Role of E2F1–Cyclin E1-Cyclin E2 Circuit in Human Coronary Smooth Muscle Cell Proliferation and Therapeutic Potential of Its Downregulation by siRNAs

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Aberrant coronary vascular smooth muscle cell (CSMC) proliferation is a pivotal event underlying intimal hyperplasia, a phenomenon impairing the long-term efficacy of bypass surgery and angioplasty procedures. Consequently research has become focused on efforts to identify molecules that are able to control CSMC proliferation. We investigated downregulation of CSMC growth by small interfering RNAs (siRNAs) targeted against *E2F1*, *cyclin E1*, and *cyclin E2* genes, whose contribution to CSMC proliferation is only now being recognized. Chemically synthesized siRNAs were delivered by two different transfection reagents to asynchronous and synchronous growing human CSMCs cultivated either in normo- or hyperglycemic conditions. The depletion of each of the three target genes affected the expression of the other two genes, demonstrating a close regulatory control. The clearest effects associated with the inhibition of the E2F1–cyclin E1/E2 circuit were the reduction in the phosphorylation levels of the retinoblastoma protein pRB and a decrease in the amount of cyclin A2. At the phenotypic level the downmodulation of CSMC proliferation resulted in a decrease of S phase matched by an increase of G1-G0 phase cell amounts. The antiproliferative effect was cell–donor and transfectant independent, reversible, and effective in asynchronous and synchronous growing CSMCs. Importantly, it was also evident in hyperglycemia, a condition that underlies diabetes. No significant aspecific cytotoxicity was observed. Our data demonstrate the interrelation among E2F1–cyclin E1-cyclin E2 and the pivotal role this circuit exerts in CSMC proliferation. Additionally, our work validates the concept of utilizing anti–E2F1–cyclin E1-cyclin E2 siRNAs to develop a potential novel therapy to control intimal hyperplasia.

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INTRODUCTION

In adult blood vessels, vascular smooth muscle cells are mainly involved in controlling vessel tone to regulate blood flow distribution and blood pressure. In these conditions, the cell proliferation rate is very low. However, in response to external stimuli induced, for example, by vascular injury, the growth rate dramatically increases and is accompanied by a number of other cellular modifications collectively known as "phenotypic modulation" (1). Whereas augmented growth rate is a relevant physiological process in vascular repair, its aberrant increase has also been implicated in the pathogenesis of various hyperproliferative vascular diseases such as vein graft occlusion, coronary bypass surgery, in-stent restenosis (ISR), atherosclerosis, and hypertension (2). In these conditions, vascular smooth muscle cell proliferation, together with a number of other pathophysiological events (3), is responsible for the thickening of the intimal vessel layer (intimal hyperplasia [IH]) with the consequent reduction of blood flow. In the case of coronary ISR (3), the downregulation of coronary smooth muscle cell (CSMC) growth by potent antiproliferative and proapoptotic drugs released from endoluminal stents (drug-eluting stent) has been shown to significantly reduce IH (4). This benefit is limited to low-risk patients, however,

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with discrete, *de novo* lesions in native coronary vessels. In high-risk patients, such as diabetics, IH occurrence is significantly higher (5). Moreover, the drug-eluting stent in current use has been associated with an increased risk of late stent thrombosis (6). Thus, the understanding of the molecular mechanisms controlling vascular smooth muscle cell proliferation, a pivotal event in IH generation, is crucial in the management of ISR as well as in other hyperproliferative vascular diseases.

E2F1 is a transcription factor directly implicated in the regulation of many cellular processes, including cell proliferation (7). When retinoblastoma protein (pRb) is phosphorylated by cyclin-dependent kinase (Cdk)-bound cyclin D, E2F1 is released from the pRb-E2F1 complex. The free E2F1 induces the transcription of cyclin E1, which, bound to its Cdk, phosphorylates pRb and further increases the amount of free E2F1, which in turn induces the transcription of many S-phase genes. The existence of this E2F1–cyclin E1 positive feedback loop promoting cell growth in different cell types is well known (8). However, despite recent data on the contribution of E2F1 and cyclin E1 to CSMC proliferation (9,10), specific information on their interrelationship is missing. Importantly, nothing is known about the role of cyclin E2, the second component of the cyclin E family (11), to the E2F1–cyclin E1 feedback loop and thus to CSMC proliferation. To investigate these aspects, we used specific small interfering RNAs (siRNAs) to deplete E2F1 and cyclin E1 and E2 in cultured human CSMCs. The effects on CSMC proliferation were evaluated in asynchronous and synchronous growing CSMCs obtained from different donors using different transfectants and culturing conditions. In addition, the consequence for the other two genes from the depletion of each of the target genes was also investigated, and the effects on the other cell-cycle relevant genes related to the E2F1–cyclin E loop were determined.

MATERIALS AND METHODS

Cell Culture and siRNA Selection

Primary cultures of human CSMC (Promocell, Heidelberg, Germany), isolated from two different donors (donor 1, white female, age 58 years; donor 2, white male, age 35 years) were used. For all experiments, cells in the third passage were considered. CSMCs were cultured as described (12). siRNAs directed against cyclin E1 (siCycE1-1415 sense 5′-GAGCGGUAAGAAGCAGAGCdTdT-3′, siCycE1-827 sense 5′-GAGGAAAUCU AUCCUCCAAdTdT-3′), cyclin E2 (siCycE2-647 sense 5′-GCUUGCAGUG AAGAGGAUAdTdT-3′, siCycE2-946 sense 5′-GGAGUGGGACAGUAUUUCAd TdT-3′), and E2F1 (siE2F1-1324 sense 5′-GAGGAGUUCAUCAGCCUUUdTdT-3′, siE2F1-1117 sense 5′-GCCACCAUAG UGUCACCACdTdT-3′) were chemically synthesized (Eurogentec SA, Seraing, Belgium) and selected according to previously reported guidelines (13). Additionally, a control siRNA directed against the *luciferase* gene (siRNAGL2 sense 5′-CGUACGCGGAAUACUUCGAdTdT-3′) was used.

siRNA Transfection

CSMCs were seeded at a density of 3.3×10^3 cells/cm^2 in 6-cm diameter plastic dishes in the presence of 4 mL of complete medium containing 15% fetal calf serum. In the experiments performed with asynchronized cells, transfections were conducted the d after seeding, whereas in the case of synchronized cells, a starvation time of 48 h in serum-free medium preceded transfection. Five μg of each siRNA were mixed at different weight ratios with the selected liposomes (Cellfectin, Invitrogen, Basel, Switzerland; Metafectene; Biontex Laboratories GmbH, Munich, Germany) in 200 μL of serum-free medium, and complexes were allowed to form for 20 min at room temperature. Afterward, 1.3 mL of serum-free medium was added to the 200 μL, mixed, and applied to the cells previously washed with phosphate-buffered saline (PBS) to reach an siRNA concentration of 230 nmol/L. After 2 h at 37°C, transfection medium was removed, cells were washed with PBS, and 4 mL of complete medium or PBS was added to the cells, depending on whether they were used for proliferation inhibition experiments or for uptake studies, respectively.

Uptake Studies

Transfections were performed using the siRNAGL2 control siRNA carrying the 5′ end of the sense strand labeled by fluorescein isothiocynate (FITC). Living cells were observed under a fluorescence microscope (Leica Microsystem, Wetzlar, Germany) and photographed. Subsequently, cells were trypsinized and resuspended in 500 μL of PBS, and the number of FITC-positive cells was evaluated by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA; DIVA software, Dialogic, Montreal, Canada).

Western Blot

Protein extraction and immunoblotting were performed as previously described (14) using 20 μg of complete cell lysate. The antibodies mouse anti-CdK 2 (1 μg/mL), mouse anti-p16^{INK4} (1 μg/mL), and rabbit anticyclin E2 (1 μg/mL) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), BD Biosciences, and Lab Vision (Fremont, CA, USA), respectively.

Quantitative Reverse-Transcription–Polymerase Chain Reaction (QRT-PCR)

Total RNA extraction; the evaluation of RNA quality, integrity, and quantification; reverse transcription; and polymerase chain reaction amplification were performed as previously described (15), except that the primers for cyclin E2, 2′,5′-oligoadenylate synthetase 1 (OAS1) and 28S ribosomal RNA were forward 5′-CTTCCAAACTTGAGGAAATC-3′/reverse 5′-TCCATCCTTAAGATATCCTC-3′, forward 5′-TCCAAGGTGGTAAAG GGTGG-3′/reverse 5′-AGGTCAGCGT CAGATCGGC-3′, and forward 5′-

TGGGAATGCAGCCCAAAG-3′/reverse 5′-CCTTACGGTACTTGTTGACTATGC-3′, respectively. The annealing temperatures for each primer couple were 56°C, 62°C, and 60°C, respectively. All amplification reactions were conducted in triplicate, utilizing SYBRGreen Master Mix buffer (Applied Biosystems, Foster City, CA, USA) and 1 μL of cDNA. The relative amounts of the mRNA of target genes were normalized by 28s rRNA content according to Pfaffl (16).

Cytotoxicity, Apoptosis Cell Cycle, and Migration Tests

Cytotoxicity was evaluated by lactate dehydrogenase assay kit according to manufacturer instructions (BioVision, Mountain View, CA, USA). Triton X-100–treated cells (1% of final concentration) were used as a positive control. Apoptosis was evaluated by the Annexin V test (Bender MedSystems, Burlingame, CA, USA) as described (14) followed by flow cytometry (FACSCanto, BD; DIVA software). Cell-cycle phase evaluation and BrdU incorporation were performed as described (12) by flow cytometry. The amount of incorporated BrdU in siRNA-treated cells over time was described by the following equation:

$$\text{BrdU incorporation} = a^*t^2 + b^*t + c \quad \text{(eq 1)},$$

where t is time (in d) and a, b, and c are fitting parameters that corresponded to 9.2 ± 1.2/–57.5 ± 9.0/135.0 ± 13.0 for siE2F1-1324–treated cells, to 7.2 ± 1.6/–50.7 ± 11.6/156.0 ± 17.4 for siCyE1-1415–treated cells, and 7.4 ± 0.1/–48.9 ± 0.5/136.5 ± 0.8 for siCyE2-647–treated cells. The fitting parameters define the behavior of BrdU incorporation in all the different conditions considered.

CSMC migration assay was performed by the fluorescence-assisted transmigration motility assay as described (17). In brief, 3 d after transfection CSMCs were trypsinized, stained by the vital dye Fast DiI™ (Molecular Probes, Eugene, OR, USA), and seeded on the upper side of Transwell-like inserts (HTS FluoroBlock™ inserts, BD) with a porous polyethylene terepthlate membrane (8-μmol/L pores) previously coated by collagen IV; the amount of cells that migrated from the top side to the bottom side of the porous membrane was detected by a Tecan Infinite 200 microplate fluorometer (Tecan Group, Mannerdorf, Switzerland) up to 5 h post seeding.

Statistics

Values are expressed as mean ± SEM. Statistical significance was determined by one-way analysis of variance and the appropriate t test; a value of $P < 0.05$ was considered to be statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Uptake studies (supplementary material 1) indicated that both transfection reagents considered (Cellfectin and Metafectene) had an excellent transfection efficacy in CSMCs as evaluated by using a 5′fluorescein (FITC)-labeled siRNAGL2 (siGL2, control siRNA directed against the *luciferase* gene).

siRNA Effects on the Protein and mRNA Levels of the Respective Targets

Our experiments were performed using CSMCs obtained from two independent donors. For each target gene, two siRNAs were synthesized without any chemical modifications. Three d after transfection, using Cellfectin on CSMCs of donor 1, the selected siRNAs (230 nmol/L) could be demonstrated to reduce the protein levels of each target (Figure 1A). Comparable results were obtained using the most active siRNAs (siRNAE2F1-1324, siRNACycE1-1415, and siRNACycE2-647 directed against E2F1, cyclin E1, and cyclin E2, respectively), in the presence of the transfection reagent Metafectene (Figure 1B). The cumulative data obtained from Cellfectin and Metafectene transfections are reported in Figure 1C. Importantly, these results were repeated with donor 2 CSMCs (data not shown).

Inhibition of target gene expression was confirmed at the mRNA level for both transfectants (Figure 1D–E). Comparable results were obtained, from the qualitative point of view, with the less active siRNAs (supplementary material 2) siRNAE2F1-1117, siRNACycE1-827, and siRNACycE2-946.

siRNA Effects on CSMC Death, Proliferation, and Migration

Three d after transfection, the selected siRNAs significantly reduced CSMC number (supplementary material 3) down to 57%, 84%, and 83% for siRNAE2F1-1324, siRNACycE1-1415, and siRNACycE2-647, respectively, compared with controls. This observation, together with the clear effects on target gene expression reported above, was not accompanied by an increase in OAS-1 gene expression and therefore could not be ascribed to an aspecific induction of the interferon response (18) (supplementary material 4A). Additionally, neither significant cytotoxicity nor apoptosis were induced (supplementary material 4B–C). Similar results were observed at shorter (2 d) and longer (5 d) time points after transfection (data not shown).

Consistent with the reduction in cell number, a clear reduction in cell proliferation was observed. Representative dot plots for donor 1 (Figure 2A) and the cumulative data obtained for donor 1 and 2 CSMCs using Cellfectin as transfectant (Figure 2B) indicated a marked reduction of S-phase and a parallel increase of G1/G0-phase cells. Comparable results were obtained using Metafectene as transfectant (data not shown).

The time course effects of the selected siRNAs on cell proliferation (BrdU incorporation) indicated a reversible inhibition of CSMC proliferation (Figure 3A). Despite a quantitative difference among the three siRNAs, as indicated by eq. 1 parameters (see Materials and Methods), the maximum of BrdU incorporation inhibition [occurring in correspondence of parabola minimum, *i.e.* for $t = -b/(2^*a)$] was similar for all the siRNAs considered and occurred just after d 3 (3.1 d for

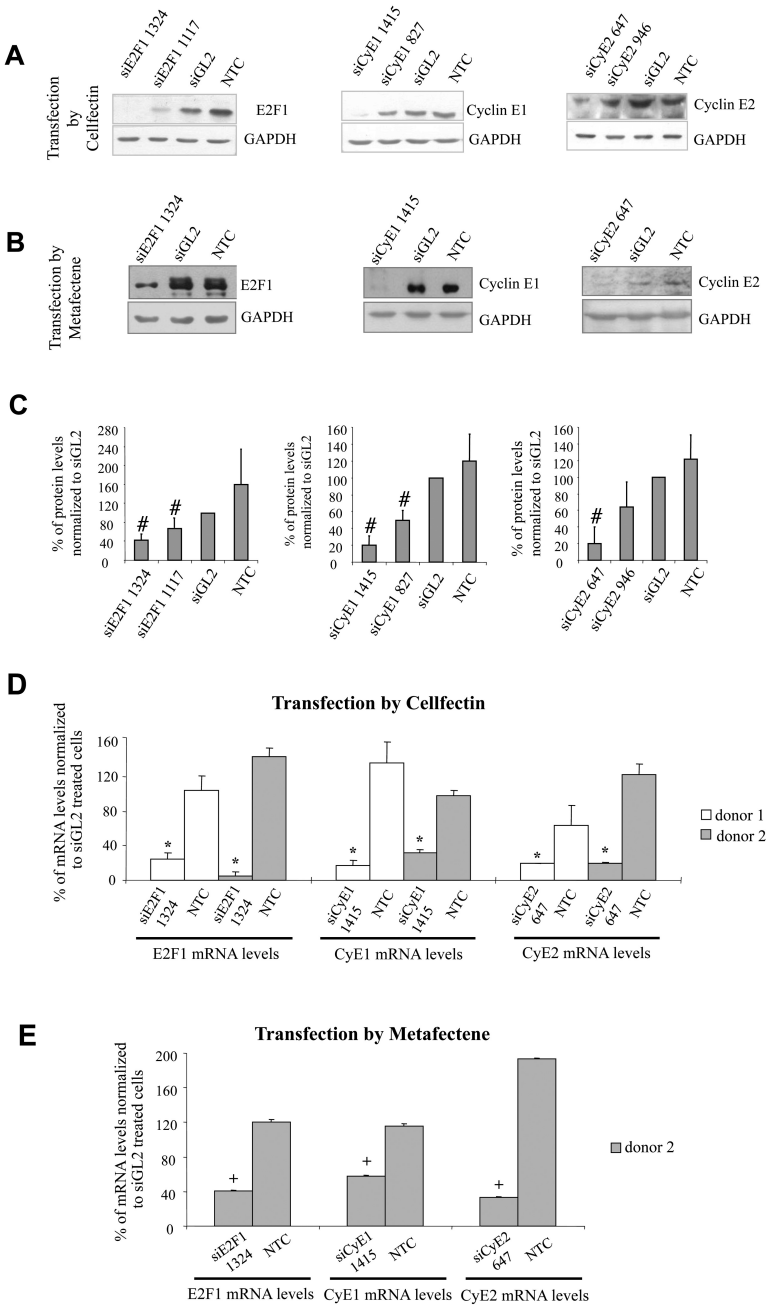


Figure 1. siRNA effects on the protein and mRNA levels of the respective targets. (A) Two siRNAs for each target gene were delivered to CSMCs from donor 1 by Cellfectin and the protein levels evaluated 3 d after transfection; representative blots are reported. (B) The most effective siRNA among each couple was delivered by Metafectene and the protein levels evaluated (CSMCs from donor 1), shown are representative blots. (C) Cumulative data obtained from both Cellfectin and Metafectene transfection are indicated; data, reported as mean ± SEM, n = 5, were normalized to *GAPDH* and expressed as ratio of siGL2 treatment, $^{\#}P < 0.02$ compared with control. (D) Target mRNA levels were evaluated after siRNA delivery either by Cellfectin (CSMCs from donors 1 and 2) or (E) Metafectene (CSMCs from donor 2); *28S* transcript levels were used to normalize the data; NTC, non-treated cells; siGL2, control siRNA-treated cells, data are expressed as mean ± SEM, n = 4. $^{*}P < 0.001$ and $^{+}P < 0.05$ compared with control.

siE2F1-1324, 3.5 d for siCyE1-1415, and 3.3 d for siCyE2-647). Notably, the decrease of BrdU incorporation was paralleled by a consistent reduction in the target protein levels (Figure 3B–D).

Augmented CSMC proliferation in diabetics is a relevant element responsible for the increased risk of developing severe hyperproliferative vascular diseases (19). Thus, our siRNAs were tested in CSMCs cultured in 22 mmol/L glucose (Figure 4A), a concentration commonly adopted to mimic the condition of poorly controlled diabetes (14). Notably, under these conditions, BrdU incorporation into CSMCs exceeds that of CSMCs cultured in normal glucose of 21% ± 12%. Cumulative data from donor 1 and 2 CSMCs indicate the antiproliferative efficacy of the selected siRNAs under a high glucose concentration (approximately fourfold higher than the normal one corresponding to 5 mmol/L).

The experiments presented so far were conducted on starved CSMCs (synchronous cells) to which growth factors (serum) were added just after the end of siRNA administration. This experimental setup was intended to mimic the condition of *in vivo* resting CSMCs exposed to blood growth factors and antiproliferative drugs at the time of recanalization interventions, a condition typically occurring during ISR treatment. However, the presence of variable amounts of proliferating CSMCs prior to recanalization interventions (20) prompted us to investigate siRNA efficacies on actively growing cells (asynchronous cells). Cumulative data from donor 1 and 2 CSMCs confirmed that antiproliferative effects of the selected siRNAs also occurred in asynchronous cells (Figure 4B).

In addition to the augmented CSMC proliferation, increased CSMC migration is a relevant element responsible for the development of hyperproliferative vascular diseases (3). We therefore evaluated the antimigratory potential of the selected siRNA by using the fluorescence-assisted transmigration motility assay (17). As shown in Figure 4C, our siRNAs were able to reduce the migration of

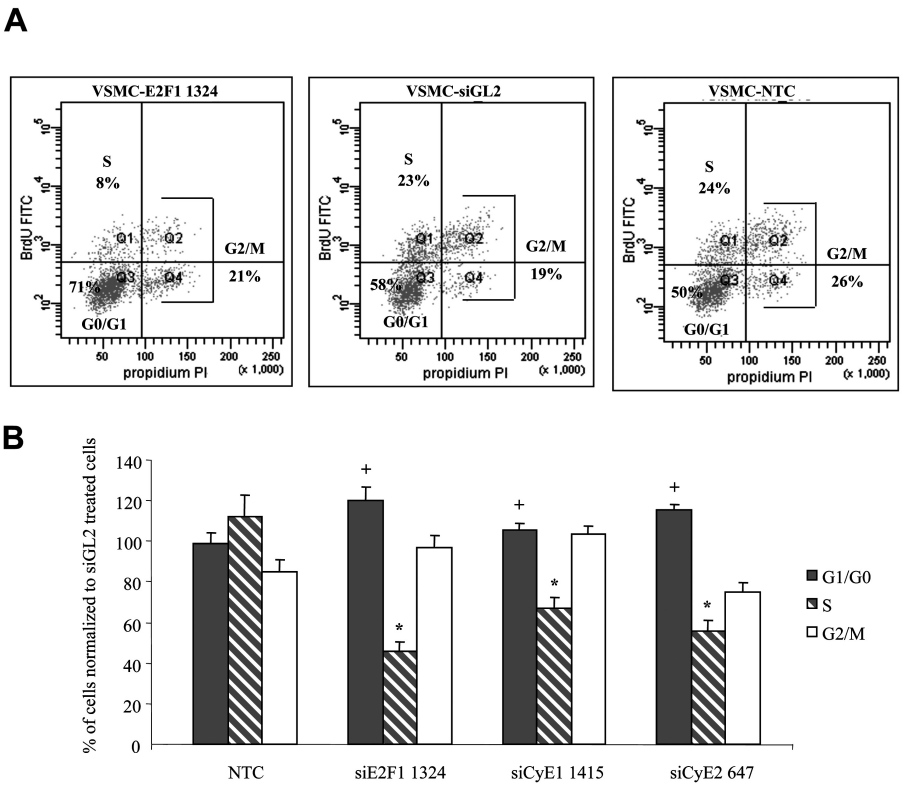


Figure 2. siRNA effects on cell proliferation. (A) Cell-cycle phase distribution data were obtained 3 d after siRNA transfection: a representative example relative to siE2F1-1324–treated CSMCs (donor 1) and its control is reported; BrdU FITC, Ig anti-BrdU labeled by FITC; PI, propidium-iodide staining. (B) Cumulative data for donors 1 and 2 indicate, for all siRNAs, a decrease of S-phase and an increase of G1-G0–phase cells; data for each cell-cycle phase are reported as percentage of the values measured in siGL2-treated cells and are expressed as means ± SEM, n = 9. $^{*}P < 0.001$ and $^{+}P < 0.05$ compared with control.

synchronized CSMCs compared with controls.

Effects of Cyclin E1 and E2 and E2F1 Depletion on the Expression Levels of Each Other

To elucidate the mechanisms leading to the observed impairment of the G1–S phase transition and to define the interrelation among E2F1–cyclin E1/E2, we studied the effects of the targeting of each of the E2F1–cyclin E1/E2 on the other two (Figure 5). Cyclin E1 depletion resulted in the decrease of both cyclin E2 and E2F1 protein (Figure 5A) and mRNA levels (Figure 5B). Cyclin E2 depletion gave comparable results on the protein and mRNA levels of cyclin E1 and E2F1 (Figure 5A). Three d after siE2F1-1324 transfection, E2F1 depletion generated a considerable reduction in cyclin E1/E2 mRNA levels but not in the protein levels (Figure 5A). However, cyclin E1/E2 protein levels decreased at d 5 post transfection (approximately down to 60% of control). Notably, the less active siRNAs gave, from the qualitative point of view, similar results (data not shown).

Finally, upon depletion of each of the E2F1–cyclin E1/E2 target proteins, a reduction in the cyclin A2 protein level was observed (Figure 6A). In addition, the levels of the hyperphosphorylated form of the protein pRb displayed an evident reduction in E2F1/cyclin E2–depleted cells; a tendency to the reduction was observed in cyclin E1–depleted cells. Only in the case of E2F1 depletion was a clear decrease of Cdk2 observed. We also observed that siE2F1-1324 treatment produced the transcriptional downregulation of cyclin A2 and Cdk2 (Figure 6B). By contrast, no major variations were detected for protein levels of cyclin D1, $p21^{cip1}$, $p16^{INK4}$, and $p27^{kip1}$ (Figure 6A).

Combined Administration of siRNAs

Our demonstration of the relationship between E2F1–cyclin E1/E2 and the contribution of these proteins to the proliferation of CSMCs led us to investigate whether the contemporary targeting of these genes might have produced a more effective inhibition of CSMC proliferation. With a final siRNA concentration of 230 nmol/L, however, no appreciable differences were observed for all the combinations tested (Figure 6C). Similar results were observed when we increased the total siRNA concentration up to 320 nmol/L (data not shown). At higher siRNA concentrations, the transfection-induced cytotoxicity interfered with experimentation.

DISCUSSION

The data presented indicate the potency of the selected siRNAs in reducing the target mRNA and protein levels (Figure 1). Notably, similar efficacies were observed for CSMCs obtained from two independent donors. This fact suggests a patient-independent effect that increases the significance of our findings. It was also clear, from the qualitative point of view, that comparable effects were obtained using either the most or the least active siRNAs (supplementary material 2). This finding makes the possibility that the observed results depend on an siRNA off-targeting effect very unlikely. Additionally, the activity of the selected siRNAs was not dependent on the transfectants (Figure 1), an observation that favors the concept of a pure siRNA-mediated mechanism of action. This hypothesis supports the possibility that the selected siRNAs may also maintain their efficacy when delivered by more complex systems, that is, those required for

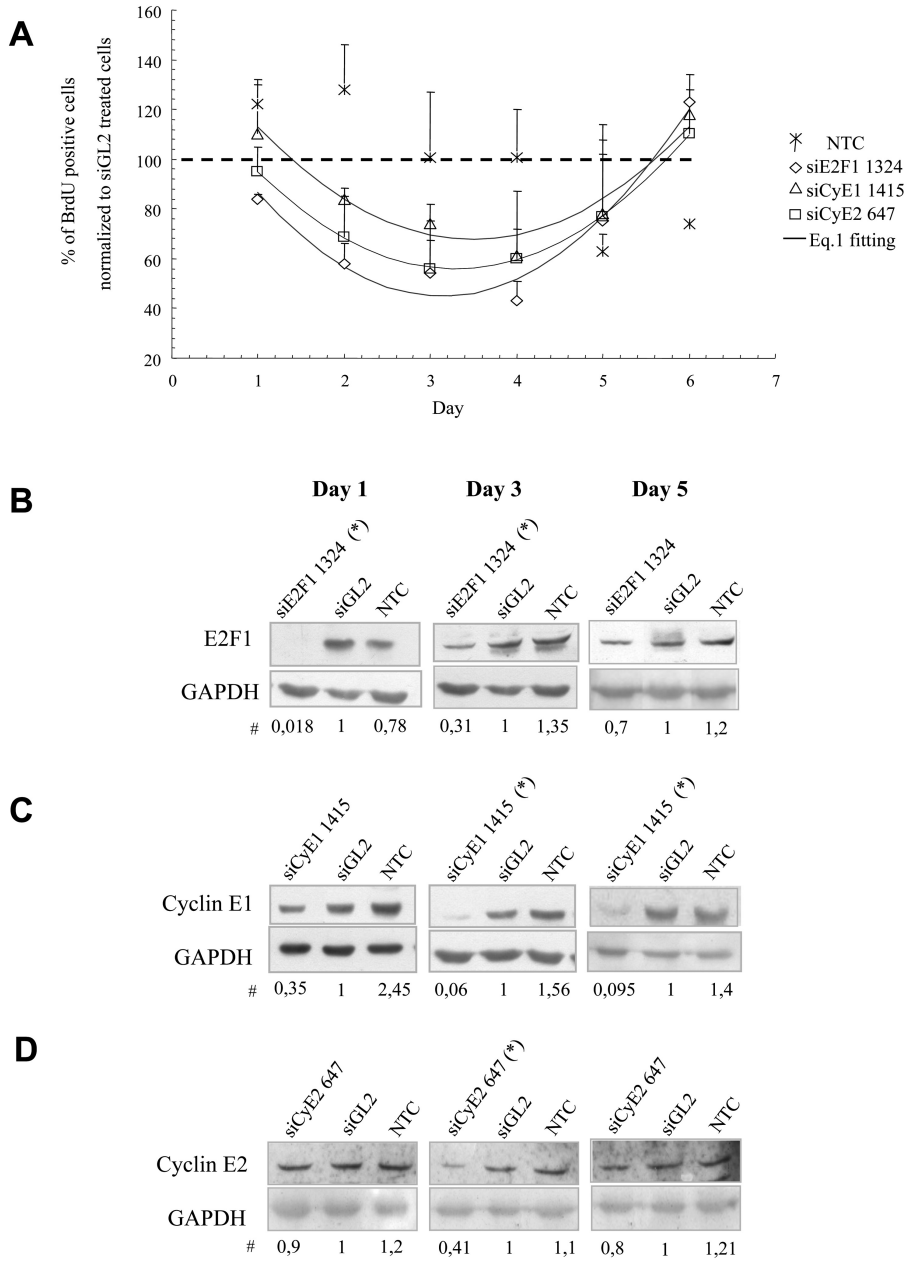


Figure 3. Time course effects of the selected siRNAs on CSMC proliferation. (A) Inhibition of CSMC proliferation, as evaluated by BrdU incorporation, lasted up to d 5 post transfection; the kinetics of cell proliferation reduction for siRNA-treated cells was mathematically evaluated by equation 1 (black lines); cumulative data from both donors are expressed as mean ± SEM, n = 6. (B–D) Proliferation inhibition was paralleled by a concomitant reduction of target proteins; shown are representative blots from donor 1 performed using Cellfectin as transfectant; #all the numbers reported below the blots derive from band quantification normalized to *GAPDH* and are expressed as the ratio of siGL2 treatment; *$P < 0.02$ compared with controls, n = 4; NTC, nontreated cells; siGL2, control siRNA-treated cells.

in vivo delivery (21,22). Indeed, *in vivo*, to overcome siRNA washout by the blood, a robust method of delivery is necessary. In addition, given the relatively short effects of the siRNAs (up to 5 days, Figure 3), a prolonged delivery is of utmost importance, because it is commonly accepted that *in vivo* the antiproliferative effect should be exerted for some months to downmodulate IH developing during ISR.

The target gene inhibition resulted in a downregulation of CSMC proliferation characterized by a significant reduction in the amount of S-phase cells with an increase of G1-G0–phase cells (Figure 2). In addition to being patient independent, the extent of this phenomenon is extremely encouraging for future *in vivo* application. Of interest is the observation that the siRNA effect is reversible (Figure 3A) and is associated with neither significant cytotoxicity nor apoptosis (supplementary material 4B–C). Thus, the impact on CSMC biology is specific, an observation that is also supported by the failure to detect any evidence of induction of the interferon response (supplementary material 4A) (18). This feature may confer to our siRNAs an advantage over the commonly used drugs for ISR, a hyperproliferative vascular disease commonly treated with stents able to release drugs such as rapamycin (23). This kind of molecule, in addition to having an antiproliferative action, displays a potent apoptotic effect (24), which in the long term can cause excessive cell death—a fact that might partially explain its reduced efficacy in high-risk patients (5) and the recently reported problems of late stent thrombosis (6).

In conditions of high glucose concentrations, CSMCs grow faster than in normal glucose concentrations (25). This observation is a contributing factor in understanding why in diabetic patients hyperproliferative vascular diseases such as ISR develop faster and more frequently than in normoglycemic patients (26). To explore the effectiveness of our siRNAs in the unfavorable conditions of high glucose concentration, siRNA antiproliferative efficacy was tested in CSMCs grown in the presence of 22 mmol/L glucose (Figure 4A). This glucose concentration was chosen to mimic the conditions associated with poorly controlled

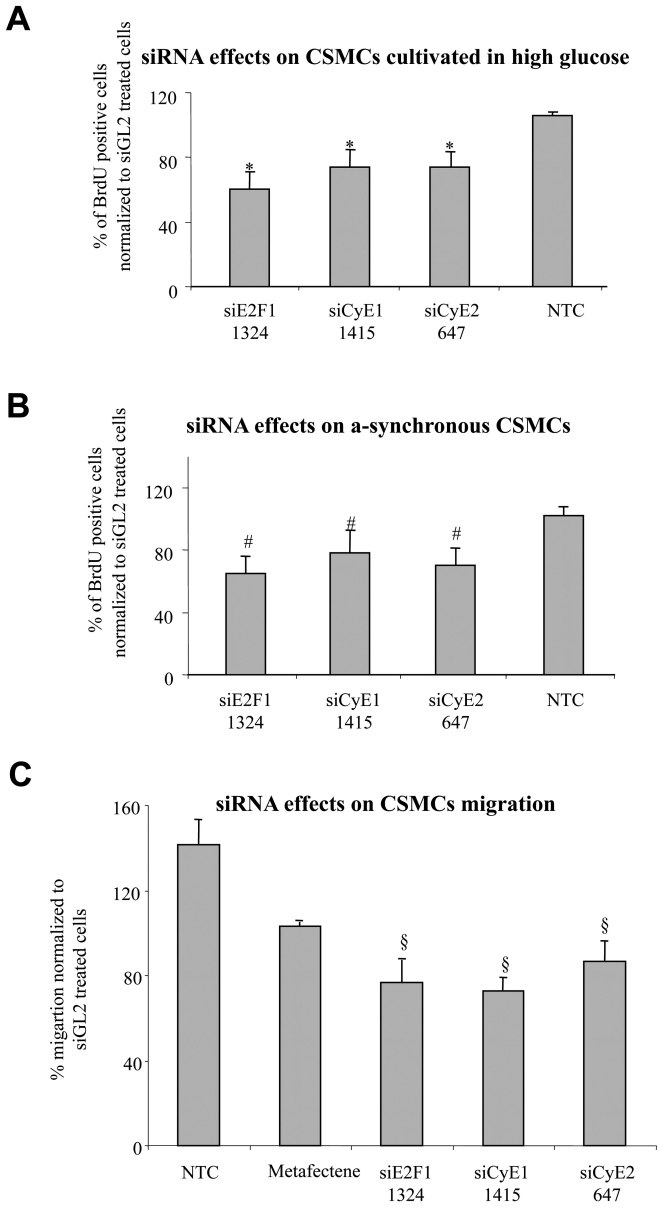


Figure 4. siRNA effects on CSMC proliferation in conditions of hyperglycemia or in asynchronous cells and on CSMC migration. (A) Three d after transfection, selected siRNAs were able to reduce the proliferation of CSMCs cultivated in conditions of high glucose (22 mmol/L) compared with controls (*$P < 0.05$). (B) Selected siRNAs were able to reduce the proliferation of asynchronous CSMCs ($^{\#}P < 0.05$, comparing siRNA-treated to control asynchronous cells); NTC, nontreated cells; siGL2, control siRNA-treated cells; cumulative data from both donors are expressed as mean ± SEM, n = 4. (C) Three d after transfection, the migration rates of siRNA-treated cells were tested as detailed in Materials and Methods; the selected siRNAs were able to reduce the migration of synchronous CSMCs cultivated in conditions of normal glucose compared with controls ($^{\S}P < 0.04$); cumulative data from both donors are expressed as mean ± SEM, n = 15.

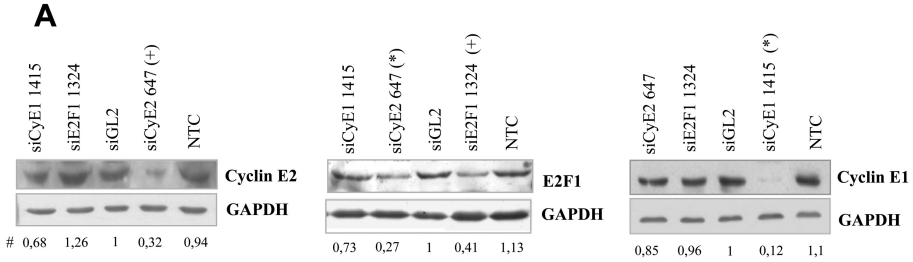
diabetes (14). Although we accept that the simulation of the diabetic condition we adopted may not completely reproduce the complexity of the diabetic patient, the data we present do indicate the efficacy of the selected siRNAs in downmodulating CSMC growth in hyperglycemia, thus extending their potential effectiveness to diabetics.

Another positive feature of our approach is the fact that the selected siRNAs also achieved CSMC proliferation inhibition when applied to asynchronous growing cells (Figure 4B). We believe this information is potentially relevant for future *in vivo* applications in which drugs will encounter both resting and actively growing CSMCs in the vessel wall (20). Our study also revealed that E2F1 knockdown reduces CSMC migration (Figure 4C), a pathophysiological event that greatly contributes to the development of hyperproliferative vascular diseases (3). These data are in agreement with the observed reduced migration in E2F1 knockout keratinocytes (27). Notably, the impaired migration detected upon cyclin E1/2 knockdown (Figure 4C) may be dependent on the concomitant downregulation of E2F1 expression (Figure 5). Future investigations will further clarify the molecular mechanisms ruling this phenomenon.

We observed that the transient depletion of cyclin E1 or E2 reduces the growth rate both in asynchronous (Figure 4B) and synchronous CSMCs (cells reentering the cell cycle) (Figure 2). However, cyclin E1 or E2 knockout mouse embryonic fibroblasts (MEFs) can grow and develop as efficiently as wild-type MEFs and can retain a normal capacity to reenter the cell cycle from quiescence (28). This discrepancy with our data may be attributable to the fact that cyclin E1 or E2 knockout MEFs adapted to cyclin E1/E2 absence. In contrast, in our CSMCs, the transient depletion was able to subvert the proliferation processes. Additionally, differences among species (mouse and human) cannot be excluded. In this sense, in contrast to MEFs, cyclin E2 knockout *Xenopus laevis* cells cannot develop and proliferate (29).

Whereas the relevance of E2F1 in IH generation has been recently reported (10), its relation with cyclin E1/E2 in CSMCs has never been investigated. Our

A



	E2F1 protein levels	Cyclin E1 protein levels	Cyclin E2 protein levels
siE2F1-1324		§ 99% ± 20	§ 100% ± 11
siCyE1-1415	68% ± 14		64% ± 15
siCyE2-647	41% ± 4	82% ± 16	

B

	E2F1 mRNA levels	Cyclin E1 mRNA levels	Cyclin E2 mRNA levels
siE2F1-1324		* 51% ± 14	* 46% ± 11
siCyE1-1415	* 18% ± 9		* 12% ± 5
siCyE2-647	* 47% ± 9	* 45% ± 10	

Figure 5. Interrelation among E2F1, cyclin E1 and E2 in CSMCs. Three d after transfection, each of the target genes was depleted by the respective siRNAs and the protein levels (A) of the other two target genes evaluated: in the upper panel, representative blots from three independent experiments are reported (CSMCs from donor 2), in the lower panel the total data are shown (CSMCs from donors 1 and 2); #all the numbers reported below the blots are derived from band quantification normalized to *GAPDH* and are expressed as a ratio of siGL2 treatment; §protein levels were reduced at d 5 post transfection; in (B) the mRNA levels corresponding to the data shown in (A) are reported (*28S* transcript levels served as normalizator); cumulative data from both donors are expressed as mean ± SEM, n = 6; *$P < 0.05$ compared with control.

data support the direct transcriptional control of E2F1 on cyclin E2 and cyclin E1 (Figure 5B). Moreover, our finding that cyclin E2 depletion is paralleled by a reduction of E2F1–cyclin E1 mRNA levels (Figure 5B) and by the reduction of the amount of the hyperphosphorylated form of pRb (Figure 6A) strongly supports the idea that cyclin E2 actively participates in the E2F1–cyclin E1 positive feedback loop (8) in human CSMCs. Furthermore, the observation that cyclin E1 depletion results in the transcriptional downregulation of cyclin E2–E2F1 further stresses the interrelation among these three genes, an observation we also confirmed using the less active siRNAs (data not shown). In agreement with the mutual interaction among E2F1–cyclin E1/E2, we observed that the depletion of each of these genes reduced the expression of cyclin A2, a known transcriptional target of E2F1 (Figure 6A). Less evident, although present, were the effects on another E2F1-regulated gene, *Cdk2*. These observations together with the reduction in the amounts of the hyperphosphorylated form of pRb are compatible with the G1-S–phase block we observed (Figure 2).

Surprisingly, the simultaneous targeting of two members of the E2F1–cyclin E1/E2 circuit did not improve the antiproliferative effects compared with the single targeting of each of them (Figure 6C). A competition between the different siRNAs might account for this observation (30). Alternatively, a suboptimal amount of the siRNA used (115 nmol/L each) cannot be excluded. This hypothesis is in line with the fact that when we combined the three siRNAs (73 nmol/L each), proliferation inhibition tended to be even less pronounced. Unfortunately, the *in vitro* setup did not allow a marked increase in the total siRNA concentrations owing to an aspecific transfection-related toxicity (data not shown). Finally, because the targeting of each of the three members induces the downregulation of the expression of the other two (Figure 5), it is possible that an additional downregulation by specific siRNAs cannot further impair the E2F1–cyclin E1/E2 circuit and thus cell proliferation. According to this hypothesis, targeting the E2F1–cyclin E1/E2 circuit is relevant and effective for the purpose of inhibiting proliferation.

In conclusion, our data demonstrate, for the first time, the interrelation among E2F1–cyclin E1/E2 in human CSMCs. Importantly we also show that E2F1–cyclin E1/E2 circuit inhibition by siRNAs resulted in a remarkable downregulation of human CSMC proliferation. This effect is donor and transfectant independent, reversible, effective in asynchronous and synchronous CSMCs and in normo- and hyperglycemia, and does not induce cell death. Thus, our results provide the rationale for future experi-

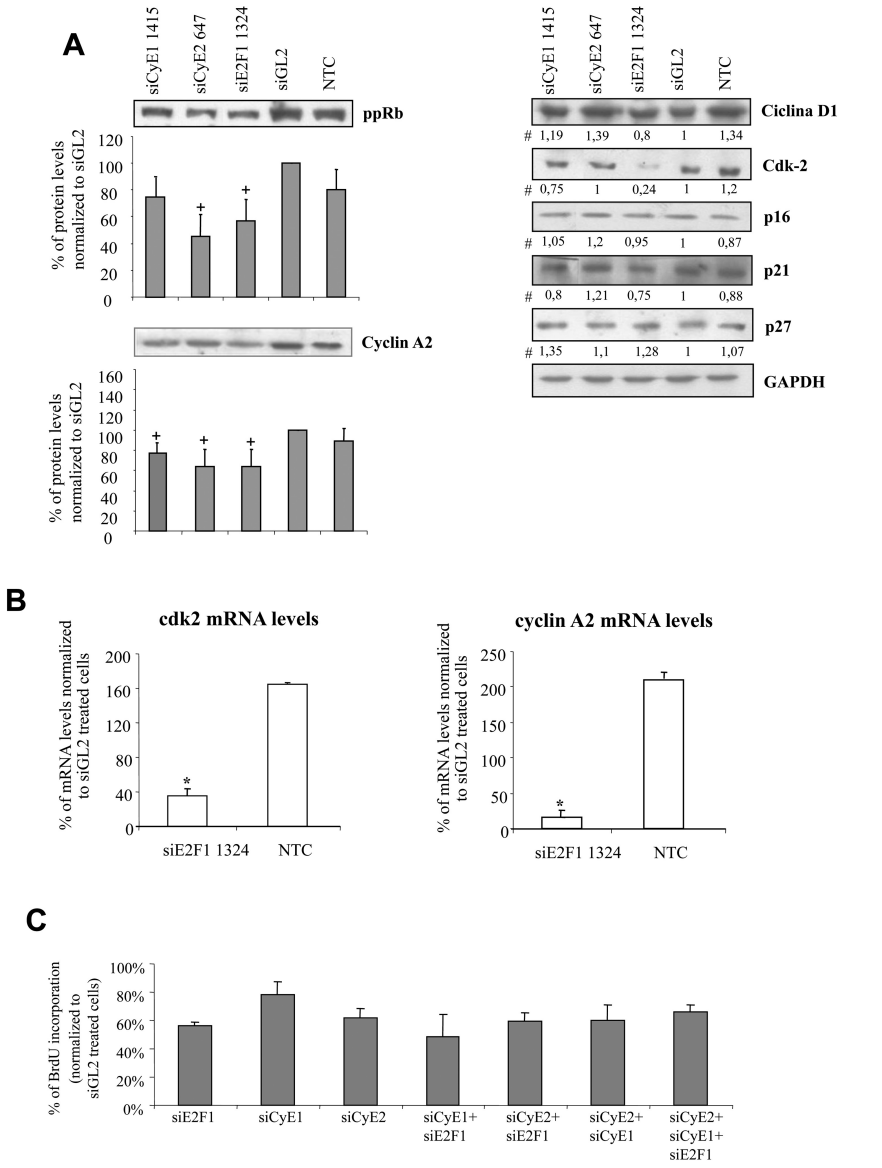


Figure 6. Expression levels of different cell cycle regulators following E2F1 and cyclin E1/E2 depletion and effect of the combined administration of different siRNAs on BrdU incorporation. (A) Three d after transfection, the protein levels of different cell cycle regulators were measured in protein extracts obtained from CSMCs of donor 2: shown are representative blots. #All the numbers reported below the blots derive from band quantification normalized to *GAPDH* and are expressed as a ratio of siGL2 treatment; for ppRb (hyperphosphorylated pRB) and cyclin A2, whose phosphorylation status and protein levels, respectively, were modified, cumulative data are reported as ratio to *GAPDH*; data are expressed as means ± SEM, n = 3, $^{+}P < 0.05$ compared with control. (B) mRNA levels of cyclin A2 and Cdk2 were measured in CSMCs treated with siE2F1-1324 in total RNA extracts obtained from both donors (*28S* transcript levels served as normalizator); results are expressed as means ± SEM, n = 6; $^{*}P < 0.05$ compared with control. (C) CSMCs were treated by a mix of the different siRNAs (115 or 76 nmol/L each in the case of a combination of two or three siRNA, respectively) and CSMC proliferation evaluated by BrdU incorporation; the effects of the combined administration were compared with an equimolar concentration of each of the single siRNAs (230 nmol/L each) 3 d after transfection; NTC, nontreated cells; siGL2, control siRNA treated cells; cumulative data from donor 1 and 2 CSMCs are expressed as means ± SEM, n = 3.

mentation in animal models aimed at the development of novel therapeutic approaches for hyperproliferative vascular diseases.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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