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A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*

(restenosis/cell cycle-regulatory genes/hemagglutinating virus of Japan/antigene)

RYUICHI MORISHITA*, GARY H. GIBBONS*, MASATSUGU HORIUCHI*, KRISTIN E. ELLISON*, MASATOSHI NAKAJIMA*, LUNAN ZHANG*, YASUFUMI KANEDA†, TOSHIO OGIHARA‡, AND VICTOR J. DZAU*§

*Division of Cardiovascular Medicine, Falk Cardiovascular Research Center, Stanford University School of Medicine, Stanford, CA 94305-5246; and †Institute for Cellular and Molecular Biology and ‡Department of Geriatric Medicine, Osaka University Medical School, Osaka, Japan

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ABSTRACT **The application of DNA technology to regulate the transcription of disease-related genes *in vivo* has important therapeutic potentials. The transcription factor E2F plays a pivotal role in the coordinated transactivation of cell cycle-regulatory genes such as c-*myc*, *cdc2*, and the gene encoding proliferating-cell nuclear antigen (PCNA) that are involved in lesion formation after vascular injury. We hypothesized that double-stranded DNA with high affinity for E2F may be introduced *in vivo* as a decoy to bind E2F and block the activation of genes mediating cell cycle progression and intimal hyperplasia after vascular injury. Gel mobility-shift assays showed complete competition for E2F binding protein by the E2F decoy. Transfection with E2F decoy inhibited expression of c-*myc*, *cdc2*, and the PCNA gene as well as vascular smooth muscle cell proliferation both *in vitro* and in the *in vivo* model of rat carotid injury. Furthermore, 2 weeks after *in vivo* transfection, neointimal formation was significantly prevented by the E2F decoy, and this inhibition continued up to 8 weeks after a single transfection in a dose-dependent manner. Transfer of an E2F decoy can therefore modulate gene expression and inhibit smooth muscle proliferation and vascular lesion formation *in vivo*.**

Abnormal growth of cardiac and vascular cells plays an essential role in the pathogenesis of cardiovascular diseases such as cardiac hypertrophy, atherosclerosis, and intimal hyperplasia. Accordingly, a molecular strategy that effectively inhibits abnormal cell proliferation has important therapeutic potentials. Intimal hyperplasia develops in large part as a result of vascular smooth muscle cell (VSMC) proliferation and migration induced by a complex interaction of multiple growth factors that are activated by vascular injury (1–5). When released from a regulatory complex with cyclin A and Cdk2, the transcription factor E2F induces a coordinated transactivation of genes involved in cell cycle regulation, including those encoding c-Myc, c-Myb, Cdc2, proliferating-cell nuclear antigen (PCNA), and thymidine kinase (6–13) (Fig. 1*A*). Indeed, the antiproliferative effects of the retinoblastoma gene product (RB) appear to depend on its capacity to bind to E2F and thereby prevent this transcription factor from binding to the E2F cis element within the promoters of several genes (14–16).

The transcriptional factor E2F binds with high specificity and affinity to double-stranded DNA containing an 8-bp consensus sequence (17). Previous studies have reported that synthetic double-stranded DNA with high affinity for a target transcription factor may be introduced into target cells as decoy cis elements to bind the factor and alter gene transcription (18, 19). Accordingly, we hypothesize that transfection of VSMCs with sufficient quantities of decoy ODNs containing the E2F binding site would effectively bind E2F, preventing the transactivation of essential cell cycle-regulatory genes and thereby inhibiting VSMC proliferation and neointima formation *in vivo* (Fig. 1*A*).

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MATERIALS AND METHODS

Decoy ODN Sequences. The sequences of the phosphorothioate ODNs used are shown below.

Decoy	Sequence
14-mer E2F decoy	5′-CTAGATTTCCCGCG-3′ 3′-TAAAGGGCGCCTAG-5′
14-mer mismatch control decoy	5′-CTAGATTTCGAGCG-3′ 3′-TAAAGCTCGCCTAG-5′
30-mer E2F decoy	5′-GATCAAAAGCGCGAATCAAAAGCGCGAATC-3′ 3′-CTAGTTTTCGCGCTTAGTTTTCGCGCTTAG-5′
30-mer mismatch control decoy	5′-GATCAAAGAACTGAATCAAAGAACTGAATC-3′ 3′-CTAGTTTCTTGACTTAGTTTCTTGACTTAG-5′
Scrambled-sequence control decoy	5′-TCCAGCTTCGTAGC-3′ 3′-GAAGGATCGATCG-5′
PRE decoy	5′-GATCCTGTACAGGATGTTCTAGCTACA-3′ 3′-CTAGGACATGTCCTACAAGATCGATGT-5′

The 14-mer E2F ODN contains the 8-bp cis element (underlined) that has been characterized in the c-*myc* promoter. The 30-mer E2F ODN contains the 8-bp cis element (underlined) that has been defined in the adenovirus E2 gene promoter. Both have been shown to bind E2F (8, 17).

***In Vitro* Transfection.** Hemagglutinating virus of Japan (HVJ)–liposome complexes containing ODN at 3 μM were formed and quiescent cultures of rat aortic VSMCs (passage 4–8) were transfected with these liposome complexes as described (20–22). After transfection, cells were maintained in fresh defined serum-free medium or medium with 5% serum, and cell numbers were determined by Coulter Counter at 3 days after transfection. One day after transfection, Northern blot analysis for c-*myc* mRNA was performed with 3′-end-labeled c-*myc* oligonucleotide probes (Oncogene Science). PCNA and Cdc2 kinase protein levels in VSMCs were measured by ELISA (Paracelsian, Ithaca, NY) at 3 days after transfection.

Gel Mobility-Shift Assay. Nuclear extract was prepared from VSMCs as described (23). E2F, mismatched, and PRE decoy (25) phosphorothioate ODN primers were ^{32}P-labeled with a 3′-end-labeling kit (kindly donated by Clontech) and purified by Nick column (Pharmacia). Labeled primer (0.5–1 ng, 10,000–15,000 cpm) and 1 μg of poly(dI-dC)·poly(dI-dC) (Sigma) were incu-

Abbreviations: HVJ, hemagglutinating virus of Japan; ODN, oligodeoxynucleotide; PCNA, proliferating-cell nuclear antigen; PRE, progesterone-responsive element; VSMC, vascular smooth muscle cell.

§To whom reprint requests should be addressed at: Falk Cardiovascular Research Center, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305-5246.

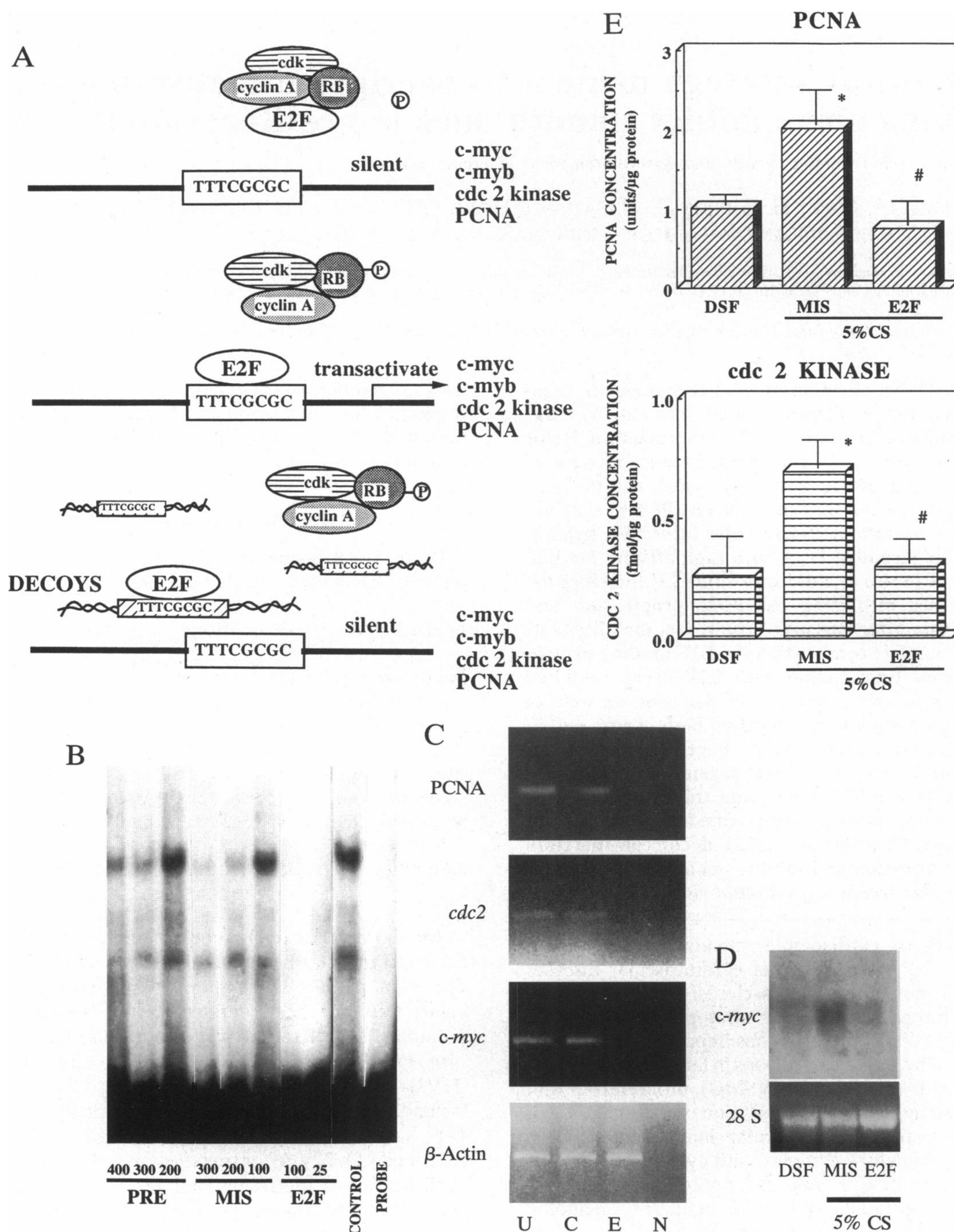

FIG. 1. (*A*) Scheme of E2F decoy strategy. TTTCGCGC, consensus sequence for the E2F binding site. In the quiescent-cell state, E2F is trapped by the combination of RB, cyclin A, and cdk (*Top*). Phosphorylation of RB releases free E2F, which binds to cis elements of the cell cycle-regulatory genes, resulting in the transactivation of these genes (*Middle*). The E2F decoy cis-element double-stranded oligodeoxynucleotide (ODN) binds to free E2F, preventing E2F transactivation of the cell cycle-regulatory genes (*Bottom*). (*B*) Gel mobility-shift assay for E2F binding site. PROBE, ^{32}P-labeled E2F decoy ODN without nuclear extract; CONTROL, nuclear extract incubated with ^{32}P-labeled E2F decoy ODN for 30 min at room temperature without any competitor; PRE, CONTROL plus progesterone-responsive element (PRE) ODN (200×, 300×, and 400× molar excess); MIS, CONTROL plus mismatched sequence ODN (100×, 200×, and 300× excess); E2F, CONTROL plus E2F decoy ODN (25× and 100× excess). (*C*) Effect of decoy ODN transfection with HVJ–liposome complex on *in vitro* VSMC expression of PCNA, *cdc2* kinase, c-*myc* and β-actin mRNA detected by RT–PCR. U, untreated; C, treated with mismatched decoy ODN; E, treated with E2F decoy ODN; N, amplification without RNA (negative control). (*D*) Effect of decoy ODN transfection with HVJ–liposome complex on *in vitro* VSMC expression of c-*myc* as detected by Northern blot. DSF, untreated VSMCs (in defined serum-free medium); MIS, VSMCs treated with mismatched decoy ODN; E2F, VSMCs treated with E2F decoy ODN. Treatments were given in medium with 5% calf serum (CS). As a control for equal loading of RNA, 28S rRNA is shown. (*E*) Effect of decoy ODN transfection with HVJ–liposome complex on PCNA and Cdc2 kinase protein concentration as assessed by ELISA. DSF, untreated VSMCs; MIS, VSMCs treated with mismatched decoy ODN; E2F, VSMCs treated with E2F decoy ODN. *, $P < 0.01$ vs. DSF; #, $P < 0.01$ vs. MIS.

bated with 10 μg of nuclear extract for 30 min at room temperature and then loaded onto a 5% polyacrylamide gel. The gels were subjected to electrophoresis, dried, and preincubated with parallel samples 10 min before the addition of the labeled probe.

***In Vivo* Transfer of ODNs.** Rats were anesthetized and a no. 2 French–Fogarty catheter was introduced into the left common carotid artery to induce vascular injury in male Sprague–Dawley rats (400–500 g) as described (20). After vascular

injury, the distal injured segment of the common carotid artery (≈1 cm proximal to the carotid bifurcation) was transiently isolated by temporary ligatures. The HVJ–liposome complex was infused into the distal common carotid segment via a cannula introduced from the external carotid within 10 min after injury and incubated within the lumen for 15 min. After transfection, the infusion cannula was removed, and blood flow to the common carotid was restored by release of the ligatures. We observed a thrombosis rate of ≈10% and a mortality rate of ≈1%. Animals were eliminated from analysis in cases of vessel thrombosis or death prior to scheduled sacrifice. The overall thrombosis rates and mortality rates were similar across all treatment groups under study.

Reverse Transcription (RT)–PCR. RNA was extracted from cell cultures at 6 or 24 hr after serum stimulation, and RT–PCR was run with primers specific for PCNA, Cdc2 kinase, and β-actin (Clontech). Primers for c-Myc (forward, 5′-TCT-CCG-AGA-AGC-TGG-CC-3′; reverse, 5′-GAG-AAG-GCC-CTG-GAA-TC-3′) were also used to produce an amplification product of ≈300 bp (24). Aliquots of RNA were amplified simultaneously by PCR (30 cycles) and compared with a negative control (primers without RNA) and PCR amplification with the β-actin primers. Vessels were harvested at 6 hr (for c-Myc) and 1 day (for Cdc2 kinase and PCNA) after transfection. RNA was extracted from uninjured or injured vessels treated with mismatched or E2F decoy ODNs for RT–PCR analysis ($n = 4$ for each group). Aliquots of RNA were amplified simultaneously by PCR (25, 30, and 40 cycles) and compared with a negative control (primers without RNA). To minimize the influence of genomic DNA contamination in the RT–PCR products, the primers selected spanned exon–intron junctions.

Measurement of DNA Synthesis. Rats were treated with 5-bromo-2′-deoxyuridine (BrdUrd, 100 mg/kg subcutaneously and 30 mg/kg intraperitoneally 18 hr before sacrifice and 30 mg/kg intraperitoneally 12 hr before sacrifice) and sacrificed on day 4 after transfection. The carotid artery was fixed with 4% paraformaldehyde and processed for immunohistochemistry in a standard manner using anti-BrdUrd antibodies (Amersham). The proportion of BrdUrd-positive cells was determined by cell counts under light microscopy by investigators blinded to the treatment groups ($n = 6$).

Morphometric Analysis. At 2, 4, and 8 weeks after transfection, rats were sacrificed and vessels were fixed by perfusion with 4% paraformaldehyde. Computer-assisted planimetry was performed in a blinded manner on three 6-μm sections, spaced 150 μm apart, from the middle of the transfected segment. Average values were compared with measurements of three sections from the middle section of the adjacent injured untransfected region.

Statistical Analysis. Values are expressed as mean ± SEM. Analysis of variance with subsequent Dunnett's test was used to determine significant differences in multiple comparisons.

RESULTS

Serum stimulation of confluent, quiescent VSMCs induced the mRNA expression of cell cycle-regulatory genes (c-*myc*, *cdc2*, PCNA) in association with cell proliferation. Gel shift analysis documented further that serum stimulation resulted in increased E2F binding activity as compared with unstimulated controls. This increased E2F binding was abolished by excess quantities of the unlabeled 14-mer double-stranded ODN E2F cis element (Fig. 1*B*; ref. 17). Transfection of the 14-mer E2F cis-element decoy ODN markedly inhibited VSMC proliferation in response to serum stimulation (Fig. 2), as well as expression of c-*myc*, *cdc2*, and PCNA as assessed by RT–PCR, Northern blot, or ELISA (Fig. 1 *C–E*). In contrast, the control missense E2F cis-element ODN containing base-pair substitutions that eliminate E2F binding (17) failed to inhibit the

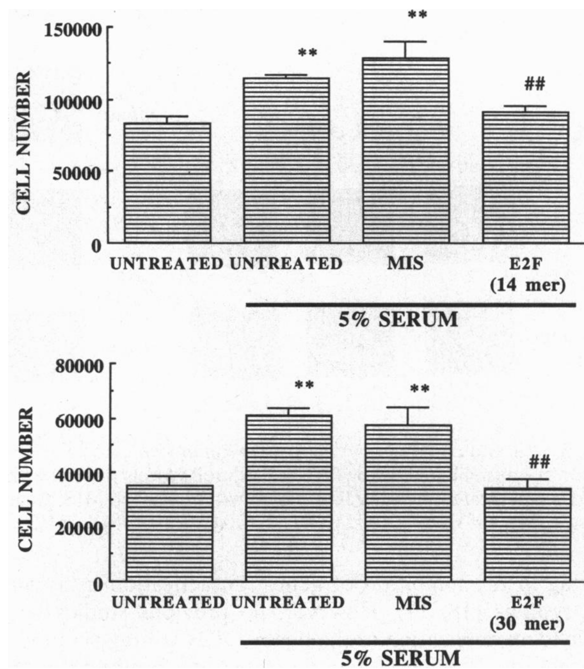

FIG. 2. Effect of 14-mer (*Upper*) and 30-mer (*Lower*) E2F decoy ODNs on VSMC growth at 3 days after transfection. MIS, VSMCs treated with mismatched ODN; E2F, VSMCs treated with E2F ODN. **, $P < 0.01$ vs. untreated; ##, $P < 0.01$ vs. mismatched.

induction of c-*myc*, *cdc2*, and PCNA expression or VSMC proliferation in response to serum stimulation. Similar results were achieved with a 30-mer E2F decoy which contained two 8-bp E2F cis elements (Fig. 2; ref. 8). After 1 week, the *in vitro* inhibitory effect was attenuated, indicating that the E2F decoy exerts a specific and reversible growth inhibitory effect rather than nonspecific cytotoxicity.

Next, we examined the effect of an *in vivo* antigene strategy using the E2F decoy ODN in the rat carotid balloon injury model. After balloon injury, levels of c-*myc*, *cdc-2*, and PCNA mRNA were elevated in carotid vessels transfected with the control missense E2F ODN as detected by RT–PCR (Fig. 3 *A* and *B*). However, *in vivo* transfection of the 14-mer E2F decoy ODN resulted in a marked decrease in c-*myc*, *cdc2*, and PCNA mRNAs. Moreover, the E2F decoy ODN significantly inhibited BrdUrd incorporation (a marker of DNA synthesis) within the vessel wall 4 days after injury (Fig. 3*C*) and markedly suppressed neointima formation 2 weeks after injury when compared with untreated or control-treated vessels (Fig. 4 *A* and *B*). The selectivity of the E2F decoy ODN effect was confirmed further by the demonstration that the inhibition of neointima formation was limited to the area of intraluminal transfection (neointima/medial ratio for transfected segments was 0.291 ± 0.061 and that for untransfected segments was 1.117 ± 0.138, $P < 0.01$). Comparisons of the untreated injured segments across the various treatment groups failed to demonstrate significant differences in the degree of neointima formation induced by balloon injury.

Finally, we examined the long-term efficacy of this strategy. A single administration of E2F decoy ODN resulted in a sustained inhibition of neointima formation up to 8 weeks after transfection, in an apparently dose-dependent manner (Fig. 4*C*). There was no significant difference in neointima formation of E2F decoy (3 μM)-transfected arteries between 2 and 8 weeks after vascular injury.

DISCUSSION

Synthetic double-stranded decoy ODNs can block the binding of nuclear factors to promoter regions of targeted genes,

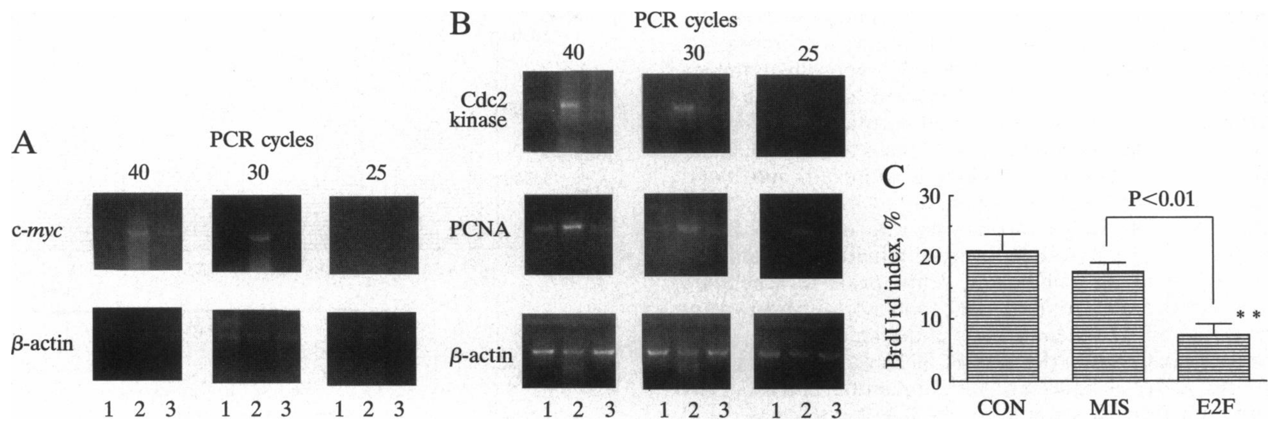

FIG. 3. (*A* and *B*) Effect of decoy ODN on *in vivo* carotid artery gene expression. Lanes: 1, untreated intact vessels; 2, injured vessels treated with control mismatched decoy ODN; 3, injured vessels treated with E2F decoy ODN. (*C*) Effect of decoy ODN on BrdUrd labeling index. CON, injured vessel treated with HVJ–liposome without ODN; MIS, treatment with control mismatched ODN (3 μM); E2F, treatment with E2F decoy ODN (3 μM). **, $P < 0.01$ vs. CON. $P > 0.05$ for CON vs. MIS.

resulting in the inhibition of gene transactivation in *in vitro* assay systems (18, 19). However, no previous studies have described an application of the decoy ODN strategy as *in vivo* gene therapy. This study demonstrates the feasibility of the decoy strategy in the treatment of intimal hyperplasia, the pathological process that underlies restenosis, atherosclerosis, and vascular graft occlusion. Although the rat carotid model does not precisely simulate human restenosis, it is a useful animal model to assess the determinants of VSMC proliferation and migration *in vivo.* The transcription factor E2F is proposed to play a pivotal role in the regulation of cell proliferation by inducing a coordinated transactivation of genes involved in cell cycle regulation (9–13). Accordingly, we demonstrated that decoy binding of E2F in VSMCs can prevent gene expression and thereby inhibit VSMC proliferation and neointima formation *in vivo.*

The specificity of the inhibitory effect of the decoy ODN against E2F on neointima formation is supported by several lines of evidence: (*i*) two different E2F decoy ODNs completely inhibited VSMC proliferation *in vitro*, whereas the mismatched ODN did not, (*ii*) E2F decoy ODN inhibited the expression of the targeted cell cycle-regulatory genes *in vitro* and *in vivo* in a sequence-specific manner, (*iii*) E2F decoy ODN inhibited a quantitative marker of cell cycle progression *in vivo* (BrdUrd labeling), (*iv*) the administration of E2F decoy ODN, but not mismatched or scrambled ODN or the cis-element decoy ODN for the PRE, markedly inhibited neointima formation up to 8 weeks after a single intraluminal

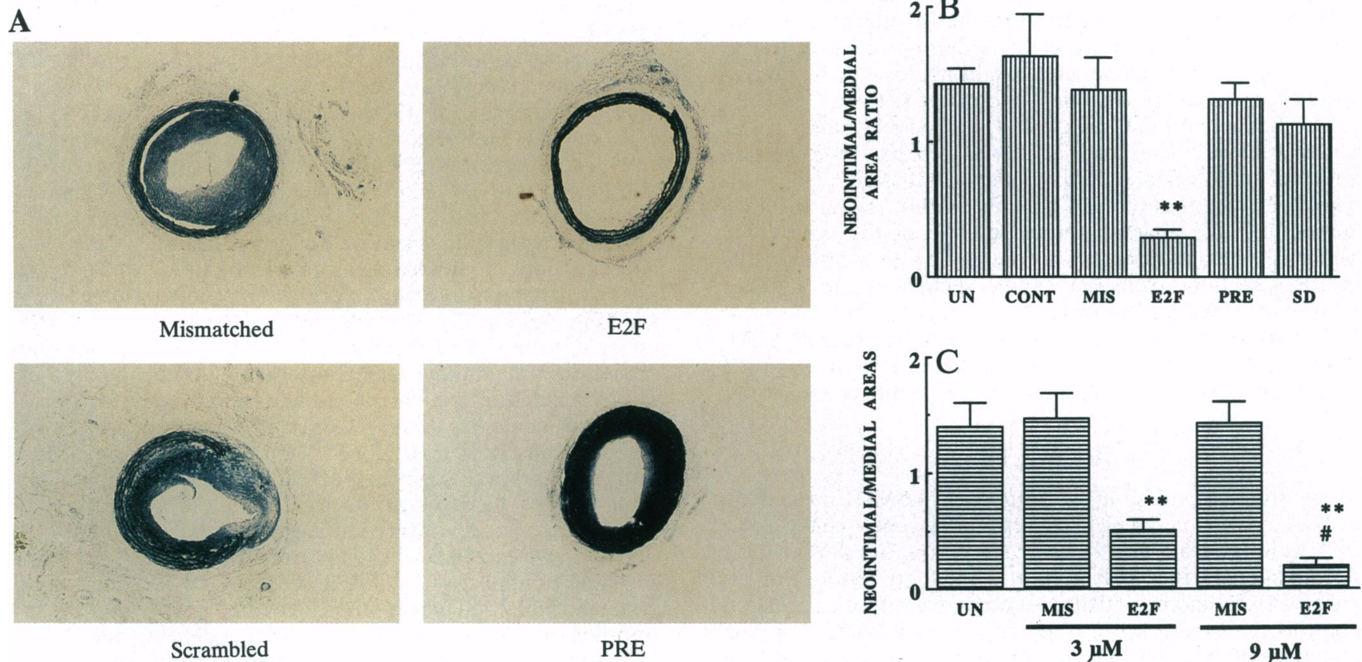

FIG. 4. (*A*) Effect of decoy ODN on neointima formation in injured carotid treated with mismatched ODN, scrambled-sequence ODN, E2F decoy ODN, or PRE decoy ODN. (×40.) (*B*) Effect of decoy ODN on intimal/medial area ratio at 2 weeks after transfection. UN, untreated injured vessels ($n = 5$); CONT, injured vessels treated with HVJ–liposomes without ODN ($n = 5$); MIS, injured vessels treated with mismatched ODN ($n = 8$); E2F, injured vessels treated with E2F decoy ODN ($n = 8$); PRE, injured vessels treated with PRE decoy ODN ($n = 6$); SD, injured vessels treated with scrambled-sequence ODN ($n = 8$); **, $P < 0.01$ vs. other groups. (*C*) Sustained effect of decoy ODN at 8 weeks after transfection. UN, untreated injured vessels; MIS, injured vessels treated with mismatched ODN (3 μM and 9 μM); E2F, injured vessels treated with E2F decoy ODN (3 μM and 9 μM) ($n = 5$). **, $P < 0.01$ vs. MIS and UN; #, $P < 0.05$ vs. 3 μM.

administration, and (*v*) the prevention of neointima formation was limited to the area transfected with the E2F decoy ODN. These converging lines of evidence indicate sequence-specific efficacy of the decoy ODN in the blockade of gene expression and cell proliferation. This approach is attractive because (*a*) the potential drug targets (transcription factors) are plentiful and readily identifiable, (*b*) the synthesis of the sequence-specific decoys is relatively simple, (*c*) knowledge of the exact molecular structure of the targeted transcription factor is unnecessary, and (*d*) decoy ODNs may be more effective than antisense ODNs in blocking constitutively expressed factors as well as multiple transcription factors that bind to the same cis element (27, 28).

The sustained effectiveness of a single administration of the E2F decoy in preventing neointima formation is an intriguing observation that is similar to our previous report describing the efficacy of antisense ODNs directed against the expression of cell cycle-regulatory genes (20). It appears that blockade of the initial wave of medial cell replication within the first week after vascular injury in this model provides sustained efficacy in preventing neointima formation. We speculate that the arrest of cell cycle progression due to the blockade of cell cycle-regulatory gene expression also influences other processes critical to the neointima formation, such as cell migration and the balance of matrix protein production and degradation. Moreover, we speculate that indirect influences on re-endothelialization and vascular remodeling that occur subsequently may also contribute to the long-term inhibition of neointima formation *in vivo* (26).

In this study, we report a therapeutic strategy to prevent neointimal hyperplasia. Although studies of this technology in several other animal models (e.g., the pig and primate) remain to be performed, our data show that the transcription factor decoy strategy can modulate *in vivo* gene transcription and vascular pathobiology in the intact animal. Further studies are necessary to enhance cell targeting and minimize effects on endothelial cell replication at the periphery of the injured transfected area. This technology offers great promise as a tool for defining biological processes and treating pathological conditions.

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