Nucleolar stress is an early response to myocardial damage involving nucleolar proteins nucleostemin and nucleophosmin

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Edited by Eric N. Olson, University of Texas Southwestern, Dallas, TX, and approved March 5, 2011 (received for review November 30, 2010)

Nucleolar stress, characterized by loss of nucleolar integrity, has not been described in the cardiac context. In addition to ribosome biogenesis, nucleoli are critical for control of cell proliferation and stress responses. Our group previously demonstrated induction of the nucleolar protein nucleostemin (NS) in response to cardiac pathological insult. NS interacts with nucleophosmin (NPM), a marker of nucleolar stress with cytoprotective properties. The dynamic behavior of NS and NPM reveal that nucleolar disruption is an early event associated with stress response in cardiac cells. Rapid translocation of NS and NPM to the nucleoplasm and suppression of new preribosomal RNA synthesis occurs in both neonatal rat cardiomyocytes (NRCM) and cardiac progenitor cells (CPC) upon exposure to doxorubicin or actinomycin D. Silencing of NS significantly increases cell death resulting from doxorubicin treatment in CPC, whereas NPM knockdown alone induces cell death. Overexpression of either NS or NPM significantly decreases caspase 8 activity in cultured cardiomyocytes challenged with doxorubicin. The presence of altered nucleolar structures resulting from myocardial infarction in mice supports the model of nucleolar stress as a general response to pathological injury. Collectively, these findings serve as the initial description of myocardial nucleolar stress and establish the postulate that nucleoli acts as sensors of stress, regulating the cellular response to pathological insults.

nucleolus | rRNA | hypoxia | hypertrophy

Nucleoli act as sensors of stress in noncardiac cells (1) in addition to their well documented function as coordinators of transcription, processing and assembly of rRNA with ribosomal proteins to form mature ribosomal subunits (2). Nucleoli rapidly lose compact organization with nucleolar proteins delocalizing to the nucleoplasm upon exposure to cytotoxic stimuli in a phenomenon termed "nucleolar stress" associated with impairment of preribosomal RNA (pre-rRNA) transcription (3), increased levels of p53 and apoptosis (4).

Nucleoli possess several characteristic proteins including nucleostemin (NS), which rapidly shuttles between nucleolus and nucleoplasm (5). Functional activities of NS are linked to proliferation (6–8), embryogenesis, cell cycle progression (9, 10), and pre-rRNA processing (11), as well as involvement in regulation of p53 pathway in tumor cell lines (12–15). Although expression in the myocardium decreases shortly after birth, NS is induced following pathological injury in adult myocardium and elevated NS levels correlate with cardioprotection in mice (16). NS interacts with another nucleolar protein called nucleophosmin (NPM; ref. 17), a well known nucleolar marker and multifunctional phosphoprotein with antiapoptotic properties (18, 19) implicated in ribosome biogenesis (20, 21) and p53 pathway regulation (22, 23). NPM delocalization from nucleoli is widely accepted as a marker of nucleolar stress in noncardiac cells (1, 24, 25). However, nucleolar stress and the consequences for cell viability following cytotoxic insults remain unexplored in the myocardial context.

Nucleoli are sensitive to pathological conditions that prompt delocalization of NS and NPM as an early response to stimuli in neonatal rat cardiomyocytes (NRCM) as well as cardiac progenitor cells (CPC), supporting the premise of nucleoli as sensors of stress as originally described in noncardiac cells (1, 26, 27). Furthermore, impairment of nucleolar function by knocking down either NS or NPM increases sensitivity of both NRCM and CPC to apoptotic stimuli. Our results identify a mechanistic basis for maintenance of nucleoli in cardiomyocytes and point toward nucleoli as another coordinator of p53 activity, affording new possibilities for development of therapeutic approaches to protect the heart from stress.

Results

Nucleolar Disruption Is a Common Response to Stress. Nucleoli serve as sensors of stress in noncardiac cells (1, 4, 24, 26), but the involvement and dynamic regulation of nucleoli in cardiomyocytes needs to be established. Nucleolar disruption has been studied in nonmyocytes thorough use of a selective inhibitor of polymerase I transcription known as actinomycin D (ActD; ref. 27) or the well known chemotherapeutic drug doxorubicin (DOX; refs. 14 and 27), which promotes cardiomyocyte apoptosis (16). Nucleolar disruption in cultured NRCM was induced following exposure to either DOX or ActD (Fig. 1*A*). Localization of NS was assessed in relationship to NPM by confocal microscopy, with diffuse delocalization of both NS and NPM to the nucleoplasm within 4 h of DOX or ActD treatment. Nucleolar decondensation is associated with reduction of nucleolar size and diminished signal intensity for both NS and NPM. Because NS is highly expressed in proliferating CPC (16) and CPC viability is impaired by exposure to DOX (28), the induction of nucleolar stress in CPCs was assessed following exposure to DOX with ActD as a positive control (Fig. 1*B*). Resultant nucleolar delocalization as evidenced by loss of NS and NPM immunolabeling initially occurs after 8 h of exposure to DOX or ActD, indicating that CPCs possess greater resistance to nucleolar stress relative to NRCM. Confocal microscopy for NS correlates with immunoblot findings quantitated over an 8- to 24-h time course of DOX treatment in NRCM,

Author contributions: D.A. and M.A.S. designed research; D.A., B.B., C.T.C., B.S., A.J., M. McGregor, N.G., A.Z., and M. Konstandin performed research; N.G., S.T., M. Khan, S.M., M.V., H.T., M. Mason, Z.C., S.D., R.A., and K.F. contributed new reagents/analytic tools; D.A. analyzed data; and D.A. and M.A.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1017935108/-/DCSupplemental.

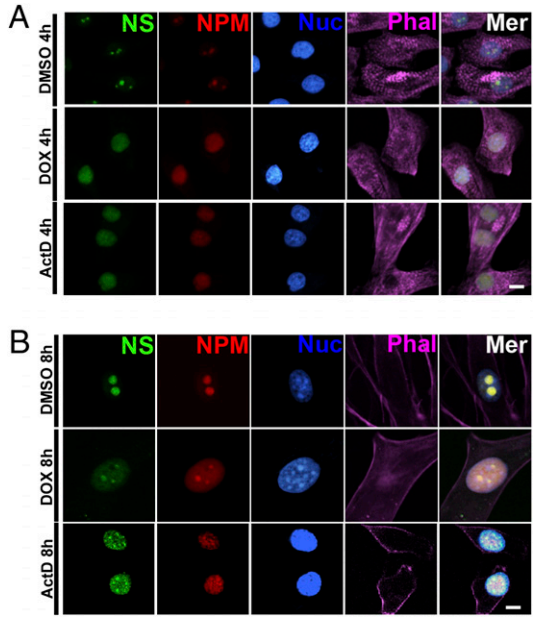

Fig. 1. Nucleolar disruption is a common response to stress in neonatal rat cardiomyocytes and cardiac progenitor cells. (*A* and *B*) Single channel confocal scanning and merge showing NRCMs after 4 h (*A*) and CPCs after 8 h (*B*) of treatment with DMSO (*Top*), 1 μM DOX (*Middle*) or 0.05 μg/mL ActD (*Bottom*). NS (green) and NPM (red) colocalize within nucleoli of DMSO-treated cells and delocalize into the nucleoplasm of DOX- and ActD-treated cells. Phalloidin (Phal, purple) for cell structure and sytox blue (Nuc, Blue) for nuclei are shown as a merged image (Mer). (Scale bars: 10 μm.)

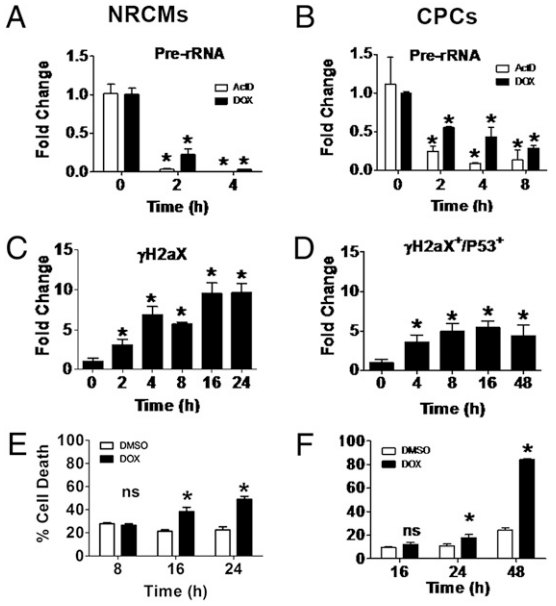

Fig. 2. Nucleolar disruption is associated with impairment of new rRNA synthesis and precedes apoptosis. DOX and ActD inhibit new rRNA synthesis in NRMCs (*A*) and CPCs (*B*). Levels of pre-rRNA were measured by qRT-PCR and normalized to 18S expression ($n = 3$ for each group, $^*P < 0.05$ vs. 0). (*C*) Phosphorylation of histone H2AX (γH2AX), assessed by FACS analysis, significantly increases at early time points and in parallel with nucleolar stress in NRCMs ($n = 3$ for each group, $^*P < 0.05$ vs. 0). (*D*) γH2AX levels increased similarly in CPCs and in parallel to p53 as assessed by measuring $\gamma H2AX^+/p53^+$ cells ($n = 3$ for each group, $^*P < 0.05$ vs. 0). (*E* and *F*) Cell death, estimated by FACS analysis, increases with time in DOX-treated NRMCs (*E*) and CPCs (*F*; $n = 3$ for each group, $^*P < 0.05$ vs. DMSO).

where NS levels decrease by 22.2 ± 9.0% at 24 h (Fig. S1*A*). In comparison, CPC are relatively resistant to nucleolar stress after DOX treatment for 24 h, with a 34.0 ± 3.6% decrease in NS at 24 h of exposure (Fig. S1*B*). In contrast, NPM protein levels remained unchanged in either NRCM or CPC during the 24 h exposure, indicating that delocalization need not necessarily correspond to loss of protein content (Fig. S1 *A* and *B*). NPM interacts with $p19^{ARF}$ to mediate nucleolar sequestration under steady state conditions (23), and DOX exposure induces $p19^{ARF}$ mobilization to the nucleoplasm (Fig. S1*C*), similar to findings with NS and NPM. Collectively, these results demonstrate nucleolar stress as a common response in cardiac cells to transcriptional and genotoxic stress resulting from exposure to ActD or DOX. However, CPC resist induction of nucleolar stress relative to NRCM when exposed to identical challenges.

Nucleolar Disruption Impairs New rRNA Synthesis and Precedes Apoptosis. rRNA transcription is essential to produce ribosomes required for protein synthesis. Mature rRNA derives from a single precursor (pre-rRNA) that is cleaved to generate 18S, 28S, and 5.8S products (Fig. S2*A*). Nucleolar stress is associated with inhibition of new rRNA synthesis as determined by quantitative RT-PCR (qRT-PCR) measuring the ratio between signals derived from amplification of a fragment of pre-rRNA relative to the level of 18S mature rRNA, which does not change (Fig. S2 *B* and *C*) under our experimental condition and is used as internal standard (29, 30). pre-rRNA synthesis is lost following treatment of NRCM with either DOX or ActD for 4 h (Fig. 2*A*). In comparison, pre-rRNA levels in CPCs were reduced by DOX and ActD, but transcriptional inhibition was still incomplete after 8 h (Fig. 2*B*). DNA damage assessed by flow cytometry of phosphorylated H2AX (γH2AX) levels shows significant increases following exposure to DOX for 2 h in NRCMs versus 4 h in CPC (Fig. 2 *C* and *D*). Increased p53 expression in the CPC population parallels that of γH2AX as assessed by measuring $\gamma H2AX^+/p53^+$ with flow cytometry (Fig. 2*D*) and apoptosis increases in both NRCM and CPC after DOX treatment (Fig. 2 *E* and *F*). Collectively, these results demonstrate nucleolar stress as an early response to genotoxic stress activated upstream of DOX-induced apoptosis.

NS and NPM Influence Cell Survival in Response to Apoptotic Challenge. Functional activities of NS and NPM in CPC under basal and stress conditions were assessed by silencing expression with lentiviral vectors producing specific shRNA for NS (ShNS), NPM (ShNPM), or a scramble sequence (ShSc) under control of the human U6 promoter (31). Specificity of the shRNAs is supported by findings that NS silencing did not affect NPM expression and vice versa (Fig. S3*A*). We examined the consequences of diminished NS or NPM upon cell survival and nucleolar stress by using CPC expressing ShSc, ShNS, or ShNPM treated with DMSO or DOX in conjunction with Annexin V labeling-based flow cytometry. NS silencing did not significantly increase CPC apoptosis under basal conditions, but instead sensitized them to DOX treatment with significant increases in cell death after 16 h relative to normal CPCs, which maintain survival at the same time point (Fig. 3*A*). In contrast, increases in CPC apoptosis result from NPM silencing under basal conditions before DOX exposure, with apoptosis only slightly increased after additional DOX challenge (Fig. 3*A*). Major alterations of nucleolar structure observed with NPM silencing (32) were evident in CPCs, with nucleolar fragmentation and delocalization of NS (Fig. 3*B*) as well as $p19^{ARF}$ (Fig. S3*B*) evident in nucleoplasm with ShNPM. In the converse experiment, NS knockdown did not affect NPM localization (Fig. 3*B*), supporting a central role for NPM, but not NS, in regulating global architecture of nucleoli (11, 12, 15). Down-regulation of either NS or NPM inhibits

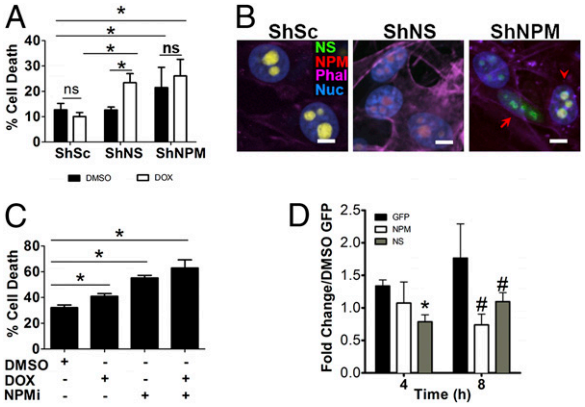

Fig. 3. Gain and loss of function studies. (*A*) Cell death is increased in CPCs silenced for NS (ShNS) and then challenged with DOX for 16 h. NPM silencing (ShNPM) increases cells death even under basal conditions. CPCs overexpressing a scramble shRNA (ShSc) were used as control ($n = 4$, $^*P < 0.05$). (*B*) Confocal images showing CPCs infected with ShSc, ShNS ShNPM with a cell showing fragmented nucleoli (arrow), and a nearby cell infected with low efficiency showing regular nucleoli and normal levels of NS and NPM (arrowhead). NS (green), NPM (red), Phalloidin (Phal, purple), nuclei (Nuc, blue). (Scale bars: 10 μm.) (*C*) Increased cell death in NRCMs challenged for 16 h with the NPM-specific inhibitor NSC348884 (NPMi). Cotreatment with DOX does not further increase cell death ($n = 3$, $^*P < 0.05$). (*D*) NRCMs overexpressing NS or NPM show decreased caspase 8 activation compared with GFP-overexpressing cells after 4 and 8 h of DOX treatment ($n = 3$,$^*P < 0.05$).

pre-rRNA synthesis and induces expression of p53 target genes p21 and mdm2 (Fig. S3*C*). Activation of p53 was confirmed by using a p53 luciferase reporter assay (Fig. S3*D*). This result validates the importance of NS and NPM in regulating rRNA biogenesis and controlling p53 pathway. Pharmacologic inhibition of NPM with NSC348884 (NPMi), previously reported to induce apoptosis in cancer cells (33), also significantly increases apoptosis in NRCMs (Fig. 3*C*), thus confirming a key role of NPM for cell survival.

Antiapoptotic capabilities of NS and NPM overexpression were tested using adenoviral vectors encoding GFP alone or GFP fused to NS (NSGFP) or NPM (NPMGFP) after validation of appropriate expression and localization (Fig. S4 *A* and *B*).

NRCM were infected overnight with NSGFP or NPMGFP vectors and challenged with DOX the following day to determine inhibition of apoptosis (Fig. 3*D*). Apoptotic signaling was decreased by either NSGFP expression relative to GFP following DOX challenge as determined by caspase-8 activity at 4 h; signaling was decreased by NPMGFP expression as determined by caspase-8 activity at 8 h. NSGFP- or NPMGFP-mediated protection is lost after 16 h of DOX exposure (Fig. S4*C*), when delocalization of GFP signal is evident within the nucleus (Fig. S4*D*). Because NPM silencing induces apoptosis in CPCs under basal conditions, we tested whether NS overexpression could protect against the loss of NPM. Apoptosis remains unchanged in CPC where NPM is silenced regardless of whether NS or GFP is overexpressed (Fig. S4*E*), indicating that NS cannot compensate for NPM functional loss.

Collectively, these data demonstrate that physiological levels of NPM are necessary to maintain viability, presumably by maintaining nucleolar integrity. NS overexpression cannot rescue the apoptosis in ShNPM CPCs. Although not essential for cell survival, forced down-regulation of NS reduces the threshold of apoptotic resistance in CPCs, implicating NS in controlling the DNA damage response. The protective effects of NS and NPM in overexpression studies indicate that NS or NPM delay (but cannot abrogate) nucleolar disruption resulting from apoptotic challenge with DOX.

DOX Induces Nucleolar Stress in Vivo. Companion experiments to the in vitro demonstrations of DOX-induced nucleolar disruption (Fig. 1 *A* and *B* and Fig. S1*C*) were performed in vivo using postnatal mice at 1 wk of age, when myocardial NS and NPM are still highly expressed (Fig. S5 *A–D*). Myocardium shows colocalization of NS and NPM within cardiomyocyte nucleoli in controls sections that is lost after DOX challenge, prompting delocalization of NPM to the nucleoplasm (Fig. 4*A*) and loss of NS signal (Fig. S6*A*), in agreement with prior in vitro findings (Fig. 1 *A* and *B* and Fig. S1 *A* and *B*). Additional indices of nucleolar stress include up-regulation of p53 (Fig. 4*B*) after DOX challenge, which was transcriptionally active, as demonstrated by the increased expression of target genes p21 and mdm2 (Fig. 4*D*). Also, pre-rRNA levels were reduced (Fig. 4*C*), as previously seen in vitro (Fig. 2 *A* and *B*). Validation of DNA damage induced by DOX challenge was confirmed by TUNEL assay to confirm pathological effects of the treatment (Fig. S6*B*). Thus, nucleolar stress occurs in vivo as a response to pathologic stress associated with p53 pathway activation and apoptosis.

Nucleostemin and Nucleophosmin Are Concurrently Induced After Myocardial Infarction and Trans Aortic Constriction. Myocardial infarction (MI) and trans aortic constriction (TAC) induce NS (16), so experiments were performed to determine the effects on NPM and correlate pathological damage with nucleolar stress in the experimental model of infarction or TAC challenge. NS and NPM expression were evaluated in hearts subjected to MI versus controls (CON). Several cardiomyocytes within the border zone (BZ) show swollen and enlarged nucleoli characterized by irregular shape where both NS and NPM appear delocalized (Fig. 5*A*), although the majority of cells display normal localization of both proteins (Fig. S7*A*). NS and NPM expression levels peak respectively at 4 and 7 d post-MI, with persistent elevation of NS for up to two weeks (Fig. 5*B* and Fig. S7*B*). pre-rRNA level peaks at 4 d after MI and returns to normal expression level within 14 d (Fig. 5*C*), which follows similar changes in expression of NS and NPM protein during this time course. These differences were restricted to the BZ, as no changes between MI and CON were observed in the remote region (Fig. S7*C*). We obtained similar results by culturing NRCMs under hypoxia/hypoxia-reperfusion conditions, as an in vitro counterpart to myocardial infarction. Although the majority of cells display regular nucleoli, groups of NRMCs show small nucleoli with NS and/or NPM signals partially delocalized

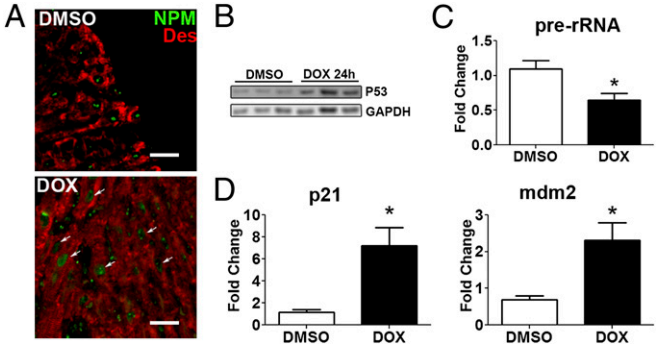

Fig. 4. Doxorubicin induces nucleolar stress in vivo. (*A*) Confocal images of heart sections from mice at postnatal day 7 injected with DMSO (*Upper*) or 15 mg/kg DOX (*Lower*) and killed 24 h later. NPM (green), desmin (red). Cardiomyocytes from DOX-treated mice display delocalization of NPM (arrows). (Scale bars: 40 μm.) (*B*) Representative immunoblot showing p53 accumulation in heart from DOX-treated mice. (*C*) pre-rRNA expression was down-regulated in DOX-treated mice ($n = 3$; $^*P < 0.05$ vs. DMSO). (*D*) Up-regulation of mdm2 and p21 genes confirming the activation of p53 pathway in DOX-treated hearts ($n = 3$; $^*P < 0.05$ vs. DMSO).

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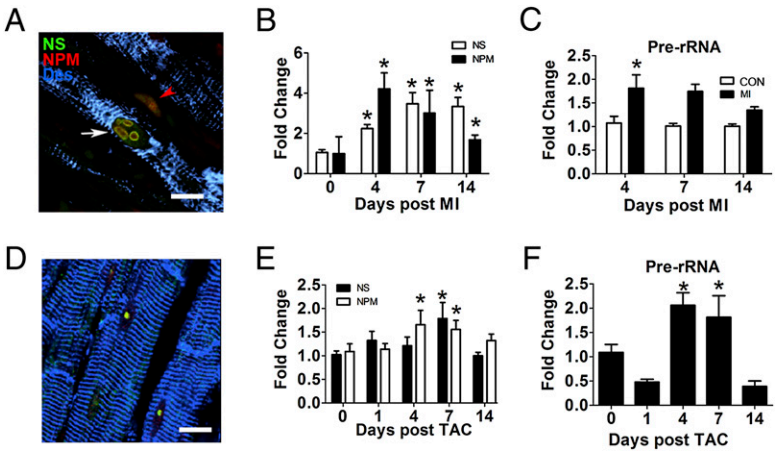

Fig. 5. NS and Nucleophosmin are induced after pathological challenge in the heart. (*A* and *D*) Confocal images showing adult cardiomyocytes in infarcted (*A*, MI) and in TAC (*D*, TAC) hearts 4 d after surgery. NS (green), NPM (red), and desmin (Des, blue) are shown. (Scale bars: 10 μm.) (*B* and *E*) Immunoblot quantifications of MI (*B*) and TAC (*E*) heart lysates showing significant increase of NS and NPM levels over time. ($n = 3$ mice per group for each experiment, $^*P < 0.05$). (*C*) qRT-PCR showing increase in pre-rRNA level in LV from MI hearts in comparison with time matching LV from sham operated mice (CON; $n = 3$, $^*P < 0.05$). (*F*) qRT-PCR showing increase in pre-rRNA level in LV from TAC hearts in comparison with LV from control mice (0; $n = 3$,$^*P < 0.05$).

into the nucleoplasm when cultured in hypoxic conditions for 24h (Fig. S8*A*). An additional 24 h of reperfusion further reduces the number of cells overall with spared NRCMs displaying normal nucleoli (Fig. S8*A*). Immunoblot data show NPM expression decreases under hypoxia and returns to control levels upon reperfusion. NS expression does not change significantly under these experimental conditions (Fig. S8*B*). Both NS and NPM are induced by hypoxia at the mRNA level, consistent with published results for NPM in cancer cells (Fig. S8*C*) (19).

The relationship among NS, NPM, and pre-rRNA synthesis was further addressed by TAC as a model of pathological hypetrophy associated with elevated protein synthesis. NS and NPM display normal localization within nucleoli of cardiomyocytes from TAC mouse hearts (Fig. 5*D* and Fig. S7*D*). NPM expression level peaks at 4 d after TAC, and NS expression level peaks at 7 d after TAC, when both proteins are significantly elevated (Fig. 5*E* and Fig. S7*E*). pre-rRNA level follows a similar trend, peaking at 4 and 7 d and decreasing at 14 d post TAC (Fig. 5*F*). Ongoing hypertrophic signaling in left ventricle of TAC mice is confirmed by the elevated mRNA levels of hypertrophic markers, such as ANP, BNP, and β-MHC genes (Fig. S7*F*). Similar results are observed in vitro treating NRCMs with 100 nM phenylephrine (PE) for 24 h. NS and NPM colocalize within the nucleolus of PE-treated NRCMs, which appear bigger in size compared with control cells (Fig. S8*D*). NS and NPM protein levels are increased by PE although only NPM reaches the statistical significance compared with control (Fig. S8*E*). NS and NPM mRNA levels are also significantly increased using this in vitro model as assessed by qRT-PCR (Fig. S8*F*). Thus, maintenance of pre-rRNA expression indicates activation of nucleolar metabolism concomitant with induction of NS and NPM protein expression following MI or TAC challenge, although NS and NPM delocalization can be seen in a select subpopulation of stressed cardiomyocytes in the BZ surrounding the infarct region as well as under hypoxic conditions in vitro.

Discussion

The traditional view of nucleoli serving primarily as a factory for assembling ribosomes is rapidly moving toward a new level of complexity at the center of key regulatory pathways controlling important biological processes such as cell cycle, proliferation, stress response, and even cellular aging (34–36). The finding that perturbation of nucleolar activity by genetic ablation of ribosome biogenesis initiates neurodegeneration in adult mice by activation of an endogenous response leading to p53 activation (24) is one example of an emerging series of findings that support the idea that the nucleolus acts as a sensor of cellular stress (1, 3, 25–27). The nucleolar protein NS, normally associated with the proliferative status of stem and cancer cells, is reinduced after pathological challenge within the adult heart and is potentially associated with cardioprotective pathways (16). The findings presented here represent a demonstration of a nucleolar stress response in the cardiac context and open up possibilities and questions on the role of nucleolar regulation in preserving cardiomyocyte and CPC survival.

The nucleolar stress in cardiomyocytes and CPC is induced by exposure to DOX, previously shown to induce nucleolar disruption in noncardiac cells (14). DOX-mediated cardiotoxicity is mediated by multiple mechanisms that are still not fully understood despite years of investigation (28). Rapid delocalization of NS and NPM within the nucleoplasm of cardiac cells induced by DOX is associated with nucleolar stress both in vitro and in vivo, as shown by the concurrent decline of new rRNA synthesis. Nucleolar disruption consequently releases several proteins within the nucleoplasm that become available to interact with p53 (4), as shown for $p19^{ARF}$ (Fig. S2*C*). Upon delocalization into the nucleoplasm, $p19^{ARF}$ interacts with and antagonizes mdm2, suggesting a direct connection between nucleolar stress and activation of p53 pathway (23). Promotion of p53 activity, already shown in other biological contexts, is prompted by nucleolar stress in an early cellular response preceding p53 activation and the induction of a suicide signal program (1, 3, 4, 26, 27). Although the importance of multiple pathways to explain DOX-mediated pathogenesis should not be underestimated, data presented herein implicate nucleolar stress as an important mechanism of DOX cardiotoxicity and corroborate reports that DOX cardiotoxic effects are ameliorated by p53 inhibition (37–39).

Substantiating the concept of nucleoli molecular signals participating as sensors of stress and regulators of survival, apoptotic signaling was induced by either down-regulation of NPM in CPC or the inhibition of NPM function in NRCM (Fig. 3*A*). NPM knockdown is also associated with nucleolar distortion and partial delocalization of NS and $p19^{ARF}$ within the nucleoplasm (Fig. S3*C*). These observations directly link nucleolar stress with induction of apoptosis and point toward loss of nucleolar in-

tegrity as a pivotal event of the cellular stress response. Indeed, overexpression of NS or NPM may be interpreted in the cellular context as a form of nucleolar disruption, which may account for why neither NS nor NPM were capable of preventing DOX-induced cardiotoxicity and loss of nucleolar integrity in prolonged treatment experiments. NS or NPM could exert protective effects against stress on a molecular level unrelated to preservation of nucleolar integrity, but further studies are necessary to address the mechanisms involved.

The role of NS in preservation of nucleolar structure and inhibiting stress responses remains less straightforward than NPM. Unlike NPM, inhibition of NS expression does not promote cell death (Fig. 3*A*), suggesting that DOX-mediated downregulation of NS is not sufficient to induce apoptosis but could facilitate progression, possibly by elevating basal level of p53 expression (Fig. S3 *C* and *D*). Also, nucleolar structure appears unaffected by NS silencing (Fig. 3*B*). This finding is consistent with other studies (11, 12, 15) that indicate NS is not essential for preservation of nucleolar integrity. However, impaired pre-rRNA synthesis, coupled with activation of p53 target genes observed in NS silenced CPC (Fig. S3 *D* and *E*), supports a role for NS to maintain nucleolar function and help resist pathological stress.

In support of this premise, NS expression is increased following MI concomitant with increased levels of new pre-rRNA synthesis (Fig. 5 *B* and *C* and Fig. S7*B*). Although myocardial sections represent brief "snapshots in time" of ongoing cellular processes, the consistent occurrence of irregular nucleoli and delocalization of NS/NPM immunolabeling in several BZ cardiomyocytes of MI hearts indicates ongoing nucleolar stress following infarction (Fig. 5A and Fig. S7*A*) in the damaged region. It is tempting to speculate that the increased expression of NS and NPM observed following MI challenge represents a compensatory reinduction of a fetal/embryonic stress response to preserve and enhance nucleolar structure and function. In line with this observation, NS transcript is increased under in vitro hypoxia most likely as a compensatory mechanism of the cells to keep the nucleolus functional under stressed conditions. NS and NPM expression increases in concomitance with pre-rRNA level both in vitro and in vivo upon hypertrophic stimuli, which further suggests their central role in sustaining nucleolar metabolism. The generation of a transgenic mouse with cardiac-specific overexpression of NS is of pivotal importance to fully address NS cardioprotective properties as well as NS role in nucleolar metabolism in vivo.

Collectively, our findings demonstrate nucleolar stress as a molecular mechanism contributing to anthracycline-mediated cardiotoxicity involved in control of p53 activity. The peculiar property of nucleoli serving as a regulatory node for the p53 pathway and ribosome biogenesis has important implications for influence over aging and senescence of the heart. Given the tight association between rRNA and protein synthesis (4), it is reasonable to presume that impaired nucleolar function would be associated with senescence of cardiac cells. Circumstantial evidence for nucleoli-associated regulation of aging involving NS and NPM comes from multiple directions: (*i*) myocardial aging is characterized by reduction of protein synthesis and decreases in ribosome functional efficiency (40); (*ii*) functional impairment of nucleoli induces neurodegeneration although p53 activation (4, 24); (*iii*) genetic ablation of NS promotes replicative senescence of cultured MEFs (10); (*iv*) NS overexpression in mouse CPCs leads to increased levels of TERT and telomere regulatory proteins (16); and (*v*) NPM maintains genomic integrity (32) and antagonizes senescence in cells suffering from genomic instability (41).

In conclusion, myocardial nucleolar stress introduces a unique perspective for investigation of stress response, apoptosis and senescence, as well as cell cycle and proliferation control in the heart. Cell proliferation depends upon NS and NPM (9, 18), which can serve as markers of cell cycle reentry similar to Ki67, another nucleolar protein (42). Thus, the process of myocardial regeneration after pathologic injury likely requires reactivation of nucleolar metabolism associated with induction of NS and NPM expression as documented in this study. Understanding the biological mechanism(s) and signaling regulating nucleolar dynamics will be of fundamental importance to identify new strategies maintain nucleolar integrity, promote survival, and increase regeneration of injured heart.

Materials and Methods

Animal Studies. The Institutional Animal Care Committee of San Diego State University approved all animal protocols. Detailed experimental procedures are reported in *SI Methods*.

Cell Culture and in Vivo Treatments. Primary neonatal rat cardiomyocytes (NRCMs) and cardiac progenitor cells (CPCs) were isolated and cultured as described (1, 2). Nucleolar stress or apoptosis were induced with 0.05 μg/mL actinomycin D (ActD), 1 μM doxorubicin (DOX), or 5 μM NSC348884 (NPMi), all from Sigma. Details are provided in *SI Methods*. Mice were injected intraperitoneally with a single dose of 15 mg/kg DOX or DMSO diluted in saline solution. Hearts were collected 24 h later and used for successive analysis.

Viral Vectors. Lentiviral vectors carrying the shRNA to specifically downregulate either mouse NS or NPM as well as the scramble control were purchased from Sigma. Lentiviral vectors were prepared and concentrated as described (2). Adenoviral vectors overexpressing GFP, NSGFP, and NPMGFP genes were produced and used as described (1). Further details are provided in *SI Methods*.

Real-Time PCR. Total RNA was isolated from frozen heart or cultured cells by using Quick-RNA MiniPrep (Zymo Research) and reverse-transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was performed on all samples in triplicate using iQ SYBR Green (Bio-Rad) according to the manufacturer's instructions. Primer sequences are provided in Table S1.

Immunoblots. Immunoblotting was performed as described (43). Details regarding sample preparation are reported in *SI Methods*.

Immunohistochemistry. Immunofluorescence staining, TUNEL assay, and immunohistochemistry were performed as described (4). Additional details are provided in *SI Methods*.

Flow Cytometry. Cell death was measured by Annexin V staining (BD Biosciences) according to manufacturer's instructions. Sytox blue (Invitrogen) at concentration of 1:1,000 vol/vol. Intracellular staining was performed as described (44). A list of primary and secondary antibodies are provided in Table S2. Cytometry was performed by using a BD FACSAria Flow Cytometer (BD Biosciences).

Statistical Analysis. All experiments were repeated independently at least three times. All data are expressed as mean ± SEM. Comparisons were performed using Student *t* test, one-way ANOVA, or two-way ANOVA according to the experimental design. *P* values less than 0.5 were considered statistically significant. Statistical analysis was performed using Graphpad Prism v 5.0 software.

ACKNOWLEDGMENTS. We thank members of the M.A.S. laboratory—in particular, Lauren Neidig and Kathleen Bradley—for helpful discussion and comments. M.A.S. is supported by National Institutes of Health Grants 1R21HL102714-01, 2 R01 HL067245, IR37 HL091102-01, P01HL085577-05, RC1HL100891-02, R21 HL102613-01, and 1 R21 HL104544-01. B.B. is supported by the San Diego State University Rees-Stealy Research Foundation. C.T.C is supported by the Rees-Stealy Research Foundation, the Achievement Rewards for College Scientists Foundation, American Heart Association Predoctoral Fellowship 10PRE3060046, and an Inamori Foundation Fellowship. M.V. and M. Konstandin are supported by Deutsche Forschungsgemeinschaft (DFG) Grants MV 1659 1/1 and KO 3900/1-1. H.T. is supported by the UEHARA Memorial Foundation.

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