Conditional Ablation of Nonmuscle Myosin II-B Delineates Heart Defects in Adult Mice

Xuefei Ma, Kazuyo Takeda, Aman Singh, Zu-Xi Yu, Patricia Zerfas, Anthony Blount, Chengyu Liu, Jeffrey A. Towbin, Michael D. Schneider, Robert S. Adelstein, Qize Wei

Rationale: Germline ablation of the cytoskeletal protein nonmuscle myosin II (NMII)-B results in embryonic lethality, with defects in both the brain and heart. Tissue-specific ablation of NMII-B by a Cre recombinase strategy should prevent embryonic lethality and permit study of the function of NMII-B in adult hearts.

Objective: We sought to understand the function of NMII-B in adult mouse hearts and to see whether the brain defects found in germline-ablated mice influence cardiac development.

Methods and Results: We used a loxP/Cre recombinase strategy to specifically ablate NMII-B in the brains or hearts of mice. Mice ablated for NMII-B in neural tissues die between postnatal day 12 and 22 without showing cardiac defects. Mice deficient in NMII-B only in cardiac myocytes (B\textsuperscript{NMHC}\textsubscript{NMII-B} mice) do not show brain defects. However, B\textsuperscript{NMHC}\textsubscript{NMII-B}/B\textsuperscript{NMHC} mice display novel cardiac defects not seen in NMII-B germline-ablated mice. Most of the B\textsuperscript{NMHC}/B\textsuperscript{NMHC} mice are born with enlarged cardiac myocytes, some of which are multinucleated, reflecting a defect in cytokinesis. Between 6 to 10 months, they develop a cardiomyopathy that includes interstitial fibrosis and infiltration of the myocardium and pericardium with inflammatory cells. Four of 5 B\textsuperscript{NMHC}/B\textsuperscript{NMHC} hearts develop marked widening of intercalated discs.

Conclusions: By avoiding the embryonic lethality found in germline-ablated mice, we were able to study the function of NMII-B in adult mice and show that absence of NMII-B in cardiac myocytes results in cardiomyopathy in the adult heart. We also define a role for NMII-B in maintaining the integrity of intercalated discs. (Circ Res. 2009;105:1102-1109.)

Key Words: nonmuscle myosin II-B ▪ cardiomyopathy ▪ intercalated discs
regulated by the neural cell–specific nestin promoter to ablate NMHC II-B in the nervous system.\textsuperscript{11} In separate experiments, we crossed the NMHC II-B floxed mice with a line of mice expressing Cre recombinase under control of the α-mysosin heavy chain (αMHC) promoter to ablate NMII-B in cardiac myocytes.\textsuperscript{12} Below, we present results showing that NMII-B plays distinct physiological roles in the brain and heart and provide evidence that absence of NMII-B in the cardiac myocytes (and not in the nonmyocytes) results in myocyte enlargement and cardiomyopathy. Moreover, we demonstrate a role for NMII-B in the intercalated disc (ID) of adult mice.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Animals**

All experiments were conducted following animal protocols approved by Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. Nestin-Cre transgenic mice were from The Jackson Laboratory (no. 003771).

**Histology, Microscopy, and Immunoblotting**

Hematoxylin/eosin (H&E) and immunofluorescence staining, electron microscopy, and immunoblotting were performed as described previously.\textsuperscript{5}

**Measurement of the Cross-Sectional Area of the Cardiac Myocytes**

The size of cardiac myocytes was measured following wheat germ agglutinin staining using a Zeiss measuring tool.

**Echocardiography**

Echocardiography was performed using an Acuson Sequoia 256c imaging system with the 15L8 multifrequency transducer. Quantitation was performed using M-mode with Prosolv Software version 3.0.

**Electrocardiography**

Three-lead electrocardiograms were recorded with a model MAC 1200 (GE Medical Systems).

**Data and Statistical Analysis**

The data were expressed as means±SD. Student’s $t$ test was used to compare data between 2 groups.

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>Cre</td>
<td>Cre recombinase</td>
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<tr>
<td>DORV</td>
<td>double outlet of the right ventricle</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin/eosin</td>
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<td>ID</td>
<td>intercalated disc</td>
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<tr>
<td>MHC</td>
<td>cardiac myosin heavy chain</td>
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<td>NMII</td>
<td>nonmuscle myosin II</td>
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<td>NMHC</td>
<td>nonmuscle myosin heavy chain</td>
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<td>VSD</td>
<td>ventricular septal defect</td>
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**Results**

**Generation of Neural- and Cardiac-Specific NMHC II-B Knockout Mice**

To ablate NMHC II-B specifically in the brain or in the heart, we used a loxP/Cre recombinase strategy to delete exon 2, the first coding exon of Myh10 (see Figure 1). Using the gene-targeting method, we generated a mouse line designated B\textsuperscript{loxP}/B\textsuperscript{loxP} in which both the neomycin resistance (Neo') expression cassette and exon 2 of Myh10 are flanked by loxP sites (Figure 1c). B\textsuperscript{loxP}/B\textsuperscript{loxP} mice express normal amounts of NMHC II-B protein and are indistinguishable from wild-type mice. We crossed B\textsuperscript{loxP}/B\textsuperscript{loxP} mice with 2 different transgenic lines expressing Cre recombinase under control of different promoters. To ablate NMHC II-B in neural tissue, we used a mouse line expressing Cre recombinase with a nestin promoter and a nervous system–specific enhancer\textsuperscript{11} (Figure 1e) to generate B\textsuperscript{loxP}/B\textsuperscript{nest} and B\textsuperscript{nest}/B\textsuperscript{nest} mice. To ablate NMHC II-B in cardiac myocytes, we used a mouse line expressing Cre recombinase driven by the αMHC promoter\textsuperscript{12} (Figure 1f) to generate B\textsuperscript{loxP}/B\textsuperscript{αMHC} and B\textsuperscript{αMHC}/B\textsuperscript{αMHC} mice. The transgenic mouse lines nestin-Cre and αMHC-Cre are well characterized and demonstrate that nestin-Cre mice express functional Cre recombinase under control of different promoters. To ablate NMHC II-B in neural tissue, we used a mouse line expressing Cre recombinase with a nestin promoter and a nervous system–specific enhancer\textsuperscript{11} (Figure 1e) to generate B\textsuperscript{loxP}/B\textsuperscript{nest} and B\textsuperscript{nest}/B\textsuperscript{nest} mice. To ablate NMHC II-B in cardiac myocytes, we used a mouse line expressing Cre recombinase driven by the αMHC promoter\textsuperscript{12} (Figure 1f) to generate B\textsuperscript{loxP}/B\textsuperscript{αMHC} and B\textsuperscript{αMHC}/B\textsuperscript{αMHC} mice. The transgenic mouse lines nestin-Cre and αMHC-Cre are well characterized and demonstrate that nestin-Cre mice express functional Cre recombinase specifically in cardiac myocytes starting at E10.5\textsuperscript{11} and that αMHC-Cre mice express functional Cre recombinase specifically in cardiac myocytes starting at E9.0 and at high levels by E11.5.\textsuperscript{13}

![Figure 1](http://circres.ahajournals.org/)  
Figure 1. Schematic representation of the strategy used to generate neural- or cardiac myocyte–specific NMHC II-B knockout mice. a, Portion of the wild-type NMHC II-B gene locus including exon 2. b, Targeting construct. Neomycin-resistance cassette (Neo') and exon 2 are flanked by loxP sites. c, B\textsuperscript{loxP} allele is generated after homologous recombination. d, NMHC II-B–ablated allele (B\textsuperscript{nest} or B\textsuperscript{αMHC}) lacking exon 2 is generated in the presence of Cre recombinase. e, The Cre expression cassette under the control of nestin promoter in the neural-specific Cre transgenic mice. f, The Cre expression cassette under the control of the αMHC promoter in the cardiac myocyte–specific Cre transgenic mice. Black boxes in a, b, and c indicate exon 2; arrowheads, lox P sites.
Nestin-Cre–Specific Ablation of NMHC II-B in the Mouse Brain

Immunoblot analysis of Bnest/Bnest mice on postnatal day (P)18 shows that NMHC II-B protein is markedly reduced in the cerebellum (Figure 2A, compare lanes 1 and 2), as well as throughout the entire brain (data not shown) but not in the hearts of Bnest/Bnest mice (Figure 2A, lanes 3 and 4). Immunofluorescence staining using an antibody for NMHC II-B confirms that NMHC II-B protein is ablated in the cerebellum of these mice at P18 (Figure 2B). As shown in the figure, Purkinje cells in Bfl/Bfl mice express high levels of NMHC II-B (red; Figure 2B, a). However, the NMHC II-B protein level is significantly reduced in the cerebellar Purkinje cells of Bnest/Bnest mice (Figure 2B, compare a with c). In contrast, staining for calbindin (Purkinje cell marker) is unaltered in these cells (Figure 2B, compare b with f). Figure 2B (d and h) confirms the loss of NMHC II-B (yellow in d and green in h) in the cerebellar Purkinje cells. Both immunoblot analysis and immunofluorescence staining confirm that NMHC II-B protein is also significantly reduced in the cerebral cortex of Bnest/Bnest mice (data not shown).

All of the Bnest/Bnest mice die between P12 and P22 as a result of a severe hydrocephalus, which causes enlargement of the lateral ventricles and a paper-thin cortex, with absence of most brain cortical tissue (Figure 2C, compare b and d). Figure 2C (c) also shows an underdeveloped cerebellum (ellipse), which correlates with defects in motor activity in these mice and which was also seen in hypomorphic mice that have a point mutation in NMHC II-B,14 The arrows in Figure 2C (c) point to deformities following the decompression of the lateral ventricles and loss of cerebral–spinal fluid. Of note is our finding that, similar to hypomorphic mice with a point mutation in NMHC II-B,15 the spinal canal of Bnest/Bnest mice is completely obliterated at P7 (Figure 2D). This is consistent with a role for NMII-B in cell–cell adhesion in the spinal canal.

Sectioning of the hearts confirms that there is none of the cardiac abnormalities in the Bnest/Bnest mice that are found in B+/B− mice. Specifically, there is no evidence for a ventricular septal defect (VSD), double outlet of the right ventricle (DORV), myocyte hyper trophy, decreased myocyte number, or increased cardiac myocyte binucleation, as seen in B+/B− mice.8,16 This demonstrates that ablation of NMHC II-B in the nervous system does not affect cardiac development. B+/Bnest mice appear normal in all respects.

Pathological Changes in the Heart Following αMHC-Cre–Specific Ablation of NMHC II-B

To address the role of NMII-B in heart development and in the adult mouse, we crossed the Bfl/Bfl mice with αMHC-Cre mice (Figure 1f), so that ablation would occur specifically in the cardiac myocytes starting at midgestation or after approximately E11.5 to avoid the early lethality found in germline-ablated B+/B− mice. Similar to other investigators who have used this particular line of mice expressing Cre recombine,17 we found no adverse effects of the enzyme on the tissues in which it was expressed (see also Figures 4 and 6 and Online Figure I). The NMII-B level is significantly reduced in the hearts of NMC/BαMHC/BαMHC mice compared to Bfl/Bfl mice at P0, as demonstrated in the immunoblot in Figure 3A. However, the NMII-B level is not affected in the brain of BαMHC/BαMHC mice (Figure 3A). The presence of residual NMHC II-B in the heart of BαMHC/BαMHC mice can be attributed to nonmyocytes in the heart, which continue to express wild-type amounts of NMHC II-B. Immunofluorescence staining of E13.5 mouse heart sections using antibodies to NMHC II-A, II-B, and II-C helps to clarify the ablation of NMHC II-B from the myocytes alone. It also demonstrates that ablation of NMHC II-B in cardiac myocytes has no effect on NMHC II-A and II-C expression. Figure 3B (a and d) shows that NMHC II-A (green) is only present in the nonmyocytes in the heart. This is indicated by the lack of green signal coincident with desmin (red) in the cardiac myocytes, because NMHC II-A is not present at this age in these cells. In contrast, in Figure 3B (b), NMHC II-B (green) and desmin (red) costain the myocytes (yellow) and stain the
Figure 3. NMHC II-B expression levels in B<sup>MHC</sup>/B<sup>MHC</sup> mice at E13.5. A, Immunoblots of whole tissue lysates from the heart and the brain of B<sup>MHC</sup>/B<sup>MHC</sup> were probed using antibodies specific for NMHC II-B, actin, or β-tubulin, as indicated. Note that the NMHC II-B protein level does not significantly change in the brain of B<sup>MHC</sup>/B<sup>MHC</sup> mice. In contrast, the NMHC II-B protein level is significantly reduced in the heart of B<sup>MHC</sup>/B<sup>MHC</sup> mice. B, Immunofluorescence staining of heart sections from B<sup>MHC</sup>/B<sup>MHC</sup> (a through c) and B<sup>MHC</sup>/B<sup>MHC</sup> (d through f) mice at E13.5 using antibodies specific for NMHC II-A (green) (a and d), NMHC II-B (green) (b and e), NMHC II-C (green background) (c and f), and desmin (red) (all images). Desmin is a marker for cardiac myocytes. Note the costaining of NMHC II-B and desmin in the cardiac myocytes (yellow) (b). NMHC II-B staining in the myocytes disappears after ablation but persists in the nonmyocytes (arrows) (b and e). c and f confirm the low expression of NMHC II-C in the heart at this age. Note that there is no change in the staining for NMHC II-A and II-C in the B<sup>MHC</sup>/B<sup>MHC</sup> heart. DAPI (blue) stains the nuclei.

nonmyocytes green, showing that in the B<sup>MHC</sup>/B<sup>MHC</sup> heart NMHC II-B is expressed in both cell types. The arrows are pointing to the nonmyocytes (green). However, in the B<sup>MHC</sup>/B<sup>MHC</sup> heart the cardiac myocytes now appear red because NMHC II-B has been ablated, but desmin remains. Again, the arrows point to the nonmyocytes, which stain green for II-B, indicating that NMII-B is not ablated in nonmyocytes. Figure 3B (c and f) shows that there is very little NMHC II-C in these hearts and that this does not change after II-B is ablated.

H&E staining on the day of birth (P0) (Figure 4) shows evidence of an increase in the size of the cardiac myocytes in B<sup>MHC</sup>/B<sup>MHC</sup> hearts (Figure 4f) compared to B<sup>B</sup>/B<sup>MHC</sup> hearts (Figure 4c). Figure 4f also shows examples of abnormally shaped nuclei (arrows), reflecting an abnormality in cytokinesis attributable to the loss of NMHC II-B. A VSD in a B<sup>MHC</sup>/B<sup>MHC</sup> heart (Figure 4d and enlarged in 4e) is also shown. These abnormalities were not seen in B<sup>B</sup>/B<sup>B</sup> or B<sup>B</sup>/B<sup>MHC</sup> mice (Figure 4a and 4b). Unlike B<sup>B</sup>/B<sup>B</sup> mice, in which the heart phenotype of a membranous VSD and DORV is almost 100% penetrant, only 2 of 9 B<sup>MHC</sup>/B<sup>MHC</sup> mice examined were born with a VSD and neither displayed a DORV. We did not see any difference in the deletion of NMII-B in the B<sup>MHC</sup>/B<sup>MHC</sup> cardiac myocytes with or without the presence of a VSD. However, 5 of 9 newborn B<sup>MHC</sup>/B<sup>MHC</sup> mice examined had an obvious increase in cardiac myocyte size along with nuclear changes. The absence of a DORV and the small percentage of mice with VSDs compared to B<sup>B</sup>/B<sup>B</sup> mice are likely attributable to the timing of the loss of NMHC II-B from the cardiac myocytes.

An advantage of these cardiac myocyte–specific NMHC II-B knockout mice is that most of them survive to adulthood permitting analysis of the role of NMII-B in the adult heart. H&E-stained sections of B<sup>MHC</sup>/B<sup>MHC</sup> hearts examined at 6 months, similar to hearts examined at P0 (Figure 5a and 5e), show evidence for myocyte hypertrophy (Figure 5b and 5f). This hypertrophy is even more evident by 10 months (Figure 5c and 5g). Figure 6 shows wheat germ agglutinin staining to more easily visualize and quantify the increase in cardiac myocytes size in B<sup>MHC</sup>/B<sup>MHC</sup> hearts at 4 and 6 months of age. As shown in Figure 6f, there is a progressive increase in the size of the cardiac myocytes. In addition to cardiac myocyte hypertrophy, B<sup>MHC</sup>/B<sup>MHC</sup> mice at 10 months also display additional pathological changes including interstitial fibrosis (Figure 5d and 5h). There is.
infiltration with inflammatory cells, such as lymphocytes, plasma cells, and macrophages in the interstitium of the myocardium and the pericardium of B^αMHC/B^αMHC mouse hearts. Some of these changes can be seen as early as 6 months of age in B^αMHC/B^αMHC mice (Figure 5f) but are much more prominent at 10 months of age (Figure 5d and 5h; n=4). We also observed vacuolation in cardiac myocytes, suggesting that the myocytes have undergone marked degeneration (Figure 5h, arrow). The presence of vacuolated cells in the heart prompted us to examine them for evidence of an increase in apoptosis. Online Figure II shows the results of a TUNEL assay comparing B^αMHC/B^αMHC and B^lox/B^lox mouse hearts. There is a small increase in the number of cells undergoing apoptosis in the mutant heart, as indicated by the arrows in the figure.

The presence of the inflammatory response in these hearts raises the possibility of a viral myocarditis. Of note, however, we did not observe inflammation in the hearts of B^lox/B^lox littermates. Analyses of both control and B^αMHC/B^αMHC mice for the presence of viruses associated with myocarditis were negative for enterovirus (Coxsackie viruses and echo virus; data not shown). In 2 of 8 B^αMHC/B^αMHC mice, thrombi were seen in the H&E-stained sections of the left atrium of the heart at 10 months of age, consistent with severe pathological changes in the heart and compromised cardiac function (see Online Figure III).

We also addressed the question of whether the fetal cardiac program was reactivated in the hearts of these mice by performing both immunoblot analyses and immunofluorescence microscopy on the NMII-B–ablated and normal hearts. In contrast to our previous findings for mice that were rendered hypomorphic for NMII-B, in which there is a 40-fold increase in the expression of β-cardiac myosin,\textsuperscript{10} we found only a 2- to 3-fold increase in the expression of βMHC in the 6-month-old B^αMHC/B^αMHC mouse heart. Microscopy confirmed this and showed that the increase was only detected in relatively few cardiac myocytes and did not correlate with the extent of myocyte hypertrophy (data not shown).

**B^αMHC/B^αMHC Mice Develop a Cardiomyopathy With Abnormalities in the ID**

To obtain information about the cardiac function of B^αMHC/B^αMHC mice, we carried out echocardiography at 4, 6, and 10 months of age. The Table shows that although there are no significant differences between B^lox/B^lox and B^αMHC/B^αMHC mice at 4 and 6 months, there are differences at 10 months of age. These include an increase in left ventricular internal diameter at the end of systole and a marked decrease in the percentage of fractional shortening from 44±8% (n=7) for B^lox/B^lox to 29±9% (n=12) for B^αMHC/B^αMHC mice. These results confirm that cardiac function is significantly compromised in the B^αMHC/B^αMHC mice at 10 months and are consistent with a cardiomyopathy. We also performed ECGs (3 standard leads) to determine whether abnormalities could be detected in B^αMHC/B^αMHC mice at 4 and 6 months, there are differences at 10 months of age. These include an increase in left ventricular internal diameter at the end of systole and a marked decrease in the percentage of fractional shortening from 44±8% (n=7) for B^lox/B^lox to 29±9% (n=12) for B^αMHC/B^αMHC mice. These results confirm that cardiac function is significantly compromised in the B^αMHC/B^αMHC mice at 10 months and are consistent with a cardiomyopathy. We also performed ECGs (3 standard leads) to determine whether abnormalities could be detected in B^αMHC/B^αMHC mice at this age. The electric axis ranged from +30° to +90° for the control animals. However, 4 of 5 of the B^αMHC/B^αMHC mice displayed an abnormal right axis deviation, ranging from +90° to more than +210° and in 3 of them, the severity of the deviation (more than 120°) is consistent with an abnormality in cardiac conduction (Online Figure IV).

To understand a possible cause of the defect in conduction in B^αMHC/B^αMHC hearts, we carried out an electron micros-
copy study. Previous work from this laboratory has demonstrated that in adult mice NMII-B was detected in the IDs in the heart. Moreover, deletion and mutation of proteins associated with the IDs are often associated with defects in cardiac conduction. It was, therefore, of interest to see whether the IDs of the Bflox/Bflox mice were normal in structure. Figure 7A is electron micrographs showing that the IDs of Bflox/Bflox mice are widened and distorted (4/5 mice examined) compared to Bflox/Bflox mice. Approximately 20% of the IDs of Bflox/Bflox mice show this abnormality, which is not found in Bflox/Bflox mice. Careful inspection of the affected IDs shows that whereas the adhesion junctions are severely disrupted (Figure 7A, b, large arrow, and c), the desmosomes (arrowheads) and gap junctions (arrows) remain mostly intact and are less affected (Figure 7A, b and c).

To gain insight into the cause of the disruption of the ID, we carried out an immunoblot analysis of a number of proteins known to be present at the disc at 6 months. Figure 7B shows that of the proteins analyzed, including a number of adhesion molecules, only the actin-binding protein mXina is decreased (a decrease of 78.5±4.8% compared to the wild type; n=2 mice, performed in triplicate). In contrast, expression of connexin 43 is increased, most likely because of cardiac myocyte hypertrophy. Figure 7C shows the distribution of both mXina and connexin 43 in wild-type and Bflox/Bflox hearts at 10 months using confocal immunofluorescence microscopy. The staining confirms the decreased expression of mXina at the ID. It also shows that, unlike the wild-type disc, mXina is not uniformly associated with connexin 43 in many of the discs. We propose that the loss in NMII-B at the ID is the primary cause of the disruption of cell–cell adhesion in the NMII-B–ablated heart. Moreover, the loss (NMII-B) and decrease (mXina) of 2 actin-binding proteins at the ID, the latter of which also binds to β-catenin, are expected to contribute to instability at the adhesion junction (see Figure 6. Wheat germ agglutinin staining of the cardiac myocytes. Staining of cardiac myocytes with wheat germ agglutinin (red) at 4 months (a and b) and at 6 months (c through e) and quantification of myocyte size (f) showing a progressive increase in myocyte size in BαMHC/BαMHC hearts. *P<0.01 (n=3 mice). There is no significant difference in myocytes size at 6 months between the Bflox/BαMHC heart and the Bflox/Bflox heart. DAPI (blue) stains the nuclei.

| Table. Echocardiographic Results From Bflox/Bflox and BαMHC/BαMHC Mice |
|-----------------|---------------|----------------|----------------|-----------------|------------------|----------------|
| Age (mo)        | HR (bpm)      | LVEDD (mm)     | LVESD (mm)     | IVS (mm)        | LVPW (mm)        | FS (%)          |
| Bflox/Bflox (n=4) | 4             | 486±17         | 4.0±0.8        | 2.6±0.5         | 0.6±0.1          | 0.8±0.2         | 36±4           |
| BαMHC/BαMHC (n=4) | 4             | 516±73         | 4.8±0.7        | 3.0±0.1         | 0.6±0.1          | 0.7±0.8         | 36±4           |
| Bflox/Bflox (n=7) | 6             | 500±59         | 4.8±0.2        | 3.0±0.4         | 0.9±0.7          | 0.6±0.2         | 37±7           |
| BαMHC/BαMHC (n=7) | 6             | 490±77         | 4.9±0.6        | 3.1±0.4         | 0.6±0.1          | 0.5±0.1         | 37±3           |
| Bflox/Bflox (n=7) | 10            | 518±47         | 4.6±0.5        | 2.5±0.5         | 0.6±0.1          | 0.6±0.1         | 44±8           |
| BαMHC/BαMHC (n=12)| 10            | 426±129        | 4.9±0.5        | 3.5±0.5*        | 0.9±0.3          | 0.6±0.1         | 29±9*          |

*P<0.01. FS indicates fractional shortening; HR, heart rate in bpm; IVS, interventricular septal thickness; LVEDD, left ventricular end diastolic dimension; LVESD, left ventricular end systolic dimension; LVPW, left ventricular posterior wall thickness.
Discussion. Of note, no defects in the brain or other organs were found in BαMHC/BαMHC mice at any age.

Discussion

We have previously reported that, as early as E11.5, global ablation of NMHC II-B in mice resulted in hydrocephalus associated with defects in cell–cell adhesion of the neural epithelial cells lining the spinal canal and cerebral ventricles.9,15 In Bnest/Bnest mice, ablation of NMHC II-B was initiated at E10.5, controlled by the nestin promoter, which is consistent with the delayed onset of hydrocephalus. Of particular note, despite the death of these mice between 12 to 22 days of age, most likely resulting from severe hydrocephalus, there were no abnormalities found in the heart.

In addition to learning whether the defects we found in the B /B mouse hearts were related directly or indirectly to those found in the nervous system, we also wanted to study the role of NMII-B in the adult mouse heart. Most BαMHC/ BαMHC mice manifested progressive cardiac abnormalities, starting with myocyte hypertrophy, which was apparent as early as P0 and increased during postnatal development to 6 and 10 months of age. At 10 months, there was also evidence of myocyte vacuolation and cell degeneration, interstitial fibrosis, and an infiltration of the cardiac tissue with inflammatory cells. We hypothesize that the cardiac phenotype in fibrosis, and an infiltration of the cardiac tissue with inflammatory cells are secondary to the primary abnormality in the cardiac myocytes, which is most likely myocyte degeneration.

The pathological changes in the hearts of cardiac-specific NMHC II-B knockout mice are in agreement with the echocardiographic and ECG studies. The marked decrease in the fractional shortening at 10 months is consistent with the compromised contractility of cardiac muscle. The abnormalities noted in ECGs (an abnormal electrical axis) could reflect the striking defects found in the IDs. Previous work has shown that in the adult heart NMII-B is localized to the Z-lines and IDs.18 The IDs are composed of adherens junctions, desmosomes, and gap junctions that form cell–cell boundaries and connections between cardiac myocytes and allow the myocardium to function in synchrony. As noted above, work from a number of laboratories has shown that NMIIIs play an important role in cell–cell adhesion7,17,20,22 and that abnormalities in a number of adhesion proteins result in either loss or structural changes in the cardiac IDs.20,23,24 Figure 7A provides evidence that loss of NMII-B primarily affects the adhesion junctions rather than the gap junctions or desmosomes. Moreover, BαMHC/ BαMHC hearts at 6 months show a milder defect in the adhesion junction of the IDs and no defects in the desmosomes and gap junctions (data not shown).

We have analyzed the expression of a number of ID proteins and found a significant decrease in the expression of mXinα in BαMHC/BαMHC hearts compared to the wild-type hearts. Mice ablated for mXinα also show abnormal IDs.19 Unlike the mXinα knockout hearts, NMII-B–ablated hearts show no decrease in expression levels of N-cadherin or β-catenin. Moreover, there was no change in the distribution of β-catenin. We, therefore, attribute the primary cause of the disruption of the IDs to the loss of NMII-B. We speculate that the decrease of mXinα is secondary to the loss of NMII-B, and the mechanism of this decrease is of ongoing interest. The decrease in both of these proteins, one of which (mXinα) has been demonstrated to also bind to β-catenin,21 would explain the marked disruption of the IDs.
The finding of a role for NMII-B in the cardiac II is similar to the findings for NMII-B in the spinal canal. Our hypothesis is that NMII exerts tension and stabilizes actin filaments, which, in turn, are required for maintenance of adhesion complexes between cells or, in this case, between the cardiac myocytes. The loss of NMII-B from the adhesion complex could, therefore, result in the gradual deterioration in the cardiac adhesion complex, including the loss of mXin, over a period of time, and this would account for our failure to observe abnormal discs in B/B mice, which died before birth. Interestingly, generation of mice in which NMII-A replaced NMII-B did not produce defects in the IDs. This is consistent with the hypothesis that in cases in which NMII-A and NMII-B are apparently playing a structural rather than a motor role, 1 isoform is more likely to substitute for the other in vivo, as well as in cultured cells. When myosin is playing more of a motor role, for example, in neural cell migration, because of significant differences in the kinetics of MgATP hydrolysis and actin-binding properties between the myosin isoforms, successful substitution is much less likely, at least in vivo. These findings further support the idea that disruption of the IDs in B/H11002 mice is attributable to the loss of NMII and is secondary to the development of the cardiomyopathy.

These conditionally ablated mice demonstrate that the defects we observed in the hearts and brains of the B/B mice are independent of each other. The availability of NMHC II-B floxed mice will allow conditional ablation of NMHC II-B in a variety of tissues and cells and thus help to further define its role both in situ and in vivo.

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Disclosures

None.

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SUPPLEMENT MATERIAL

Detailed Materials and Methods

Generation of targeting vector and B\textsuperscript{flox}/B\textsuperscript{flox} mice

The targeting vector includes a 6.5-kb genomic DNA fragment containing the first coding exon ( exon2) of NMHC II-B. A 1.8-kb PGK-Neomycin resistance (Neo\textsuperscript{r}) expression cassette was inserted into a unique BstEII site, 230bp upstream of exon2. Both the PGK-Neo\textsuperscript{r} expression cassette and exon2 were flanked by loxP sites (Figure 1b). The linearized targeting vector was electroporated into CMT-1 embryonic stem (ES) cells derived from 129S6SvEv mice. G418-resistant (0.20 mg/ml) ES cell clones were screened for successful homologous recombination by restriction enzyme digestion with BamHI and Southern blot analysis using the BamHI-SpeI fragment (indicated in Figure 1c) as a probe. One positive ES cell clone was microinjected into blastocysts which were transplanted into pseudo-pregnant mice to generate chimeric mice. The chimeras were crossed to C57BL/6J mice to produce germ-line transmitted, heterozygous mice. Mice were genotyped by PCR analysis (primers: 5'GACCCTACTATTCCAATTCAT and 5'CAGAGAAACGATGGGAAAGAC). The products are 250bp for wild type and 350bp for the floxed allele.

Histology, immunofluorescence microscopy and immunoblot analysis

Brains and hearts were fixed in 3.7% paraformaldehyde in PBS or frozen in OCT. H&E staining, immunofluorescence staining and immunoblotting were performed according to standard procedures as described previously.\textsuperscript{1} The antibodies used for immunofluorescence staining were: NMHC II-B (rabbit, 1:1000),\textsuperscript{2} calbindin (mouse, 1:1000, Sigma), connexin 43 (rabbit, 1:100, Cell Signalling), and desmin (mouse, 1:100, Dako). The antibodies used in immunoblot analysis were: α-actinin (mouse, 1:200, Abcam), connexin 43 (rabbit, 1:1000, Cell Signalling), N-cadherin (mouse, 1:2000, Invitrogen), α-catenin (mouse, 1:500, BD Biosciences), β-catenin (mouse, 1:1000, Zymed), γ-catenin (rabbit, 1:1000, Cell Signalling), mXin\textalpha (rabbit, gift from Dr. Jim Lin, Departments of Biological Sciences, University of Iowa, 1:10,000), NMHC II-B (rabbit, 1:50,000), tubulin (rabbit, 1:1000, Sigma) and actin (mouse, 1:5000, Sigma), GAPDH (mouse, 1:5000, Abcam).

Wheat germ agglutinin (WGA) staining and measurement of the cross-sectional area of the cardiac myocytes

Paraffin embedded heart sections were first dewaxed and rehydrated by standard procedures. Following antigen retrieval in citric acid buffer (10 mM, pH 6.0), the sections were blocked with 1% BSA/5% goat serum in PBS for 1 hour at room temperature and then incubated with Alexa Fluor® 594-WGA (10 µg/ml, Invitrogen) and DAPI in blocking solution for 1 hour at room temperature. The slides were then washed and mounted using Prolong Anti-fade mount media (InVitrogen). Confocal images were captured using a Zeiss LSM 510 Meta Confocal Microscope and the cross sectional area of the cells was measured with a Zeiss measuring tool. We measured all myocytes in the confocal image and calculated their average size.

TUNEL assay

The TUNEL assay was carried out using the In Situ Cell Death Detection Kit, Fluorescein following the manufacturer’s instructions (Roche Applied Science). After TUNEL staining, the heart sections were stained with antibodies to desmin to identify cardiac myocytes and counter stained with DAPI to visualize nuclei.
**Echocardiography**

The mice were anesthetized with 1.5% isoflurane and imaged in the supine position. Three standard lead echocardiography was performed using an Acuson Sequoia 256c imaging system with the 15L8 multi-frequency transducer. All images were obtained at a frequency of 15MHz. Multiple 2-D and M-mode images of the left ventricle (LV) were obtained on each subject. Quantitative assessments of LV size, function and wall thickness were performed using M-mode echocardiography. The M-mode measurements were performed off-line on Prosolv Software Version 3.0. Three measurements were performed for each parameter and averaged.

**Electrocardiography**

The mice were anesthetized with isoflurane and analyzed in a supine position after echocardiography. Electrocardiograms were recorded with a model MAC 1200, G.E. Medical Systems.

**Electron Microscopy**

Heart tissue was fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, postfixed with 1% OsO₄ in 0.1 M phosphate buffer and embedded in Polybed 812 (Polysciences). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEOL).

**Data and Statistical Analysis**

The data were expressed as mean ± SD. Student’s t-test was used to compare the data between two groups.
Online Figure I  H&E staining of heart sections from 6 month old mice showing hypertrophy of the \(B^{\alpha\text{MHC}}/B^{\alpha\text{MHC}}\) cardiac myocytes, but not in the other three genotypes. The absence of enlarged myocytes in panels b and c suggests that Cre expression at this age has no effect on cardiac myocyte size.
Online Figure II TUNEL staining reveals an increase in apoptotic myocytes (arrows, green) in 10 month old βαMHC/βαMHC mouse hearts compared to wild type hearts. Desmin (red) indicates cardiac myocytes, DAPI (blue) stains the nuclei.
Online Figure III  Organized thrombus in the left atrium (*, magnified 5x on right) of a 10.5 month old B^{aMHC}/B^{aMHC} mouse heart stained with H&E.
Online Figure IV  EKG of a 10 month old B$^{\alpha\text{MHC}}$/B$^{\alpha\text{MHC}}$ mouse showing marked right deviation of the electrical axis compared to a wild type B$^{\text{flox}}$/B$^{\text{flox}}$ mouse.
References
