, intravascular microthrombosis and ultimately leads to ischemic cardiomyocyte death (24). Also, repression of NFkB transcription in models of ischemia-reperfusion injury results in reduced cardiomyocyte death (24,25). The potential contribution of this inflammatory mediator to cardiac pathology has been well-described (26).
The importance of inflammatory processes in the causation of human cardiovascular disease has become increasingly apparent. Elevated ICAM1 in serum has specifically been found to be significantly associated with increased risk for myocardial infarction and for peripheral arterial disease (27–29). Our observations that wa3 mutant mice have elevated levels of Icam1 expression associated with thrombosis of coronary arteries, myocardial ischemia and progressive ventricular dilation suggest that it would be a useful model for studying the pathophysiology of this process and for testing potential anti-inflammatory and anti-adhesive therapies. Furthermore, Nkip1 represents a gene that specifically modulates an important inflammatory mediator in the heart; this result identifies a novel pathway that could contribute to heritable predisposition for cardiovascular disease, and it may be an appropriate candidate for association analysis in population studies.

METHODS

Mice

Mutant mice are maintained at Brigham and Women’s Hospital on a C57BL/6 (B6) background and a hybrid line containing B6 and DBA/2J (D2) alleles. C3H/HeJ (C3H), B6 and D2 mice were bred onsite, obtained from The Jackson Laboratory or Charles River. All animals were handled according to IACUC approved guidelines.

PCR

Primers to Ckmm and Dm15 were described previously. The marker D7Bwg1 was identified in mouse genomic sequence (L47235) and amplified with the primers: 5'-ATA CATTAAAGCCCCTACCTATCTTTACA-3' and 5'-TACCCA GCCCCTCTTGTGGTT-3'. Other informative genetic markers were selected from the WIGR mouse genetic map (www.genome.wi.mit.edu/mouse_index.html).

Mapping

Two mapping crosses were performed. First, 20 F2 progeny with open eyelids at birth were obtained from an intercross of F1 D2/C3H wa3/+ mice. Genomic DNA was isolated from tail biopsies as previously described (30). Informative markers from the ends of each chromosome were then tested as part of a haplotype analysis strategy (4). Linkage to proximal Chr. 7 was identified and 86 additional homozygous mutant mice were tested for informative markers spanning Chr. 7. Marker order was determined from previous data and full-length mouse genomic sequence (AC073787) (5).

Northern analysis

One-day-old and nine-day-old mice were sacrificed by CO₂ asphyxiation and heart and skin samples were snap frozen in liquid nitrogen. Total RNA was isolated from wa3/wa3 or normal tissue using the RNeasy purification system (Qiagen). Ten micrograms of total RNA was loaded on NorthernMax™ formaldehyde gels and run according to the manufacturers protocol (Ambion). RNA was transferred to a Genescreen™ plus membrane (NEN). Hybridizations were performed with Expresshybe (Clonetech) at 65°C and were washed to a stringency of 55°C in 0.1 x SSC and 0.1% SDS. Mouse cDNA clones were selected from their respective Unigene entries and purchased from Research Genetics. Mouse northern blots and cDNA panels were obtained from Origene technologies.

Sequencing

First strand cDNA was generated from 1 µg total heart RNA from wa3/wa3 and normal mice. Nkip1 was amplified from first strand cDNA generated from total mouse heart RNA with the following primers: 5'-CCATGGCACAGCGAAGC ATTCCAG-3' and 5'-CCTCAACAGACTTGTCTCCC-3', 5'-CAGAATGCTTTCTGGGAACATGG-3' and 5'-GTGCC AGTCAGATCTGGTT-3'. Amplified products were subcloned into PCR2.1 vector (Invitrogen) and sequenced. Sequence was assembled with the Sequenccher alignment program (Genecodes) and pairwise comparisons of mutant and normal sequences were performed. Primers flanking the eighth intron were used to amplify 50 ng of genomic DNA and were sequenced in a similar fashion.

Histology

Tissue samples from CO₂ asphyxiated mice were excised and fixed in Bouin solution for hematoxylin and eosin (H&E) sections or 4% paraformaldehyde for in situ analysis at 4°C for a minimum of 24 h. Tissues were paraffin embedded as described elsewhere (31) and sectioned at 6 µm. H&E staining and Masson trichrome staining were performed as described elsewhere (32).

In situ analysis

In situ analysis was performed using 10 µm paraffin-embedded sections prepared as previously described (30). Radiolabeled RNA probes were generated from a 3' fragment of Nkip1, subcloned in pT3T7 vector (Stratagene) and digested with NotI or Eco RI for T7 (antisense) or T3 (sense) transcription, respectively.

Echocardiography

Transthoracic echocardiography was performed using a 6–15 MHz linear array probe and a Sonos 5500 ultrasonograph (Hewlett-Packard, Andover, MA, USA) as described (1,32). In brief, two-dimensional images of the LV in short-axis and long-axis views were obtained with mice oriented in a left lateral decubitus or supine position. M-mode interrogation was performed using the short-axis two-dimensional images. LV end-diastolic (LVEDD) and end-systolic (LVESD) chamber dimensions and wall thickness were obtained from M-mode tracings using measurements averaged from three separate cardiac cycles. LV fractional shortening was calculated using the formula: \( \frac{LVEDD - LVESD}{LVEDD} \). Orthogonal left atrial dimensions were measured from two-dimensional images in the long-axis view.
Generation of Nkip1 antibodies
A 1395 bp fragment of the Nkip1 cDNA was generated with the primers 5'-CCATGGGACAGCAAGCATTCC-3' and 5'-CACAGACTTGTCCTCCAGGCG-3' corresponding to amino acids 1–443 of the translated product. An additional 1299 bp C-terminal fragment was generated with the primers 5'-CATGAATGGGCTTCTCA-3' and 5'-GCAMAAATCTAGTAGGCT-3'. Both fragments were subcloned into PC R2.1 (Invitrogen) and sequenced. Verified clones were subcloned into Pet30c R1 and subcloned into Pet30c (Novagen) and transformed into BL21(DE3)pLysS (Novagen). Cells were grown to an OD of 600 and induced with 1 mM IPTG and grown an additional 4 h. Cells were isolated, lysed and Nkip1 peptides were purified with a nickel column (Pierce). Purified protein was sent to Prosci (www.prosci.com) for generation of rabbit polyclonal antibodies according to the manufacturers protocols.

Western

Adult mouse heart was homogenized in 1 ml lysis buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1% NP-40, 1 mM DTT, 5% glycerol, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM PMSF). Tissue lysates were centrifuged for 20 min at 4°C. Supernatants were separated by SDS–PAGE (5–10 µl tissue lysate was loaded in each lane) and transferred to PVDF membranes. Blots were probed with affinity-purified Nkip1 antibodies, and bound antibodies were detected by SuperSignal chemiluminescence (PIERCE).

Rel A immunoprecipitation
Skin from the bodies of two 1-day-old mice was thoroughly homogenized in 1 ml lysis buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1% NP-40, 1 mM DTT, 5% glycerol, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM PMSF). Tissue lysates were centrifuged for 20 min at 4°C. Twenty micrograms of Nkip1 antibody was incubated with 40 µl protein G agarose bead for 2–4 h at 4°C, and washed four times with wash buffer (25 mM Tris–HCl, 0.15 M NaCl, pH 7.2). Tissue lysate was incubated with the antibody bound protein G agarose beads overnight at 4°C. Immunoprecipitate was washed six times with wash buffer and heated with same volume of 2 × SDS loading buffer at 100°C for 5 min. The heated mixture was run in SDS–PAGE for western detection.

Real-time quantitative PCR
Relative expression levels were determined by quantitative RT–PCR with the ABI PRISM 7000 sequence detection system. Genes tested included Nppa, Bcl2l1, Nppb, Gja1, Cxcl1, Icam1, Nfkbia, Il10, Mef2c, Mxs2, Myl2, Nmyc1, Tryp3, Tnf, Tnfsf6, Vcam1, Vegf, Hmger, Psmb2 and Chordc1. Each reaction contained 2.5 ng of cDNA, 5 µM forward and reverse primers and SYBR Green PCR Master Mix (Applied Biosystems). The primers used included: Icam1F 5'-CGCGAGGACCTTTAAGCTGACT-3', Icam1R 5'-CAGGGGACCATAGCGAGCTCT-3', VcamF 5'-CTAAAGGTCATGCATCTCCAGGAT-3', VcamR 5'-CTCGCGGGAAGGTCTATT-3' and AnfF 5'-AGCA GGTTCCTTGAATCCATCAG-3', AnfR 5'-CCAGGCT ATATTGAGCAAT-3'. (Other primer sequences available upon request.) Each sample was amplified 40 cycles. The cycle threshold (Ct) value for each template was normalized to the Ct values of the control gene: Psmb2F 5'-TGAAT GTGTCCTCCACAGCAG-3' and Psmb2R 5'-GCCCTCTCATG TCGTGTATAG-3'. Statistical analysis was performed with StatView for windows software. An analysis of variance (ANOVA) was conducted on the relative input amounts from day 1, heart and skin respectively, with the normal and wa3 phenotypes as between-subject factors.

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