Ablation and Mutation of Nonmuscle Myosin Heavy Chain II-B Results in a Defect in Cardiac Myocyte Cytokinesis

Kazuyo Takeda,* Hiroko Kishi,* Xuefei Ma,* Zu-Xi Yu, Robert S. Adelstein

Abstract—We have identified a novel form of cardiac myocyte enlargement in nonmuscle myosin heavy chain II-B (NMHC II-B) ablated mice, based on a partial failure in cytokinesis. In contrast to most cells, cardiac myocytes lack NMHC II-A, and ablation of NMHC II-B results in a heart with 70% fewer myocytes at embryonic day 14.5 (E14.5) than control mice (B+/B− and B+/B+). In addition, B-/B− cardiac myocytes show a marked increase in binucleation at E12.5, reflecting the occurrence of karyokinesis in the absence of cytokinesis. An increase in binucleation and cell size is also found in hypomorphic, homozygous mice harboring a single amino acid mutation (R709C) in the gene encoding NMHC II-B. The nonmyocytes in B-/B− hearts and homozygous mutant hearts, all of which contain NMHC II-A, do not show either of these abnormalities. B+/B− cardiac myocytes at E14.5 show a decreased bromodeoxyuridine (BrdU) labeling index compared with controls, consistent with the decrease in myocyte proliferation. This decreased BrdU labeling is not seen in nonmyocyte cells in the heart. In addition to these changes, both B+/B− mice as well as homozygous mutated mice show an increase in cyclin D2 and D3 reflecting an abnormality in earlier steps in the cell cycle. Whereas cardiac myocytes completely ablated for NMHC II-B show enlargement and binucleation, mice expressing as little as 6% of the normal amount of wild-type NMHC II-B in the heart do not show these abnormalities.

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Key Words: cell cycle | heart development | immunohistochemistry | myocyte enlargement | bromodeoxyuridine labeling index

Nonmuscle myosin II is present in all mammalian cells and plays an important role in cell motility, morphology, and cytokinesis (see reviews1–4). The nonmuscle myosin II molecule is composed of homodimeric heavy chains (200 kDa) and two pairs of light chains (20 and 17 kDa).5 At present, there are three genes known to encode nonmuscle myosin II heavy chains (NMHCs) in mammalian cells; the protein products are referred to as NMHC II-A, II-B, and II-C.6

Ablation of NMHC II-B provided evidence that this protein plays an important role in cardiac and brain development.7,8 Most B+/B− mice die by embryonic day 15 (E15). The brain defects include a severe hydrocephalus with evidence for abnormal migration of developing neurons,8,9 The cardiac abnormalities include a membranous ventricular septal defect, dextroposition of the aorta, and an unusual form of cardiac myocyte enlargement, which has its onset at E12 or earlier and is found in all four chambers of the heart.7

In addition to generating mice ablated for NMHC II-B, we produced mice with markedly decreased amounts of this protein.10 These B+/B− mice, referred to as mild hypomorphs, were generated by replacing a neuron-specific exon in the NMHC II-B gene with the gene encoding neomycin resistance. Despite expressing only 12% of the amount of NMHC II-B expressed by B+/B− mice in their hearts, B+/B+/B− mice survived and were fertile, but developed myocyte hypertrophy after 11 months. Crossing B+/B+/B− mice with B+/B− mice resulted in B+/B− mice, which are severe hypomorphs. These mice express only 6% of the amount of myosin expressed by B+/B− mice in the heart and develop myocyte hypertrophy 24 days after birth (P24). In contrast to the enlargement found in cardiac myocytes of the B+/B− mice, the skeletal and smooth muscle cells were normal in size. This difference could be related to the finding that, unlike cardiac myocytes, skeletal11 and smooth muscle12 cells contain NMHC II-A, in addition to NMHC II-B.

This article addresses the mechanism of cardiac myocyte enlargement in the B+/B− heart during development. We demonstrate that B+/B− cardiac myocytes lacking both NMHC II-A and II-B show evidence for decreased proliferation. There are fewer of these cells in B+/B− hearts and they show a marked increase in binucleation compared with

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control hearts. Moreover, mice, which are homozygous for a single amino acid mutation (R709C) in NMHC II-B and have decreased expression of the mutant myosin, also show an increase in cardiac myocyte binucleation and cell size. On the other hand, the presence of as little as 6% of the normal amount of wild-type NMHC II-B in the heart is sufficient to prevent binucleation and the associated increase in myocyte size. We suggest that the underlying cause of the decrease in the number of cardiac myocytes and increase in binucleation in the NMHC II-B ablated and mutated mice is a defect in cytokinesis.

**Materials and Methods**

**Generation of NMHC II-B Mutant Mice**

NMHC II-B ablated and hypomorphic mice were generated as previously reported.\(^7\)\(^-\)\(^10\) For generation of R709C (Arg 709 in NMHC II-B replaced by Cys) mice, the genomic fragment containing exon 17 and the surrounding region of the NMHC II-B gene was obtained from 129SV vector genomic library (Stratagene, Cedar Creek, Tex.). The point mutation R709C was introduced into exon 17 using recombiant PCR. The targeting construct consisting of a 6.5-kb genomic fragment, including R709C, the Neomycin resistance cassette in an intron 5\(^9\) to the mutation and the Diphtheria Toxin cassette at the 3' end was used to replace the native gene by homologous recombination after electroporation of ES cells. After generation of heterozygous ES cells, the mutation was confirmed by nucleotide sequencing and three positive ES cell clones were injected into blastocysts derived from C57BJ/6 mice. All three clones gave rise to chimeric mice that transmitted the targeted allele into the germline. As demonstrated later, the expression of the mutated allele was decreased compared with the wild-type allele. This is due to the presence of the gene encoding neomycin resistance in the intron 5\(^9\) to the mutated exon. The designation for the mutant allele is BCN, where C indicates the Arg→Cys mutation and N indicates the cassette expressing neomycin resistance.

**Preparation of Tissues**

Mouse embryos taken at E12.5 to P0 were fixed with 4% paraformaldehyde, embedded in paraffin, and 4- to 6-μm sections were cut for morphological analysis. For frozen sections, unfixed embryos were snap frozen in OCT compound. Serial sections, 5 to 10 μm thick, were cut and every tenth section was stained with hematoxylin-eosin (H&E). The remaining sections were snap frozen in OCT compound. Serial sections, 5 to 10 μm thick, were cut and every tenth section was stained with hematoxylin-eosin (H&E). The sections were stained with DAPI (Sigma), followed by the appropriate FITC- or Rhodamine-conjugated secondary antibodies (Vector Labs). The characteristics of the NMHC II antibodies have been described.\(^11\)\(^-\)\(^13\) Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) or propidium iodide (Vector Labs). For immunofluorescence detection of cyclin D3 in myocytes, anti-cyclin D3 rabbit polyclonal antibody (Santa Cruz Biotechnology) and anti-desmin mouse monoclonal antibody (clone D33, DAKO) were used.

**Morphometric Analysis**

The frequency of occurrence of binucleation and the transverse diameters of mononucleated and binucleated myocytes were evaluated and compared for B-/B' and control (B'/B' and B'/B') hearts. The transverse diameters were measured at the level of the nuclei in 60 randomly selected ventricular myocytes using paraffin sections stained with H&E. One hundred cells from each heart were evaluated for binucleation. For cultured myocytes, digital images of dual immunostaining for NMHC II-A and sarcomeric α-actinin were taken using confocal microscopy and the top surface area of individual cells was measured using Leica Qwin Software. At least 15 randomly selected cultured myocytes were measured for each experiment. Data are shown as mean±SD; Student's t test was used to evaluate the significance of differences in the values obtained for the two groups.

**Determination of BrdU Labeling Index in E14.5 Mouse Embryo Tissues**

5-Bromo-2′-deoxyuridine (BrdU, Sigma) was injected intraperitoneally into B/B' intercrossed mice, pregnant with E14.5 embryos, at a dose of 250 μg/g body weight. After 1 hour, embryos were removed and fixed with 70% EtOH and embedded into paraffin for sectioning.

For detection of BrdU in hearts, sections were subjected to dual immunofluorescent staining for anti-BrdU mouse monoclonal antibody and anti-desmin goat polyclonal antibody (Santa Cruz Biotechnology) to distinguish between cardiac myocytes and non-myocytes. The sections were incubated with a mixture of anti-BrdU antibody and anti-desmin antibody, diluted, followed by incubation with a mixture of Texas Red-conjugated horse anti-mouse IgG antibody (Vector Labs) and fluorescein (FITC)-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratory). The sections were counterstained with 0.01% DAPI (Molecular Probes).

The BrdU labeling index was determined as [the number of BrdU-positive and desmin-positive cells]/[the number of desmin-positive cells]×100%. The BrdU labeling index of nonmyocytes was determined as [the total number of BrdU-positive cells minus the number of BrdU-positive and desmin-positive cells]/[total number of cells minus the number of desmin-positive cells]×100%. The BrdU labeling index was scored from 7 different embryos (3 B'/B', 4 control). The images were analyzed using IDL Software, version 5.4 (programmed by Christian A. Combs, NHLBI).

**Western Blot Analysis**

Hearts were snap-frozen on dry ice and homogenized in Nonidet P-40 (NP-40) buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.0, 1 μg/mL aprotinin, 50 μg/mL leupeptin, 2.5 μL/mL protease inhibitor mix [0.1 g pepatin A, 0.2 g chymostatin, 0.1 g TLCK, 0.1 g TPOCK in 10 mL DMSO], 0.1 mmol/L PMSF and 1% vol/vol NP-40). After centrifugation at 16 000g for 10 minutes, the supernatant was analyzed by immunoblot analysis as previously described.\(^10\) The following antibodies were all from Santa Cruz: anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-182), and anti–cyclin A (sc-596). Anti–CDK 4 (sc-260) and anti–NMHC II-B were from Covance. A monoclonal antibody to actin (MAB 1501) was from Chemicon. The signals were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce). Immunoblots were quantified using a Personal Densitometer SI (Molecular Dynamics).
Results

Evidence for a Defect in Cytokinesis in B<sup>−/−</sup> Hearts

Figures 1a and 1b show that the ventricular wall of the B<sup>−/−</sup> heart (Figure 1a) differed from that of a control heart (Figure 1b, B<sup>+/+</sup>) at E12.5 in being composed of 2 to 3 myocyte layers rather than 4 to 5 layers. This suggested that the B<sup>−/−</sup> hearts contained fewer myocytes and that the myocyte enlargement we previously observed in E12.5 B<sup>+/−</sup> hearts might be due to a defect in cytokinesis.

We quantified binucleation and myocyte size in E12.5 B<sup>−/−</sup> cardiac myocytes and compared these parameters to those found in control hearts. Table 1, Knockout, shows that, whereas only 1% of control ventricular myocytes are binucleated at this age, 23% of the B<sup>−/−</sup> cardiac myocytes are binucleated, and the transverse diameter of B<sup>−/−</sup> myocytes is significantly larger than that of the control myocytes. The two nuclei in the E12.5 B<sup>−/−</sup> myocytes are often arranged in parallel or diagonally at the same level in the cell (arrows, Figure 1a), rather than being aligned along the longitudinal axis as is usually the case in binucleated adult heart cells. Importantly, other types of cells including fibroblasts, endothelial cells, smooth muscle cells, and mesothelial cells in the B<sup>−/−</sup> hearts, all of which contain NMHC II-A, show neither binucleation nor an increase in cell size. In addition to giving the average size of the cardiac myocytes, Table 1 (data line 1, right side) lists the transverse diameter of the mononucleated and binucleated cells separately and shows that, in general, the binucleated cells are larger.

We also generated mice with a single amino acid mutation in NMHC II-B after homologous recombination. This mutation, R709C, was chosen because it occurs in a conserved area of the MHC sequence and because a similar mutation, which decreases, but does not eliminate myosin activity, has already been described in humans in NMHC II-A.14,15 Figures 1c and 1d show an H&E stained section from a mutant (c) and

![Figure 1. Increased binucleated cardiac myocytes in B<sup>−/−</sup> and B<sup>+/−</sup>/B<sup>+/−</sup> mouse hearts. a and b, Sections of ventricular myocardium from E12.5 B<sup>−/−</sup> (a) and B<sup>+/−</sup> (b) mice showing that, whereas the B<sup>−/−</sup> ventricular wall is composed of 2 to 3 layers of myocytes, the equivalent section in the B<sup>−/−</sup> heart consists of 4 to 5 layers. c and d, Sections of ventricular myocardium from E14.5 B<sup>+/−</sup>/B<sup>+/−</sup> (c) and B<sup>−/−</sup> (d) mice. Arrows in a and c indicate binucleated cells, which are quantitated in Table 1. H&E stained. Bars, as indicated.](http://circres.ahajournals.org/)

### Table 1. Cell Size and Nuclear Status of Cardiac Myocytes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cell Size</th>
<th>Mononucleated</th>
<th>Binucleated</th>
<th>Mitotic</th>
<th>Mice (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact hearts</td>
<td>Diameter, μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knockout</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sup&gt;−/−&lt;/sup&gt; (E12.5)</td>
<td>7.37 ± 0.11†</td>
<td>74% (6.58 ± 0.5 μm)‡</td>
<td>23% (11.94 ± 1.29 μm)¶</td>
<td>2%</td>
<td>4</td>
</tr>
<tr>
<td>B&lt;sup&gt;+/−&lt;/sup&gt;, B&lt;sup&gt;−/−&lt;/sup&gt; (E12.5)</td>
<td>4.94 ± 0.26†*</td>
<td>95%</td>
<td>1%</td>
<td>4%</td>
<td>6</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sup&gt;+/−&lt;/sup&gt;/B&lt;sup&gt;+/−&lt;/sup&gt; (E14.5)</td>
<td>9.74 ± 3.7‡</td>
<td>71.5% (8.42 ± 2.3 μm)¶</td>
<td>26% (13.39 ± 3.7 μm)¶</td>
<td>2.5%</td>
<td>2</td>
</tr>
<tr>
<td>B&lt;sup&gt;−/−&lt;/sup&gt;, B&lt;sup&gt;−/−&lt;/sup&gt;/B&lt;sup&gt;+/−&lt;/sup&gt; (E14.5)</td>
<td>6.72 ± 1.93‡</td>
<td>91%</td>
<td>6%</td>
<td>3%</td>
<td>3</td>
</tr>
<tr>
<td>Hypomorph</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sup&gt;+/−&lt;/sup&gt; (E13.5)</td>
<td>6.95 ± 1.51‡</td>
<td>97%</td>
<td>2%</td>
<td>1%</td>
<td>3</td>
</tr>
<tr>
<td>B&lt;sup&gt;−/−&lt;/sup&gt;, B&lt;sup&gt;−/−&lt;/sup&gt;/B&lt;sup&gt;+/−&lt;/sup&gt; (E13.5)</td>
<td>6.59 ± 1.47‡</td>
<td>98%</td>
<td>1%</td>
<td>1%</td>
<td>2</td>
</tr>
<tr>
<td>Cultured myocytes</td>
<td>Surface area, μm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sup&gt;−/−&lt;/sup&gt; (E14.5, 24 hours)</td>
<td>3923§</td>
<td>47%§</td>
<td>53%§</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B&lt;sup&gt;−/−&lt;/sup&gt;, B&lt;sup&gt;−/−&lt;/sup&gt;/B&lt;sup&gt;+/−&lt;/sup&gt; (E14.5, 24 hours)</td>
<td>1059§</td>
<td>95%§</td>
<td>5%§</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.000001 vs B<sup>−/−</sup>
†60 myocytes/mouse were measured; ‡100 myocytes/mouse were analyzed; §15 myocytes/mouse were measured; ¶cell diameter.
wild-type litter mate (d) at E14.5. Note the binucleated myocytes in the B^CN/B^CN heart indicated by the arrows. Table 1 shows that E14.5 mice homozygous for this mutation demonstrate an increase in the size of their cardiac myocytes compared with wild-type and heterozygous mice as well as an increase in binucleation similar to B^2/B^2 mice (Table 1, Mutant). As noted in Materials and Methods, B^CN/B^CN mice were hypomorphic with respect to their expression of NMHC II-B. They expressed approximately 15% of the R709C mutated myosin compared with the amount of NMHC II-B found in wild-type littermates (see next section). In contrast to the B^2/B^2 mice, the B^CN/B^CN mice can survive for up to 15 days.

**Lack of Binucleation in Hypomorphic Mice**

Previously, we reported that hypomorphic mice expressing only 6% (B^Di/B^2) or 12% (B^Di/B^Di) of the normal amount of wild-type NMHC II-B in their hearts developed myocyte hypertrophy, but in contrast to B^2/B^2 mice, this did not occur during embryonic development.10 To see whether a relatively small content of wild-type NMHC II-B could prevent binucleation in embryonic hearts, we quantified the extent of binucleation in stained sections of the ventricular myocardium from B^Di/B^2 E13.5 mouse hearts using B^1/B^Di litter mates as controls. Table 1, Hypomorph, shows that there was no increase in binucleation or in cell size in the B^Di/B^2 myocytes at E13.5 compared with B^2/B^2 litter mates, in contrast to the findings above for B^2/B^2 myocytes. This implies that the presence of a relatively small amount of wild-type NMHC II-B is sufficient to prevent the embryonic binucleation and myocyte enlargement seen in B^2/B^2 mice.

**Binucleation and Enlargement of Cultured Cardiac Myocytes (E13.5 to E14.5)**

Figures 2a through 2f demonstrate that when myocytes from E14.5 B^2/B^2 (Figures 2a through 2c) or control hearts (Figures 2d through 2f) are placed in culture, the increase in binucleation and top surface area in B^2/B^2 myocytes, which was observed in the intact hearts as increased diameters, is present at 24 hours. Quantification of binucleation and myocyte size are shown in Table 1, Cultured Myocytes. Identification of the cardiac myocytes was confirmed by staining with sarcomeric α-actinin (Figures 2b, 2c, 2e, and 2f, red). The phenotype of the cultured B^2/B^2 cardiac myocytes is particularly striking, showing markedly enlarged cells with loss of cell polarity. Of note is the appearance of NMHC II-A in both B^2/B^2 and control myocytes, but only after they were placed into culture (Figures 2a, 2c, 2d, and 2f, green).

In contrast to the cultured cardiac myocytes shown in Figures 2a through 2f, Figure 2g reveals that B^2/B^2 cardiac myocytes in an intact heart do not stain with antibodies against myosin II-A. The increased fluorescence staining for NMHC II-A in both B^2/B^2 and control myocytes, but only after they were placed into culture (Figures 2a, 2c, 2d, and 2f, green).

![Figure 2](http://circres.ahajournals.org/lookup/doi/10.1161/01.RES.0000125584.25445.91)
NMHC II-B (green), which detects this protein in both cardiac and non-myocyte cells of the intact B\textsuperscript{1}/B\textsuperscript{1} heart.

Figure 3 shows a number of representative cardiac myocytes undergoing cell division after 12 hours in culture. Figure 3a shows a B\textsuperscript{1}/B\textsuperscript{1} or B\textsuperscript{1}/B\textsuperscript{2} cardiac myocyte at the end of normal cytokinesis and karyokinesis. Antibody staining detects NMHC II-A (green) and \(\alpha\)-actinin (red) and nuclei are stained with DAPI (blue). Figure 3b shows a B\textsuperscript{2}/B\textsuperscript{2} cell that has also completed cytokinesis and karyokinesis. In contrast to Figure 3b, Figure 3c shows a B\textsuperscript{2}/B\textsuperscript{2} cell that underwent karyokinesis in the absence of cytokinesis, resulting in binucleation. Figure 3d shows three myocytes, two of which are binucleated (arrowheads) and one of which is mononucleated (arrow).

To further characterize the B\textsuperscript{CN}/B\textsuperscript{CN} mutant heart, we stained cultured cardiac myocytes prepared from E14.5 mice with antibodies to NMHC II-B (Figures 4a and 4c, green) and \(\alpha\)-actinin (Figures 4b and 4c, red). The figure shows that, similar to wild-type NMHC II-B,\textsuperscript{11} the mutant isoform is distributed diffusely throughout the cell. Moreover, at P0,

![Figure 3. Cytokinesis in B\textsuperscript{−}/B\textsuperscript{−} and control cultured cardiac myocytes. Antibodies to NMHC II-A were visualized with FITC (green), antibodies to \(\alpha\)-actinin with rhodamine (red), and the nuclei with DAPI (blue). a, Cytokinesis in a B\textsuperscript{−}/B\textsuperscript{−} or B\textsuperscript{+}/B\textsuperscript{−} cardiac myocyte. b, Cytokinesis in a B\textsuperscript{−}/B\textsuperscript{−} cardiac myocyte which did not result in binucleation. c, Abnormal cytokinesis in a B\textsuperscript{−}/B\textsuperscript{−} cardiac myocyte resulting in binucleation. d, A group of three cardiac myocytes, two of which show binucleation (arrowheads) and one of which is mononucleated (arrow).](image)

![Figure 4. Localization of NMHC II-B and \(\alpha\)-actinin in a binucleated B\textsuperscript{CN}/B\textsuperscript{CN} cultured cardiac myocyte. a, Antibodies to NMHC II-B show that mutant myosin (green) is distributed throughout the cytoplasm (nucleus stained with DAPI, blue). b, Antibodies to \(\alpha\)-actinin (red) show its localization to nascent Z-lines and stress fibers in the same cardiac myocyte. c, Merging panels a and b reveals that some of the mutant myosin is present in stress fibers along with \(\alpha\)-actinin (yellow). d, Differential interference contrast image of the same cell.](image)
Localization with the same antibodies shows that the mutant myosin is present in the Z-lines of cardiac myocytes in the intact heart (data not shown). This is consistent with the mutation being present in the motor domain and not in the rod portion of the molecule, which governs myosin localization.

**Decreased Cell Number and BrdU Labeling Index in B^2/B^2^ Cardiac Myocytes**

As noted above, the decrease in the number of myocyte layers in the ventricular wall of B^2/B^2^ hearts suggested the presence of fewer cardiac myocytes. We quantified the number of nuclei belonging to B^2/B^2^ and wild-type cardiac myocytes (DAPI staining) using immunofluorescent staining with antibodies to desmin to distinguish myocytes from non-myocytes. Table 2, left side, shows that the number of nuclei from E14.5 B^2/B^2^ mouse cardiac myocytes is significantly lower than that from B^1/B^2^ and B^1/B^1^ cardiac myocytes, whereas there is no difference between B^2/B^2^ and control mice in the number of nuclei from cells that are not myocytes. Moreover, because the number of nuclei belonging to B^2/B^2^ cardiac myocytes includes binucleated cells, the actual number of cardiac myocytes is even lower (by approximately 23%) in the B^2/B^2^ heart, resulting in a total decrease of approximately 70%. The finding of fewer cardiac myocytes in the B^2/B^-- heart compared with control hearts is consistent with a defect in myocyte cell proliferation.

To quantify cell proliferative activity in B^2/B^- cardiac myocytes, we analyzed BrdU incorporation in B^2/B^- (Figure 5a) and control mouse hearts (Figure 5b) and other tissues such as liver at E14.5 (Table 2). The figure shows that immunofluorescent staining with anti-BrdU (magenta) and anti-desmin (green) antibodies allowed us to distinguish proliferating cardiac myocytes (desmin-positive) from non-myocytes (desmin-negative). Figures 5a and 5b also shows that staining for BrdU fluorescence is visualized in both mononucleated (arrows) and binucleated myocytes (arrowhead), indicating that the latter cells differ from those seen in the adult mouse heart after birth in that they are actively synthesizing DNA.

We calculated the BrdU labeling index for E14.5 mouse tissues including the heart and liver. As shown in Table 2 (right side), the BrdU labeling index in B^2/B^- cardiac myocytes is significantly lower than B^2/B^-- and B^2/B^+ cardiac myocytes. On the other hand, there is no significant difference in the BrdU index between B^2/B^- and control mice with respect to nonmyocyte cells in the heart or cells in the liver. This finding suggests that the cell proliferative activity is specifically impaired in B^2/B^- cardiac myocytes, consist-

### Table 2. Total Nuclei and BrdU Labeling Index in E14.5 Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total No. of Nuclei (per Section, Mean±SD)</th>
<th>BrdU Labeling (%), Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cardiac Myocytes</td>
<td>Nonmyocytes</td>
</tr>
<tr>
<td>B^2/B^- (n=3)</td>
<td>268.3±68.6</td>
<td>596.3±61.0</td>
</tr>
<tr>
<td>B^1/B^+, B^1/B^- (n=4)</td>
<td>744.5±36.2*</td>
<td>612.5±100.9</td>
</tr>
</tbody>
</table>

*P<0.0001 vs B^2/B--; †P<0.01 vs B^2/B--.

Figure 5. a and b, Dual immunofluorescent staining for BrdU and desmin in E14.5 mouse hearts. Representative sections of B^2/B^- (a) and B^1/B^- (b) mouse hearts stained with antibodies to BrdU (magenta, due to dual staining with DAPI) and desmin (green). BrdU-negative nuclei are stained with DAPI (blue). Arrows and arrowheads indicate mononucleated and binucleated cardiac myocytes labeled with BrdU, respectively. Quantified in Table 2. c and d, Localization of cyclin D3 in E14.5 hearts. Images from confocal microscopy show that cyclin D3 (magenta) is increased in intensity and in the number of nuclei stained in cardiac myocytes in B^2/B^- hearts (c) compared with B^2/B^- hearts (d). Cardiac myocytes were identified by staining for desmin (green). No increase in cyclin D3 was noted for the nonmyocytes. Nuclei were counterstained with DAPI (blue).
tent with the lower number of these cells in the B2/B2 heart. To rule out the possibility that increased apoptosis plays a role in the decrease in cardiac myocytes, we performed TUNEL analysis in B2/B2 and control hearts. We found no evidence for increased cell death in the cardiac myocytes in the B2/B2 heart (data not shown).

Increases in Cyclin D2 and D3 in B−/B− and BCN/BCN Hearts

To look for other possible cell cycle defects in cardiac myocytes of B−/B− mice, we analyzed heart extracts for a number of related proteins. Figure 6a is an immunoblot analysis of tissue extracts from B−/B− and control hearts at E14.5. Two different amounts of extract were analyzed for each of the proteins and actin was used to normalize sample loading. The figure shows that cyclin D2 and D3, but not cyclin A or cyclin-dependent kinase 4 (CDK 4), are increased in B−/B− E14.5 hearts. Quantitation of the immunoblots indicated that the increase in cyclin D2 is 3.28±0.85 (n=4, mean±SD) and cyclin D3 is 3.81±1.70 (n=4) times that found for B+/B+ hearts.

Because the immunoblot analysis in Figure 6a was performed with cardiac extracts, it was important to investigate whether the increase in cyclin D2 and D3 reflected an increase in these proteins in cardiac myocytes, nonmyocytes, or both. Figure 5b shows dual immunofluorescent staining of a B−/B− heart (left) demonstrating that the increase in cyclin D3 is confined to the nuclei of cardiac myocytes. In contrast to the cardiac myocytes, the nonmyocytes showed no increase in cyclin D3.

We also studied the possible increase in cyclin D2 in the hearts of BCN/BCN mice at P0. Figure 6b demonstrates the decreased expression of R709C mutant myosin in the BCN/BCN mouse heart. Compared with B+/B+ hearts, only 15% of B−/B− myosin is expressed. The figure also shows an approximate 2-fold upregulation of cyclin D2 in the BCN/BCN hearts. A similar increase was also found for cyclin D3 (data not shown).

Discussion

We provide evidence that, during mouse embryogenesis, B−/B− hearts exhibit a novel form of cardiac myocyte enlargement based on a partial failure of these cells to undergo cytokinesis. To support this conclusion, we note that myocyte enlargement was present throughout the atria and ventricles of B−/B− hearts and was seen at E12.5, a time when these myocytes are normally capable of proliferation. This resulted in hearts with a higher proportion of nonmuscle cells than muscle cells, because the former are capable of undergoing normal cytokinesis due to the presence of NMHC II-A. Further evidence of a defect in cytokinesis is the unusual presence of binucleation in 23% of the muscle cells at E12.5, which reflects karyokinesis in the absence of cytokinesis. Moreover, we show that binucleation and myocyte enlargement can be eliminated by the presence of as little as 6% of the normal complement of wild-type nonmuscle myosin II-B in the B−/B− cardiac myocytes. It is noteworthy that Robinson et al16 found that only approximately 10% of the total cellular myosin II in Dictostelium appeared to localize to the cleavage furrow during cytokinesis.

One obvious point is that we did not find a complete failure in cytokinesis in the B−/B− heart. We attribute this to the following: previous work with Dictostelium has shown that
there is only a complete failure in cytokinesis in cells grown in suspension because attached cells are capable of undergoing a traction-mediated form of cytokinesis. Because cardiac myocytes are attached in a three-dimensional network, we speculate that this could prevent a complete failure in cytokinesis. The dividing cultured cardiac myocytes shown in Figure 3b appear as if they could have undergone traction-mediated cytokinesis. However, we have also found that embryonic cardiac myocytes contain a third isoform of nonmuscle myosin II, nonmuscle myosin II-C (K. Takeda and R.S. Adelstein, unpublished data, 2003). Therefore, its presence may account for partial, rather than complete, failure in cytokinesis in cardiac myocytes. Of course, a combination of both cell-cell and cell-matrix contacts, as well as the presence of NMHC II-C, is also possible.

It is noteworthy that, despite the expression of nonmuscle myosin II-A in cultured cardiac myocytes, these cells continued to manifest a defect in cytokinesis as reflected by increased binucleation (53% versus 23% for cardiac myocytes in the intact heart) after 24 hours in culture. The B+/B+ and B+/B− cardiac myocytes also showed an increase in binucleation from 1% to 5% under the same culture conditions (Table 1, Cultured Myocytes).

The marked increase in binucleation that we found in cardiac myocytes in the B+/B+ heart has led us to attribute the decreased number of myocytes to a defect in cytokinesis. This is also consistent with the well-documented role of myosin II in this process. Thus, it is possible that the other defects we have observed in the cell cycle could be secondary to a defect in cytokinesis. However, we cannot rule out the possibility that these defects may also reflect a role for myosin II in other steps in the cell cycle.

Murine cardiac myocytes normally undergo increasing binucleation beginning 4 days after birth, reaching a constant level of 95% by day 10. Li et al19 have provided evidence that the actin-myosin contractile ring is formed, despite the lack of cytokinesis, during the process of binucleation. This raises the possibility that B+/B− embryonic cardiac myocytes are exhibiting early withdrawal from the cell cycle and this could explain the binucleation. However, we found BrdU-positive cells among the binucleated myocytes (see Figure 5), indicating that some of the binucleated myocytes are still actively participating in the cell cycle, differing from the binucleated nuclei normally found in murine hearts after birth.

In this article, we also describe the effects of introducing a single amino acid mutation in the motor domain of NMHC II-B using homologous recombination. As shown above, the expression of the mutant (R709C) myosin is decreased due to the presence of the gene encoding Neomycin resistance in a nearby intron. Although it is tempting to attribute the defect in cardiac myocytes in B+/B− mice to the mutation alone, we have preliminary evidence that this may not be the case, because removal of the Neo cassette, which restores normal levels of B+/B− expression, “rescues” the cardiac myocytes from enlargement and binucleation (X. Ma and R.S. Adelstein, unpublished observation, 2003). This suggests that both gene dosage and the mutation play a role in generating the binucleated cells.

Previously, we have described two different types of NMHC II-B−deficient mice with cardiac myocyte enlargement. In this article, we address the mechanism of myocyte enlargement found in B+/B− and hypomorphic B+/B− mice, which had its onset during embryonic development. A different mechanism must underlie the cardiac myocyte hypertrophy found in the hypomorphic B−/B− and B+/B− mice, because it does not involve binucleation of myocytes, it has its onset after birth, and it is accompanied by reexpression of β-cardiac myosin. The difference in mechanisms may be related to the change in NMHC II-B localization during embryonic development and in adult mice. Whereas NMHC II-B is present throughout the cytoplasm of embryonic myocytes, it is confined to the Z-line and intercalated disks of adult myocytes. Thus, it will be of interest to determine how the decreased content of NMHC II-B in these structures in adult mice could result in the myocyte hypertrophy seen in these hypomorphic mice. However, as far as we are aware, the present study is the first report of a decrease in cardiac myocyte proliferation during embryonic development due to a defect in cytokinesis.

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References


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