Prx1 Controls Vascular Smooth Muscle Cell Proliferation and Tenasin-C Expression and Is Upregulated With Prx2 in Pulmonary Vascular Disease

Frederick S. Jones, Robyn Meech, David B. Edelman, Rebecca J. Oakey, Peter Lloyd Jones

Abstract—Prx1 and Prx2 are homeobox transcription factors expressed during vasculogenesis. To begin to elucidate how Prx1 and Prx2 are regulated and function in the adult vasculature, in situ hybridization studies were performed. Prx1 and Prx2 mRNAs were not detected in normal adult rat pulmonary arteries; however, both genes were induced with vascular disease, colocalizing to sites of tenasin-C (TN-C) expression. Because catabolism of the extracellular matrix (ECM) is a critical step in the development of vascular disease, we investigated whether changes in vascular smooth muscle cell (SMC)–ECM interactions regulate Prx1 and Prx2. A10 SMCs cultured on native type I collagen showed low levels of Prx1 and Prx2 mRNA expression, whereas cells cultured on denatured collagen showed higher levels of expression of both genes. At a functional level, transfection of SMCs with a Prx1 expression plasmid showed significant increased growth. Because TN-C also promotes SMC growth and its expression is also upregulated by denatured collagen, we tested and thereafter showed that Prx1 expression significantly enhances TN-C gene promoter activity 20-fold. Similar experiments conducted with truncated Prx1 proteins showed that the N-terminal portion and the homeodomain of Prx1 were necessary to induce the bulk of TN-C promoter activity. These findings support the hypothesis that Prx genes are regulated by changes in SMC adhesion and play key morphoregulatory roles during the development and progression of pulmonary vascular disease in adults. (Circ Res. 2001;89:131-138.)

Key Words: tenasin-C ■ homeobox genes ■ pulmonary

Homeobox transcription factors guide formation of the body plan during embryogenesis. Although homeobox genes also function during postnatal development and in adult disease, little is known about their roles in vascular remodeling. Prx1 and Prx2 represent paired-related homeobox genes, which characteristically bind class I homeodomain binding sites (HBSs) containing an ATTA core motif. Prx1 and Prx2 are expressed during embryogenesis, predominantly in mesenchyme-specific patterns. In the developing cardiovascular system, Prx1 and Prx2 are evident in the endocardial cushions and valves, the epicardium, and the wall of the great arteries and veins. In avian embryos, Prx1 and Prx2 are first expressed within the primary vessel wall of muscular coronary and pulmonary arteries (PAs), but as the vessels mature, their expression is restricted to non-muscle cells in the adventitia and outer media.

Among the targets that may be regulated by Prx proteins is the ECM protein tenasin-C (TN-C). In support of this, expression of Prx1, Prx2, and TN-C overlap in several settings including epithelial-mesenchymal transformation and vasculogenesis. TN-C is also expressed in remodeling adult tissues, including injured PAs, where it surrounds proliferating cells at the adventitial-medial boundary. Functionally, TN-C promotes growth and survival in cultured smooth muscle cells (SMCs) and in hypertensive PAs. On the basis of these findings, identifying the factors that control TN-C expression represents a potentially important step toward treating pulmonary vascular disease.

Multiple factors regulate TN-C, including ECM-degrading proteases. For example, inhibition of matrix metalloproteinase (MMP) activity suppresses TN-C expression and PA SMC growth, and reduces the severity of vascular lesions. These studies indicate that MMPs are upstream in an adhesion-dependent signaling pathway that controls TN-C. Consistent with this, we have shown that the TN-C gene promoter contains an ECM-responsive element that is silenced in SMCs cultivated on native type I collagen but is activated on the denatured form of this substrate. This ECM-responsive element harbors an HBS containing an ATTA core motif, which suggests that induction of TN-C expression by MMPs and denatured collagen might be controlled by homeobox proteins.

Here, we show that Prx1 and Prx2 are upregulated during the development of pulmonary vascular disease in adult rats,
localizing to sites of TN-C expression. We also report that
Prx gene expression is regulated by changes in SMC adhesion
to type I collagen and that Prx1 controls SMC growth and
TN-C gene transcription. These findings support the hypoth-
esis that Prx proteins play key roles in the development of
pulmonary vascular disease by controlling SMC proliferation
and the composition of the vascular ECM.

Materials and Methods

Cell Culture

A10 vascular SMCs were maintained in M199. All experiments
were performed in triplicate, unless otherwise stated, in M199 containing
2% FBS. Collagen substrates were prepared as published.19 TN1-
GFP cells were generated by transfecting A10 SMCs with the
TN1-pEGFP construct. Cell lines were selected on the basis of
resistance to G418 and via fluorescence-activated cell sorting for
GFP. To assess proliferation, SMCs were maintained in medium
supplemented with bromodeoxyuridine (BrdU) for the final 4 hours
of the designated experiment.

Prx Expression Vectors

Prx cDNAs were isolated by reverse transcribing total RNA isolated
from A10 SMCs. Truncated forms of rat Prx1 and Prx2 were also
prepared using reverse transcriptase–polymerase chain reaction (RT-
PCR) and Pfu polymerase. cDNAs were cloned into a modified
pcDNA3 expression vector containing an N-terminal c-myc epitope tag.

In Situ Hybridization

Adult Sprague-Dawley rat lung tissues were used for in situ
hybridization studies (a kind gift from Dr Marlene Raboinvitch,
The Hospital for Sick Children, Toronto, Ontario, Canada). Riboprobes
were generated using SP6 or T7 polymerase and [35 S]dUTP. Sections
were incubated with riboprobes before signal detection with photo-
emulsion and were counterstained with Hoechst dye for visualization
of nuclei by epifluorescence. Prx mRNA expression
was visualized using dark-field microscopy.

mRNA Expression Studies

RT-PCR reactions were performed with cDNAs using primer pairs
for Prx1, Prx2, and GAPDH. For Northern analysis, 1 μg of A10
SMC Poly(A’’ ) RNA was separated and transferred to a nylon
membrane. Hybridizations were performed with [32 P]-random-labeled
Prx1 and Prx2 cDNA probes. Autoradiograms were analyzed using
ImageQuant software. A loading control for RNA was also carried
out by comparing Prx mRNA expression with that of rat GAPDH.

Western Immunoblotting

Protein was separated on 4% to 15% polyacrylamide gels and
transferred to polyvinylidene difluoride membranes. The amount of
protein loaded was determined on the basis of transfection effi-
ciency. To detect GFP and c-myc, membranes were incubated with
anti-GFP and anti-myc mouse monoclonal antibodies. Membranes
were then incubated with a horseradish peroxidase–conjugated goat
anti-mouse antibody and detected on Kodak X-Omat film by
enhanced chemiluminescence.

Immunostaining

For detection of Prx1, SMCs were incubated with an anti–c-myc
antibody, or with control IgG, and thereafter with an FITC-
conjugated species-specific antibody. Nuclei were detected with
DAPI. For BrdU detection, a cell proliferation assay kit was used.
BrdU-positive nuclei were scored in control- and Prx1-transfected
SMCs, and the percentage increases in BrdU incorporation deter-
dined after accounting for differences in transfection efficiency.

Site-Directed Mutagenesis

A 4173-bp fragment of the murine TN-C gene promoter was ligated
into pEGFP, a promoterless vector encoding GFP. This vector was
designated TN1-pEGFP. To assess whether the ATTA HBS is
involved in regulating TN-C promoter activity, site-directed mu-
tagenesis was performed using mutant oligonucleotide primers.

Cotransfection and Luciferase Assays

The following plasmids were introduced using LipofectAMINE:
Prx1-pcDNA3 myc, TN1-pEGFP (wild-type), TN1-pEGFP (mu-
ant), pcDNAmyc empty vector, and pSV-β-galactosidase control
reporter vector. Cells were harvested at 48 hours after transfection
and evaluated for transfection efficiency on the basis of β-galacto-
sidase activity. Western immunoblotting was then used for detection
of GFP and myc proteins.

For luciferase reporter assays, A10 SMCs were transfected using
Fugene reagent with either empty pcDNAmyc vector or the Prx1
expression vector, together with a TN-C promoter/luciferase reporter
vector (TN7), containing the −247/+121 region of the murine TN-C
gene containing the proximal promoter and a segment of the first
exon.25 In these experiments, a lacZ reporter construct (CMV β) was
cotransfected to provide an internal reference standard for transfe-
cion efficiency. Luciferase activity was measured using the Millipore
Cytolucifer 2450 system. To demonstrate that comparable levels of
different Prx proteins are expressed after transfection of A10 cells,
Western blots using the myc-tag antibody were performed on lysates
of SMCs transfected with each Prx expression construct and the
CMV β plasmid. In these experiments, the amount of lysate analyzed
was first normalized to the β-galactosidase activity.

Electromobility-Shift Assays

Wild-type and mutant oligonucleotides encompassing the TN-C
promoter HBS were end-labeled with [γ-32 P]ATP, 0, 0.5, 1.5, and 4.5
μg of nuclear protein were incubated with 20,000 cpm of [32 P]-labeled
wild-type or mutant probe in binding buffer. For supershift assays,
Prx1-transfected A10 SMCs were preincubated with anti-myc anti-
body before the addition of radiolabeled wild-type or mutant
oligonucleotide probes. Samples were resolved on 7% nondenaturing
acrylamide gels, and DNA:protein complexes were visualized by
autoradiography.

Statistical Analyses

Results were compared by 1-way ANOVA and Student-Newman-
Keuls post hoc analysis. A P value of <0.05 was considered
statistically significant.

An expanded Materials and Methods section can be found in an
online data supplement available at http://www.circresaha.org.

Results

Cloning of Rat Prx1 and Prx2

To isolate rat Prx genes, RT-PCR experiments were performed
using A10 SMC total RNA. A full-length Prx1 cDNA
of 810 bp was isolated containing an open reading frame of
651 bp, encoding a 217–amino acid protein. Comparison of
this sequence with other Prx gene sequences indicated that it
was 100% identical to rat Prx1 (Rhox) but also contained an
additional exon of 72 bp that is found in several mammalian
Prx1 genes. This additional sequence is inserted at position
651 bp, encoding a 217–amino acid protein. Comparison of
this sequence with other Prx gene sequences indicated that it
was 100% identical to rat Prx1 (Rhox) but also contained an
additional exon of 72 bp that is found in several mammalian
Prx1 genes. This additional sequence is inserted at position
927 in the Rhox sequence. The Prx1 cDNA sequence is 97%
identical to that of mouse Prx1. The Prx2 cDNA from A10
cells was 570 bp and encodes a protein that is 94% identical
to mouse Prx2. This cDNA represents a novel form of Prx2
that has a deletion of 57 amino acids within the N-terminal
region.
Induction of Prx1 and Prx2 With Pulmonary Vascular Disease

To determine the expression patterns of Prx mRNAs, ribo-probes were hybridized to pulmonary tissue isolated from adult rats injected with saline (control), or monocrotaline (MCT), an alkaloid toxin that induces pulmonary hypertension. Prx antisense riboprobes did not hybridize to normal tissue (Figure 1), whereas these genes were expressed at the adventitial– outer medial boundary in hypertensive animals by 21 days after injection (Figure 1). In addition, the airways and surrounding tissue expressed Prx1 mRNA. By 28 days, Prx1 and Prx2 mRNAs were expressed in the PA adventitia and within the media and subendothelium (Figure 1). Extensive Prx1 mRNA expression was also evident in the airways and lung interstitium (Figure 1). In contrast, Prx1 and Prx2 sense riboprobes did not hybridize with either control or hypertensive tissue (data not shown). Thus, Prx1 and Prx2 mRNAs are induced and upregulated with the progression of pulmonary vascular disease.

Changes in SMC Adhesion Regulate Prx1 and Prx2

Because remodeling of the PA ECM is critical to the progression of vascular disease, we next sought to determine whether alterations in vascular SMC-ECM interactions control Prx mRNA expression. As a model system, SMCs were cultured on native and denatured type I collagen. As a model system, SMCs were cultured on native and denatured type I collagen. A10 SMCs were used for these studies because they behave in a manner that is identical to that of primary PA SMCs in terms of their phenotypic and gene expression responses to type I collagen. RT-PCR studies showed that Prx1 and Prx2 mRNAs were expressed in the PA adventitia and within the media and subendothelium (Figure 1). Extensive Prx1 mRNA expression was also evident in the airways and lung interstitium (Figure 1). In contrast, Prx1 and Prx2 sense riboprobes did not hybridize with either control or hypertensive tissue (data not shown). Thus, Prx1 and Prx2 mRNAs are induced and upregulated with the progression of pulmonary vascular disease.

Figure 1. Expression of Prx genes in pulmonary vascular disease. Dark-field photomicrographs showing expression patterns of Prx mRNAs (in gold) in lung tissue derived from normal (saline-injected) and hypertensive (MCT-injected) adult male Sprague-Dawley rats. Cell nuclei were visualized with Hoechst dye (in blue). 21d and 28d indicate 21 and 28 days, respectively. Bar=25 μm.

Figure 2. Control of Prx1 and Prx2 mRNA expression by ECM. A, Prx mRNA expression was assessed via RT-PCR assays using cDNAs generated from A10 SMCs cultivated on native (Nat) or denatured (DN) type I collagen. Parallel RT-PCR reactions using GAPDH primers served as control. B, Northern blot for Prx1, Prx2, and GAPDH mRNAs in A10 SMCs maintained either on native (Nat) or denatured (DN) collagen. C, Densitometric assessment of Prx mRNA expression levels derived from duplicate Northern blots.

Scanning densitometry of duplicate Northern blots, normalized to GAPDH, showed that Prx1 and Prx2 were both upregulated ~3-fold in SMCs maintained on denatured collagen compared with those maintained on native collagen (Figure 2C). These results demonstrate that alterations in SMC adhesion to the ECM control Prx1 and Prx2 mRNA expression.

Prx1 Promotes SMC Growth

To begin to establish a function for Prx1, SMCs cultivated on denatured collagen were transiently transfected with a Prx1 expression plasmid containing an N-terminal c-myc tag. Protein expression was examined by Western blotting of A10 SMC nuclear extracts and by indirect immunofluorescence using an antibody against the c-myc tag. A Prx1 protein (~26 kDa) was expressed in nuclei of transfected cells (Figure 3A). Immunofluorescence studies showed that Prx1 was expressed in SMCs that appeared to be undergoing division (Figure 3A). Taken together with our observation that Prx1 is upregulated in remodeling PAs, we hypothesized that Prx1 controls SMC growth. To test this, A10 SMCs cultivated on native collagen were cotransfected either with the parental c-myc vector or with the Prx1 vector and a β-galactosidase–encoding expression plasmid in the presence of BrdU to assess cell prolifer-
When normalized for transfection efficiency, overexpression of Prx1 significantly increased BrdU incorporation by 78.1% \((P<0.03)\) (Figure 3C).

**Expression of TN-C Requires an HBS Within Its Gene Promoter**

Previously, we showed that TN-C is first expressed at the adventitial-medial boundary of MCT-treated hypertensive rat PAs, as well as in the airways and the interstitium.\(^{17}\) This expression pattern is identical to that observed for Prx1 and Prx2 mRNAs (Figure 1). Also consistent with our present results obtained for Prx1 and Prx2 mRNA expression (Figure 2), TN-C mRNA expression is reduced by native collagen and is upregulated by denatured collagen.\(^{19,21}\) Collectively, these data indicate that TN-C is upregulated with Prx1 and Prx2 in pulmonary vascular lesions and that changes in vascular SMC adhesion alters the expression of all three genes.

We have also shown that transcriptional induction of TN-C in SMCs cultivated on denatured collagen requires a 122-bp DNA element in the TN-C promoter containing an HBS.\(^{21}\) To determine whether the HBS is a key component in the ECM responsiveness of the TN-C promoter, GFP-reporter constructs containing either the wild-type or an HBS-mutated TN-C promoter were transiently transfected into SMCs cultivated on native or denatured collagen. Mutation of the TN-C HBS inhibited TN-C promoter activity in SMCs cultured on denatured collagen, as compared with wild type-transfected SMCs (Figure 4A). No GFP expression was observed in wild type- or mutant- transfected SMCs cultivated on native collagen (Figure 4A).

As a first step toward characterizing the protein(s) that may bind to the HBS, electrophoretic mobility-shift assays were performed. Radiolabeled oligonucleotides containing the wild-type and mutated HBS were incubated with nuclear extracts from SMCs maintained on native and denatured collagen incubated in binding reactions with radiolabeled oligonucleotide containing the wild-type or mutant TN-C promoter HBS. Arrows indicate binding complexes. C, Gel-shift assay in which increasing amounts of nuclear extract (NE) \((0, 0.5, 1.5, \text{ and } 4.5 \mu g)\) were incubated with the wild-type radiolabeled TN-C HBS (left). Specificity of binding of nuclear extracts to the HBS was tested by incubation with a \(\times 20\) and \(\times 200\) molar excess of cold competitor (CC) oligonucleotide.
experiments, the intensity of DNA:protein complexes observed with the wild-type HBS increased with greater concentrations of nuclear extract; formation of these complexes was abolished in reactions containing excess unlabeled HBS (Figure 4C). These experiments indicate that the TN-C promoter HBS interacts with different proteins in an ECM-dependent manner.

To determine whether Prx1 protein binds directly to the HBS, experiments were performed using radiolabeled HBS probes and nuclear extracts from cells transfected with the c-myc-tagged Prx1 expression vector. When compared with mock-transfected SMCs, no differences in DNA:protein complex formation were observed (data not shown). Preincubation of binding reactions with a c-myc antibody did not block or supershift DNA:protein complexes. Recombinant Prx1 protein also failed to bind to the wild-type HBS (data not shown). These results indicate that Prx1 either interacts weakly or does not bind directly to the core TN-C promoter HBS sequence.

Regions of the Prx1 Protein That Contribute Toward TN-C Promoter Activation

Although Prx1 protein did not directly bind to the probe containing the HBS, the coincident induction of Prx genes and TN-C in pulmonary vascular disease and in isolated SMCs prompted us to investigate whether Prx1 transactivates the TN-C gene promoter. For these experiments, an A10 SMC line (TN1-GFP) was generated in which a TN-C promoter-GFP reporter was stably integrated. As shown in Figures 5A and 5B, TN-C gene promoter activity was suppressed on native collagen and activated on denatured collagen. Transfection of TN1-GFP cells cultured on native collagen with the Prx1 expression plasmid led to increased Prx1 protein production and TN-C promoter activity (Figure 5C).

To quantify the level of TN-C promoter activation by Prx1 and to determine the regions of Prx1 required for this activation, cotransfection experiments were performed using a TN-C promoter/luciferase gene reporter plasmid (construct TN7) and three different myc-tagged Prx1 expression constructs (Figure 6A). These Prx constructs expressed either the full-length Prx1 protein, a truncated form (PNL) containing the N-terminal half of the protein with the Prx domain and nuclear localization sequence, or a truncated form (PHD) containing the N-terminal portion of Prx1 and the homeodomain.

To demonstrate that the constructs were expressed at equivalent levels, Western blots were performed on extracts from A10 cells transfected with Prx1, PNL, and PHD
constructs. Cellular lysates were first adjusted to an internal reference standard of β-galactosidase activity. In a total of six experiments, no differences in Prx protein expression levels were noted. A representative Western blot is shown in Figure 6B.

In luciferase reporter assays, Prx1 induced a 20-fold induction of TN-C promoter activity relative to that observed in cells transfected with empty pcDNA3-myc vector (Figure 6C). The PHD construct led to a significant 10-fold activation of the TN-C, whereas the PNL construct produced a <4-fold induction (Figure 6B). These data indicate that the C-terminal portion of Prx1 contributes ~50% of the level of TN-C promoter activation. However, the remaining segment of Prx1 containing the N-terminal portion and homeodomain of Prx1 is required for high levels of TN-C promoter activation. These data were collected from four separate experiments performed in triplicate (n=12).

Discussion

Although homeobox transcription factors control a range of cellular activities during embryonic development, little is known about their regulation and functions in adult tissues. We have shown that expression of two paired-related homeobox genes, Prx1 and Prx2, is induced during the development of pulmonary vascular disease in adult rats. This induction of Prx1 and Prx2 coincides with that of the ECM protein, TN-C. In addition, alterations in SMC adhesion were shown to regulate Prx gene expression. Because structural remodeling of the ECM also regulates SMC proliferation, TN-C biosynthesis, and the severity of pulmonary vascular disease, we assessed whether Prx1 could modulate these functions. Expression of Prx1 promoted SMC growth and induced TN-C expression. Finally, we showed that the ability of Prx1 to transactivate the TN-C promoter relies on distinct regions of this homeobox protein. These experiments support the idea that Prx proteins and concomitant alterations in the expression of particular ECM proteins (ie, TN-C) are likely to be important factors in the genesis of pulmonary vascular disease.

A tenable hypothesis based on this study is that expression of Prx and TN-C genes is controlled by the same factors. In keeping with this, bone morphogenetic proteins and angiotensin II have each been shown to regulate Prx and TN-C expression. In this study, we focused on the role of changes in SMC adhesion as a factor that controls Prx genes and TN-C. This direction was based on a growing body of evidence implicating cell adhesion as an important factor regulating vascular disease. Moreover, our previous work demonstrated that remodeling of native collagen activates a β3 integrin–dependent extracellular signal–regulated kinase mitogen-activated protein kinase (ERK MAPK) signaling cascade that results in TN-C gene transcription. The present study indicates that Prx genes are also regulated by changes in the structure of type I collagen. It will therefore be important to determine whether β3 integrin and ERK MAPKs also control the expression and/or posttranslational processing of Prx genes and proteins.

The expression of other homeobox genes has also been shown to depend on the surrounding ECM. For example, endothelial cell HoxD3 expression is suppressed by basement membrane proteins during acquisition of an angiogenic phenotype. Similarly, HoxB7 expression in mammary epithelial cells is incompatible with basement membrane–directed lactational differentiation. Because HoxB7 is also expressed in fetal but not adult SMCs, it would be interesting to determine whether alterations in SMC HoxB7 expression are also influenced by changes in the vascular ECM.

Our studies show that Prx1 activates TN-C transcription. This suggests that Prx proteins not only respond to changes in cell adhesion, but they can also act in a reciprocal manner to regulate the composition of the ECM. This type of “inside-out” control has already been described for the Gax homeobox gene, which promotes a quiescent SMC phenotype by suppressing expression of α,β and α,β integrins. Given that SMC α,β integrins also interact with denatured collagen (which we have now shown promotes Prx1 and Prx2 expression), it is possible that an inverse relationship exists between Gax and Prx genes in developing and remodeling arteries.

The appearance of Prx1 and Prx2 in the adventitia of hypertensive PAs suggests that these genes might regulate the behavior of nonmuscular cells. Consistent with this, developmental studies show that Prx gene expression and recruitment of SMCs first takes place in the surrounding loose mesenchyme or primordial adventitia. Whether further growth of the vessel wall involves proliferation of medial SMCs or recruitment and growth of undifferentiated adventitial cells is presently unknown. However, cell labeling studies clearly show that adventitial fibroblasts represent a component of the neointimal layer within injured adult systemic arteries. In addition to Prx1 and Prx2 expression, induction of α-smooth muscle (SM) actin represents another hallmark of activated fibroblasts in different remodeling tissues, and it has been shown that Prx1 can transactivate the α-SM actin gene promoter. In light of the present results, it will be important to determine whether Prx genes also control adventitial fibroblast behavior in hypertensive PAs via their ability to modulate α-SM actin.
Our study also demonstrates that different Prx1 domains contribute to TN-C gene transcription. One way that the N- and C-terminal portions of Prx1 might participate in this transactivation event is via interaction with a protein domain on another transcription factor, or they might modulate the interaction of the Prx1 homeodomain with other factors. Also, Prx1 activation of TN-C promoter activity with lack of direct binding of Prx1 protein to the HBS suggests several possibilities for transactivation. First, Prx1-HBS interactions might require an accessory DNA element in the 247-bp TN-C promoter, which binds a protein cofactor that is important to stabilize interaction of Prx proteins with the HBS. Alternatively, Prx1 might not bind to the HBS but instead interact with other proteins that are assembled at other elements within the TN-C promoter. Such elements in the 247/+121 TN-C promoter include binding sites for nuclear factor κB, POU homeodomain proteins, nuclear factor-eB, and a TRE/AP-1 element that binds to fos, jun, and other bZip proteins. A tenable hypothesis is that Prx1 activates TN-C transcription via protein-protein interactions with these factors. For instance, Prx1 is known to form complexes with the Maf oncoprotein, a bZip transcription factor family member. A Maf-recognition element, which is also a consensus TRE/AP-1 element, is found at −114 bp in the TN-C promoter. Maf forms heterodimeric combinations with Prx1, as well as with fos and jun; such interactions may modulate the activity of TN-C and other genes that are targets of these factors. Additionally, Prx1 may interact directly with components of the basic transcription machinery assembled at the TATA box. For instance, Prx1 is known to interact with the serum response factor and RB, proteins that are capable of interacting with the basic transcription machinery. Alternatively, it is possible that Prx1 transactivates TN-C via its ability to modulate the expression of transcription factors that bind directly to the TN-C gene promoter.

Mice bearing null mutations in both Prx1 and Prx2 have been informative in elucidating the role of these genes during development.38–42 Mutant animals die 24 hours after birth and display skeletal and limb defects, as well as vascular anomalies, including abnormal positioning and awkward curvature of the aortic arch, and a misdirected and elongated ductus arteriosus. These defects are conceivably due to deregulated ECM synthesis.42 Whether this relates to altered TN-C expression, however, has not been determined.

In summary, our findings support the hypothesis that changes in SMC adhesion to the ECM are related to the expression and functions of Prx genes. In addition, the present work provides a foundation for studies aimed at deciphering the gene networks and signal-transduction events that are responsible for Prx gene expression through changes in the vascular ECM, as well as the mechanisms by which TN-C gene transcription and cellular proliferation are controlled under these conditions.

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Frederick S. Jones, Robyn Meech, David B. Edelman, Rebecca J. Oakey and Peter Lloyd Jones

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Supplementary Materials and Methods

Cell Culture

A10 vascular SMCs, a fetal rat thoracic aorta cell line, were routinely maintained in Medium 199 (M199) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 10 units/mL penicillin G sodium, 10 mg/mL streptomycin sulfate, 0.85% saline (Gibco-BRL). Cells were passaged by trypsinization using 0.05% trypsin/EDTA (Gibco-BRL). Vitrogen-100 type I collagen substrates were prepared based on our published methods\(^1\). For native type I collagen, fibrillogenesis was initiated overnight at 37°C. For the assessment of the effects of altering SMC adhesion to the ECM on A10 SMC gene expression, type I collagen was heated for in 0.02 M acetic acid, neutralized with 0.1 M NaOH, and then air dried onto tissue culture dishes or glass coverslips. Before plating cells, collagen substrates were rinsed extensively with serum-free M199.

Preparation of Prx expression vectors

In order to isolate rat Prx1 and Prx2, A10 SMC total RNA was reverse transcribed using the first strand cDNA synthesis (Gibco-BRL). PCR reaction for Prx1 and Prx2 were then carried. The primer sequences were as follows: Prx 1 (forward: 5'-ACC TCC AGC TAC GGG CAC GTT CTG-3', reverse: 5' ACC TCA GTT GAC TGT TGG CAC-3') and Prx 2 (forward: 5'-GGA ATT CGA CAG CGC GGC CGC CGC CTT-3', reverse: 5'-CCG CTC GAG TCA GTT CAC TGT GGG CAC CTG GCT GTG GT-3'). Truncated forms of rat Prx1 and Prx2 were also prepared using RT-PCR and Pfu polymerase. All fragments were cloned into a modified pcDNA3 expression vector (InVitrogen, Carlsbad, CA) containing an N-terminal c-myc epitope tag that allowed
tracking of the protein in immunohistochemical procedures. All constructs were sequenced and selected when confirmed to be in the correct 5' to 3' orientation.

_in situ_ hybridization

_in situ_ hybridization for Prx1 and Prx2 mRNAs was performed on normal and hypertensive lung tissue sections. Adult male Sprague-Dawley rats (Charles River Breeding Laboratories Inc, Montreal, Quebec, Canada) were assigned at random to receive either monocrotaline or physiological saline (0.9% NaCl). Adult rats were injected at 8 weeks of age. All injections were administered sub-cutaneously in the hind flank; the dose of monocrotaline (Transworld Chemicals) was 60 mg/kg body wt, and the saline injection was of an equal volume. At the end of the designated experimental time point, animals were killed by overdose with sodium pentobarbital (300 mg/kg), and lungs were removed, rinsed in PBS and fixed overnight in 2% paraformaldehyde before embedding and sectioning. These tissues were kindly provided by Drs. Marlene Rabinovitch and Kyle Cowan at The Hospital for Sick Children, Toronto, Canada.

In order to generate Prx1 and Prx2 antisense and sense riboprobes, pCDNA-myc-tagged were linearized and thereafter transcribed and labeled with SP6 or T7 polymerase in the presence of $^{35}$S-dUTP. Sections were de-paraffinized with xylene and ethanol, acid treatment and proteinase K. Sections were post-fixed in paraformaldehyde, washed in acetic anhydride and left to air dry. Sections were then incubated overnight with probes in hybridization solution containing the probes in an humidified chamber overnight at 50°C, and then washed before signal detection by dipping in a photographic emulsion and exposing for 1-2 weeks. Slides were developed
and counter-stained with Hoechst dye for visualization of nuclei. Prx1 and Prx2 mRNA expression was visualized under dark field or fluorescence microscopy.

**Semi-quantitative RT-PCR for Prx1, Prx2, TN-C and GAPDH**

Semi quantitative RT-PCR reactions were performed with A10 cDNAs cultivated on native and denatured type I collagen using the following primer pairs; Prx1 (forward 5'-ACCTCCAGCTACGGGCACGTT-3' and reverse 5'-TAGCCATGGCGCTTTTCAGTG-3'), Prx2 (forward 5'-ATGGCAGTGGCAAACGG-3' and reverse 5'-GTTGCAGGACTTGACCTCC-3') and GAPDH (forward 5'-TGGGGCCAAAAGGTCATCATCTC-3' & reverse 5'-GCCGCTGCTTCCACCACCTTCTT-3').

**Northern blot analysis**

Poly(A⁺) RNA was isolated from A10 cells cultured on either native or heat denatured type I collagen using an Oligotex Direct mRNA Kit (Qiagen). One μg of poly(A⁺) RNA was separated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham Life Sciences Inc) by capillary transfer before UV cross-linking. Hybridization was performed in QuickHyb Solution (Stratagene) for 2 hours with ³²P-random labeled cDNA probes for Prx 1 and Prx2. Membranes were washed with 2X SSC containing 0.1% SDS at room temperature for 2 x 15 minutes, and thereafter in 0.1X SSC containing 0.1% SDS at 68°C for 30 minutes. Membranes were exposed to X-ray film at −70°C and autoradiographic results were scanned and analyzed using Imagequant software (Molecular Dynamics). A loading control for RNA
was also carried out by comparing Prx 1 and Prx2 mRNA expression with GAPDH via hybridizing the Prx-hybridized membranes with a $^32$P-random labeled cDNA probe for rat GAPDH.

**Western immunoblot analysis**

A10 SMC extracts were harvested in ice-cold PBS buffer (pH7.4) containing 1 mmol/L benzamidine, 1 mmol/L PMSF, 1 μg/mL aprotinin, 5 μg/mL leupeptin, 2 mmol/L NaF, 2 mmol/L Na$_3$VO$_4$ and 1 mmol/L MgCl$_2$, and then lyzed in RIPA buffer as previously described$^2$. Samples were quantified and separated on 4-15% linear gradient polyacrylamide gels (Bio-Rad) in SDS-PAGE sample buffer (Bio-Rad). In order to confirm that equal amounts of protein were loaded, duplicate SDS-PAGE gels were run in parallel with experimental gels and then stained with Coomassie Blue. Proteins transferred to polyvinyl fluoride membranes were rinsed in wash buffer (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl and 0.1% Tween-20), and then blocked for 2 hours at 25°C in wash buffer, supplemented with 5% Non-Fat Milk. To detect GFP, membranes were incubated with an anti-GFP mouse monoclonal antibody (Clontech) diluted 1:500 in blocking buffer. To detect c-myc protein, membranes were incubated with an anti-myc mouse monoclonal antibody (InVitrogen) diluted 1:5000 in wash buffer, Rinsed membranes were then incubated with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Gibco-BRL). Thereafter, GFP and myc proteins were visualized by enhanced chemiluminescence (Amersham), before exposure to Kodak X-Omat film.
**Immunodetection of Prx1 and BrdU**

For tissue culture studies, A10 SMCs were cultured on glass coverslips coated with type I collagen. SMCs transiently transfected with myc-tagged empty vector, or with the myc-tagged Prx1 expression vector in Medium 199 plus 2% fetal calf serum were fixed in methanol. Cell cultures were maintained for a total of 72 hours in culture. For assessment of cell proliferation, SMC cultures were also co-transfected with a β-galactosidase expression vector for normalization of transfection efficiency, and maintained in Medium 199 plus 0.1% fetal calf serum, which was supplemented with BrdU for the final 4 hours of the experiment. For Prx1 protein detection, fixed SMCs were pre-incubated in wash buffer (PBS/1% BSA) supplemented with 10% normal goat serum, and thereafter with an anti-c-myc mouse antibody (Sigma), or with an or with appropriate control antisera. After being washed, coverslips were incubated with a fluorescein-conjugated species-specific secondary antibody. After washing, cells were stained with DAPI for detection of nuclei. After washing, all coverslips were mounted on glass slides using Antifade reagent (Molecular Probes). Observations and photomicrographs were obtained using epifluorescence. For BrdU detection, a cell proliferation assay system utilizing anti-BrdU antibodies and a species-specific peroxidase-conjugated antibody were used as per the manufacturers’ instruction (Amersham). BrdU positive nuclei were counted in both control and Prx1-transfected SMCs, and the total numbers of proliferating cells were scored after accounting for differences in transfection efficiency.

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**Site-directed mutagenesis of the TN-C gene promoter**
A 4173 bp fragment of the mouse TN-C gene promoter was ligated into pEGFP, a promoterless vector containing a coding region for green fluorescent protein (GFP) (Clontech). This vector was designated TN1-pEGFP. To assess whether the ATTA HBS, located −57 bp upstream from the RNA start site, is involved in regulating TN-C promoter activity in A10 SMCs, we performed site-directed mutagenesis using the Quikchange method (Stratagene). Briefly, the HBS in TN1-pEGFP was mutated to ACTC using the following oligonucleotide primers: forward: 5’-GGA GGC GAG GCG TCC CAC TCC AGA-3’ and reverse: 5’-CCT CCG CTC CGC AGG GTG AGG TCT CCT TCC TCG-3’, in conjunction with Pfu Turbo DNA polymerase. The PCR product was digested with DpnI to remove methylated plasmid template DNA. Introduction of the ACTC mutation into the wild type promoter was confirmed by sequencing.

**Generation of A10 TN1GFP cell lines**

The response of A10 SMCs to changes in type I collagen structure, and to Prx1 gene expression, was accomplished by generating an A10 cell line that stably harbors the TN-C gene promoter placed upstream and in-frame from a GFP reporter gene protein. Briefly, A10 SMCs were transfected on tissue culture plastic with the TN1-pEGFP construct via Lipofectamine (Life Technologies). Stable cell lines were selected based on their resistance to G418 (Gibco-BRL). Further, cells expressing high levels of GFP were further selected by fluorescence activated cell sorting (Children's Hospital of Philadelphia Core Service).
Cellular co-transfection and luciferase reporter assays

Transient transfection experiments were performed by plating either A10 or A10 TN1-GFP cells at a density of $5 \times 10^5$/dish in M199 containing 2% FBS and 10 units/mL penicillin G sodium, 10 mg/mL streptomycin sulfate, 0.85% saline on 60 mm diameter dishes with native or denatured collagen substrates as described above. At 24 hours medium was aspirated and cells were rinsed in serum-free, antibiotic-free M199 (3x 1 hour). Transient transfection experiments were performed using a total of 3 μg of DNA, Lipofectamine according to the manufacturer's instructions and serum-free, antibiotic-free M199. The following plasmid constructs used in transient transfections were Prx1-pcDNA3myc, Prx2-pcDNAmyc, TN1-pEGFP, TN1-pEGFP (Mut), pcDNAmyc empty vector, pEGFP empty vector and pSV-β–galactosidase control reporter vector. Cells were incubated in the presence of DNA-Lipofectamine complexes for 4 hours at which time the antibiotic-free M199 containing DNA-Lipofectamine complexes was replaced with antibiotic-free M199 containing 2% FBS. Cells were harvested at 48 hours post-transfection for evaluation of transfection efficiency based on β–gal activity as well as for western immunoblot detection of GFP and myc proteins.

For luciferase reporter assays using the full length and truncated Prx1 expression vectors, A10 SMCs were transfected with either empty pcDNA3 vector or the full length Prx1 expression vector, together with a luciferase reporter vector (either the parent pGL3basic promoterless luciferase vector or the vector containing the −247/+121 region of the mouse TN-C gene containing the proximal promoter and a segment of the first exon3. In all experiments, a lacZ reporter construct (CMVBeta; Clontech) was co-transfected to provide an internal reference standard for transfection efficiency. Cells
were transfected with Fugene reagent in a 1:6 ratio of DNA to transfection reagent as recommended by the manufacturer. Cells were harvested 72 h later and re-suspended in lysis buffer (100 mmol/L Tris-acetate pH 7.8, 10 mmol/L magnesium acetate, 1 mmol/L EDTA, 1% Triton X100, and 1 mmol/L DTT). Beta galactosidase activity was assayed using the Fluoreporter kit (Molecular Probes) and fluorescence was measured using the Millipore Cytofluor 2450 System.

**Gel Electromobility-Shift Assays**

To generate nuclear extracts, A10 SMCs were harvested in low salt buffer (0.6% NP40, 150 mmol/L NaCl, 10 mmol/L HEPES pH 7.9, 1 mmol/L EDTA, 0.5 PMSF) before centrifugation to pellet nuclei. Isolated nuclei were re-suspended in high salt buffer (25% glycerol, 20 mmol/L HEPES pH 7.9, 420 mmol/L NaCl, 1.2 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 2 mmol/L benzamidine, 5 μg/mL pepstatin, 5 μg/mL leupeptin and 5 μg/mL aprotinin). Samples were then centrifuged briefly at 13,000 rpm and supernatants were collected for quantification using the BCA protein assay (PIERCE) and subsequent storage in liquid nitrogen. Wild type (forward 5'-GGCGTCCCATTACAGAGGAA-3' and reverse 5'-CCGCAGGGTAATGTCTCCTT-3') and mutant (forward 5'-GGCGTCCCACTCCAGAGGAA-3' & reverse 5'-CCGCAGGGGTAGGTCTCCTT3') oligonucleotides encompassing a homeodomain region of the TN-C promoter were end-labeled with [γ-32P]ATP. 0, 0.5, 1.5 and 4.5 μg of nuclear protein were incubated with 20,000 cpm of 32P labeled wild type or mutant probe for 30 minutes. This binding reaction was performed in a binding buffer at a total volume of 10 μl, resulting in a final concentration of 10 mmol/L HEPES, pH 7.9; 3
mmol/L MgCl₂; 50 mmol/L NaCl; 10% glycerol; 0.5 mmol/L DTT; 0.5 mmol/L EDTA; and 1 μg of poly(dI-dC). For super-shift assays, A10 SMCs transiently transfected with Prx1 expression vector and a β-galactosidase reporter gene were pre-incubated with an anti-myc antibody (Invitrogen) prior to the addition of radiolabeled TN-C wild type or mutant oligonucleotide probes. For competition reactions, a 20 and 200-fold excess of unlabeled TN-C wild type or mutant oligonucleotide probe was added to reaction mixtures 10 minutes prior to addition of radiolabeled oligonucleotide probes. All samples were resolved on 7% non-denaturing acrylamide gels in 0.25 Tris-borate/EDTA (TBE) buffer. The gel was vacuum-dried and exposed to Kodak X-Omat film.

**Statistical Analyses**

All statistical assessments were compared by one-way analysis of variance and Student Newman Keuls post-hoc analysis, A *P* value of less than 0.05 was considered statistically significant.
References

