GC Factor 2 Represses Platelet-Derived Growth Factor A-Chain Gene Transcription and Is Itself Induced by Arterial Injury

Levon M. Khachigian, Fernando S. Santiago, Louise A. Rafty, Onza L.-W. Chan, Gabrielle J. Delbridge, Alex Bobik, Tucker Collins, Alfred C. Johnson

Abstract—Platelet-derived growth factor (PDGF) is a mitogen and chemoattractant for a wide variety of cell types. The genes encoding PDGF A chain (PDGF-A) and PDGF B chain (PDGF-B) reside on separate chromosomes and are independently regulated at the level of transcription. Regulatory events underlying inducible PDGF-A expression have been the focus of much investigation. However, mechanisms that inhibit transcription of this gene are not well understood. In this study, we report the capacity of a newly cloned DNA binding factor, GC factor 2 (GCF2), to repress expression driven by the human PDGF-A promoter. 5' Deletion and transient cotransfection analysis in vascular endothelial cells revealed that GCF2 repression is mediated by a nucleotide region located in the proximal region of the PDGF-A promoter. Electrophoretic mobility shift assays demonstrate that GCF2 binds to this region in a specific and dose-dependent manner. Interestingly, the site bound by GCF2 overlaps those for specificity protein-1 (Sp1) and early growth response factor-1 (Egr-1), zinc finger transcription factors that direct basal and inducible expression of the PDGF-A gene. Gel shift experiments revealed that GCF2 competes with these factors for interaction with the PDGF-A promoter. Overexpression of GCF2 suppressed endogenous PDGF-A expression in vascular endothelial cells and smooth muscle cells. GCF2 was induced on mechanical injury of cells in culture as well as after balloon injury of the rat carotid artery wall. Time course studies revealed the sustained induction of GCF2 after injury while PDGF-A levels sharply returned to baseline. Smooth muscle cell proliferation was inhibited by GCF2, an effect reversed by the addition of exogenous PDGF-AA. These findings demonstrate negative regulation of PDGF-A expression by GCF2. This is the first report of the induction of an endogenous transcriptional repressor in the rat vessel wall. (Circ Res. 1999;84:1258-1267.)

Key Words: GC factor 2 ▪ platelet-derived growth factor-A ▪ DNA binding protein ▪ transcription ▪ injury
transcriptional activators themselves. Activity of the PDGF-B promoter can be repressed by the zinc finger protein ZNF174, cloned originally from a human fetal library, likely by passive means. Second, active repression, involves the intrinsic inhibitory activity of the repressor. WT-1, an active repressor, can inhibit PDGF-A promoter activity as well as expression driven from the promoters of a large number of other genes. These include insulin-like growth factor type II, transforming growth factor-β1, colony-stimulating factor-1, and insulin-like growth factor-1 receptor. Despite the capacity of WT-1 to repress reporter expression driven by multiple growth factor promoters, the relevance of WT-1 repression to the regulation of the authentic gene has not been established. Additionally, the limited pattern of WT-1 expression and the transient nature of growth factor induction suggests that other repressors may exist with the ability to negatively regulate growth factor gene expression.

GCF is a transcriptional repressor so termed because of its preference for G+C-rich elements in DNA. GCF is a 91-kDa protein that interacts and downregulates expression driven by the epidermal growth factor receptor (EGFR) promoter, which lacks TATA and CAAT motifs. A second member of the GCF family, GCF2, was recently isolated from an ovarian carcinoma cDNA library. GCF2 shares homology with GCF in a 309-bp region located at positions 1382-1690 within the proximal region of the promoter. Cells were cotransfected with 2 µg of pTKGH to correct for transfection efficiency. CAT reporter activity was normalized to levels of growth hormone (GH) secreted into the conditioned medium. Error bars represent SEM from at least 2 independent determinations.

Figure 1. GCF2 represses the activity of the PDGF-A promoter. Endothelial cells were transfected with 15 µg of PDGF-A promoter CAT construct ΔSac and 5 µg of pcDNA3, GCF2-pcDNA3, or Egr-1-pcDNA3 as indicated. The cells were cotransfected with 2 µg of pTKGH to correct for transfection efficiency. CAT reporter activity was normalized to levels of growth hormone (GH) secreted into the conditioned medium. Error bars represent SEM from at least 2 independent determinations.

dependent manner and competes with transcription factors directing expression of the gene for overlapping binding sites. GCF2 represses PDGF-A promoter-dependent expression and can inhibit the expression of the endogenous gene itself. In addition, we provide evidence for an inverse relationship between the repressor and the growth factor in the artery wall after mechanical injury.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells were purchased from Cell Applications, Inc, and grown in DMEM (pH 7.4) containing 10% FBS, 50 µg/mL streptomycin, and 50 IU/mL penicillin at 37°C in a humidified atmosphere of 5% CO₂. Rat aortic smooth muscle cells were obtained from Cell Applications, Inc, and grown in Waymouth’s medium (pH 7.4) containing 10% FBS and antibiotics. Cells were routinely passaged with trypsin/EDTA and not used in experiments beyond passage 6.

Transient Transfection Analysis and Chloramphenicol Acetyltransferase (CAT) Assay

Endothelial cells in 100-mm-diameter dishes at 70% confluence were transfected with the indicated amounts of cesium chloride-purified plasmid DNA using a modification of a calcium phosphate precipitation method. After transfection, the cells were incubated overnight in an atmosphere of 3% CO₂, washed twice with PBS (pH 7.4) and incubated for an additional 24 hours at 5% CO₂. Cell lysates were assessed for CAT activity as previously described. Conditioned medium was sampled at the time the cells were harvested and assayed for human growth hormone (Biocline) to correct for transfection efficiency. Total protein concentration in the lysates was assessed using a modified Bradford assay (Bio-Rad) and used to normalize transfection data.

Electrophoretic Mobility Shift Assay (EMSA)

Binding reactions for gel shift assays were performed in 20 µL of 10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 mM PMSF, 0.75 µg salmon

Figure 2. GCF2 repression of the PDGF-A promoter is mediated by a G+C-rich sequence in the proximal region of the promoter. Cells were transfected with 15 µg of PDGF-A promoter CAT construct f28 or f36 and either 5 µg of pcDNA3 or GCF2-pcDNA3. The cells were cotransfected with 2 µg of pTKGH, normalized to levels of growth hormone in the supernatant. Error bars represent SEM from at least 2 independent determinations.
sperm DNA, 5% sucrose, 0.75 mg poly dI-dC, 10 mg of BSA, 32P-labeled oligonucleotide probe (150,000 cpm), and 1.5, 3, or 6 mg of in vitro transcribed/translated GCF2 protein. The reaction was incubated for 35 minutes at 22°C. In competition gel shifts, 6 mg of nuclear extract was prepared as described and combined with 6 mg of GCF2 protein. As a control, an equal amount of BSA protein was substituted for GCF2. Bound complexes were separated from free probe by loading the samples onto a 5% nondenaturing polyacrylamide gel (PAGE) and electrophoresing at 200 V for 2 to 3 hours. The gels were dried and subjected to autoradiography overnight at −80°C.

Figure 3. GCF2 interacts with the proximal PDGF-A promoter. A, Specific and dose-dependent interaction of GCF2. EMSA was performed with in vitro transcribed/translated GCF2 protein and the 32P-labeled oligonucleotides indicated in the figure as described in Materials and Methods. B, DNA binding domain of GCF2 is critical for interaction with proximal PDGF-A promoter. In vitro transcribed/translated GCF2Δ510 lacks the complete 309-bp DNA binding domain of GCF2 (1382-1690). Arrows indicate monomeric and dimeric forms of bound GCF2. The nucleotide sequence of Oligo A is 5′-GGG GGG GGC GGG GGG GGC GGC GGC GGA GG-3′ and LKmdc is 5′-GCT GTC TCC ACC CAT TGT TCG CAC TCT-3′; Oligo Am is 5′-GGG GGC GGC GGC GGC GGG GGG GCg GGG GGA GG-3′ (sense strand shown; bases indicated in lowercase denote insertions).

Northern Blot Analysis
Endothelial cells or smooth muscle cells were grown to 70% confluency in 100-mm-diameter Petri dishes and transfected with 10 μg pcDNA3 or GCF2-pcDNA3 using FuGENE6 reagent (Boehringer Mannheim) at a FuGENE6:DNA ratio of 3:1 (vol:μg), per the manufacturer’s instructions. After 24 hours, total RNA was extracted with TRIzol reagent (Life Technologies), and 20 μg of each sample was resolved by electrophoresis on 1% formaldehyde/agarose/MOPS gels. RNA was transferred overnight onto Hybond nylon membranes before hybridization with a 32P-labeled 260-bp fragment of PDGF-A cDNA in ExpressHyb (Clontech). Vacuum-dried gels were autoradiographed for 1 week at −80°C, then stripped and reprobed with a 32P-labeled 1.1-kb fragment of GCF2 cDNA, and later with a 32P-labeled fragment of β-actin cDNA followed by reexposure.

Injury and Western Blot Analysis
Endothelial cells or smooth muscle cells were rendered growth-quiescent by incubation overnight in medium containing 1% or 0.1% FBS, respectively. The cells were injured by repeated scraping with a sterile stainless steel comb and solubilized in RIPA buffer. Extracts were resolved by 10% SDS-PAGE and transferred to nylon membranes by electrophoretic blotting. Nonspecific binding sites on the membrane were blocked with nonfat skim milk before probing with affinity-purified rabbit polyclonal antibodies to PDGF-A (Genzyme) or GCF2. This was followed by chemiluminescent detection using HRP-linked swine anti-rabbit secondary antiserum (NEN-DuPont).

Assays of Cell Proliferation
Cells were seeded into 96-well titer plates (Nunc-InterMed) at 2500 cells per well and allowed to grow for 48 hours in complete medium. The cells were transfected with 1 μg of the indicated plasmid in complete growth medium using FuGENE6 (Boehringer Mannheim) in accordance with manufacturer’s instructions. At the time of transfection, and again after 24 hours, the cells were rinsed with PBS (pH 7.4) and trypsinized, and the suspension was quantitated using an automated particle counter (Coulter Electronics).

Rat Carotid Artery Injury and Immunohistochemical Analysis
Sprague-Dawley rats (400 g) were obtained from the Biology Research Unit, Baker Medical Research Institute, Melbourne, Aus-
tralia. Left common carotid arteries of anesthetized rats (ketamine 80
mg/kg; xylene 10 mg/kg) were subjected to balloon catheter injury
using a 2F Fogarty catheter as previously described. Briefly, the
saline-inflated balloon catheter was passed along the length of the
common carotid four times. Various times after injury (0, 4 hours, 6
hours, 24 hours, and 10 days), rats were anesthetized with sodium
pentobarbitone (100 mg/kg). The vessels were perfused with saline
and gently dissected free of surrounding tissue, frozen in OCT
compound (Tissue-Tek), and sectioned (5 µm). Sections were fixed
in 4% formalin for 2 minutes, then absolute ethanol for 10 minutes.
For immunohistochemical analysis, sections were incubated with
affinity-purified rabbit polyclonal IgG raised against PDGF-AA
(1:100) (Genzyme) or in vitro transcribed/translated GCF2 (1:200).17
Secondary detection was performed using the avidin-biotin complex
(ABC) kit.22 Deendothelialization was confirmed by vital staining
with Evans blue (60 mg/kg IV). All surgical procedures were
approved by the Baker Medical Research Institute and Alfred
Hospital Animal Experimentation Committee.

Results

GCF2 Represses PDGF-A Promoter-Dependent Expression

To determine whether GCF2 can influence expression driven
by the PDGF-A promoter, we transfected endothelial cells with
cells bearing a GCF2 expression vector driven by the cytomegaloviral
(CMV) promoter (construct GCF2-pcDNA3), together with a
CAT reporter construct, pGL3, bearing 643 bp of PDGF-A
promoter sequence. CAT activity was normalized for transfection
efficiency after assessment of human growth hormone
levels secreted into the culture medium. Overexpression of
GCF2 in this cotransfection setting strongly inhibited reporter
expression driven by the PDGF-A promoter (Figure 1). GCF2
repression of PDGF-A promoter-driven CAT activity was also observed in rat
vascular smooth muscle cells (data not shown). In contrast, overexpression of a
transcriptional activator of PDGF-A, Egr-1, with the same backbone (pcDNA3)
stimulated CAT activity 2-fold (Figure 1).

Repression by GCF2 Is Mediated by Nucleotide Elements Located in the Proximal Region of the PDGF-A Promoter

GCF2 binds preferentially to nucleotide sequences rich in
guanines and cytosines.17 The proximal region of the
PDGF-A promoter is intensely G+C-rich.1 We hypothesized
that GCF2 repression of the PDGF-A promoter may involve elements located in the proximal region. Transient cotransfection experiments revealed that expression driven by con-
struct f28, which contains 71 bp of PDGF-A promoter sequence, was inhibited by GCF2 coexpression (Figure 2). However, when GCF2-pcDNA3 was cotransfected with con-
struct f36, which bears 55 bp of promoter sequence, repression by GCF2 was no longer observed (Figure 2). These findings suggest that GCF2 repression of the PDGF-A prom-
er is mediated by nucleotide elements located between the 5’
promoter end points of f28 and f36. The GCF2 binding site in the epidermal growth factor receptor promoter has a core
binding sequence of 5'-AGC CCC CCG CG-3’ (or 5’-CG
CCG GGG GCC GCT-3’).17 The nucleotide sequence between the
f28 and f36 5’ end points bears the 5’-CGG GGG C-3’ motif
(Figure 2). Separate experiments revealed that a CAT reporter
construct driven by 153 bp of the PDGF-B promoter,23 which
does not bear the GCF2 core binding sequence, was unaf-

fected by cotransfection with GCF2-pcDNA3 (data not shown).

GCF2 Interacts With Proximal PDGF-A Promoter

To determine whether GCF2 binds directly to the PDGF-A
promoter, we incubated in vitro transcribed/translated GCF2
with a 32P-labeled probe, Oligo A, whose sequence spans the 5’
end points of f28 and f36, and resolved the adducts by
electrophoresis under nondenaturing conditions. GCF2 bound to the oligonucleotide in a dose-dependent manner as two
distinct nucleoprotein complexes (Figure 3A). The upper and more intense complex represents dimeric GCF2, whereas the lower complex is the monomeric form.17 Neither complex was observed when an oligonucleotide bearing an unrelated
sequence, 5’-P-LKmdc, was substituted for 32P-Oligo A (Figure
3A). Furthermore, neither complex formed when 32P-
Oligo Am was substituted for 32P-Oligo A (Figure 3A). Oligo
Am was generated by altering the 5’-CGG GGG C-3’
repeated motif in Oligo A by insertion of additional guanines
(5’-CGG GGG gcc-3’) thus disrupting the promoter sequence.

Figure 5. Mechanical injury induces endothelial expression of
GCF2 and PDGF-A. Vascular endothelial cells were grown to
confluence and rendered quiescent by incubation in 1% FBS for
24 hours. Monolayers were injured by repeated scraping with a
sterile stainless steel comb. Cells were then allowed to recover
for 24 hours, and samples were collected every 4 hours for
5 days. Gene expression was determined using quantitative
reverse transcription PCR. Results are presented as the
mean ± SEM of triplicate determinations. A, level of GCF2 gene
expression; B, level of PDGF-A gene expression; C, level of
PDGF-B gene expression.

Downloaded from http://circres.ahajournals.org/ by guest on September 22, 2017
When wild-type $^{32}\text{P}-\text{Oligo A}$ was incubated with GCF2Δ510, an in vitro transcribed/translated form of GCF2 that carries a deletion in its DNA binding domain, nucleoprotein complex formation was no longer observed (Figure 3B). Additional experiments revealed that wild-type GCF2 failed to interact with a $^{32}\text{P}$-labeled G+C-rich RNA oligomer composed of 23 bases (data not shown). Taken together, these findings demonstrate that GCF2 interacts with the proximal PDGF-A promoter in a dose-dependent and specific manner.

### Competition Between Several Zinc Finger Binding Proteins and GCF2 for Binding Sites in the Proximal PDGF-A Promoter

The preceding findings demonstrate the capacity of GCF2 to bind to the PDGF-A promoter and inhibit promoter-dependent reporter gene expression. We next addressed the question of the mechanism underlying GCF2 repression. Previous studies by our group have shown that a number of zinc finger transcription factors interact with the GCF2 binding site and mediate basal and inducible expression of the PDGF-A gene. These nuclear factors include Sp1 and Egr-1. We explored the possibility that GCF2 competes with these factors for binding to the PDGF-A promoter. Endothelial extracts were incubated with $^{32}\text{P}-\text{Oligo A}$ in the absence or presence of GCF2 or a corresponding amount of BSA, before resolution of $^{32}\text{P}$-labeled nucleoprotein complexes by electrophoresis. This produced the characteristic profile of $^{32}\text{P}$-labeled nucleoproteins composed of Sp1, Egr-1, Sp3, and other nuclear proteins (Figure 4). Inclusion of GCF2 in the binding mixture markedly attenuated the intensity of the Sp1, Egr-1, and Sp3 complexes, among other complexes (Figure 4). In contrast, these complexes were unaffected by substitution of GCF2 with an identical amount of BSA (Figure 4). Thus, GCF2 competes with these positive regulatory transcription factors for overlapping binding sites in the PDGF-A promoter.

### Mechanical Injury Stimulates PDGF-A and GCF2 Expression In Vitro

To determine the potential pathophysiological relevance of the above findings to vascular disorders, we examined the relationship between GCF2 and PDGF-A expression in an in vitro model of cell injury. Endothelial cell monolayers were scraped repeatedly with a sterile stainless steel comb, and lysates were assessed for levels of PDGF-A. PDGF-A was weakly expressed in unmanipulated endothelial cells (Figure 5A). However, within 4 hours of injury, PDGF-A levels increased 3-fold (Figure 5A, open bars), consistent with a previous report of inducible PDGF-A expression in aortic endothelium within 4 hours of mechanical injury. By 8 hours, PDGF-A levels increased almost 10-fold and returned to preinjury levels by 12 hours (Figure 5A).

We next determined whether GCF2 was induced by injury in this model. GCF2 is basally expressed in resting cells, and...
levels increased 4 hours and 8 hours after injury (Figure 5B). By 12 hours, however, GCF2 levels increased 3-fold (Figure 5B, closed bars), at which time PDGF-A levels had returned to background (Figure 5A). The inverse temporal correlation between GCF2 and PDGF-A after peak expression of the growth factor in this model suggests a regulatory role for GCF2 in the expression of PDGF-A after injury.

Mechanical Injury of Arteries In Vivo Stimulates GCF2 Expression

To provide evidence for a similar relationship between these genes in the artery wall, levels of PDGF-A and GCF2 were assessed in the arterial media by immunohistochemical analysis various times after balloon catheter injury of the rat carotid. PDGF-A antigen was weakly, if at all, expressed in the unmanipulated artery wall (Figure 6A). Within 4 hours of injury, PDGF-A was clearly expressed in the media (Figure 6B, arrow) as previously reported. Expression was only transient, because PDGF-A immunoreactivity was not detected at 6 hours (Figure 6C) or after 10 days (Figure 6D), when a significant neointima had formed, a hallmark of this model.

In contrast to PDGF-A, GCF2 was basally expressed in normal artery wall (Figure 7A). Injury dramatically increased GCF2 expression in the media within 4 hours (Figure 7B). Unlike PDGF-A, GCF2 levels remained elevated over the next 18 hours (Figures 7B through 7D). Indeed, intense GCF2 expression even 24 hours after injury (Figure 7D) contrasts dramatically with the rapid decline in PDGF-A levels between 4 and 6 hours (Figures 6B through 6C). Ten days after injury, GCF2 was still expressed at levels comparable to those in the uninjured vessel wall (compare Figure 7E to 7A). Staining for both GCF2 and PDGF-A was specific, because neither the secondary antibody nor components of the immunohistochemical detection system produced positive staining in the absence of primary antibody. These findings provide a spatial and inverse temporal correlation between GCF2 and PDGF-A after peak PDGF-A expression after injury to the vessel wall.

We performed Western blot analysis for GCF2 protein with lysates of cultured smooth muscle cells prepared various times after mechanical injury to verify findings from immunostaining of the injured artery wall (Figure 7). GCF2 protein was inducibly expressed within 1 hour of injury; levels continued to increase at 4 hours and 6 hours (Figure 8). These data are consistent with the temporal pattern of inducible GCF2 protein expression in medial smooth muscle cells of the injured vessel wall (Figure 7).

Overexpression of GCF2 Attenuates Endogenous PDGF-A Expression

The temporal relationship between GCF2 and PDGF-A in these models suggests that GCF2 may downregulate the expression of endogenous PDGF-A. Because our earlier

Figure 7. Sustained activation of GCF2 by injury. Immunohistochemical analysis was performed on 5-μm cross sections of balloon-injured rat carotid arteries using affinity-purified rabbit polyclonal antibodies raised against in vitro transcribed/translated GCF2. A, Uninjured artery. B, Four hours after injury. C, Six hours after injury. D, Twenty-four hours after injury. E, Ten days after injury. F, Uninjured artery without GCF2 (primary) antibody but with secondary (goat anti rabbit IgG) antibody and components of ABC kit. Brown staining indicates specific signal. The blue stain is a consequence of Evans blue infusion before sacrifice. Sections were counterstained with hematoxylin to identify cell nuclei. Magnification ×200.
studies used PDGF-A promoter reporter constructs in an overexpression setting, we performed Northern blot analysis using total RNA of two different vascular cell types 24 hours after transfection with GCF2-pcDNA3 or the backbone alone. Because this approach requires high transfection efficiency, we used a lipid-based formulation that enabled transfection of virtually the entire cell population (data not shown). PDGF-A mRNA, as expected, was expressed at higher levels in smooth muscle cells than endothelial cells28 (Figure 9A). In both cell types, however, endogenous PDGF-A expression was completely inhibited by GCF2-pcDNA3 transfection (Figure 9A). To confirm the expression of GCF2 on transfection of this plasmid, the blot was stripped and reprobed with a32 P-labeled GCF2 cDNA fragment. Strong hybridization was observed in both cell types with an mRNA species of \( \approx 2.3 \text{ kb} \) \(^{17}\) (Figure 9A), consistent with the size of GCF2 cDNA insert present in the vector. In contrast, \( \beta \)-actin mRNA levels were unaffected by GCF2 overexpression (Figure 9A). These findings demonstrate the capacity of GCF2 to suppress native PDGF-A expression in two vascular cell types. To demonstrate expression of GCF2 protein, as well as mRNA, after transfection with GCF2-pcDNA3, we prepared nuclear extracts of cells transfected with this construct as well as the backbone alone. EMSA, using these extracts together with32 P-labeled Oligo A, indicates that in vivo expressed GCF2 protein can interact with its binding site in the proximal PDGF-A promoter (Figure 9B).

**Overexpression of GCF2 Inhibits Smooth Muscle Cell Replication**

Finally, we determined whether exogenous GCF2 had the ability to influence cell proliferation. Endothelial cells or smooth muscle cells transfected with either GCF2-pcDNA3
or pcDNA3 were resuspended by trypsinization and quantitated. Endothelial cell replication after 24 hours was unaffected by GCF2 transfection (Figure 10A). However, proliferation was inhibited in smooth muscle cells transfected with GCF2 (Figure 10B). Cell growth in this group 24 hours after transfection was 37% less than the population of cells transfected with pcDNA3 alone (Figure 10B). In contrast, proliferation of smooth muscle cells transfected with construct GCF2A428-pcDNA3, which lacks the GCF2 DNA binding domain, was not inhibited (Figure 10B). Interestingly, addition of PDGF-AA (30 ng/mL) rescued the cells from growth inhibition by GCF2 (Figure 10B). Morphological inspection of both cell types as well as trypan blue exclusion experiments revealed that differences in cell number were not due to cytotoxicity (data not shown). Therefore, although GCF2 suppressed endogenous PDGF-A expression in endothelial cells and smooth muscle cells, proliferation of only the latter cell type was attenuated as a consequence.

**Discussion**

The specificity and efficiency with which eukaryotic genes are transcribed depend on specific, ordered interactions between transcription factors and cis-acting nucleotide elements in promoter regions. Models of inducible gene transcription involve the formation of 3-dimensional stereospecific enhancer complex composed of transcription factors that can bend DNA and components of the basal transcriptional machinery. Transactivation is also associated with alterations in chromatin structure. Repression is less well understood but is, nonetheless, an equally important aspect of transcriptional regulation. Here, we have described the ability of a newly cloned DNA binding factor, GCF2, to repress expression driven by the human PDGF-A promoter. Briefly, deletion and transient transfection analysis revealed that GCF2 repression is mediated at a region located between the 5’ end points of PDGF-A promoter constructs f28 and f36. Gel shift experiments demonstrated that GCF2 interacts with this region of the promoter in a specific, dose-dependent manner and competes with activating transcription factors for overlapping binding sites. GCF2 is activated by mechanical injury in culture as well as in the artery wall. GCF2 can suppress endogenous PDGF-A expression in both endothelial cells and smooth muscle cells but attenuates proliferation in only the latter cell type.

The precise mechanism(s) with which GCF2 regulates PDGF-A transcription, or the transcription of other genes, is not clear. Active repressors, such as WT-1, and the human Kruppel-related factor, YY1, possess intrinsic repressing activity and the capacity to downregulate transcription through modular domains. On the other hand, passive repressors downregulate transcription by modulating the activity of positive regulatory factors. An example of a passive repressor is GCF, which may compete with transcription factors for common binding sites or interact directly with these proteins. In addition, NAB1 and NAB2 repress Egr-1-dependent transactivation by interacting with this zinc finger protein. GCF2 may also function as a passive transcriptional repressor, because it competes with Sp1 and Egr-1 for common binding sites in the PDGF-A promoter, and these transcription factors each activate PDGF-A transcription. Alternatively, or in addition, GCF2 may interact with DNA to destabilize bound factors or interact directly with factors to prevent binding or increase off rates. GCF2, like some repressors, may have the capacity to activate transcription under certain conditions. For example, whether WT-1 functions as a transcriptional repressor or activator seems to be dependent on physical interactions with p53. Although the present findings define a transcriptional regulatory role for GCF2, we cannot yet rule out the possibility that GCF2 controls gene expression by additional mechanisms, such as by regulation of mRNA stability. Detailed structural characterization of GCF2 should provide more detailed insights into the functional properties of this transcriptional repressor.

Many genes, whose products can influence chemotactic, mitogenic, adhesive, and thrombotic events are activated in response to mechanical injury (reviewed in References 38 through 42). Several of these genes are under the transcriptional control of factors, such as c-Fos, c-Jun, c-Myc, c-Ets-1, nuclear factor-κB, and Egr-1, which are themselves activated by injury to the vessel wall. In contrast to positive regulatory factors, the present study is the first to report the inducible expression of an endogenous transcriptional repressor after mechanical injury of vascular cells in the rat model. Previously, Weir et al demonstrated downregulation of the growth arrest homeobox gene, gax, after balloon injury to the rat carotid. Recently, Aoyagi et al demonstrated p53 induction in the rabbit carotid artery wall after balloon denudation. Because GCF2 inhibits both PDGF-A promoter-dependent and endogenous PDGF-A mRNA expression, the repressor could influence the course of PDGF-A expression after injury. The capacity of GCF2 to inhibit smooth muscle cell replication without influencing endothelial cell growth is consistent with the notion of autocrine growth loop(s) involving PDGF in the former cell type. Smooth muscle cell responsiveness can be inhibited by substances such as suramin, antisense oligonucleotides directed at PDGF-A mRNA, and α1-macroglubulin, which block the PDGF ligand–PDGF receptor circuit. GCF2 may interfere with autocrine growth involving PDGF-A at the earliest stage in the cycle by inhibiting the production of the growth factor. That GCF2 inhibition of smooth muscle cell proliferation could be rescued by addition of exogenous PDGF-AA is consistent with an autocrine role for the PDGF A chain. Unlike smooth muscle cells, large vessel endothelial cells in culture do not express PDGF receptors. It is therefore not surprising that GCF2 could not inhibit the growth of cells lacking receptors for PDGF-A.

The promoters of many growth factor genes induced by injury, such as transforming growth factor-β1, fibroblast growth factor-2, and PDGF-B, also contain G+C-rich elements that are bound by Sp1 and Egr-1 and collagen expression. COL1A1 and collagen accumulation in the neointima occurs when GCF2 levels have already returned to background (Figure 8), it is conceiv-
able that GCF2 may also play a regulatory role in the composition of the matrix in the neointima.

The mechanisms underlying the induction of GCF2 itself, in the context of injury or any other stimulus, are not known. Elucidation of the genomic structure of GCF2 should provide valuable insights for the regulation of this gene. Future studies should determine whether positive regulatory factors such as Sp1 and Egr-1 can influence the inducible expression of this repressor and whether GCF2 can repress its own expression.

Acknowledgments
This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia, National Heart Foundation of Australia, and NSW Health Department. L.M.K. is a recipient of an R. Douglas Wright Fellowship from the NHMRC. This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia.

References
GC Factor 2 Represses Platelet-Derived Growth Factor A-Chain Gene Transcription and Is Itself Induced by Arterial Injury
Levon M. Khachigian, Fernando S. Santiago, Louise A. Rafty, Onza L.-W. Chan, Gabrielle J. Delbridge, Alex Bobik, Tucker Collins and Alfred C. Johnson

Circ Res. 1999;84:1258-1267
doi: 10.1161/01.RES.84.11.1258

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/84/11/1258

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/