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Endothelial nitric oxide synthase promotes neonatal cardiomyocyte proliferation by inhibiting tissue inhibitor of metalloproteinase-3 expression

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Abstract

Objective: We have recently demonstrated that endothelial nitric oxide synthase (eNOS) promotes cardiomyocyte proliferation. However, mechanisms by which eNOS regulates cardiomyocyte proliferation are not fully understood. The goal of the present study was to investigate the role of tissue inhibitor of metalloproteinase-3 (TIMP-3) in eNOS-mediated cardiomyocyte proliferation.
Methods and results: Experiments were conducted in cultured neonatal mouse cardiomyocytes. TIMP-3 expression was significantly decreased in wild-type (WT) cardiomyocytes treated with an adenoviral eNOS (Ad-eNOS). Furthermore, TIMP-3 levels were significantly decreased in cardiomyocytes derived from eNOS transgenic mice. Conversely, TIMP-3 transcript levels were significantly elevated in eNOS$^{-/-}$ cardiomyocytes. The inhibitory effect of NO on TIMP-3 expression was dependent on *S*-nitrosylation of c-*jun*, a subunit of AP-1. Cell proliferation was increased in TIMP-3$^{-/-}$ cardiomyocytes while recombinant TIMP-3 decreased proliferation in both TIMP-3$^{-/-}$ and WT cardiomyocytes. Furthermore, the decline in proliferation observed in eNOS$^{-/-}$ cardiomyocytes was abrogated when TIMP-3 expression was blocked by an anti-TIMP-3 antibody. *In vivo* cardiomyocyte proliferation was assessed by Ki67 immunostaining on postnatal day 1 hearts. Ki67-positive cardiomyocytes were decreased in eNOS$^{-/-}$, but increased in eNOS-Tg and TIMP-3$^{-/-}$ hearts compared to WT controls.
Conclusions: Our study suggests that eNOS promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression.

Keywords: Nitric oxide; Endothelial nitric oxide synthase; Cardiomyocyte; Tissue inhibitor of metalloproteinase-3; AP-1

1. Introduction

NO is produced by a family of NO synthase (NOS) enzymes from the guanidino group of L-arginine in an NADPH-dependent reaction. There are three isoforms of NOS encoded by distinct genes and expressed in a variety of tissues and cell types: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [1]. All three NOS isoforms are expressed in the heart with eNOS as the predominant isoform, which is expressed in the endothelium, endocardium and in cardiomyocytes [1,2]. NO produced from eNOS is an important signaling molecule involved in cardiomyogenesis, angiogenesis and cell survival [3–5]. We recently demonstrated that eNOS is important in fetal and postnatal heart development [6,7].

During fetal and early neonatal development, the heart grows through active cardiomyocyte proliferation and hypertrophy [8,9]. This is followed by eventual cessation of cardiomyocyte proliferation in the adult heart, where all

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remaining growth occurs by cardiomyocyte hypertrophy. The switch from hyperplastic to hypertrophic growth occurs in about a week after birth in rodents [9,10]. NO production from eNOS promotes cardiomyocyte proliferation and contributes to heart development in early postnatal life [6,7]. However, mechanisms by which eNOS regulates cardiomyocyte proliferation are not fully understood.

The tissue inhibitor of metalloproteinases (TIMPs) are a family of four homologous proteins that regulate extracellular matrix homeostasis through regulation of matrix metalloproteinases (MMPs) [11]. All four TIMPs are expressed in the myocardium. The expression of TIMP-1 and -3 is inducible and regulated by AP-1 [12]. TIMP-2 is constitutively expressed and TIMP-4 expression is mostly cardiac-specific [13]. Unique among the TIMP family, TIMP-3 is sequestered in the extracellular matrix, expressed at high levels in the heart, and is a potent inhibitor of all known MMPs as well as of several a disintegrin and metalloproteinase (ADAM) enzymes implicated in cardiac pathology [14]. Deficiency of TIMP-3 leads to progressive dilated cardiomyopathy in aged mice with characteristic matrix degradation, cytokine activation, and cardiac remodeling [15]. Furthermore, TIMP-3 appears to play a role in cell survival and cell cycle regulation [15,16]. However, the role of TIMP-3 in cardiomyocyte proliferation is not known.

S-nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine, has been recognized as an important mechanism for dynamic, post-translational modulation of protein function [17]. NO can inhibit the activity of the transcription factor AP-1 through *S*-nitrosylation of its subunits (c-*jun*/c-*fos*) [18]. Since AP-1 is an important transcription factor for TIMP-3, it is possible that NO may regulate TIMP-3 expression *via* *S*-nitrosylation of AP-1. In the present study, we hypothesized that NO produced from eNOS inhibits TIMP-3 expression *via* *S*-nitrosylation of AP-1. We further hypothesized that inhibition of TIMP-3 promotes cardiomyocyte proliferation. To test these hypotheses, a primary cell culture model of neonatal mouse cardiomyocytes was employed. Neonatal cardiomyocytes were obtained from eNOS$^{-/-}$, eNOS over-expressing mice, TIMP-3$^{-/-}$ and corresponding wild-type mice. Changes in cell proliferation were further confirmed *in vivo*. Our study suggests that eNOS promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression.

2. Methods

2.1. Animals

Wild-type (WT), eNOS$^{-/-}$ and iNOS$^{-/-}$ mice of the genetic background C57BL/6 were obtained from Jackson Laboratories (Bar Harbor, ME). TIMP3$^{-/-}$ and cardiac specific eNOS transgenic (eNOS-Tg, line 23) mice were generated as described previously [19,20]. The eNOS-Tg mice were backcrossed to C57BL/6 for more than 15 generations. A breeding program was carried out to generate neonates for this study. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Isolation and culturing of neonatal mouse cardiomyocytes

The neonatal cardiomyocyte cultures were prepared from mice born within 24 h as in our previous reports [7,21–24]. Non-cardiomyocytes were removed through 1 h of preplating after which cardiomyocytes were plated in M199 medium containing 10% fetal calf serum (FCS) on culture plates pre-coated with 1% gelatin (Sigma, Oakville, ON). Neonatal cardiomyocytes were incubated at 37 °C and 5% CO_2 in a humidified chamber. A subconfluent monolayer of spontaneously beating cardiomyocytes was formed within 2 days. Purity of cardiomyocytes determined by cardiac muscle sarcomeric α-actinin staining was 95% after 72 h of cell culture [7,22].

2.3. Cellular proliferation

To determine the effect of TIMP-3 on cardiomyocyte proliferation, neonatal cardiomyocytes isolated from WT, TIMP-3$^{-/-}$ eNOS$^{-/-}$, and eNOS-Tg mice were cultured as described above and seeded at a cell density of 100,000 cells/well in 96 well plates precoated with 1% gelatin. Eighteen hours following cell seeding, cardiomyocytes were treated with 1 μg/ml recombinant mouse TIMP-3 (rTIMP-3; EMD Biosciences, San Diego, CA) or TIMP-3 antibody (1:75 dilution, Santa Cruz Biotechnology Inc, CA) in M199/10% FCS and cultured for another 54 h. Cellular proliferation was first assessed by cell counts using a haemocytometer by two investigators. Cellular proliferation was also measured using a fluorescence-based assay (CyQUANT Cell Proliferation Assay Kit; Molecular Probes, Eugene, Oregon) as per manufacturer's instructions. Fluorescence was measured in a Victor3 multilabel microplate reader (Wallac) using excitation and emission wavelengths of 480 and 520 nm, respectively. A standard curve was created to convert sample fluorescence values into cell numbers.

In vivo cardiomyocyte proliferation was determined by nuclear Ki67 immunostaining on postnatal (day 1) heart sections similar to our previous report [7]. Briefly, heart sections were incubated with primary antibodies, anti-Ki67 (1:400, Lab Vision, Fremont, CA) and anti-atrial natriuretic peptide (ANP, a marker for cardiomyocytes, 1:200, Santa Cruz, CA). This was followed by incubations of respective secondary fluorescent antibodies. Hoechst 33342 was used to stain nuclei. Data are expressed as the percentage of Ki67-positive nuclei in cardiomyocytes.

2.4. Adenoviral infection of neonatal mouse cardiomyocytes

Overexpression of eNOS in WT neonatal cardiomyocytes was accomplished through the introduction of adenoviral vectors containing the bovine eNOS gene as we described [24].

Viral vectors containing green fluorescence protein (GFP) served as negative controls. Cells were infected with 1 plaque forming unit/cell and incubated for an hour in the presence of minimal volume of M199 with 2% FCS containing the adenoviruses. Following the 1 h incubation, full volume of 10% FCS M199 was applied. Cells were incubated for an additional 24 h and subsequently harvested.

2.5. Measurement of NO production

NO production was measured in living cardiomyocytes using a membrane-permeable fluorescent indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA, Molecular Probes, Eugene, Oregon) as we recently described [22]. The fluorescence produced by NO production was measured using a Victor3 multilabel microplate reader (Wallac) using excitation and emission wavelengths of 485 and 515 nm, respectively. The results are expressed as arbitrary units of fluorescence.

2.6. Immunoprecipitation and determination of S-nitrosylation

To analyze *S*-nitrosylation of AP-1, immunoprecipitation was performed. Briefly, 1 mg protein lysate from cardiomyocytes was incubated with a rabbit polyclonoal c-*jun* antibody (1:200, Cell Signaling Technology, MA) for 3 h. The immunocomplex was captured using protein G-agarose (Santa Cruz Biotechnology, CA). The immunoprecipitated material was eluted and samples were incubated with 100 μM mercuric chloride ($HgCl_2$) for 10 min at 37 °C to release NO from *S*-nitrosylated AP-1. The levels of NO were measured using DAF-FM (1 μM). In addition, *S*-nitrosylation of immunoprecipitated c-*jun* was directly measured by non-reducing Western blot analysis. Samples were run on 10% polyacrylamide gels using anti-*S*-nitroso-cysteine antibody (1:1000, Sigma, Oakville, ON). The levels of *S*-nitrosylated c-*jun* protein were determined using densitometry.

2.7. Determination of TIMP-3, p21 and p27 mRNA by real-time RT-PCR

Total RNA was extracted from cardiomyocytes using Trizol as previously described [23,24]. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Burlington, ON). Real-time PCR was conducted using SYBR Green PCR Master Mix as per manufacturer's instructions (Eurogentec, San Diego, CA). 28S rRNA (house keeping gene) was used as a loading control. The primer sequences were as follows: TIMP-3 upstream 5′ GAT GCC TTC TGC AAC TCC GA 3′ and downstream 5′ CGG TCC AGA GAC ACT CAT TC 3′; p21 upstream 5′AGT GTG CCG TTG TCT CTT CG3′ and downstream 5′ACA CCA GAG TGC AAG ACA GC 3′; p27 upstream 5′ TCG CAG AAC TTC GAA GAG G 3′ and downstream 5′ TGA CTC GCT TCT TCC ATA TCC 3′ [25]; 28S rRNA upstream 5′ TTG AAA ATC CGG GGG AGA G 3′ and downstream 5′ ACA TTG TTC CAA CAT GCC AG 3′. Samples were amplified for 34 cycles using MJ Research Opticon Real-Time PCR machine. The levels of TIMP-3, p21 and p27 were compared to that of 28S rRNA, and the relative expression of these genes was obtained.

2.8. Western blot analysis

TIMP-3 protein levels were assessed by Western blotting. Briefly, cell lysates containing 50 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% gels, followed by electrotransfer to polyvinylidene difluoride membranes. Blots were probed with antibodies against TIMP-3 (1:200; Santa Cruz Biotechnology, CA) and α-actinin (1:2000; Sigma, Oakville, ON) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology, CA). Detection was performed using an ECL chemiluminescence detection method. Signals were determined by densitometry and levels of TIMP-3 to α-actinin are presented.

2.9. Measurement of apoptosis

Caspase-3 activity was assessed using the BIOMOL QuantiZyme Assay System (AK-700) as described previously [6,22]. Briefly, cell lysates containing 20 μg of protein were incubated at 37 °C for 16 h with the caspase-3 substrate Ac-DEVD-AMC in the presence or absence of the inhibitor Ac-DEVD-CHO. Fluorescent intensity was measured with the Victor3 multilabel microplate reader (Wallac) using excitation and emission wavelengths of 360 and 460 nm, respectively. Data are presented as the amount of AMC substrate cleaved/μg protein.

Terminal deoxynucleotidyl transferase d-UTP nick end labeling (TUNEL) staining on cultured cardiomyocytes was performed using an In Situ Cell Death Detection Kit (Roche Diagnostics, Switzerland) as our previous reports [21,22]. Data are expressed as the average percent of TUNEL positive nuclei by two independent observers.

2.10. Statistical analysis

Data are presented as mean ± SEM or ± SD. Student's *t* test, one- or two-way ANOVA followed by Student–Newman–Keuls test were performed. $P<0.05$ was considered statistically significant.

3. Results

3.1. NO from eNOS inhibits TIMP-3 mRNA expression in cardiomyocytes

To determine whether NO produced from eNOS can modulate TIMP-3 expression, mouse neonatal cardiomyocytes were infected with a bovine eNOS adenovirus (Ad-eNOS; 1 plaque forming unit/cell) and TIMP-3 mRNA levels were

subsequently analyzed by real-time RT-PCR. As shown in Fig. 1A and B, infection of cardiomyocytes with Ad-eNOS inhibited TIMP-3 expression and increased NO production ($P<0.05$). Treatment of cardiomyocytes with a pharmacological NO donor SNAP (10 μM) for 4 h increased NO levels by about 3 fold ($P<0.05$) and resulted in a significant reduction in TIMP-3 expression ($P<0.05$, Fig. 1C). These findings were further confirmed by transgenic eNOS overexpression (eNOS-Tg). TIMP-3 expression was significantly reduced in neonatal cardiomyocytes isolated from eNOS overexpressing mice ($P<0.05$, Fig. 1D).

To examine if iNOS was involved in the regulation of TIMP-3 expression in the present study, cultured neonatal cardiomyocytes were treated with a selective iNOS inhibitor 1400 W (10 μM, 6 h). 1400 W did not significantly alter TIMP-3 mRNA/28S rRNA ratio in WT cardiomyocytes (0.11 ± 0.01 *vs.* 0.13 ± 0.02, $n=3$ independent experiments, $P=$n.s.). Furthermore, TIMP-3 mRNA levels were similar between WT and iNOS$^{-/-}$ cardiomyocytes (0.10 ± 0.01 *vs.* 0.11 ± 0.01, $n=3$ independent experiments, $P=$n.s.).

3.2. Elevation of TIMP-3 mRNA expression in eNOS$^{-/-}$ cardiomyocytes

Having determined that eNOS overexpression resulted in reduced levels of TIMP-3, it was predicted that deficiency in eNOS or inhibition of eNOS activity would result in elevated TIMP-3 levels. To test this hypothesis, TIMP-3 mRNA expression was measured in eNOS$^{-/-}$ and WT cardiomyocytes treated with or without an NOS inhibitor N^{ω}-nitro-L-arginine

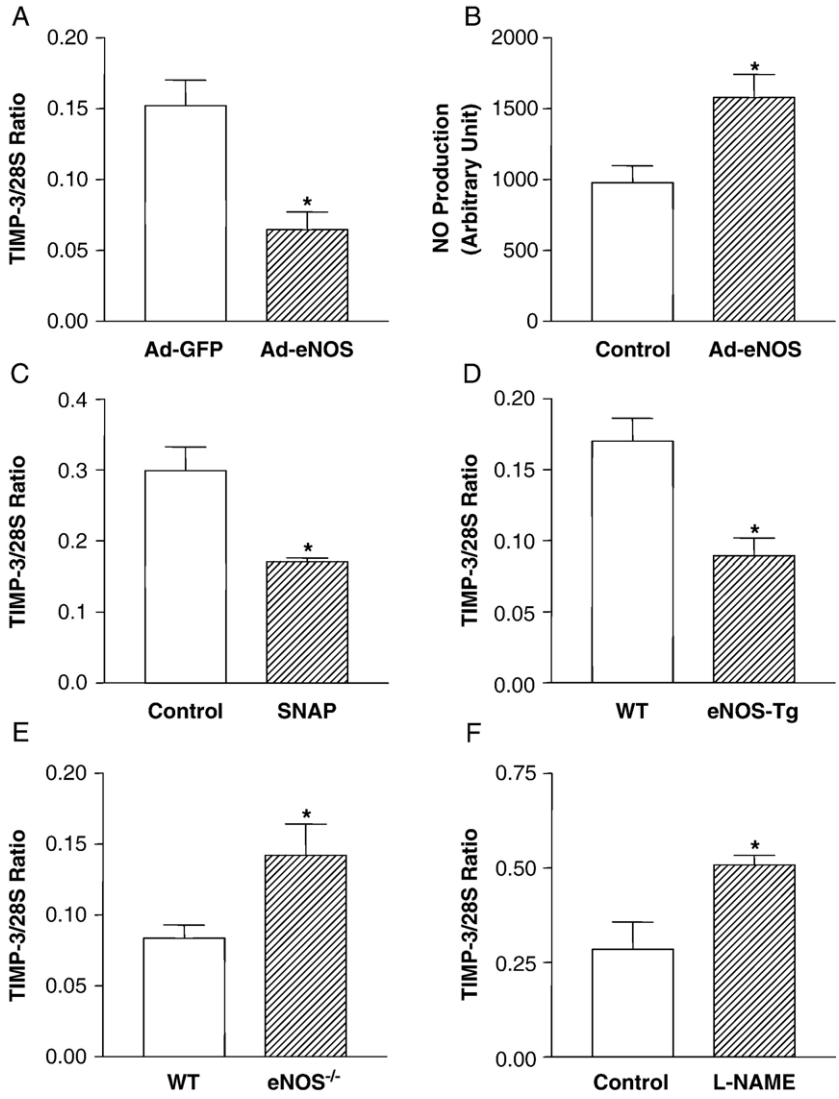

Fig. 1. Effects of NO production from eNOS on TIMP-3 mRNA levels in cardiomyocytes. A. Effects of Ad-eNOS on TIMP-3 mRNA expression as compared to Ad-GFP treated cells. B. NO production in cardiomyocytes treated with Ad-eNOS. Ad-GFP was not used in this experiment because GFP interferes with fluorescent measurement of this assay. C. Effects of SNAP (10 μM, 4 h) on TIMP-3 mRNA in WT cardiomyocytes. D. TIMP-3 mRNA expression in eNOS-Tg cardiomyocytes *vs.* WT controls. E. eNOS$^{-/-}$ *vs.* WT cardiomyocytes. F. Effects of L-NAME (300 μM, 6 h) on TIMP-3 mRNA in WT cardiomyocytes. Data are mean±SEM from 3–6 independent experiments. *$P<0.05$ *vs.* corresponding controls. Ad, adenovirus; GFP, green fluorescence protein; SNAP, *S*-nitroso-*N*-acetylpenicillamine; Tg, transgenic; WT, wild-type.

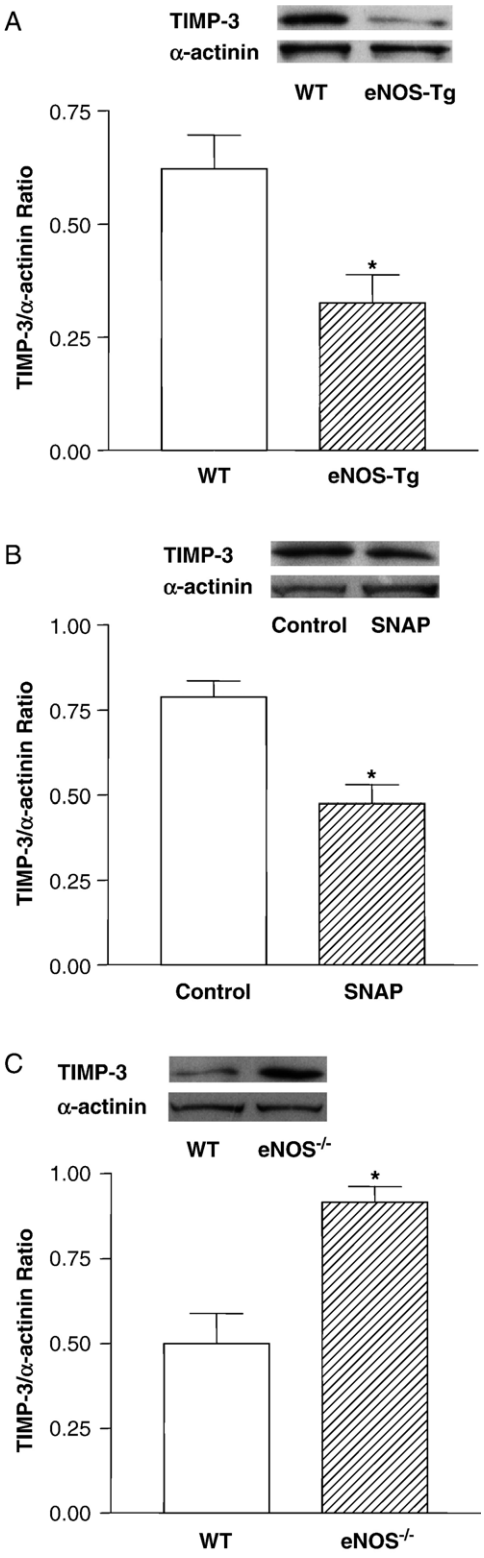

Fig. 2. Effects of NO on TIMP-3 protein expression in cultured neonatal mouse cardiomyocytes. TIMP-3 protein levels determined by Western blot analysis in eNOS-Tg cardiomyocytes (A), in WT cardiomyocytes treated with SNAP (10 μM, 18 h) (B) and in eNOS$^{-/-}$ cardiomyocytes (C) with α-actinin as a loading control. Data are mean±SEM from 3 independent experiments. *$P<0.05$ *vs.* control. SNAP, *S*-nitroso-*N*-acetylpenicillamine; Tg, transgenic; WT, wild-type.

methyl ester (L-NAME, 300 μM). The data showed that TIMP-3 mRNA levels were significantly elevated in both eNOS$^{-/-}$ ($P<0.05$, Fig. 1E) and in WT cardiomyocytes treated with L-NAME as compared to controls ($P<0.05$, Fig. 1F).

3.3. NO from eNOS regulates TIMP-3 protein expression in cardiomyocytes

TIMP-3 protein levels were determined by Western blot analysis. TIMP-3 protein expression was significantly decreased by eNOS transgenic overexpression ($P<0.05$, Fig. 2A) and by SNAP treatment ($P<0.05$, Fig. 2B). Interestingly, TIMP-3 protein levels were significantly increased in eNOS$^{-/-}$ compared to WT cardiomyocytes ($P<0.05$, Fig. 2C). These data are consistent with the changes in mRNA expression shown in Fig. 1.

3.4. NO from eNOS inhibits TIMP-3 expression via S-nitrosylation

To elucidate the mechanism by which NO modulates TIMP-3 mRNA expression, *S*-nitrosylation was examined. To that end, cardiomyocytes were treated with Ad-eNOS or Ad-GFP in the presence or absence of dithiothreitol (DTT, 200 μM), a thiol-reducing agent known to reverse *S*-nitrosylation. As shown in Fig. 3, DTT treatment reversed the effect of Ad-eNOS on TIMP-3 expression ($P<0.05$), suggesting that NO production from eNOS inhibits TIMP-3 expression *via S*-nitrosylation in mouse neonatal cardiomyocytes.

3.5. NO from eNOS induces S-nitrosylation of activating protein-1 (AP-1) and decreases TIMP-3 expression in cardiomyocytes

Studies have shown that AP-1 is a critical transcriptional regulator of TIMP-3 expression [12]. To confirm the role of

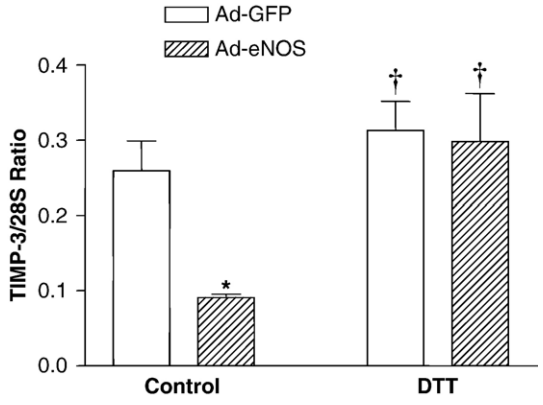

Fig. 3. NO from eNOS modulates TIMP-3 mRNA expression through *S*-nitrosylation. WT cardiomyocytes were treated with either Ad-GFP or Ad-eNOS for 24 h in the presence or absence of the thiol-reducing agent DTT (200 μM). TIMP-3 mRNA levels were determined by real-time RT-PCR analysis. Data are mean±SEM from 5–6 independent experiments. *$P<0.05$ *vs.* Ad-GFP control; †$P<0.05$ *vs.* Ad-eNOS control. Ad, adenovirus; GFP, green fluorescence protein; DTT, dithiothreitol.

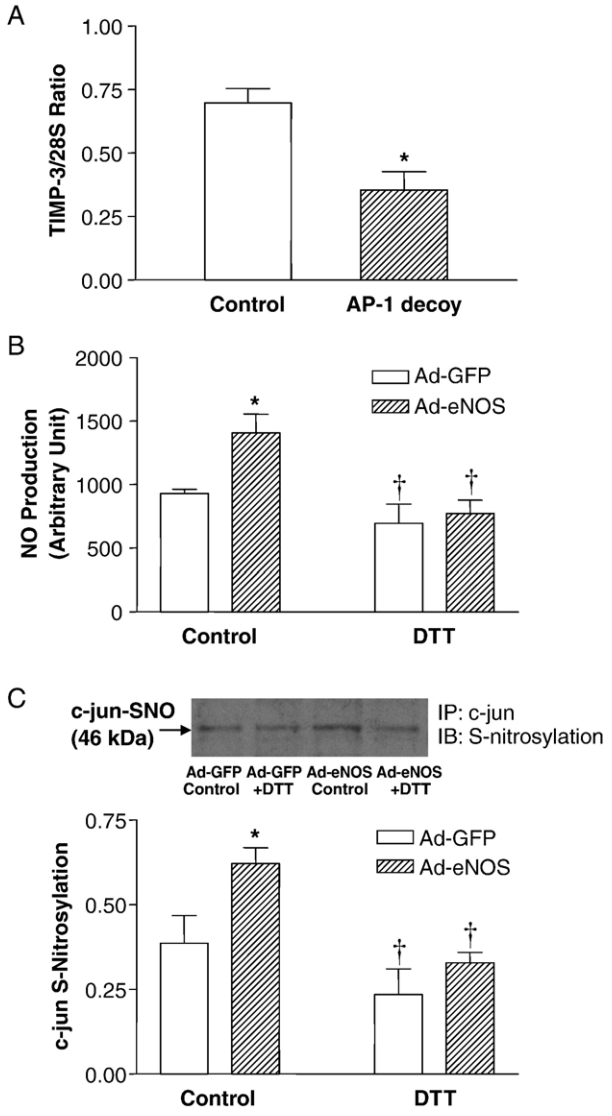

Fig. 4. NO from eNOS increases *S*-nitrosylation of AP-1 in neonatal cardiomyocytes. A. Effects of AP-1 decoy (2.5 μM) on TIMP-3 mRNA levels in WT cardiomyocytes. B. Effects of Ad-eNOS on c-*jun* *S*-nitrosylation using DAF-FM as a probe for NO production in the presence or absence of the thiol-reducing agent DTT (200 μM) in WT cardiomyocytes. C. The data obtained in B were confirmed by c-*jun* immunoprecipitation, followed by Western blot analysis. The levels of *S*-nitrosylated c-*jun* were measured by densitometry. Data are mean±SEM from 3–5 independent experiments. * $P<0.05$ *vs.* Ad-GFP control; † $P<0.05$ *vs.* Ad-eNOS control. Ad, adenovirus; GFP, green fluorescence protein; AP-1, activating protein-1; DTT, dithiothreitol; WT, wild-type.

AP-1 in the regulation of TIMP-3 expression, WT cardiomyocytes were treated with an AP-1 decoy (2.5 μM, 8 h), which consists of a double stranded oligonucleotide containing the consensus binding sites for AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′). Data showed that the AP-1 decoy significantly decreased TIMP-3 expression ($P<0.05$, Fig. 4A), suggesting an important role for AP-1 in the regulation of TIMP-3 expression in neonatal cardiomyocytes.

It is well established that NO can inhibit AP-1 activity *via* *S*-nitrosylation of its subunits (c-*jun*/c-*fos*) [18]. To determine if NO modulates TIMP-3 expression through the *S*-nitrosylation of AP-1, cardiomyocytes were treated with Ad-eNOS and Ad-GFP in the presence or absence of DTT. Total protein was extracted from these cells and samples were then subjected to immunoprecipitation with a c-*jun* antibody. Subsequently, NO was released from the *S*-nitrosylated bonds using 100 μM mercuric chloride and NO levels were measured using DAF-FM. Treatment of neonatal mouse cardiomyocytes with Ad-eNOS significantly increased *S*-nitrosylation of c-*jun*, and this effect of Ad-eNOS was reversed by DTT treatment ($P<0.05$, Fig. 4B). These results were further confirmed by the direct detection of c-*jun* *S*-nitrosylation using Western blot analysis. Ad-eNOS significantly increased the *S*-nitrosylated c-*jun* protein levels, and this was reversed by DTT treatment ($P<0.05$, Fig. 4C).

3.6. TIMP-3 inhibits proliferation in cardiomyocytes

As the current study showed that NO inhibited TIMP-3 expression in cardiomyocytes, the effect of TIMP-3 on cell proliferation was further investigated. To that end, WT and TIMP-3$^{-/-}$ cardiomyocytes were cultured and treated as described in the methods. Cell proliferation was assessed 72 h post-seeding by direct counting using a haemocytometer (Fig. 5A) and verified using the CyQUANT cell proliferation kit (Fig. 5B). Our data showed that cell number

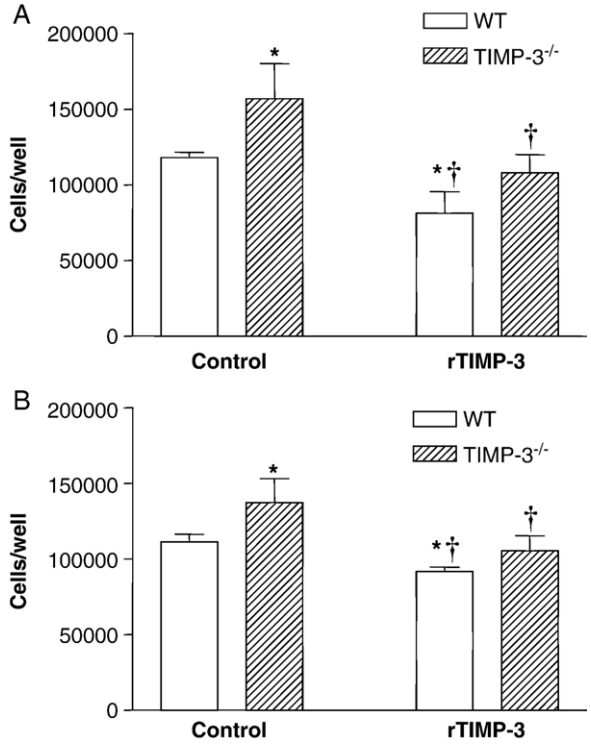

Fig. 5. TIMP-3 inhibits proliferation in cultured neonatal cardiomyocytes. WT cardiomyocytes were treated with rTIMP-3 (1 μg/ml) as detailed in methods. Cell numbers per well were determined using a haemocytometer (A) or by using the CyQUANT proliferation kit (B). Data are mean±SD from 3–4 independent experiments. *$P<0.05$ *vs.* WT control; †$P<0.05$ *vs.* respective WT or TIMP-3$^{-/-}$ control. rTIMP-3, recombinant mouse TIMP-3; WT, wild-type.

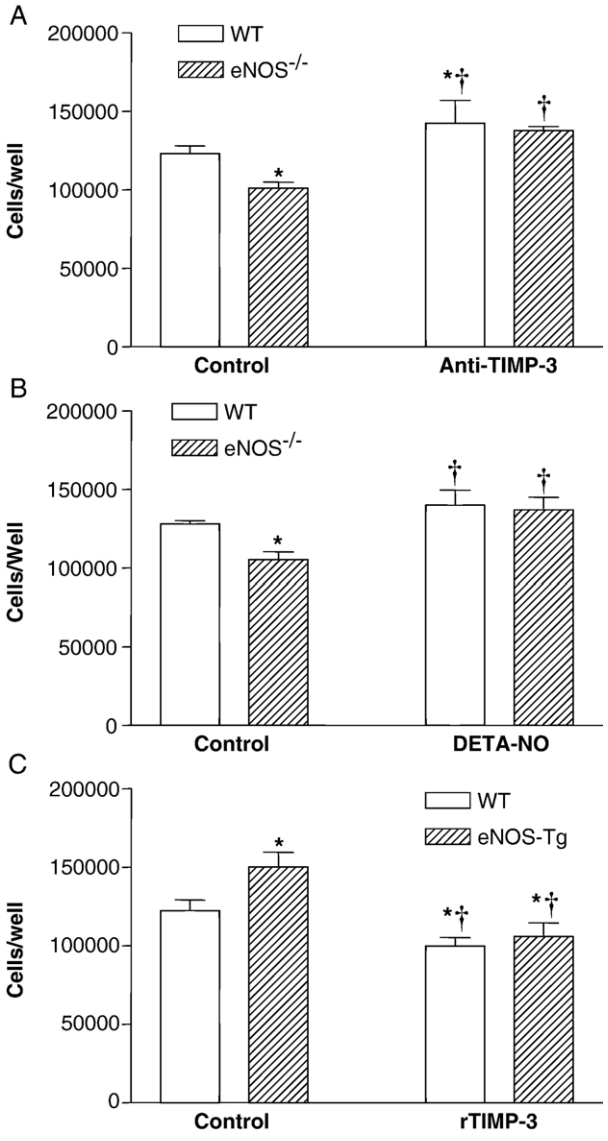

Fig. 6. NO from eNOS promotes proliferation of cardiomyocytes by inhibiting TIMP-3 expression. Cell numbers were determined using a haemocytometer. A. Effects of an anti-TIMP-3 antibody (1:75 dilution) on cell proliferation in WT and eNOS$^{-/-}$ cardiomyocytes. B. Effects of DETA-NO (2 μM) on cell proliferation in eNOS$^{-/-}$ cardiomyocytes. C. Effects of rTIMP-3 (1 μg/ml) on cell proliferation in WT and eNOS-Tg cardiomyocytes. Data are mean±SD from 3 independent experiments. *$P<0.05$ *vs.* WT control; †$P<0.05$ *vs.* respective eNOS$^{-/-}$ or eNOS-Tg control. rTIMP-3, recombinant TIMP-3; Tg, transgenic; DETA-NO, diethylenetriamine NONOate; WT, wild-type.

was significantly higher in the TIMP-3$^{-/-}$ as compared to WT cardiomyocytes ($P<0.05$, Fig. 5A and B). Furthermore, addition of recombinant mouse TIMP-3 (rTIMP-3) significantly lowered cell numbers in both WT and TIMP-3$^{-/-}$ cardiomyocyte cultures ($P<0.05$, Fig. 5A and B).

3.7. NO from eNOS promotes cardiomyocyte proliferation via inhibition of TIMP-3

We recently demonstrated that eNOS promotes cardiomyocyte proliferation [7]. Consistent with this finding, the present study showed that proliferation was decreased in eNOS$^{-/-}$ cardiomyocytes (Fig. 6A). Treatment with an NO donor DETA-NO restored proliferation in eNOS$^{-/-}$ cardiomyocytes (Fig. 6B). Furthermore, transgenic eNOS overexpression significantly increased cardiomyocyte proliferation compared to the WT controls (Fig. 6C). These data further support the notion that NO production from eNOS promotes cardiomyocyte proliferation. To investigate the role of TIMP-3 in eNOS-mediated cardiomyocyte proliferation, WT and eNOS$^{-/-}$ cardiomyocytes were treated with an anti-TIMP-3 antibody. Inhibition of TIMP-3 activity by anti-TIMP-3 antibody significantly increased proliferation in both WT and eNOS$^{-/-}$ cells (Fig. 6A). In addition, treatment with recombinant TIMP-3 significantly inhibited proliferation in both WT and eNOS-Tg cardiomyocytes (Fig. 6C).

3.8. Effects of TIMP-3 and eNOS on p21 and p27 mRNA expression in cardiomyocytes

In order to investigate mechanisms by which TIMP-3 and eNOS regulate cardiomyocyte proliferation, mRNA levels of cyclin-dependent kinase inhibitors p27 and p21 were determined by real-time RT-PCR (Fig. 7A and B). Results showed that recombinant TIMP-3 significantly increased p21 and p27 mRNA expression ($P<0.05$). Compared to WT controls, the expression of both p21 and p27 was decreased

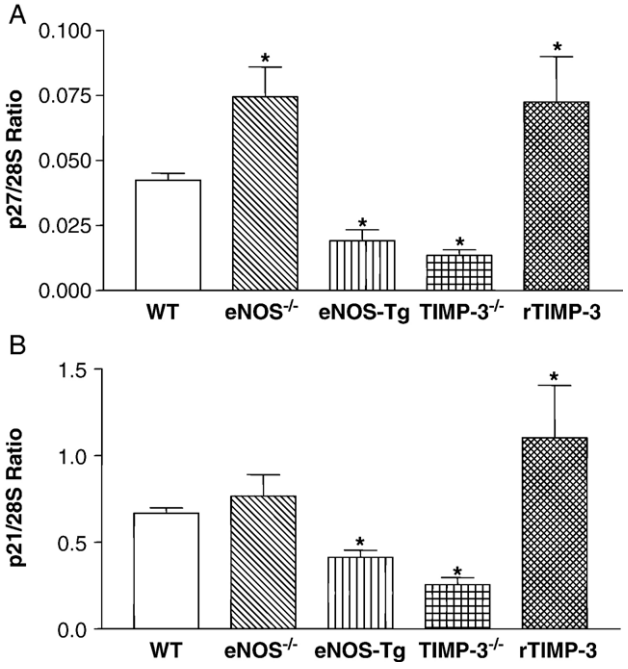

Fig. 7. NO and TIMP-3 regulate cyclin-dependent kinase inhibitors p27 and p21 in cardiomyocytes. Neonatal cardiomyocytes were isolated from WT, eNOS$^{-/-}$, eNOS-Tg or TIMP-3$^{-/-}$ mice. WT cardiomyocytes were treated with rTIMP-3 (1 μg/ml) as described in the methods. Total RNA was isolated and subjected to real-time RT-PCR analysis. A. p27 mRNA expression in relation to 28S. B. p21 mRNA expression in relation to 28S. Data are mean± SEM of 3–4 independent experiments. *$P<0.05$ *vs.* WT group. rTIMP-3, recombinant TIMP-3; Tg, transgenic; WT, wild-type.

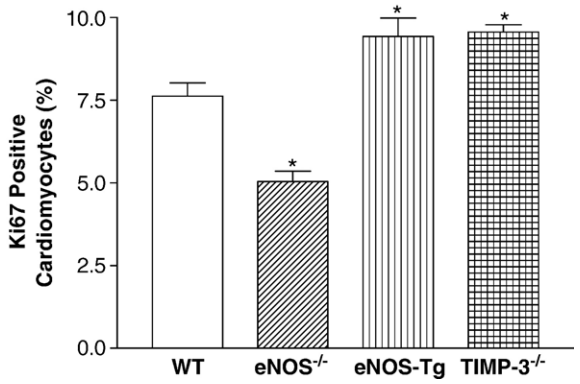

Fig. 8. *In vivo* cardiomyocyte proliferation in postnatal (day 1) hearts of WT, eNOS$^{-/-}$, eNOS-Tg and TIMP-3$^{-/-}$ mice. Cardiomyocyte proliferation as a percent of total cardiomyocytes was determined by Ki67 immunostaining as detailed in methods. Data are mean±SEM of 4–5 mice per group. *$P<0.05$ *vs.* WT mice. Tg, transgenic; WT, wild-type.

in eNOS-Tg and TIMP-3$^{-/-}$ cardiomyocytes ($P<0.05$). Interestingly, deficiency in eNOS significantly increased p27 but not p21 expression (Fig. 7A and B). These data demonstrated that TIMP-3 and eNOS modulate p21 and p27 expression in cardiomyocytes.

3.9. In vivo cardiomyocyte proliferation in neonatal hearts

To further demonstrate the effects of TIMP-3 and eNOS on neonatal cardiomyocyte proliferation *in vivo*, Ki67 immunostaining was performed in postnatal day 1 hearts. Consistent with cardiomyocyte proliferation in cell culture experiments (Figs. 5 and 6), nuclear Ki67 positive staining was decreased in eNOS$^{-/-}$ hearts, but significantly increased in eNOS-Tg and TIMP-3$^{-/-}$ hearts compared to WT controls ($P<0.05$, Fig. 8).

3.10. TIMP-3 does not increase apoptosis in cardiomyocytes

In order to determine if recombinant TIMP-3 treatment has any effect on cardiomyocyte apoptosis, WT cardiomyocytes were cultured and treated with 1 μg/ml recombinant TIMP-3 for 54 h. TUNEL staining and measurement of caspase-3 activity were performed 72 h post seeding. Our data showed that recombinant TIMP-3 had no significant effect on TUNEL staining (6.2±0.9% *vs.* 5.7±1.4%) or caspase-3 activity in cardiomyocytes (0.95±0.25 *vs.* 0.81±0.14 pmol/μg protein, $n=4$ independent experiments per group, $P=$n.s.).

4. Discussion

We recently demonstrated that eNOS promotes cardiomyocyte proliferation in the early postnatal hearts [7]. However, mechanisms by which eNOS regulates cardiomyocyte proliferation are not fully understood. The major finding of the present study is that TIMP-3 inhibits the proliferation of neonatal mouse cardiomyocytes. We demonstrated for the first time that deficiency of eNOS increases TIMP-3 expression and decreases cardiomyocyte proliferation *via* modulation of p27. NO produced from eNOS inhibits TIMP-3 expression *via S*-nitrosylation of AP-1 in cardiomyocytes. Our study suggests that eNOS is important in regulating TIMP-3 expression and cardiomyocyte proliferation.

Studies have shown that NO regulates TIMP-3 expression in several cell types. For example, treatment with an NOS inhibitor L-NAME increases TIMP-3 expression in mouse uteri during peri-implantation [26]. Similarly, NO production from murine mammary adenocarcinomal cells inhibits TIMP-2 and TIMP-3 expression [27]. Although the specific NOS isoforms responsible for the effects are not defined in these studies, the results do suggest that endogenous NO production inhibits TIMP-3 expression. To our knowledge however, there is no report on the effects of NO on TIMP-3 expression in cardiomyocytes. In the present study, overexpression of eNOS using Ad-eNOS and eNOS transgenic mice (eNOS-Tg) resulted in a significant decrease in TIMP-3 expression in cardiomyocytes. Furthermore, NOS inhibition with L-NAME or deficiency in eNOS gene significantly increased TIMP-3 expression in cardiomyocytes. Thus, our study provides strong evidence for an inhibitory effect of eNOS-derived NO on TIMP-3 expression in cardiomyocytes.

Mechanisms by which NO inhibits TIMP-3 are not known. Studies have shown that TIMP-3 expression is predominantly regulated by AP-1 in the mouse JB6 cells [12]. In the present study, treatment with an AP-1 decoy decreased TIMP-3 expression suggesting that AP-1 is a major regulator of TIMP-3 in cardiomyocytes. NO has been shown to inhibit AP-1 activity through *S*-nitrosylation [18]. We treated cardiomyocytes with DTT, a reducing agent known to reverse *S*-nitrosylation [28]. DTT restored TIMP-3 expression in the Ad-eNOS treated cardiomyocytes, suggesting that NO production from eNOS mediates its inhibitory effect on TIMP-3 through *S*-nitrosylation. AP-1 is a heterodimer consisting mainly of c-*jun* and c-*fos* subunits. To further confirm AP-1 nitrosylation by eNOS, immunoprecipitation and Western blot analysis were performed. Data showed that eNOS increased *S*-nitrosylation of c-*jun* in cardiomyocytes, and that DTT treatment reversed this effect. Taken together, our results suggest that NO from eNOS inhibits TIMP-3 expression in cardiomyocytes at least in part by inhibiting AP-1 activity *via S*-nitrosylation of c-*jun*.

TIMP-3 has been shown to regulate cell proliferation. However, depending on the cell types studied, TIMP-3 has either inhibitory or stimulatory effects on proliferation. For example, TIMP-3 inhibits proliferation of endothelial cells [29] and rat vascular smooth muscle cells [16]. In contrast to these findings, several studies have shown that TIMP-3 promotes proliferation in embryonic chicken fibroblasts *in vitro* [30] and bronchiole epithelial cells during fetal development *in vivo* [31]. A novel finding of the present study is that cell proliferation was increased in TIMP-3$^{-/-}$ compared to WT cardiomyocytes. Treatment with a recombinant TIMP-3 protein inhibited proliferation in both TIMP-3$^{-/-}$ and WT cardiomyocytes. Furthermore, *in vivo* cardiomyocyte proliferation was increased in TIMP-3$^{-/-}$ neonatal hearts compared

to WT controls. These results demonstrated an inhibitory role of TIMP-3 on cardiomyocyte proliferation.

NO production from eNOS promotes cardiomyocyte proliferation [7]. In the present study, cardiac specific eNOS overexpression increased cardiomyocyte proliferation *in vivo* in neonatal hearts, providing further support to this notion. However, mechanisms by which eNOS affects cardiomyocyte proliferation are not fully understood. Based on the finding that NO production from eNOS inhibits TIMP-3 expression and recombinant TIMP-3 inhibits cardiomyocyte proliferation, we studied the role of TIMP-3 in the proliferation of eNOS$^{-/-}$ cardiomyocytes. Cell proliferation was decreased in eNOS$^{-/-}$ cardiomyocytes. However, treatment with an anti-TIMP-3 antibody significantly increased proliferation in eNOS$^{-/-}$ cardiomyocytes. These results suggest that the decreased proliferation of eNOS$^{-/-}$ cardiomyocytes is mediated at least in part by elevated levels of TIMP-3. We recently showed that deficiency in eNOS results in congenital septal defects in mice [6]. Whether increased TIMP-3 expression contributes to these pathological changes in eNOS$^{-/-}$ mice requires further investigation.

Mechanisms by which TIMP-3 regulates neonatal cardiomyocyte proliferation are not known. Studies have shown that TIMP-3 overexpression inhibits vascular smooth muscle cell proliferation independent of protease inhibitory activity. Furthermore, TIMP-3 induces expression of the cyclin-dependent kinase inhibitor p21, which inhibits cell proliferation [16]. The cardiac cell cycle is tightly controlled by numerous regulatory proteins including cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors including p21 and p27 [32,33]. As such, we studied the effects of TIMP-3 and eNOS on p21 and p27 expression. Data showed that recombinant TIMP-3 increased p21 and p27 mRNA expression. Interestingly, p27 mRNA levels were also increased in eNOS$^{-/-}$ cardiomyocytes while both p21 and p27 mRNA levels were decreased in eNOS-Tg and TIMP-3$^{-/-}$ cardiomyocytes. Our study suggests that eNOS and TIMP-3 regulate neonatal cardiomyocyte proliferation *via* modulation of p21 and p27 expression. Since the present study was conducted on postnatal day 1 hearts, the effects and mechanisms identified herein may not be applicable to adult cardiomyocytes, which have very limited ability to proliferate. Both eNOS and TIMP-3 protein sequences share 94% and 96% homology, respectively between mouse and human. However, whether eNOS and TIMP-3 regulate neonatal cardiomyocyte proliferation in humans in the same manner as in mice remains to be determined.

Previous studies have shown that TIMP-3 induces apoptosis through a caspase-dependent pathway in HeLa cells and rat vascular smooth muscle cells [16,34]. As changes in apoptosis may affect cell proliferation, we wanted to determine whether TIMP-3 has any effect on cardiomyocyte apoptosis. To that end, we performed TUNEL staining and measured caspase-3 activity, a terminal caspase involved in TIMP-3 induced apoptosis [34]. Our data showed that recombinant mouse TIMP-3 did not increase TUNEL positivity or caspase-3 activity in cardiomyocytes. These findings indicate that the decline in cardiomyocyte proliferation by recombinant mouse TIMP-3 treatment is not due to an increase in apoptosis. Our previous studies have shown that eNOS is important in protecting cardiomyocytes from apoptosis [6]. Mechanisms of NO-mediated protection are likely *via* upregulation of Bcl-2, heme oxygenase-1 and heat shock proteins [4]. In the present study, TIMP-3 was regulated by eNOS but did not affect cardiomyocyte apoptosis, suggesting that modulation of TIMP-3 may not mediate the anti-apoptotic effect of eNOS.

In summary, the present study demonstrates that TIMP-3 inhibits the proliferation of neonatal mouse cardiomyocytes. NO inhibits TIMP-3 expression through *S*-nitrosylation of AP-1. Furthermore, deficiency in eNOS increases TIMP-3 expression and decreases cardiomyocyte proliferation. Our study suggests that eNOS promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression.

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References

[1] Balligand JL, Cannon PJ. Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. Arterioscler Thromb Vasc Biol 1997;17:1846–58.
[2] Bloch W, Fleischmann BK, Lorke DE, Andressen C, Hops B, Hescheler J, et al. Nitric oxide synthase expression and role during cardiomyogenesis. Cardiovasc Res 1999;43:675–84.
[3] Duda DG, Fukumura D, Jain RK. Role of eNOS in neovascularization: NO for endothelial progenitor cells. Trends Mol Med 2004;10:143–5.
[4] Razavi HM, Hamilton JA, Feng Q. Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. Pharmacol Ther 2005;106:147–62.
[5] Zhao X, Lu X, Feng Q. Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. Am J Physiol Heart Circ Physiol 2002;283:H2371–8.
[6] Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, et al. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. Circulation 2002;106:873–9.
[7] Lepic E, Burger D, Lu X, Song W, Feng Q. Lack of endothelial nitric oxide synthase decreases cardiomyocyte proliferation and delays cardiac maturation. Am J Physiol Cell Physiol 2006;291:C1240–6.
[8] Cluzeaut F, Maurer-Schultze B. Proliferation of cardiomyocytes and interstitial cells in the cardiac muscle of the mouse during pre- and postnatal development. Cell Tissue Kinet 1986;19:267–74.
[9] Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. Cardiomyocyte DNA synthesis and binucleation during murine development. Am J Physiol 1996;271:H2183–9.
[10] Li F, Wang X, Capasso JM, Gerdes AM. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28:1737–46.

[11] Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 2002;115: 3719–27.
[12] Kim H, Pennie WD, Sun Y, Colburn NH. Differential functional significance of AP-1 binding sites in the promoter of the gene encoding mouse tissue inhibitor of metalloproteinases-3. Biochem J 1997;324 (Pt 2):547–53.
[13] Fedak PW, Verma S, Weisel RD, Li RK. Cardiac remodeling and failure from molecules to man (Part II). Cardiovasc Pathol 2005;14: 49–60.
[14] Satoh M, Nakamura M, Saitoh H, Satoh H, Maesawa C, Segawa I, et al. Tumor necrosis factor-alpha-converting enzyme and tumor necrosis factor-alpha in human dilated cardiomyopathy. Circulation 1999;99: 3260–5.
[15] Fedak PW, Smookler DS, Kassiri Z, Ohno N, Leco KJ, Verma S, et al. TIMP-3 deficiency leads to dilated cardiomyopathy. Circulation 2004;110: 2401–9.
[16] Baker AH, Zaltsman AB, George SJ, Newby AC. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. J Clin Invest 1998;101:1478–87.
[17] Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein *S*-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol 2005;6: 150–66.
[18] Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. FASEB J 2000;14:1889–900.
[19] Brunner F, Andrew P, Wolkart G, Zechner R, Mayer B. Myocardial contractile function and heart rate in mice with myocyte-specific over-expression of endothelial nitric oxide synthase. Circulation 2001;104: 3097–102.
[20] Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, et al. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). J Clin Invest 2001;108:817–29.
[21] Song W, Lu X, Feng Q. Tumor necrosis factor-alpha induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. Cardiovasc Res 2000;45:595–602.
[22] Burger D, Lei M, Geoghegan-Morphet N, Lu X, Xenocostas A, Feng Q. Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. Cardiovasc Res 2006;72: 51–9.
[23] Peng T, Lu X, Feng Q. Pivotal role of gp91phox-containing NADH oxidase in lipopolysaccharide-induced tumor necrosis factor-alpha expression and myocardial depression. Circulation 2005;111:1637–44.
[24] Peng T, Lu X, Lei M, Feng Q. Endothelial nitric-oxide synthase enhances lipopolysaccharide-stimulated tumor necrosis factor-alpha expression via cAMP-mediated p38 MAPK pathway in cardiomyocytes. J Biol Chem 2003;278:8099–105.
[25] Kwon YH, Jovanovic A, Serfas MS, Tyner AL. The Cdk inhibitor p21 is required for necrosis, but it inhibits apoptosis following toxin-induced liver injury. J Biol Chem 2003;278:30348–55.
[26] Zhang X, Wang HM, Lin HY, Liu GY, Li QL, Zhu C. Regulation of matrix metalloproteinases (MMPS) and their inhibitors (TIMPS) during mouse peri-implantation: role of nitric oxide. Placenta 2004;25:243–52.
[27] Orucevic A, Bechberger J, Green AM, Shapiro RA, Billiar TR, Lala PK. Nitric-oxide production by murine mammary adenocarcinoma cells promotes tumor-cell invasiveness. Int J Cancer 1999;81:889–96.
[28] Schroeder RA, Cai C, Kuo PC. Endotoxin-mediated nitric oxide synthesis inhibits IL-1beta gene transcription in ANA-1 murine macrophages. Am J Physiol Cell Physiol 1999;277:C523–30.
[29] Qi JH, Ebrahem Q, Moore N, Murphy G, Claesson-Welsh L, Bond M, et al. A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. Nat Med 2003;9:407–15.
[30] Yang TT, Hawkes SP. Role of the 21-kDa protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts. Proc Natl Acad Sci U S A 1992;89:10676–80.
[31] Gill SE, Pape MC, Leco KJ. Tissue inhibitor of metalloproteinases 3 regulates extracellular matrix-cell signaling during bronchiole branching morphogenesis. Dev Biol 2006;298:540–54.
[32] Kang MJ, Kim JS, Chae SW, Koh KN, Koh GY. Cyclins and cyclin dependent kinases during cardiac development. Mol Cells 1997;7:360–6.
[33] Yoshizumi M, Lee WS, Hsieh CM, Tsai JC, Li J, Perrella MA, et al. Disappearance of cyclin A correlates with permanent withdrawal of cardiomyocytes from the cell cycle in human and rat hearts. J Clin Invest 1995;95:2275–80.
[34] Bond M, Murphy G, Bennett MR, Newby AC, Baker AH. Tissue inhibitor of metalloproteinase-3 induces a fas-associated death domain-dependent type II apoptotic pathway. J Biol Chem 2002;277:13787–95.