Commentary

NAB2: A Transcriptional Brake for Activated Gene Expression in the Vessel Wall?

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Gene expression is regulated by a hierarchy of control mechanisms that transmit signals from the cell exterior to the nucleus. These mechanisms include precision-controlled signal transduction cascades such as protein kinases that modify the location and activity of DNA-binding transcription factors. Other proteins stabilize or destabilize the expression and activity of DNA-binding transcription factors. Finally, changes in chromatin structure determine the accessibility of DNA to activated transcription factors. A great deal of knowledge has accumulated regarding the role of DNA-binding transcription factors in mediating general and cell-specific gene expression. Comparatively less is known about the proteins that function as transcriptional brakes by competitively inhibiting DNA binding of transcriptional activators or by physically associating with the transcriptional activator to decrease transactivation potential. The latter class of proteins, referred to as corepressors, do not bind DNA directly. Instead, corepressors physically associate with DNA-binding transcription factors at evolutionarily conserved domains that are thought to act as molecular bridges between the DNA-binding transcription factor and the preinitiation complex. In this Commentary, we will briefly review the early genetic response of the vessel wall to injury, then discuss how a new class of corepressors that Silverman et al have studied and now report in this issue of *The American Journal of Pathology*¹ may function to counterbalance the activity of a transcription factor that appears to be a nodal point for injury-induced vascular gene expression.

Among the earliest events to occur in an injured blood vessel is the activation of myriad signaling pathways, which converge on a cell's genome to modulate gene expression. Two classes of genes are typically studied in this context: the early response or immediate early genes (IEG), so named because their induction is independent of protein synthesis, and the secondary or delayed response genes (DRG), whose expression requires *de novo* protein synthesis.² The IEG family includes genes encoding transcription factors (eg, *c-fos*), extracellular matrix molecules (eg, thrombospondin-1), cytoskeletal proteins

(eg, β -actin), metabolic enzymes (eg, cyclooxygenase-2), and signaling molecules (eg, MKP-1). Several IEG-encoding DNA-binding transcription factors are induced in cells of the vascular wall within minutes of balloon injury.³⁻⁵ The expression of IEG is both rapid and transient, with levels of transcripts often dropping to baseline within a few hours of injury. Such dynamic gene activity is followed by the peak expression of a battery of DRG-encoding cell cycle regulators, growth factors and their receptors, proteases, and extracellular matrix proteins. The collective action of these DRG is to stimulate smooth muscle cell (SMC) proliferation, migration, and ultimately neointimal formation. The sequential activation of IEG and DRG in vascular tissue has led to speculation that the proteins encoding the former directly regulate the latter. For example, there is accumulating evidence supporting a role for the IEG product early growth response protein 1 (Egr-1) in the transcriptional activation of platelet-derived growth factor (PDGF)-A and PDGF-B in injured smooth muscle and endothelial cells.^{5,6}

Egr-1 (also called NGFI-A, Krox24, Zif268, and TIS8) is a zinc finger-containing transcription factor (class C₂H₂) involved in growth, differentiation, radiation injury, and neuronal signaling.⁷ The nature of stimuli that induce *egr-1* mRNA and its expression kinetics is essentially identical to the *c-fos* proto-oncogene, the prototypical IEG. Transcription of these IEG begins as early as 5 minutes poststimulation with levels peaking between 30 minutes and 1 hour of the stimulus. In most instances, the amount of *egr-1* mRNA that accumulates after stimulation exceeds that of the *c-fos* proto-oncogene. Egr-1 protein begins to accumulate as early as 30 minutes after stimulation and then rapidly declines by 4 hours poststimulus. Thus, Egr-1 represents one of the first genes to be induced and translated into a functional protein after growth and differentiative stimuli. Its rapid induction and nuclear localization suggest an important role for Egr-1 in orchestrating changes in vascular cell gene expression after injury to the vessel wall.⁵

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The rapid induction of IEG such as *egr-1* stimulated much interest in the regulatory steps leading to such dramatic changes in steady-state mRNA expression. Standard promoter analyses have revealed the presence of several *cis*-acting regulatory elements in the 5' promoter region of many IEGs. Two regulatory elements are worthy of comment in this context. While analyzing the regulation of the *c-fos* promoter, Treisman and colleagues defined a rather large *cis* element with dyad symmetry around an A/T-rich core that was essential for the serum inducibility of *c-fos*. This element was named the serum response element (SRE).⁸ Around the same time, the core A/T-rich region of the SRE was defined in several sarcomeric muscle promoters. The latter element came to be known as a CArG box (for CC-AT-rich-GG), and its presence in the regulatory region of skeletal and cardiac muscle-restricted genes suggested an important role for the CArG box-binding factor in muscle differentiation.⁹ The binding factor for the SRE and CArG box was subsequently cloned and named serum response factor (SRF), which, curiously, turned out to be an IEG itself.¹⁰ Careful dissection of the 5' promoter region of *egr-1* reveals six SRF-binding CArG box elements.⁷ The higher number of CArG boxes in the *egr-1* promoter as compared to other IEG promoters is thought to account for the greater level of *egr-1* transcripts observed after growth stimulation.⁷ In addition to many CArG boxes, the negative strand of the *egr-1* promoter harbors a binding site for its own translated protein.⁷ The *Egr-1* binding site is a GC-rich element with a consensus sequence of GCGKGGGCG.⁷ In addition to being found in the *egr-1* promoter, *Egr-1* binding sites are present in the 5' flanking region of several growth-related genes including PDGF A chain, PDGF B chain, basic fibroblast growth factor, and SRF.^{11,12} The *Egr-1* regulatory element bears some resemblance to and often overlaps with another GC-rich element that binds the ubiquitous, constitutively expressed transcription factor Sp1.¹³ Studies have shown that induced *Egr-1* can displace Sp1 from its binding site, leading to transcriptional activation of the PDGF-A chain gene in vascular cells.⁶

Transcription factors contain modular domains that are critical for DNA binding, nuclear translocation, transcriptional activation, and transcriptional repression. Extensive structure-function analysis of the *Egr-1* protein revealed the presence of a 34-amino acid domain which, on deletion, caused a dramatic increase in *Egr-1*'s ability to activate transcription.¹⁴ This finding suggested the existence of a protein that interacted with the 34-amino acid domain to mitigate *Egr-1*-dependent transcription. To test this hypothesis, the negative regulatory domain of *Egr-1* was used in a yeast two-hybrid screen to identify proteins that could bind the domain and modulate *Egr-1* function. One clone was shown to interact specifically with the negative regulatory domain of *Egr-1* and, in reporter expression studies, to inhibit *Egr-1*-dependent transcription. Moreover, the encoded protein resided in the nucleus, suggesting strongly that it was a corepressor. This gene was named NAB1 for NGFI-A-Binding protein.¹⁵ A second, related gene called NAB2¹⁶ also bound and inhibited the activity of *Egr-1* (as well as

Egr-2). In contrast to NAB1, however, NAB2 was induced by the same stimuli that resulted in *Egr-1* expression.¹⁶ Moreover, NAB2 expression was slightly delayed compared to *Egr-1* and was more tissue-restricted, suggesting an important function in inhibiting tissue-specific *Egr-1* activities. In this context, a recent study showed that overexpression of NAB2 blocked nerve growth factor-induced DRG such as TGF- β 1 and the subsequent differentiation of PC12 cells.¹⁷

Although many studies have defined mechanisms for the activation of genes in the vessel wall, very few reports have addressed the equally important task of defining mechanisms that repress gene transcription. In particular, a widely held (but not well supported) hypothesis is that repressors of gene transcription are important for maintaining vascular cells in a quiescent, physiologically normal state. The prototypical example of a corepressor is the unphosphorylated form of retinoblastoma-1 (Rb-1), which prevents cell cycle progression by interacting with numerous proteins, including the E2F family of DNA-binding transcription factors.¹⁸ This activity of Rb-1 was recently exploited to limit the development of neointimal thickening after experimental balloon injury to the vascular wall.¹⁹ E-selectin, which is generally considered to be an endothelial cell-restricted gene, can be induced in vascular SMC by pretreatment with cycloheximide followed by TNF- α , suggesting that a labile repressor protein exists in SMC to silence the E-selectin gene.²⁰ More recently, GC factor 2 was shown to compete with Sp1 and *Egr-1* for binding to a GC-rich region in the PDGF-A chain promoter.²¹ GC factor 2 inhibited the expression of PDGF-A chain and suppressed SMC proliferation *in vitro*. Moreover, GC factor 2 was induced in SMC after balloon injury and its expression kinetics were sustained, whereas PDGF-A chain levels returned to baseline. The function of GC factor 2 may therefore be to apply a molecular brake to the activation of growth-related genes during the vessel wall's response to injury.

In this issue of the *Journal*, Silverman et al report on their performance of an expression analysis of the corepressors NAB1 and NAB2 in vascular SMC both *in vitro* and *in vivo* after balloon injury of the rat aorta.¹ Using Northern analysis, the authors first show the sequential activation of *egr-1* and NAB2 (but not NAB1) mRNA in bovine aortic smooth muscle cells after phorbol myristate acetate stimulation. Using highly purified nuclear extracts, the expression of each protein was then verified by Western blotting. The Western study also revealed the nuclear location of the *Egr-1* and NAB2 proteins, indicating that their translation was followed by nuclear translocation. A significant finding was functional repression of *Egr-1*-dependent gene expression in cultured rat aortic SMC. Here, the authors used a synthetic promoter construct containing three consensus *Egr-1* sites and a minimal *c-fos* promoter driving the chloramphenicol acetyltransferase reporter gene (EBS3fos-CAT). This reporter gene was highly responsive to PMA and basic fibroblast growth factor stimulation. However, a 3.4-fold decrease in CAT activity was observed when similarly stimulated cells were cotransfected with a CMV-driven NAB2 expression plasmid. Though candidate *Egr-1* target genes

were not examined in their report, the authors' findings support the hypothesis that NAB2 functions to repress Egr-1-mediated gene transcription in cultured SMC.

To evaluate the expression of the NAB corepressors *in vivo*, the authors performed partial balloon injury of the rat aorta and then measured the steady-state expression of NAB1 and NAB2 mRNA by *en face in situ* hybridization.²² These elegant studies revealed dramatic induction of NAB2 mRNA in luminal SMC 8 days postinjury with levels remaining elevated up to 2 weeks postinjury. By 6 weeks postinjury, a time when the neointima has a low proliferative index, the expression of NAB2 returned to baseline. In contrast to the induction of NAB2 after balloon injury, little change in the steady-state expression of NAB1 was observed.

The paper by Silverman et al offers an attractive hypothesis for a transcriptional circuit in the vessel wall wherein injury-induced activation of Egr-1 mediates a secondary wave of transcription whose temporal expression is tempered by the early activation of a corepressor of Egr-1, NAB2. This hypothetical model suggests that NAB2 functions on Egr-1 target genes in a negative feedback loop. The authors further theorize that the constitutive expression of NAB1 may assist in maintaining a homeostatic genetic program of quiescence within the vessel wall. In the face of injurious insult, the coactivation of NAB2 would provide a necessary and perhaps more broadly acting brake on vascular cell gene expression. In this regard, it will be interesting to ascertain the full spectrum of NAB2 target genes in the vessel wall. For example, the *egr-1* promoter has an Egr-1-binding element.⁷ Thus, it is possible that NAB2 feeds back on the *egr-1* promoter to limit its continued expression. Another interesting candidate target gene for NAB2 is the SRF DNA-binding transcription factor.¹⁰ The 5' flanking region of SRF contains overlapping sites for Sp1 and Egr-1.¹² Thus, the transient nature of SRF-dependent IEG activation may also be due to limited expression of SRF through the repressive action of NAB2 on Egr-1. Such a mechanism could also help explain the transient down-regulation of several SRF-dependent SMC differentiation genes in the vessel wall after injury.²³ Although a recent report showed that mutating the Egr-1 binding site in the SRF promoter had little effect on promoter activity,²⁴ Egr-1 is known to function in a cell context-dependent manner.⁷ Thus, it is formally possible that in SMC, SRF requires Egr-1 for full activity, and the presence of NAB2 may assist in attenuating SRF transcription through NAB2's repressive action on Egr-1. The full spectrum of NAB2 (and Egr-1) target genes in the vessel wall could be pursued through the use of expression profiling in null and gain-of-function mutant cell lines and tissues. Other interesting aspects of NAB2 to study include the cloning and characterization of its promoter (is NAB2 an IEG?), as well as vascular cell expression of an alternatively spliced isoform of NAB2, which is defective for nuclear translocation.¹⁶

In summary, activation of vascular cells *in vivo*, whether by physical or chemical injury, often results in an altered genetic program that may precipitate vascular pathology. The activation of Egr-1 and other immediate early tran-

scription factors in the vessel wall likely mediates the phenotypic characteristics of an activated SMC, including cell cycle entry and traverse, cellular migration, and matrix deposition. The present study brings yet another level of regulation that may be essential for the vessel's response to injury. The panoply of autocrine and paracrine mechanisms by which the vessel responds to injury indicates the importance of these responses during evolution. Such sophisticated responses require multiple levels of regulation; NAB2, as a negative regulator of Egr-1-dependent transcription, provides another important mechanism by which the growth response of the vessel wall may be tempered. The therapeutic potential of corepressors is obvious and future experiments will soon reveal the practical value of targeting this class of proteins.

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