Myocardial Induction of Nucleostemin in Response to Postnatal Growth and Pathological Challenge

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Abstract—Stem cell–specific proteins and regulatory pathways that determine self-renewal and differentiation have become of fundamental importance in understanding regenerative and reparative processes in the myocardium. One such regulatory protein, named nucleostemin, has been studied in the context of stem cells and several cancer cell lines, where expression is associated with proliferation and maintenance of a primitive cellular phenotype. We find nucleostemin is present in young myocardium and is also induced following cardiomyopathic injury. Nucleostemin expression in cardiomyocytes is induced by fibroblast growth factor-2 and accumulates in response to Pim-1 kinase activity. Cardiac stem cells also express nucleostemin that is diminished in response to commitment to a differentiated phenotype. Overexpression of nucleostemin in cultured cardiac stem cells increases proliferation while preserving telomere length, providing a mechanistic basis for potential actions of nucleostemin in promotion of cell survival and proliferation as seen in other cell types. (Circ Res. 2008;103:89-97.)

Key Words: nucleostemin ■ cardioprotection ■ cardiomyocytes ■ stem cells ■ Pim-1 ■ telomerase

Cellular-based myocardial regeneration depends on tightly regulated signaling cascades that control survival and proliferation. In the case of stem cell populations, these signaling pathways have been predominantly defined by decades of study in hematopoietic and developmental contexts. The relatively recent advent of myocardial adult stem cells and their distinctive characteristics has prompted reexamination of the operational definition of “stem cells” and “stemness.” The traditional view of stem cell behavior as derived from classic lineage studies may not appropriately reflect the biology of stem cells in tissues characterized by slow cellular turnover such as the myocardium. For example, activation of signaling typically associated with regulation of proliferation and survival in stem cells is also observed in combination with partial or fully committed cellular phenotypes following tissue injury. These revelations have prompted dissolution of long-standing assertions related to "stem cell–associated" signaling, now viewed as regulation of tissue repair and regeneration or, in some, cases oncogenic transformation. Nucleostemin is found at high levels in various stem cells and human cancers, where it has been associated with maintenance of proliferation. Expression of nucleostemin drops precipitously during differentiation and genetic deletion of nucleostemin results in embryonic lethality at approximately day 4 postcoitum with blastocysts comprised of cells that fail to enter S phase. Similar arrest in G0/G1 phase of cell cycle was observed in HeLa cells if nucleostemin was eliminated by RNA interference. Nucleostemin has been purported to mediate cellular dedifferentiation and regenerative processes in newts. Although the molecular basis of nucleostemin-mediated actions remains controversial, evidence supports mechanisms related to inhibition of p53 or telomeric repeat-binding factor 1 (TRF1) that negatively regulates telomere length. Collectively, these characteristics point to a pivotal role for nucleostemin in maintenance of cell survival, antagonizing senescence, and promotion of regenerative potential.

Participation of nucleostemin in myocardial repair and regeneration has no precedent in the literature. Our findings establish a role for nucleostemin in response to pathological injury and demonstrate biological properties of nucleostemin expression in cardiac stem cells (CSCs), postnatal development, and response to paracrine fibroblast growth factor (FGF) treatment, as well as induction by Pim-1 kinase activity. Beneficial action depends on enhanced cell proliferation coupled with maintenance of telomeric length, which is preserved in c-kit+ CSCs by nucleostemin overexpression. Therefore, nucleostemin is a novel marker of protective signaling in the myocardium that, together with established...
links to stem cells, point to a role in myocardial repair and regeneration.

**Materials and Methods**

Details regarding nucleostemin cDNA, adenovirus, FGF treatments, antibodies, and immunoblotting are provided in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

Immunohistochemistry and confocal microscopy, including cell proliferation and telomere length measurements, were performed as previously described,\(^26,27\) with details in the online data supplement.

Stem cell and adult cardiomyocyte cultures were performed as described previously,\(^28,29\) with details in the online data supplement.

Murine surgical procedures were performed as previously described,\(^30\) with details provided in the online data supplement.

Pim-1–overexpressing transgenic mice were created as previously described.\(^31\)

All data are expressed as means±SEM. Differences in variables examined by Student \(t\) test. \(P<0.05\) was considered significant. Statistical data analyzed using Microsoft Excel software.

**Results**

**Nucleostemin Expression Declines Rapidly After Birth**

Nucleostemin is detectable within nuclei of cardiomyocytes in sections of neonatal mouse myocardium, as well as cultured neonatal rat cardiomyocytes (Figure 1). Nucleostemin expression diminishes rapidly within weeks after birth evidenced by fewer positive nuclei with lower intensity immunofluorescence in sections of older hearts relative to postnatal sections (Figure 1A). Nucleoli of cultured cardiomyocytes are labeled consistent with nucleostemin localization (Figure 1B).\(^32\) Progessive loss of nucleostemin correlated with increased age in myocardial sections (Figure 1A) and lysates showing significant (\(P<0.01\)) decreases in nucleostemin protein (Figure 1C). These results indicate nucleostemin association with young myocytes possessing proliferative potential during early postnatal growth.\(^33-36\) Exposure of neonatal rat cardiomyocytes to doxorubicin significantly increases nucleostemin levels (Figure 1A in the online data supplement), indicating cardiotoxic effects of doxorubicin may impair proliferation of young myocytes through antagonizing nucleostemin. However, nucleostemin overexpression is ineffective at antagonizing p53 protein in this system (supplemental Figure 1B).

**Nucleostemin Is Induced Following Pathological Challenge**

Relatively low-level nucleostemin expression in adult myocardium is markedly increased by acute pathological challenge or chronic heart failure (Figure 2). Myocardial infarction prompts nucleostemin expression in nuclei of cardiomyocytes primarily localized to the border zone adjacent to the ischemic region (Figure 2A). Immunoblot analyses of excised border zone/infarct regions reveal nucleostemin is increased at 24 hours after induction of myocardial infarction, with significant elevation of protein level within 48 hours that peaks at 72 hours. After 96 hours, expression of nucleostemin decreases from peak levels and returns to basal levels within 1 week (Figure 2B). In addition to cardiomyocyte expression, nucleostemin is also expressed in c-kit\(^+\) cells observed 4 days postinfarction. Two areas of enrichment for these c-kit\(^+\)/nucleostemin\(^+\) cells were the endothelial layer of healthy vessels in proximity to the infarct (Figure 2C) and individual cells in proximity to the border zone of damaged tissue (Figure 2D, at arrow).
Observations of nucleostemin expression in pathologically challenged myocardium were extended to include additional models of cardiac stress characterized by heart failure or pressure overload hypertrophy. The tropomodulin-overexpressing transgenic (TOT) mouse model is a well-characterized model of chronic dilated cardiomyopathy developed by our group.37–39 TOTs show nucleostemin expression throughout the myocardium by confocal microscopy (Figure 3A) and elevated protein level by immunoblot (Figure 3B). In comparison, pressure overload–induced hypertrophy also induced increased nucleostemin immunoreactivity in sections prepared from mice subjected to transaortic constriction. Areas of nucleostemin reactivity are restricted to cells neighboring and comprising large vessels such as endothelium lining the interior as well as cardiomyocytes surrounding vessels (Figure 3C). Quantitative immunoblot analysis of TAC-induced nucleostemin expression in the vasculature is not practical because of comparatively restricted regionalization of protein expression around large vessels relative to the whole heart.

**Nucleostemin Expression Is Induced by FGF**

At present, relatively little is known about inductive signals that mediate nucleostemin expression, but FGF-2 increases nucleostemin in adult bone marrow stem cells.21 Similarly, treatment of cultured adult mouse cardiomyocytes with FGF-2 prompts induction of nucleostemin immunoreactivity (Figure 4A). Immunofluorescence microscopy of FGF-2–treated cells show relatively preserved rod-shaped morphology of the FGF-treated cultures compared to vehicle-treated

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**Figure 2.** Nucleostemin expression is induced by myocardial infarction. A, Confocal microscopy of myocardial sections at various time points following myocardial infarction. Nucleostemin expression (green, at arrows) is observed in surviving cardiomyocytes within the border zone surrounding the infarct. Tropomyosin (red) labels sarcomeric structure and nuclei are labeled with Topro-3 stain (blue). B, Immunoblot shows time course of nucleostemin expression after myocardial infarction peaking at 72 hours postinduction. C, Confocal microscopy showing coincidence of nucleostemin (green) and c-kit (red) expression in cells lining a vessel proximal to the region of injury at 4 days postinfarction. The inset at the upper right shows the boxed region (yellow) at higher magnification. Nuclei are labeled with Topro-3 stain (blue). D, Confocal microscopy showing nucleostemin (green) and c-kit (red) expression coincident in a small cell (arrow) at the interface between the border zone (BZ) and infarct region (IR). A cell expressing c-kit but lacking nucleostemin is also shown (arrowhead). Single-channel scans that were used for creation of the color overlays are shown to the left of each image.
cells (Figure 4A). Immunoblot analyses demonstrate significant elevation of nucleostemin protein expression that peaks within 2 hours posttreatment but returns to basal levels after 8 hours (Figure 4B and 4C). In vitro findings were validated in vivo using systemic FGF-2 delivery by osmotic pump. Myocardial sections show increased nucleostemin immunoreactivity in cardiomyocytes of mice receiving osmotic pumps with FGF-2 compared to control samples (Figure 4A). This increase in myocardial nucleostemin is significant as assessed by quantitative immunoblots (Figure 4E and 4F).

Nucleostemin Is Expressed in Cardiac Stem Cells and Declines on Differentiation

Established association of nucleostemin with stem cells17,21 and c-kit+ cells in the myocardium (Figure 2C and 2D) prompted further assessment of nucleostemin expression in CSCs. Neonatal mouse myocardium, which is enriched for c-kit+ cells,26 shows colocalization between c-kit and nucleostemin immunoreactivity (Figure 5A). Furthermore, cultured CSCs express high levels of nucleostemin as observed by immunohistochemistry (Figure 5B), as well as immunoblot (Figure 5C). Expression of nucleostemin in CSCs is associated with maintenance of an undifferentiated phenotype. When induced to lineage commitment by exposure to dexamethasone,40 CSCs show a precipitous decline in nucleostemin expression that is statistically significant (Figure 5D), along with increased labeling for GATA-4 (Figure 5B) and loss of c-kit expression (data not shown).

Nucleostemin Expression Is Associated With Pim-1 Kinase Activity

Recent studies from our group have identified Pim-1 kinase as an essential regulator of cell survival downstream of Akt.31 Pim-1 is associated with cell proliferation and survival in the hematopoietic system41; therefore, experiments were performed to assess the relationship between Pim-1 activity and nucleostemin expression in myocardium. Normal mice show minimal levels of Pim-1 or nucleostemin expression (Figure 6A), whereas sections from transgenic mice created to overexpress Pim-1 kinase show accumulation of nucleostemin in cardiomyocyte nuclei (Figure 6B, at arrows). Induction of nucleostemin expression is also demonstrable by immunoblot analyses of lysates created from Pim-1–overexpressing transgenics relative to nontransgenic controls (Figure 6C). Furthermore, colocalization is observable in myocardial sections from mice at 4 days after infarction challenge, where surviving myocytes in the border zone coexpress both Pim-1 and nucleostemin (Figure 6D, at arrows).

Nucleostemin Increases TERT and Telomere Regulatory Protein Expression

The molecular basis for nucleostemin effects on telomere regulatory components was assessed by immunoblot analyses of CSC culture lysates. Nucleostemin overexpression prompted concomitant increases in levels of telomere-associated regulatory proteins TERT, TRF1, and TRF2 (supplemental Figure II). These results are consistent with pres-
Nucleostemin Increases Cardiac Stem Cell Proliferation While Preserving Telomere Length

Effects of nucleostemin overexpression on c-kit+ cultured CSCs were studied to assess consequences for cell proliferation and preservation of telomeric length. Increased nucleostemin expression was readily detected in the CSC cultures following infection with the adenoviral vector by immunoblot (supplemental Figure III). Nucleostemin overexpression in CSC promotes increased 5-bromodeoxyuridine labeling of nuclei indicative of DNA synthesis, as well as increased cell cycle progression, as demonstrated by a greater percentage of cells labeled by Ki67 (Figure 7). The number of CSCs with telomerase detectable by immunolocalization was significantly increased following nucleostemin overexpression, corresponding with a higher percentage of proliferative cells within the telomerase positive CSC population when nucleostemin is overexpressed. Despite enhanced CSC proliferation resulting from nucleostemin overexpression, average telomeric length in the CSC population was preserved and remained unchanged relative to normal control CSCs that undergo proliferative growth at a lower rate (supplemental Figure III).

Discussion

Traditional categorizations of stem cell–associated molecules, such as nucleostemin, are being redefined as these signaling cascades are discovered in partially committed or fully differentiated cells and tissues. Because nucleostemin is associated with cellular proliferation, it is not surprising that this pathway is activated in response to postnatal growth or pathological injury. Initially, our intent was to demonstrate expression of nucleostemin in regenerative processes associated with cardiac stem and progenitor cell populations. However, in addition to observing associations between nucleostemin and c-kit+ cells, we noted profound increases in nucleostemin activation in neonatal and pathologically challenged myocardium within cardiomyocytes, prompting additional studies to understand the role of nucleostemin signaling in response to myocardial injury and survival signaling. Since the discovery of nucleostemin 5 years ago, subsequent literature has focused predominantly on aspects of cancer, stem cells, and developmental biology.

Previous studies of nucleostemin establish the connection between nucleostemin and proliferative populations, either in the form of stem cells or cancer. In this context, nucleostemin appears to be a consistent marker for maintenance of a proliferative state, because expression is rapidly lost on commitment to a differentiated postmitotic phenotype and depletion of nucleostemin leads to cell cycle arrest. Conversely, nucleostemin expression is rapidly induced in response to regenerative growth in the heart and is required for embryonic development because nucleostemin-
null mice die in blastocyst stage approximately 4 days after fertilization. Loss of nucleostemin apparently renders cells incapable of DNA synthesis completion in S phase for HeLa cell cultures. In the context of myocardium, nucleostemin is enriched in postnatal myocytes, as well as cultured neonatal rat cardiomyocytes, and is downregulated in adult heart (Figure 1) or in CSCs induced to differentiation (Figure 5). These findings are consistent with expression of nucleostein.

**Figure 5.** Nucleostemin expression in CSCs declines on differentiation. A, Myocardial section from a mouse at 2 days after birth shows c-kit+ cells (green) with coincident expression of nucleostemin (magenta) at arrows. Sarcomeres are labeled with tropomyosin (red), and nuclei were detected with Topro-3 (white). Single-channel scans used for creation of overlays are shown on the left of each panel. B, Cultured cardiac c-kit+ cells (green) express high levels of nucleostemin (magenta). Cardiac stem cell cultures induced to differentiate by dexamethasone treatment show decreased nucleostemin expression and increased labeling for GATA-4 (blue). Lectin (right) is used as a cytoplasmic marker because of loss of c-kit expression. C, Decreased nucleostemin expression in CSC culture following dexamethasone treatment. D, Quantitation demonstrates a significant decrease in expression of nucleostemin in CSC cultures following dexamethasone treatment. Whole cell lysates are normalized to GAPDH to correct for minor variation in protein loading.

**Figure 6.** Pim-1 kinase activity induces nucleostemin expression. Myocardial sections from a nontransgenic (A) or transgenic mouse created with cardiac-specific expression of Pim-1 kinase (B). Single-channel scans shown to the left of each micrograph correspond to color overlays representing merged images of nucleostemin (red), Pim-1 kinase (green), or tropomyosin (blue) scans. Nucleostemin is evident in nuclei of cardiomyocytes as indicated (arrows in B). C, Immunoblot of lysates created from a nontransgenic or transgenic mouse created with cardiac-specific expression of Pim-1 kinase shows increased nucleostemin with histone bands shown to demonstrate comparable loading of protein samples. D, Myocardial section from infarcted mouse heart showing colocalization of Pim-1 and nucleostemin (at arrows) in surviving myocytes. Single-channel scans shown along left of micrograph corresponding to color overlays representing merged images of nucleostemin (magenta), Pim-1 kinase (green), or tropomyosin (red) scans.
min in a proproliferative state as neonatal myocytes are capable of limited mitotic activity. Although nucleostemin overexpression does not stimulate proliferation or hypertrophy in cultured adult cardiomyocytes (data not shown), induction of nucleostemin expression may be useful for antagonizing telomeric shortening associated with enhanced mitotic activity (Figure 7 and supplemental Figure II).

Functionally competent telomerase is restricted to a few cells in adult organism, germ cells, and stem/progenitor cells. In telomerase-competent cycling cells, detection of TERT in combination with markers of the cell cycle indicates that telomerase is active and prevents telomeric shortening. TERT expression is higher in nucleostemin-overexpressing CPCs than in control CPCs (Figure 7 and supplemental Figure II). Additionally, TERT and Ki67 colocalize in CPCs (Figure 7). Cycling CPCs that express TERT represent morphological counterparts of telomerase activity detectable with PCR-based methods. Importantly, the fraction of telomerase-competent cycling CPCs was higher in nucleostemin-infected CPCs, indicating that nucleostemin promotes CPC proliferation without affecting telomere length. In fact, by upregulating TERT expression, nucleostemin allows CPCs to undergo multiple divisions opposing telomere attrition. It is not surprising that length of telomeres did not differ in noninfected and nucleostemin expressing CPCs. Although nucleostemin overexpressing CPCs showed higher levels of TERT (Figure 7 and supplemental Figure II), control CPCs also possess telomerase. In physiological conditions, the function of telomerase is not to elongate telomeres beyond their physiological length but to prevent telomeric shortening. Finally, 3 to 5 days in culture is a very short time interval for the control cells that would not be expected to show detectable erosion of telomeres caused by rounds of replication.

TRF1 and TRF2 are 2 telomere-related protein components of a multiple protein complex, shelterin, that control homeostasis of telomeres by modulating access of telomerase to telomeres. In this regard, decreased TRF1 binding to telomeres reduce the affinity of telomerase to telomeres. TRF1 and TRF2 promote formation of T loops in which the telomere terminus is concealed to prevent its recognition as DNA strand break by DNA damage/repair machinery. This particular conformation of telomeres is nonaccessible to telomerase, thereby blocking telomere elongation. TRF1 and TRF2 are abundant in long telomeres but are absent in short telomeres, allowing telomerase to act only on short telomeres to prevent further erosion. Increases in TRF1 and TRF2 protein resulting from nucleostemin overexpression (supplemental Figure II) may indicate that 3’ telomere termini are sequestered within the T loops opposing telomerase-dependent elongation of telomeres of normal length. TRF1 and TRF2 are critical for T-loop formation, and maintenance of this specific telomere-associated molecular structure is essential for continued cellular proliferation and prevention of senescence.

Because telomerase activity is critical for maintenance of cardiac structure and function, nucleostemin may act to fine-tune endogenous telomerase activity and promote maintenance of telomere length as well as inhibit p53-associated

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**Figure 7.** Cardiac progenitor cell proliferation is increased by nucleostemin. A through H. Left and right images correspond to control and nucleostemin-overexpressing CPCs, respectively. CPCs (c-kit, yellow) (A and E) incorporate 5-bromodeoxyuridine (red) (A and E) and express the cell cycle protein Ki67 (green) (B and F) and the catalytic subunit of telomerase (white) (C and G). D and H. Merge of stainings. I. Results are mean±SD. *P<0.05 vs control.
signaling resulting from shortened telomeres. These postulates would be consistent with diminution of nucleostemin following exposure of cultured cardiomyocytes to doxorubicin (supplemental Figure 1), and lack of nucleostemin overexpression affecting p53 levels in this context may be explained by inhibition of MDM2 resulting from aberrant nucleostemin levels. Under normal circumstances where telomeric shortening is linked to senescence and possibly apoptosis, nucleostemin accumulation may serve to antagonize these processes in injured or aging myocardium, as implicated by increases in nucleostemin resulting from cardiomyopathic injury (Figures 2 and 3). In the case of stem cells, nucleostemin may enable cell cycling, as would be desirable in regenerative processes resulting from tissue injury or stress as supported by association of nucleostemin with c-kit+ cells in the myocardium and cultured CSCs under proliferative conditions (Figure 5).

Signal transduction controlling nucleostemin expression is not well documented, but FGF-2 induces nucleostemin expression in bone marrow stem cells. Interestingly, FGF-2 exerts prosurvival effects in myocardium, is a potent angiogenic molecule, and is a crucial factor for proliferation and maintenance of several cell types, including stem cell populations. Interestingly, FGF-2 promotes differentiation of resident cardiac precursors into functional cardiomyocytes, which would seem at odds with maintenance of a proliferative state unless the action of FGF-2 occurs at an early stage of commitment, when limited mitotic activity occurs in concert with lineage specification. FGF-2 stimulates Akt activity that could account for prosurvival and proproliferative effects, and Akt activation lies upstream from Pim-1 induction in cardiomyocytes. Induction of nucleostemin expression by Pim-1 activity (Figure 6) is without precedent. FGF-2 promotes differentiation of resident cardiac precursors into functional cardiomyocytes, which would seem at odds with maintenance of a proliferative state unless the action of FGF-2 occurs at an early stage of commitment, when limited mitotic activity occurs in concert with lineage specification. FGF-2 stimulates Akt activity that could account for prosurvival and proproliferative effects, and Akt activation lies upstream from Pim-1 induction in cardiomyocytes. Induction of nucleostemin expression by Pim-1 activity (Figure 6) is without precedent.

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Methods

Nucleostemin cDNA and adenovirus. The coding sequence for mouse nucleostemin was amplified with high fidelity Taq polymerase using gene specific primers containing flanking EcoRI restriction sites. Template consisted of cDNA generated from infarcted mouse heart. The PCR product was confirmed to be the correct size, digested with EcoRI and inserted by standard ligation methods into the pDC315 shuttle vector (Microbix AdMaxTM adenovirus system). Correct orientation and coding sequence was verified and the nucleostemin encoding plasmid was cotransfected with pGlox genomic adenoviral vector into HEK293 cells. Following cellular lysis, adenovirus was expanded and purified using FPLC. Neonatal rat myocytes were infected with purified virus and nucleostemin overexpression confirmed by immunoblotting using goat anti-nucleostemin antibody (RND Systems).

Immunohistochemistry and confocal microscopy including cell proliferation and telomere length measurements. Scans were taken at identical settings and optimized for high expressing cells in the case of nucleostemin in myocardial sections, so relative fluorescence of the basal state appears negative although low level fluorescence could be present.

Stem cell culture. Briefly, mouse hearts were subjected to enzymatic digestion with collagenase. Cardiac small cells were separated from myocytes by differential centrifugation and c-kit positive cardiac progenitor cells (CPCs) were selected using c-kit antibody conjugated with magnetic immunobeads (Miltenyi). Stem cells were
differentiated in differentiation media containing 250ml DMEM, 2.2g sodium bicarbonate, 10% Calf Serum for Fetal Bovine Serum and $10^{-8}$ M dexamethasone. Cells were cultured in the media for 5 days and were analyzed for differentiation markers. For studies involving nucleostamin overexpression, CPCs were infected with adenovirus carrying nucleostemin (NS) and cultured for 3 and 5 days. Different concentrations of virus were employed but only CPCs infected with viral particles (10% suspension in culture medium) showed a 5-10 fold difference in nucleostemin expression with respect to control non-infected CPCs.

**Telomere length.** Telomere length was evaluated in NS-infected and non-infected CPCs by quantitative fluorescence in situ hybridization (Q-FISH) and confocal microscopy. A fluorescein isothiocyanate-peptide nucleic acid (FITC-PNA) probe was used. The fluorescent signals measured in lymphoma cells with short (L5178Y-S, 7 kbp) and long (L5178Y-R, 48 kbp) telomeres were utilized to compute absolute telomere length.

**Murine surgical procedures.** Mice under isoflurane anesthesia were intubated and ventilated. For infarction, a lateral thoracotomy was performed the anterior surface of the heart was exposed. The anterior descending branch of the left coronary artery was ligated using 8-0 suture (Ethicon). Cyanosis and akinesia of the affected area were observed to confirm complete ligation of the LAD artery and the chest was closed. Sham operations were conducted by passing the suture under the coronary artery at the position used for ligation. For pressure overload-induced hypertrophy, an incision was made from the neck extending to the thorax and the sternum was divided up to the second rib. The aortic arch was isolated from annexed tissue and ligated between the branchiocephalic artery and the left common carotid artery with 7-0 suture material (Ethicon). Calibration of the
constriction was performed by placing a dull 27 gauge needle to the side of the artery with the ligature tied firmly to the needle and the artery. The needle was then removed leaving a calibrated stenosis of the abdominal aorta. The sternum was closed using a 6-0 silk suture (Ethicon) and the incision was closed. Sham operated mice were subjected to the same procedure without an abdominal stenosis. All experiments were replicated in triplicate.

Immunoblotting of frozen hearts. Frozen hearts were pulverized and put in sample buffer. Samples were vortexed and sonicated in a beaker of ice. Samples were boiled for 3 minutes and centrifuged at 12000 rpm at 4°C for 5 minutes. Supernatant was collected and loaded on a 10-12% gel. Proteins from NS-infected and non-infected CPCs were extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) and a cocktail of protease inhibitors (Pierce Biotechnology). Protein concentration was measured by Bradford assay (Bio-Rad). Equivalents of 30-50 µg protein were separated on 8-12% SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes, blocked with 5-10% dry skim milk in Tris-saline buffer with 0.1% Tween20 (TBST) for 1 h at room temperature, and exposed to goat polyclonal nucleostemin antibody (AF1638, R&D), rabbit polyclonal TERT antibody (H-231, Santa Cruz) 1/1000, rabbit polyclonal TRF1 antibody (ab1423, Abcam) or mouse monoclonal TRF2 antibody (clone 4A794, Upstate) in TBST overnight at 4°C. HRP-conjugated IgG were used as secondary antibodies (Pierce Biotechnology). Proteins were detected by chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology) and OD was measured. Loading conditions were determined by the expression of β-actin (Sigma). Lysates obtained from Jurkat cells were used as positive control.
**Immunocytochemistry.** In brief, tissues were formalin-fixed paraffin embedded, sectioned at 3 micrometer. The sections were deparaffinized in xylene and rehydrated through a series of alcohols in distilled water. 10mM citrate pH 6.0 was used for antigen retrieval for 14 minutes at 50% power using a 1100W microwave. Samples were allowed to cool down at 4°C for 20 minutes, washed in several changes of ddH2O and blocked for endogenous hydrogen peroxidase activity with 3% H2O2 1X TN buffer (150mM NaCl, 100mM Tris pH 7.5) for 20 minutes at room temperature. Slides were washed in several changes in TN buffer.

**Antibodies.** Primary antibodies include nucleostemin (R&D Systems #AF1638), c-kit (R&D Systems #AF1356), tropomyosin (Sigma #T9283), Pim-1 (Cell Signaling #7572) and GAPDH (Chemicon #MAB374). Primary antibodies were applied at 4°C overnight. Secondary fluorescent conjugated antibodies for Cy3, Cy5, and FITC were used at a 1:100 dilution (all from Jackson laboratories). Alexa Fluor 546 phalloidin was used to label actin filaments (Invitrogen/Molecular Probes #A22283).

**Fibroblast growth factor (FGF) treatments.** For *in vitro* studies, myocytes were isolated, plated for 1 h, put into culture media. 24 hours later, cells were treated with bFGF (10ng/ml, recombinant murine FGF-basic, PeproTech, Rocky Hill, NJ)) in culture media for 30min, 1h, 2h, 4h, 8h, 16h and 24 hours. Experiment is replicated in triplicate. For in vivo studies, osmotic pumps containing FGF-2 (10^{-4}M, also from PeproTech) were placed subcutaneous in mice for 72 hours. Hearts were harvested and prepared for immunohistochemistry and immunoblotting. The experiment was replicated in triplicate.
Proliferation assessments. To evaluate CPC proliferation, BrdU was added to the medium twice at 8 hour interval before fixation in 4% paraformaldehyde. BrdU incorporation was measured with mouse monoclonal BrdU antibody (Roche). Additionally, the expression of the cell cycle marker Ki67 was assessed (rabbit polyclonal Ki67 antibody, Vector) and its co-localization with TERT (H-231, Santa Cruz) was established. Finally, the presence of c-kit was determined with goat polyclonal c-kit antibody (R&D).
Online Figure I. Nucleostemin levels are diminished by exposure to doxorubicin (Dox) in cultured neonatal rat cardiomyocytes, but nucleostemin overexpression has no effect upon p53 induction. (A) Cultured cardiomyocytes showing decreased nucleostemin level in response to doxorubicin treatment by quantitation (top) and corresponding blot (below). GAPDH shown as loading control. * refers to p < 0.02  (B) p53 levels increased by dox are not impacted by concurrent adenoviral nucleostemin overexpression (adNS). Quantitations for p53 (top) and nucleostemin (bottom) are shown with representative immunoblot. Neonatal rat cardiomyocytes were infected with adenovirus expressing mouse nucleostemin in media containing 2% FBS. 24 hours later, cells were treated with vehicle or 1 micromolar doxorubicin overnight in media with 2% FBS. Whole cell lysates were collected for immunoblot analysis.

Online Figure II. Nucleostemin increases expression of telomerase and telomere-associated proteins. (A-D) Protein levels of TERT (A), TRF1 (B) and TRF2 (C) in control and nucleostemin expressing CPCs together with corresponding quantitation from optical density measurements of immunoblots. Jurkat cell lysates were used as positive controls (Jurkat). β-actin expression was employed to determine protein loading (D).

Online Figure III. Expression of nucleostemin (NS) in cardiac stem cells by immunoblot analysis of control versus cultures infected with the nucleostemin adenoviral vector (NS). Lanes are shown in duplicate to demonstrate reproducibility between two sets of samples prepared identically.

Online Figure IV. Maintenance of telomere length in cultured cardiac stem cells (CSC) under normal control conditions (A and C) or in cultures overexpressing nucleostemin (B and D). Quantitation bar graph at bottom of figure shows that average telomere length as measured by quantitative fluorescence is comparable between the two CSC cell populations.