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Suppression of Growth Factor Expression and Human Vascular Smooth Muscle Cell Growth by Small Interfering RNA Targeting EGR-1

Roger G. Fahmy[1,2] and Levon M. Khachigian[1,2]*

[1]Department of Pathology, Centre for Vascular Research, The University of New South Wales, Sydney, Australia
[2]Department of Haematology, The Prince of Wales Hospital, Sydney, Australia

Abstract Smooth muscle cell (SMC) proliferation and migration are key processes that occur in the reparative response to injury after percutaneous coronary intervention and in failed bypass grafts for the treatment of atherosclerosis. In the present study, we generated novel synthetic small interfering RNA (siRNA) molecules targeting the coding region of human early growth response-1 (EGR-1) mRNA that attenuate the expression of EGR-1 and that of fibroblast growth factor-2 (FGF-2) and granulocyte-colony stimulating factor (G-CSF). These agents suppressed SMC proliferation in a dose-dependent and non-toxic manner and blocked SMC regrowth from the wound edge following mechanical injury in vitro. In contrast, the scrambled counterpart did not inhibit SMC proliferation, EGR-1 protein expression or SMC regrowth after injury. These findings demonstrate that EGR-1 siRNA can serve as inhibitors of SMC proliferation and wound repair suggesting that these agents may potentially be useful in the control of vascular proliferative disorders. J. Cell. Biochem. 100: 1526–1535, 2007. © 2006 Wiley-Liss, Inc.

Key words: siRNA; EGR-1; smooth muscle cells; vascular biology

It is well established that insult to the vessel wall results in smooth muscle cell (SMC) activation and proliferation, a key alteration in phenotype that pre-empts the formation of restenotic lesions following percutaneous coronary intervention for the treatment of atherosclerosis [Popma et al., 1991; Libby et al., 1992]. As a result, the proliferation of SMCs in the endoluminal space occludes the vessel restricting blood flow to the heart.

Early growth response-1 (Egr-1/EGR-1) is an important transcriptional regulator that mediates gene expression following vascular injury, wherein the overexpression of EGR-1 in human endothelial cells results in the regulated expression of more than 300 genes [Fu et al., 2003]. The promoter region of genes involved in the activation and proliferation of SMCs following injury also contain recognition elements for the zinc finger transcription factor, EGR-1 [Gashler and Sukhatme, 1995; Khachigian and Collins, 1997]. The expression of EGR-1, a 60–80 kDa immediate early gene product, is elevated more than fivefold in human carotid lesions relative to adjacent normal tunica media [Du et al., 2000]. It is also activated by balloon injury to the artery wall before the expression of EGR-1-dependent genes in the lesion [Kim et al., 1995; Khachigian et al., 1996a; Santiago et al., 1999b]. Mice deficient in both EGR-1 and apolipoprotein E (ApoE) have less atherosclerotic lesion areas compared with mice deficient in ApoE alone [Harja et al., 2004]. Moreover, EGR-1 is up-regulated by growth factors, cytokines, hormones, and environmental stimuli [Khachigian et al., 1996a; Khachigian and Collins, 1998]. Strategies that target EGR-1 inhibit neointimal thickening in injured rat [Khachigian et al.,

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*Correspondence to: Levon M. Khachigian, PhD, DSc, Department of Pathology, Centre for Vascular Research, The University of New South Wales, Sydney NSW 2052, Australia. E-mail: L.Khachigian@unsw.edu.au

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2002a] and pig [Lowe et al., 2001] arteries, implicating EGR-1 as a key target for the treatment of in-stent restenosis and other vascular proliferative disorders.

Numerous studies have demonstrated nucleic acid-based molecules as useful tools in reducing the formation of intimal growth in various in vivo models targeting and suppressing key regulatory genes expressed by SMCs. Such molecules include DNAzymes [Santiago et al., 1999b; Lowe et al., 2001], ribozymes [Frimerman et al., 1999], and antisense phosphorothioate oligonucleotides [Morishita et al., 1993; Autieri et al., 1995; Villa et al., 1995; Pitsch et al., 1996; Bennett et al., 1997] all of which utilize Watson–Crick base pairing.

RNA interference (RNAi) has emerged as a powerful technique to suppress gene expression in a sequence-specific manner in animal, fungi, and plant cells [Fire et al., 1998; Matzke and Birchler, 2005]. Work by Fire et al. [1998] originally demonstrated that double-stranded RNA is far more potent at specifically reducing gene expression in C. *elegans* than antisense RNA [Fire et al., 1998]. Small interfering RNA (siRNA) (21–26 nts), generated by the cleavage activity of DICER, interacts (antisense strand) with RNA-binding protein(s) forming a RNA-induced silencing complex (RISC), which in turn, degrade mRNA at the antisense binding site [Hammond et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001; Matzke and Birchler, 2005]. Efforts to exploit siRNA for therapeutic applications are promising with two siRNA molecules currently in clinical trials [Tolentino et al., 2004; Shen et al., 2006].

In the present study, we designed and synthesized siRNA targeting the coding region of EGR-1. EGR-1 siRNA blocked SMC proliferation, suppressed endogenous EGR-1, FGF-2, and granulocyte-colony stimulating factor (G-CSF) expression, and inhibited SMC regrowth after in vitro injury in a sequence-specific manner.

MATERIALS AND METHODS

siRNA and DNAzyme Sequences

Two 21-nt duplex siRNA oligonucleotides were synthesized by Qiagen-Xeragon (MD). The sequences of the siRNA are: EGR-1(601) [5′-r(CAACGAGAAGGUGCUGGUG)d(TT)-3] and [5′-r(CACCAGCACCUUCUCGUUG)d(TT)-3′] and EGR-1(1516) duplex [5′-r(GAUCCACUUGCGGCAGAAG)d(TT)-3′] and [5′-r(CUUCUGCCGCAAGUGGAUC)d(TT)-3′] targeting the coding region of the human EGR-1 transcript at positions 601–621 and 1516–1536, respectively. A scrambled duplex control, EGR-1(601)SCR [5′-r(GCGAGUAGCGCUAGGAAGU)d(TT)-3′] and [5′-r(ACUUCCUAGCGCUACUCGC)d(TT)-3′], was synthesized and subjected to a BLAST search to ensure that no significant similarity existed between other sequences resulting in non-specific inhibition.

The DNAzyme, DzF, targeting the $A^{301}U$ junction of human EGR-1 mRNA, and its scrambled counterpart, DzFSCR, were synthesized by Trilink Biotechnologies (San Diego). The sequences are: (DzF) 5′-GCGGGGACAGGCTAGCTACAACGACAGCTGCAT-(3′-3′T)-3′ and (DzFSCR) 5′-GGAGCTGACGGCTAGCTACAACGAGATCGACGC-(3′-3′ T)-3′, respectively.

Cell Culture and Transfections

Human aortic smooth muscle cells (HASMCs) were obtained from American Type Culture Collection (Manassas, VA) and cultured in Waymouth's medium, pH 7.4, supplemented with 10% fetal bovine serum (FBS), 10 μg/ml streptomycin, and 10 U/ml penicillin at 37°C and 5% CO_2. Cells were passaged by washing twice in 1× PBS followed by trypsinization. Unless otherwise stated, subconfluent SMCs (75%) were growth arrested in serum free conditions for 6 h and then transfected with DNAzymes using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany). Cells were transfected a second time in the presence of 5% serum for 18 h following the initial transfection. siRNA transfections were carried out in a similar manner using RNAiFect™ (Qiagen, Germany) specific for siRNA transfections. Cells in the untransfected groups received equal volumes of either FuGENE6 or RNAiFect.

Smooth Muscle Cell Proliferation Assay

HASMCs were seeded into 96-well plates (3,000 cells/well). Subconfluent SMCs were transfected with either 1, 10, or 50 nM siRNA or DNAzyme. Forty-eight hours following the second transfection, cells were trypsinized and resuspended in 10 ml of isotone solution for counting. Cells were then counted using an automated Coulter counter (Coulter Z Series, Miami). Minor variations in DNAzyme concentration effecting 50% inhibition of

primary SMC growth were noted occasionally from cell batch-to-batch.

Smooth Muscle Cell Injury Assay

HASMCs were grown to confluence in 8-well chamber slides (Iwaki, Japan) and transfected with 0.2 μM siRNA or DNAzyme. Eighteen hours following transfection, injury was performed with a single scratch using a sterile toothpick in the presence of 5% serum. Twenty-four hours following injury, fresh medium containing 10% serum was added to the cells for a further 48 h before the cells were fixed in 5% formaldehyde (v/v), stained in hematoxylin and eosin for analysis by light microscopy. Cells in the denuded zone were counted in two different fields of view and expressed as the mean ± standard error of the mean.

Semi-Quantitative RT-PCR

EGR-1 mRNA was analyzed by semi-quantitative RT-PCR. Subconfluent SMCs were grown in 100-mm tissue culture plates and transfected with 10 nM siRNA. Total RNA was harvested using TRIzol reagent (Invitrogen), 1 h following second transfection in 5% FBS. Five micrograms of total RNA was reverse-transcribed using SUPER SCRIPT II RNase H- Reverse Transcriptase (Invitrogen). Cycle-based PCR was used to semi quantitate EGR-1 and GAPDH mRNA expression. Each PCR reaction contained 1 mM $MgCl_2$, 20 pmol of each primer, and 1 U Taq DNA polymerase (Invitrogen). Cycle-based PCR was carried out using a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk). PCR for EGR-1 expression consisted of an initial 1 min denaturing step at 95°C. Thermal cycling consisted of 1 min denaturing at 95°C, 20 s annealing at 58°C, and a 20 s extension at 72°C for 29 cycles followed by a final extension period at 72° for 1 min. PCR for the house keeping gene, GAPDH, consisted of an initial 1 min denaturing step at 95°C. Thermal cycling for GAPDH consisted of 1 min denaturing at 95°C, 30 s annealing at 58°C, and 30 s extension at 72°C for 25 cycles followed by a further extension period of 1 min at 72°C. Primer sequences were as follows: EGR-1, 5′-GCAATTGTGAGGGACATGCT-3′ (forward) and 5′-TTCTGGAGAACCGAAGCTCA-3′ (reverse); GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). The expected sizes of the PCR products for EGR-1 and GAPDH were 117 and 451 bp, respectively.

Western Blot Analysis

HASMCs were cultured in 10-mm tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes) and transfected with either 10 nM or 0.4 μM siRNA. One hour and 4 h following the second transfection in the presence of 5% serum, cells were washed twice in 1× PBS and total protein was extracted in 150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 1% sodium deoxicolate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 10 μg/ml leupeptin, 1% aprotinin, and 2 mM PMSF. One microgram of protein sample was loaded onto a 10% SDS–PAGE and electroblotted onto a PVDF nylon membrane (Millipore, Bedford). Membranes were blocked in 0.05% Tween 20 (v/v) PBS containing 5% skim milk then incubated with rabbit polyclonal Egr-1 (sc-110), actin (sc-7210), FGF-2 (sc-79), VEGF (sc-152), or G-CSF (sc-13102) primary antibodies (Santa Cruz) at a concentration of 2 μg/ml. Membranes were then incubated with a HRP linked swine anti rabbit IgG secondary antibody (Dako, Carpinteria). Protein bands of interest were visualized by chemiluminesent detection (NEN, Boston).

Quantitation of Downstream FGF-2 Expression by ELISA

FGF-2 protein expression was quantitated from total protein harvested from cell lysates 4 h following serum stimulation and transfection with EGR-1(601) using Quantikine® human FGF basic ELISA kit (R&D systems, Minneapolis). One microgram of cell lysate was used to assess FGF-2 expression in EGR-1(601), EGR-1(601)SCR, and Vehicle groups. FGF-2 concentrations were determined at 562 nm and expressed as pg/ml/μl protein.

Injury and Immunohistochemistry of Rat Carotid Arteries

Injury by ligation to rat carotid arteries and immunohistochemistry were performed as previously described [Santiago et al., 2001; Lowe et al., 2002; Khachigian et al., 2002b].

Quantitation of G-CSF Secretion Following SMC Injury

G-CSF levels, following injury to SMCs, were assessed using the Bio-Plex™ assay system (BioRad, Hercules, CA) according to

the manufacturer's instructions. Arrested HASMCs were transfected twice before injury. Supernatant was harvested 4 h following injury and concentrated for Bio-Plex™ analysis.

RESULTS

siRNA and DNAzyme Targeting EGR-1 Inhibit SMC Proliferation

SMC proliferation is a hallmark process mediating intimal thickening following acute injury. SMC proliferation assays were performed to evaluate the effect of siRNAs targeting human EGR-1 mRNA on SMC phenotype. In these studies, two siRNA molecules, EGR-1(601) and EGR-1(1516), were compared against DzF, a 9 + 9 nt DNAzyme targeting human EGR-1. "601" and "1516" refer to the position of the first nucleotide of the human mRNA targeted by the siRNA (Genbank accession X52541). Concentrations ranging from 1 to 50 nM demonstrated that siRNA inhibited serum-inducible SMC proliferation within 48 h in a dose-dependent manner (Fig. 1). EGR-1(601) and EGR-1(1516) blocked serum-inducible SMC proliferation to virtual completion at concentrations as low as 10 nM without affecting Trypan blue uptake (data not shown). Inhibition of SMC proliferation by DzF was only observed at the 50 nM concentration (Fig. 1).

To establish sequence-specificity of siRNA, we generated EGR-1(601)SCR, a scrambled version of EGR-1(601) and evaluated its ability to influence SMC proliferation. EGR-1(601)SCR had no inhibitory effect at any given concentration, consistent with cell counts in the serum-induced untransfected group (Fig. 1).

SMC Regrowth From the Wound Edge Is Blocked by siRNA Targeting EGR-1

We next used an in vitro model of injury to determine whether EGR-1(601) could influence SMC regrowth following injury. In this model, a sterile toothpick was used to perform a single scratch along a confluent monolayer of SMCs. The injury stimulates a regenerative response that results in the regrowth of SMCs from the wound edge into the denuded zone [Santiago et al., 1999b]. Figure 2A demonstrates that EGR-1(601) inhibits SMC regrowth after injury within 3 days, whereas SMC regrowth was not blocked by EGR-1(601)SCR (Fig. 2A,B).

EGR-1(601) Attenuates Serum-Inducible EGR-1 mRNA and Protein Expression in HSMCs

The preceding data established that EGR-1 siRNA could inhibit serum-inducible SMC proliferation in a sequence-specific, dose-dependent, and non-toxic manner, and suppressed regrowth from the wound edge following injury. We next determined whether EGR-1(601) influenced EGR-1 mRNA and protein by RT-PCR and Western blot, respectively. Total lysates were harvested from SMCs 1 h following serum-stimulation and transfection with either

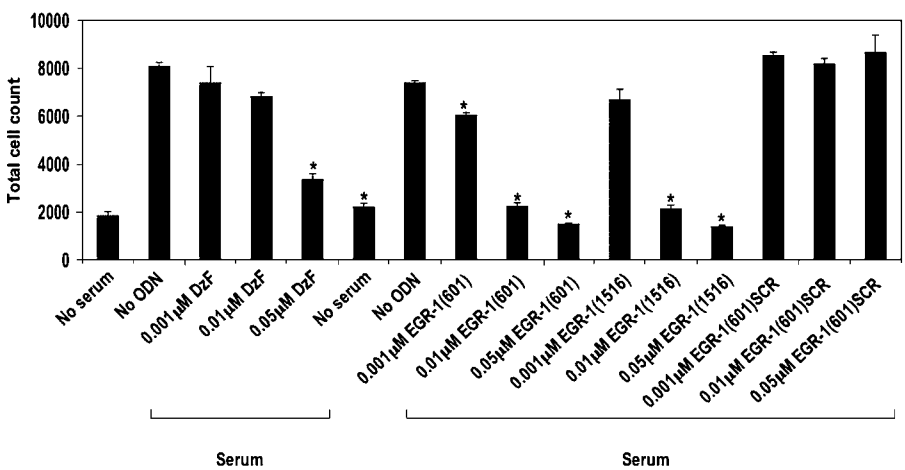

Fig. 1. EGR-1(601) inhibits serum-inducible HASMC proliferation in a dose-dependent and sequence-specific manner. Growth arrested HASMCs were transfected with 1–50 nM of either siRNA or DNAzyme, targeting EGR-1, in 96-well plates exposed to 5% serum. Cells were counted 48 h following serum induction using an automated Coulter counter. * indicates $P < 0.05$ by Student's *t*-test relative to the No ODN or EGR-1(601)SCR. No ODN denotes no oligonucleotides added.

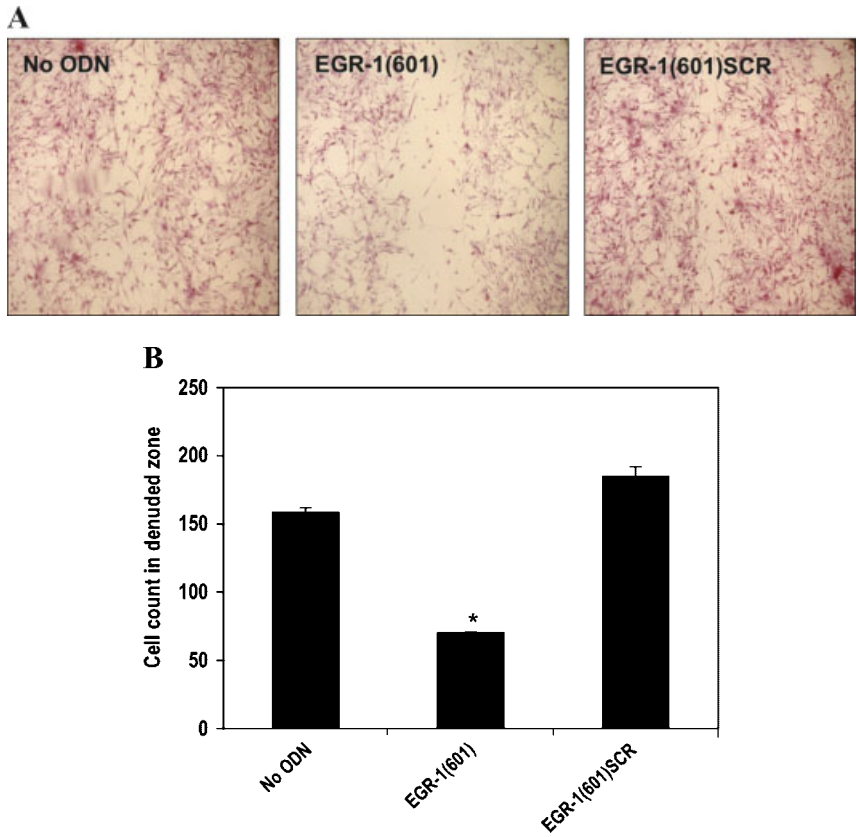

Fig. 2. **A**: EGR-1(601) inhibits cellular regrowth from the wound edge following mechanical injury to HASMCs. Growth-arrested HASMCs were transfected with 0.2 μM of either EGR-1(601) or EGR-1(601)SCR in 8-well chamber slides exposed to 5% serum for 24 h and then incubated with 10% serum for a further 48 h following injury, by scraping, with a sterile toothpick. Following the 72 h incubation in serum, the cells were fixed in 5% formaldehyde then stained in hematoxylin and eosin. **B**: Assessment of cell population in the denuded zone. Cells in the denuded zone were quantified at two different fields of view for each treatment and expressed as a mean ± SEM. No ODN denotes no oligonucleotides added. The data are representative of two independent determination, where * indicates $P < 0.05$ relative to the scrambled control using a Student's *t*-test (two-tailed, unequal variance).

EGR-1(601) or EGR-1(601)SCR. EGR-1(601), at 10 nM, inhibited serum-inducible EGR-1 mRNA (Fig. 3A, upper panel) and protein (Fig. 3A, lower panel), whereas its scrambled counterpart had no inhibitory effect. EGR-1(601) suppression of EGR-1 expression was also apparent at 0.4 μM (Fig. 3B).

EGR-1 siRNA Inhibits Induction FGF-2 Expression

Several lines of evidence indicate that FGF-2 plays a key role in vascular SMC growth. For example, FGF-2 and its receptors are expressed by SMCs in the injured artery wall. Moreover, antibodies to FGF-2 block neointima formation after balloon injury and administration of FGF-2 stimulates intimal thickening [Lindner and Reidy, 1991; Lindner et al., 1991; Brogi et al., 1993]. More recent studies have demonstrated that EGR-1 transactivates FGF-2 [Biesiada et al., 1996], consistent with our demonstration that EGR-1 DNAzymes block FGF-2 protein expression in vascular endothelium [Fahmy et al., 2003]. We explored whether siRNA inhibition of EGR-1 in vascular SMCs also compromised the expression of FGF-2 protein. Western blot analysis revealed that EGR-1(601) blocked FGF-2 expression after 4 h (Fig. 4A). In contrast, levels of VEGF were unchanged (Fig. 4A). EGR-1(601)SCR did not influence FGF-2 or VEGF expression (Fig. 4A). Quantitative analysis of FGF-2 expression using ELISA demonstrated siRNA inhibition of FGF-2. EGR-1(601) suppressed FGF-2 expression in SMCs 4 h following serum stimulation (Fig. 4B).

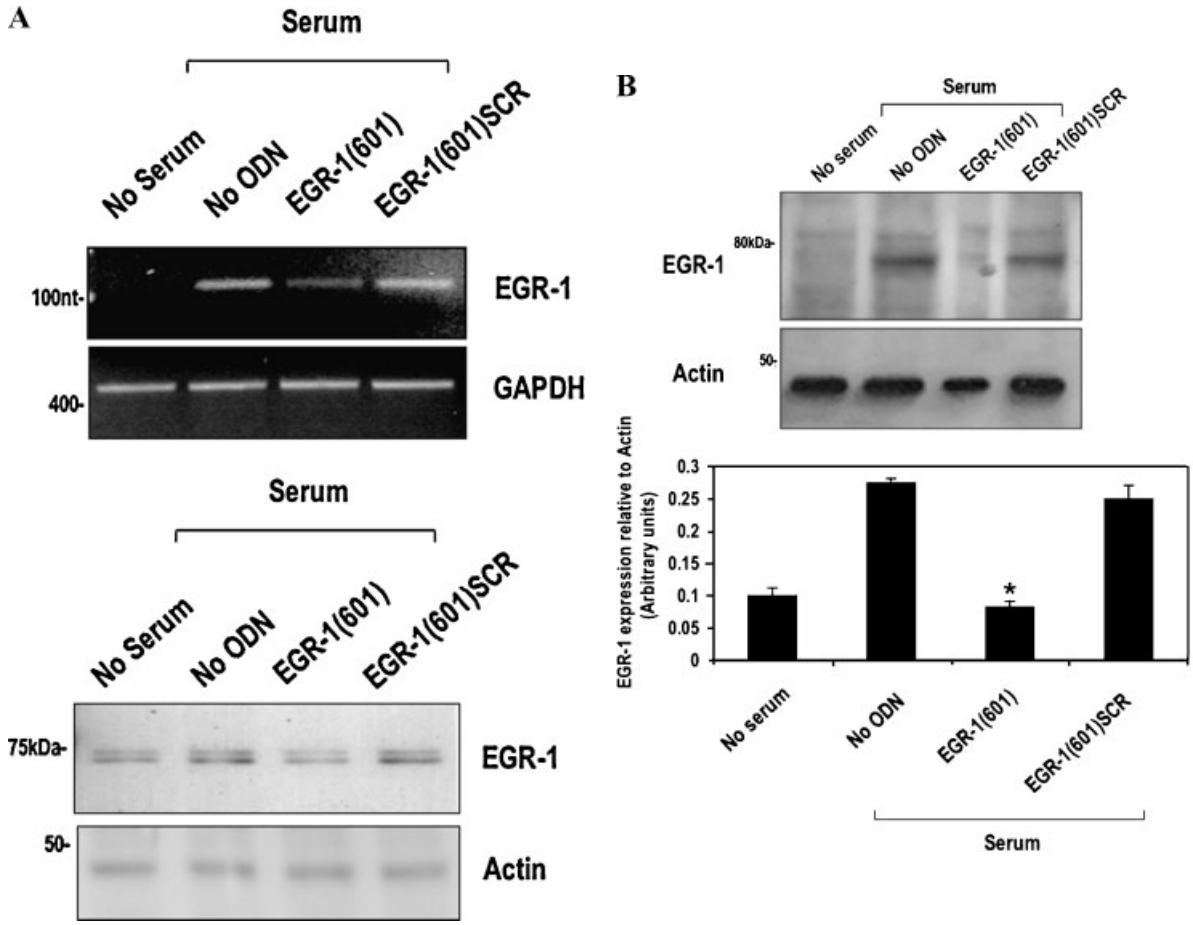

Fig. 3. siRNA blockade of EGR-1 mRNA and protein expression. HASMCs were transfected with (**A**) 10 nM (RT-PCR, **upper panel**; protein, **lower panel**) and (**B**) 0.4 μM of either EGR-1(601) or EGR-1(601)SCR in 100-mm tissue culture dishes. Cell lysates were harvested from plates 1 h following the second transfection in the presence of 5% serum. EGR-1, GAPDH, and actin levels were assessed by RT-PCR or Western blot analysis as indicated. * indicates $P < 0.05$ relative to the scrambled control using a Student's *t*-test (two-tailed, unequal variance).

EGR-1(601) Attenuates G-CSF Expression and Secretion Following SMC Injury and Stimulation, Respectively

We performed Bio-Plex™ analysis to gain additional insights into genes downstream of EGR-1 inhibited by the EGR-1 siRNA. In vitro scraping [Khachigian et al., 1996b] of the HASMCs resulted in G-CSF secretion into the supernatant within 4 h (Fig. 5A, left panel). This was not confined to an in vitro injury system. Immunohistochemical analysis demonstrated that G-CSF levels are increased in medial SMCs and the endothelium of rat carotid arteries after ligation injury (Fig. 5B), consistent with the recent demonstration of G-CSF induction in rat arteries after balloon injury [Chen et al., 2005]. EGR-1(601) attenuated G-CSF levels in supernatants of injured SMCs, whereas EGR-1(601)SCR had no inhibitory effect (Fig. 5A, left panel). In support of these observations, Western blot analysis demonstrated sequence-specific EGR-1 siRNA suppression of G-GSF protein in SMC lysates (Fig. 5A, right panel).

DISCUSSION

The present study demonstrates the ability of siRNA targeting human EGR-1 to inhibit serum-inducible human aortic SMC proliferation, endogenous EGR-1 and downstream FGF-2 and G-CSF expression, and regrowth from the wound edge following injury. Concentrations of siRNA as little at 10 nM inhibited SMC proliferation, whereas at this concentration, DzF, the DNAzyme targeting human

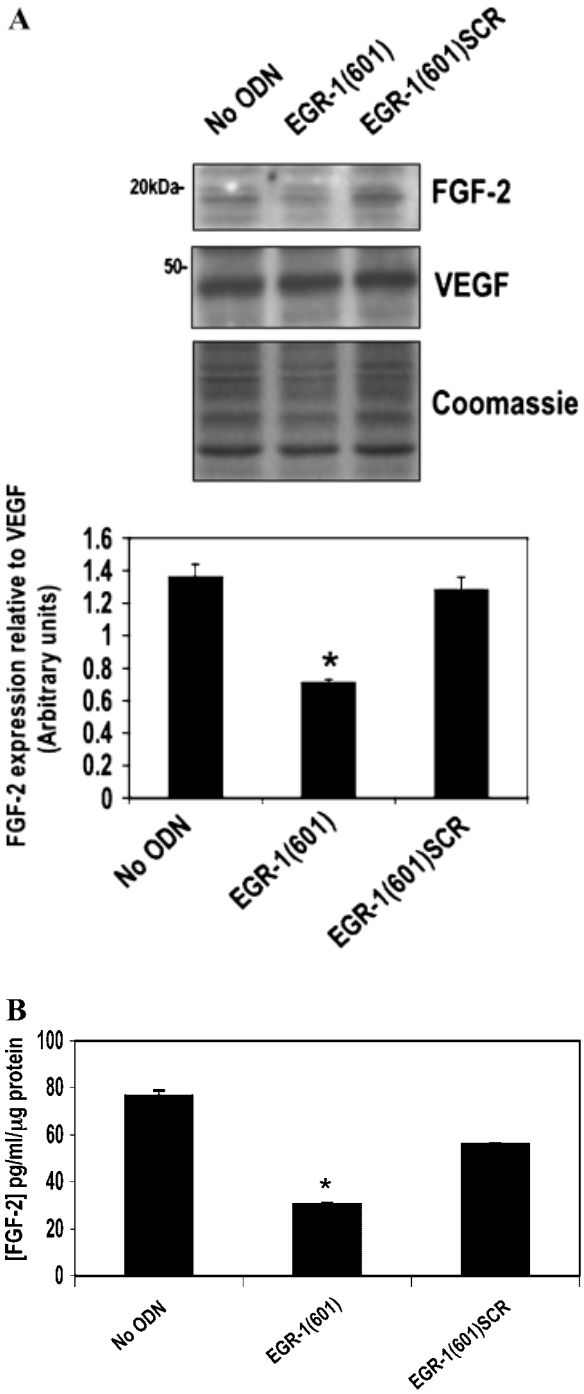

Fig. 4. **A**: Inhibition of FGF-2 following using EGR-1(601). Cell lysates were harvested 4 h following the second transfection in medium containing 5% serum. Lysates were assessed for FGF-2 and VEGF immunoreactivity by Western blot analysis using polyclonal antibodies to FGF-2 and VEGF. No ODN denotes no oligonucleotides added. **B**: Quantitative analysis of FGF-2 expression by ELISA. Total protein was extracted from HASMCs 4 h following serum stimulation and transfection with either EGR-1(601), EGR-1(601)SCR, or FuGENE6 alone (No ODN). FGF-2 concentrations were assessed by spectrophotometry at 562 nm and expressed as pg/ml/μg protein. * indicates $P < 0.05$ relative to the scrambled control using a Student's *t*-test (two-tailed, equal variance).

EGR-1, had no inhibitory effect. Our data using siRNA add further weight to the key role played by EGR-1 in SMC proliferation and wound repair previously shown using alternative small molecule nucleic acid-based strategies, such as DNAzymes [Santiago et al., 1999c] and antisense oligonucleotides [Santiago et al., 1999a].

Numerous possibilities may explain why siRNA appears to be more efficient at inhibiting SMC proliferation than DNAzyme, even though degradation of mRNA by either class of molecule relies on hybridization of target sites. For example, each technology utilizes a different mechanism that may influence the net extent of inhibition. DNAzymes, introduced into host cells, degrade mRNA through multiple turnover kinetics. siRNA, on the other hand, activate an unknown naturally-occurring enzyme that complexes with the siRNA forming RISC that increases the efficacy for targeted destruction of the transcript [Hammond et al., 2000]. Alternatively, the bioavailability of each agent within the cell and/or accessibility to their different target sites in the mRNA may influence phenotype. The present findings are, nonetheless, consistent with recent comparative studies demonstrating that siRNA is more effective at silencing target mRNA than DNAzymes [Beale et al., 2003; Yokota et al., 2004]. Studies performed between ribozymes, DNAzymes, and siRNA targeting an identical site on the EGFR mRNA revealed that siRNA inhibited A431 cancer cells overexpressing EGFR in a dose-dependent manner [Beale et al., 2003]. Furthermore, Beale and colleagues demonstrated that siRNA was more potent at inhibiting A431 cell growth at concentrations 7.5 times less than those used by ribozymes or DNAzymes [Beale et al., 2003]. Our proliferation assays demonstrated that inhibition of SMC proliferation was achieved using concentrations of siRNA five times less to that of DNAzyme.

Recent studies have shown that EGR-1 siRNA may be useful as a versatile inhibitory agent in a variety of pathologic settings [Garnett et al., 2005; Worden et al., 2005; Cron et al., 2006]. Rupp et al. [2005] demonstrated that human coronary artery SMC proliferation and Egr-1 expression after infection with *C. pneumonia* could be attenuated after transfection with siRNA targeting a different region in the coding region of the Egr-1 transcript. SMCs

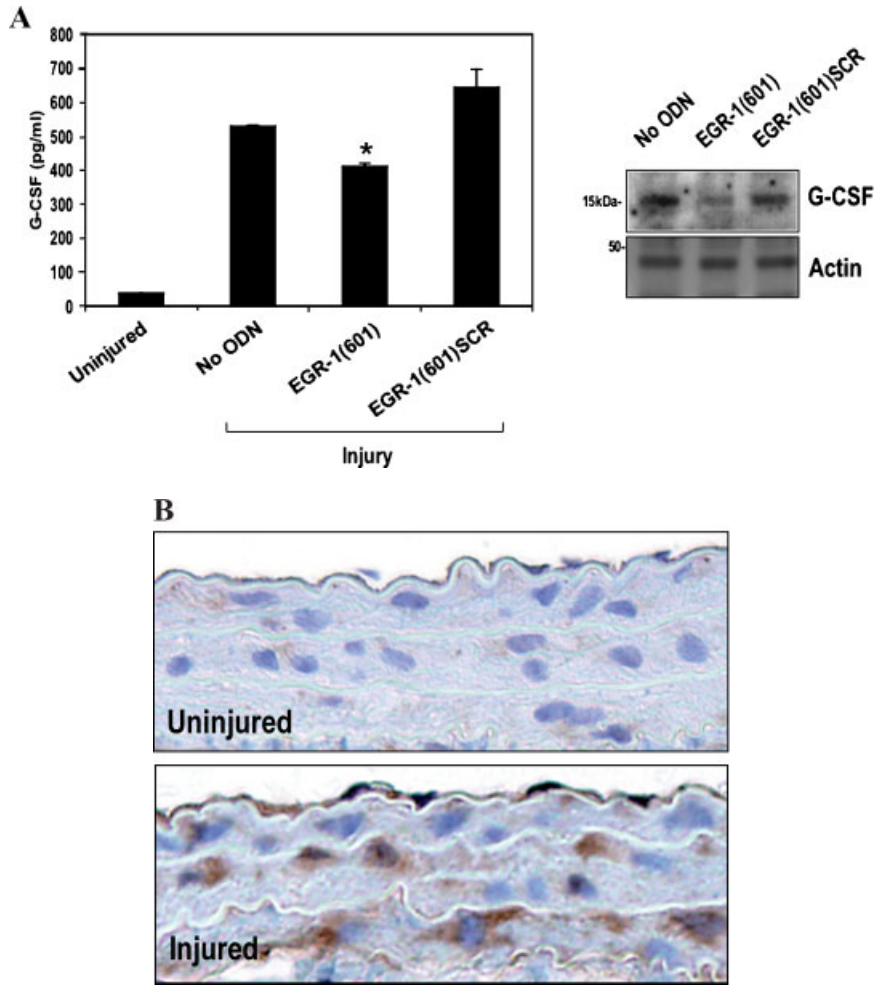

Fig. 5. **A**: EGR-1(601) inhibits G-CSF protein expression and secretion after in vitro injury. Serum-deprived HASMCs were transfected with EGR-1(601) or EGR-1(601)SCR prior to in vitro injury by mechanical scraping. The supernatant was concentrated and used to assess G-CSF levels by Bio-Plex™ assay (**left panel**). Alternatively, G-CSF immunoreactivity in the lysates 4 h after injury was determined by Western blot analysis (**right panel**). **B**: G-CSF expression is induced in medial SMCs of rat carotid arteries injured by ligation. Three hours after ligation, vessels were removed, fixed in 10% formalin in 1× PBS, embedded in wax and sectioned (5 μm) at 100 μm proximal to the tie for immunohistochemistry.

form the major cellular component of lesions of restenosis, whose pathogenesis can be suppressed by agents that inhibit SMC proliferation [Morishita et al., 1993; Villa et al., 1995; Pitsch et al., 1996; Bennett et al., 1997]. As a master regulator, EGR-1 [Khachigian, 2006] controls a wide spectrum of dependent genes, including those, like FGF-2 and G-CSF that influence SMC proliferation and intimal thickening. siRNA targeting human EGR-1 blocks SMC proliferation, regrowth after injury, and endogenous EGR-1 as well as downstream gene expression. Our findings thus further implicate EGR-1 targeting strategies as potentially useful tools in vascular occlusive disorders.

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