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RGC-32′ dual role in smooth muscle cells and atherogenesis

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ABSTRACT

Proliferation of endothelial cells (EC) and smooth muscle cells (SMC) is a critical process in atherosclerosis. Here, we investigated the involvement of sublytic C5b-9 effector Response Gene to Complement 32 (RGC-32) in cell cycle activation, phenotypic switch, and production of extracellular matrix (ECM) in SMC. Overexpression of RGC-32 augmented C5b-9-induced cell cycle activation and proliferation of SMC in an ERK1-dependent manner and silencing of RGC-32 inhibited C5b-9-induced cell cycle activation. C5b-9-induced cell cycle activation also required phosphorylation of RGC-32 at threonine 91. We found that ECM components fibronectin and collagens I-V were expressed by SMC in human aortic atherosclerotic tissue. Silencing of RGC-32 in cultured SMC was followed by a significant reduction in TGF-β-induced expression of SMC differentiation markers myocardin, SM22 and α-SMA, and that of collagens I, IV and V. These data suggest that RGC-32 participates in both sublytic C5b-9-induced cell cycle activation and TGF-β-induced ECM production.

1. Introduction

Atherosclerosis represents the most prevalent underlying cause of ischemic heart disease and stroke, the two leading causes of death in the world [1]. Cardiovascular disease accounts for 18% of disability adjusted life-years lost in high income countries and 10% in low- and middle-income countries, thus placing a substantial strain on the economies of developing countries [1,2].

Proliferation of endothelial cells (EC) and smooth muscle cells (SMC) is a critical process involved in angiogenesis and atherosclerosis [3,4]. SMC are fully differentiated cells that normally proliferate at a low rate [3]. In response to vascular injury, aortic SMC (ASMC) dedifferentiate and adopt a proliferative phenotype [5]. Proliferation occurs in response to the release of various mitogenic factors such as basic fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor 1 [6], and C5b-9, the membrane attack complex of complement [7]. In normal blood vessels, EC are in a quiescent and non-proliferative state because of cell-to-cell contact inhibition. However, EC undergo a rapid proliferative burst as a result of physical endothelial injury, which can occur after balloon angioplasty, or as a result of endothelial dysfunction that can result from elevation and modification of low-density lipoprotein (LDL) or during angiogenesis [8]. Proliferation of aortic EC (AEC) can occur as a result of many factors, one of which may be the assembly of sublytic C5b-9 on the cell membrane [9–11].

RGC-32 expression and activation play an important role in cell cycle activation [12,13]. Overexpression of RGC-32 in OLGxC6 glioma cell hybrids and ASMC leads to an increase in DNA synthesis [12–14]. RGC-32 interacts with cell division control 2 (CDC2) and enhances its activity *in vitro*. After sublytic complement C5b-9 stimulation, RGC-32 is translocated to the nucleus, where it becomes activated as a complex with CDC2 [12,15]. We have previously shown that C5b-9-induced cell cycle activation of EC is RGC-32 dependent, and in this setting RGC-32 mediates EC release of growth factors such as placental growth factor (PIGF), leptin, and regulated on activation, normal T cell expressed and secreted (RANTES) [16]. RGC-32 silencing in primary human AEC abrogates the ability of sublytic C5b-9 to induce the cell cycle, migration,

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and CDC2 activation, while suppressing Akt activation [16,17]. We have established that a significant number of genes regulated by RGC-32 (such as cyclin D1, cyclin D3, Akt, ROCK1, RhoGDI, and profilin) exhibit critical functions related to endothelial cell proliferation, migration, and cytoskeletal reorganization [17].

Our findings concerning human aortic lesions show that RGC-32 is expressed by AEC and in the media of the atherosclerotic aortic wall, where it is seen to co-localize with ASMC, immune-inflammatory cells, and C5b-9 [17]. RGC-32 expression increases with the progression of atherosclerosis in both the intima and media of aortic lesions [17]. Furthermore, RGC-32 contributes to vascular lesion formation in murine models of atherosclerosis, by stimulating the proliferation as well as the migration of ASMC [14].

The shift from the differentiated contractile SMC phenotype to a less differentiated, proliferative state and excessive ECM production, both TGF-β regulated events, are key alterations in atherogenesis [18]. Fibrous cap formation also stems from the migration and proliferation of SMC and deposition of ECM [19]. Since SMC differentiation and ECM production are crucial steps in atherosclerotic plaque development, the goal of this study was to explore the effect of RGC-32 on ASMC proliferation and on TGF-β induced differentiation of ASMC and ECM production.

We investigated the involvement of RGC-32 in cell cycle activation and the phenotypic switch in ASMC and also its role in the production of extracellular matrix by these cells. This investigation was accomplished by either overexpressing or silencing RGC-32 with siRNAs and analyzing their effect on cell cycle activation and of TGF- β-mediated ECM production. We also evaluated the impact of RGC-32 on SMC differentiation markers. We found that RGC-32 plays a dual role in ASMC, being involved in sublytic C5b-9-induced cell cycle activation and also in TGF-β-induced ECM production, while promoting an SMC synthetic phenotype.

2. Materials and methods

2.1. Culture of primary ASMC

Human ASMC from Lonza (Walkersville, MD) were cultured in SMC basal medium supplemented with 5% fetal bovine serum, 10 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin (Lonza). After three to five passages, the cells were starved for 18 h in SMC basal medium without serum and growth supplements. ASMC were then exposed to TGF-β (10 ng/ml) for 18 h.

2.2. Culture of T/G HA-VSMC cells

T/G HA-vascular SMC (VSMC) is a smooth muscle cell line derived from the normal aorta of an 11-month-old child (American Type Culture Collection, Manassas, VA). These cells were in cultured in Ham's F12K medium supplemented with 10 mM TES, 0.05 mg/ml ascorbic acid, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement, and 10% fetal bovine serum (FBS). T/G HA-VSMC were serum- and growth factor-starved in Ham's F12K medium for 18 h prior to DNA synthesis.

2.3. Activation of serum complement and assembly of sublytic C5b-9

Pooled normal human serum (NHS) from healthy adult donors was used as a source of serum complement. Pooled human serum immunochemically depleted of C7, C8, or C9 (C7D, C8D, or C9D, respectively) and purified human complement proteins were from Quidel (San Diego, CA). To assemble sublytic C5b-9, ASMC were sensitized with anti-human HLA class A, B, C monoclonal IgG (Dako, Carpinteria, CA), then exposed to NHS or K76-treated NHS (1/30 dilution). As previously described, K76 COONa (K76) (Otsuka Pharmaceuticals Co, Tokyo, Japan) prevents C5b-9 assembly in serum by binding to C5 [20,21]. Alternatively, complement-deficient serum (diluted 1/10) with or without replacement of the missing component (at 10 μg/ml when present) was added after antibody sensitization. The sublytic serum complement was previously titrated using limited doses of Ab and excess NHS, and lactate dehydrogenase release was then measured, as an indication of cell death [22].

2.4. SiRNA RGC-32 transfection and overexpression of RGC-32

2.4.1. Transient transfection of siRNA RGC-32 in human ASMC

ASMC were plated at a density of 1×10^5 cells/well in 6-well plates, then transiently transfected with RGC-32 siRNA (siRGC-32) or control siRNA (siCTR) (Santa Cruz Biotechnology) by using Viromer Blue (Lipocalyx GmbH, Germany). siRGC-32 is a pool of 3 target-specific 19–25 nucleotide siRNA designed to knock down gene expression. siCTR is non-targeting 20–25 nucleotide siRNA. 10 μM of either siCTR or siRGC-32 were diluted separately in Opti-MEM I Reduced Serum Medium (Gibco, Waltham, MA) for a final concentration of 0.28 μM and then added over 1 μl of Viromer Blue. The mixture was incubated 15 min at room temperature and then added to the cells. At 48 h after transfection, ASMC were starved in serum-free SMC medium (Lonza) overnight and then treated with 10 ng/ml TGF-β (10 mg/ml) or vehicle for the indicated periods of time. The expression of RGC-32, collagens I, IV, and V, as well as myocardin (MYOCD), smooth muscle protein 22 (SM22), and alpha smooth muscle actin (α-SMA) mRNAs were determined by real-time PCR.

2.4.2. Transfection of T/G HA-VSMC

SiRNAs were complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The complexed siCTR or siRGC-32 was added to the cells for 18 h. An equal volume of fresh growth medium was added, and the cells were further cultured for 24 h.

2.4.3. Overexpression of RGC-32 in ASMC

ASMC were transfected with the pVP22 or pVP22-RGC-32 plasmid using Effectene transfection reagent for 18 h as previously described [12]. In brief, SMC were plated at a density of 1×10^5 cells/well in 6-well plates, and 1 μg of DNA was allowed to form a complex with 10 μl of Effectene for 20 min. The transfection complex was added to the cells and incubated at 37 °C in 5% CO_2 for 24 h. The transfection complex was aspirated from the cells and replaced with fresh growth medium. The cells were further incubated at 37 °C in 5% CO_2 for 24 h. After 18 h the culture medium was removed, and the cells were synchronized in G_0/G_1-phase by starving them in serum- and growth factor-free medium for 18 h. The cells were then treated with sublytic C5b-9 (C5b-9) or K76-treated serum (C5D) for 48 h and then processed for cell cycle assays.

2.5. Cell cycle activation assay

2.5.1. [^{3}H]-thymidine incorporation

DNA synthesis was performed as in [23]. Cultured cells were synchronized in G_0/G_1-phase by starvation in basal medium. After stimulation, [^{3}H]thymidine (Perkin Elmer, Boston MA) was added to cells at a final concentration of 1 μCi/ml. Each plate was incubated at 37 °C in 5% CO_2 for 18 h. The medium was aspirated from the cells, and they were washed two times with phosphate-buffered saline (PBS). The cells were then lysed with 0.3 M NaOH and incubated on ice for 20 min. The lysate was transferred to a 1.5-ml Eppendorf tube, and the DNA was precipitated by adding 20% trichloroacetic acid and incubating the tubes on ice for 30 min. The precipitated DNA was then aspirated into a 10-cc syringe through a 22-gauge needle. The DNA was expelled through a filter apparatus onto G/FA filter paper (Fisher Scientific, Pittsburgh PA). The filter was washed with 10% TCA and then 95% ethanol. The filter paper was then air-dried and placed into a scintillation vial with 5 ml of

Table 1
List of human primers used for Real-Time PCR.

Gene symbol	Primer's sequence
RGC-32	For: 5′- AGGAACAGCTTCAGCTTCAGT -3′
	Rev.: 5′- GCTAAAGTTTTGTCAAGATCAGCA -3′
COL1A1	For: 5′- GGATTCCAGTTCGAGTATGG-3′
	Rev.: 5′- CAGTGGTAGGTGATGTTCTGG -3′
COL4A1	For: 5′- CGGGCCCTAAAGGAGATAAAG-3′
	Rev: 5′- GAACCTGGAAACCCAGGAAT -3′
COL5A1	For: 5′- GACTGCCAGATTTGGACACTAT -3′
	Rev: 5′- GGATGACCTTTACGAGGCTTAC -3′
ACTA2	For: 5′- GACCTTTGGCTTGGCTTGTC -3′
	Rev.: 5′- GCTTCACAGGATTCCCGTCT -3′
SM22	For: 5′- CCAGACTGTTGACCTCTTTGA -3′
	Rev.: 5′- CGGTAGTGCCCATCATTCTT -3′
MYOCD	For: 5′- CTCCACTCGGAACTTTCTTCTT -3′
	Rev.: 5′- GCATTCTTCAGGGTTCTCATCTA -3′
18S	For: 5′- GTAACCCGTTGAACCCCATT -3′
	Rev.: 5′- CCATCCAATCGGTAGTAGCG-3′

scintillation cocktail (RPI, Mount Prospect IL). The radioactivity was then counted.

2.5.2. Cell proliferation assay

Cultured cells were plated into 96-well plates and starved in basal medium. After stimulation, the cells were incubated for 2–3 days at 37 °C in 5% CO_2. The medium was then aspirated, and fresh growth medium was added. The cells were incubated for 1 h at 37 °C in 5% CO_2, then CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Madison WI 53711) was added to each well, and the cells were incubated for 1 h at 37 °C in 5% CO_2. The absorbance at 490 nm was recorded with a 96-well plate reader.

2.5.3. BrdU incorporation

In brief, transfected cells were stimulated for 18 h, then pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 1 h. Pulse-labeled cells were harvested by trypsinization and then permeabilized with Cytofix/Cytoperm buffer provided by the manufacturer (BD Biosciences, San Diego, CA). BrdU and DNA were stained with fluorescein isothiocyanate anti-BrdU and 7-aminoactinomycin D, respectively, according to the manufacturer's instructions. Two-color flow cytometric analysis was used to measure the total DNA content of and characterize the cells that were actively synthesizing DNA in a specific cell cycle. Flow cytometric analysis was performed using a Becton-Dickinson flow cytometer.

2.6. CDC2 kinase assay

Immunoprecipitation kinase assay was performed as previously described [7]. The medium was aspirated from the cells, and they were washed twice with PBS. The treated cells were lysed by adding 1 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 10 mM EDTA, 1% *v*/v Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF, 0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin). The cells were then scraped with a cell scraper and incubated on ice for 30 min. The lysate was transferred to a 1.5-ml Eppendorf tube and centrifuged for 30 min at 4 °C. The supernatant was transferred to a new 1.5 ml-Eppendorf tube, and the protein concentration was determined using BCA protein quantitation assay (Thermo Fisher, Rockford, IL). CDC2 was immunoprecipitated using a specific antibody against CDC2 p34 (SC-54) (Santa Cruz Biotech, Dallas, TX). Immunoprecipitated CDC2 was incubated in the presence of 5 μg of histone H1 (Roche Indianapolis, IN) with 1 μCi of [γ-^{32}P] ATP (Perkin Elmer, Boston) in the reaction buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$). The reaction mixture was incubated for 30 min at 37 °C. Phosphorylated histone H1 bands were analyzed by 10% SDS-PAGE and autoradiography.

2.7. RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated using RNeasy (Qiagen, Santa Clarita, CA) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed to obtain cDNA as previously described [24]. Real-time quantitative PCR was performed using a StepOne real-time PCR system (Applied Biosystems, Foster City, CA). Real-time PCR was performed according to the manufacturer's protocol using a LightCycler FastStart DNA MasterHybProbe (Roche) and specific primers (Table 1) designed by IDT (Coleville, IA). The -fold change in target gene samples, after normalization with the housekeeping gene (18S), was calculated as described [24].

2.8. Immunohistochemical staining of aortic tissue samples

Cross-sections of abdominal aortas dissected anterior to the bifurcation into the common iliac arteries were obtained from nine autopsy cases. The study was approved by the Committee on Research and Ethics of "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca, Romania. The specimens were fixed by immersion in neutral buffered 10% formalin and subsequently dehydrated and embedded in paraffin. The aortic atherosclerotic lesions collected from nine patients (five males and four females, age 42– 84) consisted of four fibrous plaques and five intimal thickenings (Table 2).

Table 2
Quantification for RGC-32, collagen I-V and fibronectin in human atherosclerotic wall.

N°	Type	Age	Sex	Area	RGC-32	Collagen I	Collagen II	Collagen III	Collagen IV	Collagen V	Fibronectin
1	IT	60	F	Intima	+ +	+	+ +	+ +	+ +	+ +	+ +
				Media	+ +	+ +	+ +	+ +	+ + +	+ + +	+ + +
2	IT	51	F	Intima	+ +	+ +	+ +	+ +	+ +	+ +	+ +
				Media	+ + +	+	+ +	+ +	+ +	+ +	+ +
3	IT	54	M	Intima	+ +	+ +	+ +	+ +	+ + +	+ +	+ + +
				Media	+ + +	+ +	+	+ +	+ +	+ +	+ +
4	IT	84	M	Intima	+ +	+ +	+ +	+ +	+ +	+	+ +
				Media	+ + +	+ +	+ +	+ + +	+ +	+ + +	+ +
5	IT	69	M	Intima	+ +	+ +	+ +	+	+ +	+ +	+ +
				Media	+ + +	+	+	+	+	+	+ + +
6	FP	59	M	Intima	+ +	+ +	+ + +	+ +	+ +	+ + +	+ +
				Media	+ + +	+ +	+ +	+ + +	+ +	+ + +	+
7	FP	57	M	Intima	+ +	+ + +	+ + +	+ + +	+ +	+ +	+ + +
				Media	+ + +	+ +	+ +	+ +	+ +	+ +	+ + +
8	FP	73	F	Intima	+ +	+ +	+ +	+ + +	+ +	+ + +	+ +
				Media	+ + +	+ + +	+ +	+ + +	+ +	+ + +	+
9	FP	74	F	Intima	+	+	+	+ + +	+ + +	+ +	+ + +
				Media	+ +	+ +	+ + +	+ + +	+ +	+ + +	+ +

IT = Intimal thickenings; FP = Fibrous Plaques.

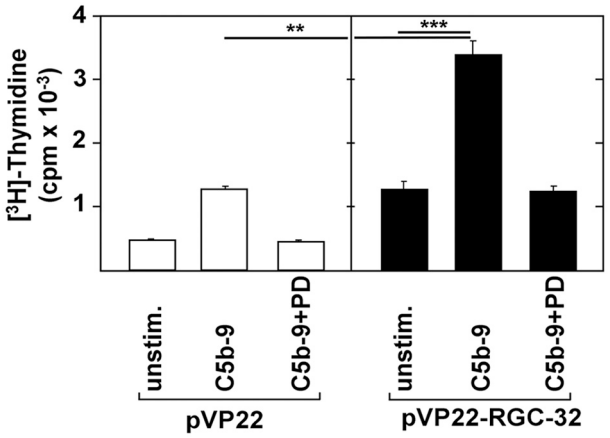

Fig. 1. Induction of cell cycle activation by RGC-32 overexpression in ASMC is ERK1-dependent. ASMC were transfected with pVP22-RGC-32 or the empty vector pVP22 using Effectene transfection reagent for 18 h. The cells were cultured in growth medium for 18 h. The cells were starved in serum- and growth factor-free medium for 18 h and then pre-treated with 50 μM PD98059 (PD) for 45 min before stimulation with sublytic C5b-9. After 18 h, DNA synthesis was assessed by [^{3}H]thymidine incorporation. RGC-32 overexpression led to an increase in thymidine incorporation in C5b-9-stimulated cells when compared to unstimulated cells or cells transfected with the empty vector pVP22. Inhibition of MEK1 by PD98059 abolished C5b-9-induced thymidine incorporation. Data are expressed as mean ± SEM ($n = 3$). ** = $p < 0.01$; *** = $p < 0.001$.

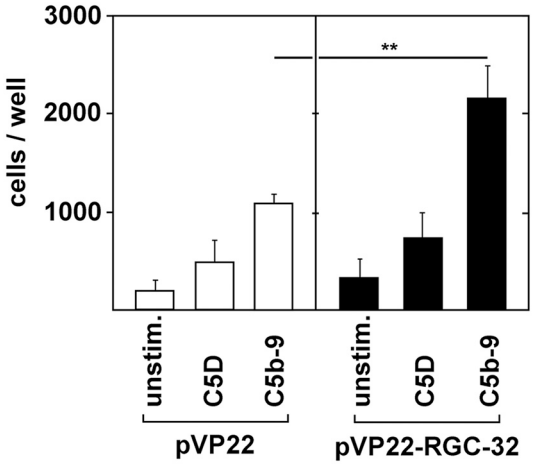

Fig. 2. Overexpression of RGC-32 leads to an increase in ASMC cell number. ASMC were transfected with pVP22 or pVP22-RGC-32 using Effectene transfection reagent for 18 h. The medium was removed, and the cells were cultured in fresh growth medium for 18 h. The cells were synchronized in the G_0/G_1-phase by starving them in serum- and growth factor-free medium for 18 h. The cells were treated with sublytic C5b-9- or K76 COONa-treated normal human serum (C5D) for 48 h. Cell number was determined by MTS assay. Transfection with RGC-32 led to an increase in cell number in sublytic C5b-9-treated cells when compared to cells transfected with pVP22. Data are expressed as mean ± SEM ($n = 3$). ** = $p < 0.01$.

The expression of RGC-32 was examined by immunohistochemical staining as previously described [17,25]. Immunohistochemistry was also used to investigate the expression of fibronectin (FN), collagens type I to V (as ECM components), and α-SMA (as a marker of SMC) in human aortic atherosclerotic lesions. In brief, paraffin sections were deparaffinized and washed in PBS, and endogenous peroxidase was quenched with 3% H_2O_2. For antigen retrieval, the deparaffinized slides were placed in Dako Target Retrieval Solution (Dako, Carpinteria, CA) and boiled for 30 min. Slides were incubated overnight at 4 °C with rabbit IgG anti-RGC-32 (Bioss Inc., Woburn, MA) diluted 1:300 in PBS, then washed three times in PBS and incubated for 1 h at room temperature with secondary HRP-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:250 in PBS Tween 0.1%. For the rest of the proteins investigated, the primary antibodies were rabbit IgG anti-collagen I, III, IV, or V (Santa Cruz Biotech) and rabbit polyclonal α-SMA antibody (R&D Systems). For visualization, the RTU Vectastain kit (Vector Labs) was used. After several PBS rinses, the bound antibody was detected using Nova RED (Vector Labs, Burlingame, CA), and the slides were subsequently counterstained with Harris hematoxylin (Sigma, St. Louis, MO) and mounted using VectaMount AQ (Vector Labs).

The staining intensity of the RGC-32 deposits was evaluated independently by two investigators in a blinded fashion. The intensity of staining was graded as follows: negative (−), slightly positive (+), positive (++), or highly positive (+++).

2.9. Double-staining immunohistochemistry

Paraffin sections of aortic atherosclerotic lesions were stained for RGC-32 and α-SMA [25]. Sections were initially processed for RGC-32 immunostaining as described above and the reactions were developed with NovaRed. The sections were treated with BLOXALL blocking solution and then incubated for 2 h at room temperature with mouse monoclonal anti-α-SMA (R&D Systems, Minneapolis, MN). The slides were washed several times in PBS and reacted with alkaline phosphatase-conjugated goat anti-mouse IgG (Vector). The reaction was developed using a Vector Alkaline Phosphatase Substrate Kit III (Vector Labs). Control sections were prepared by immunostaining without the primary antibody or by using control isotype IgG instead of the primary antibody. The immunostained slides were independently evaluated by two investigators.

2.10. Statistical analysis

Comparisons between multiple groups were performed by using either one-way or two-way ANOVA with *post hoc* correction. Comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. *p*-values <0.05 were considered significant. Statistical analysis was performed using GraphPad Prism software, version 7.

3. Results

3.1. Overexpression of RGC-32 in ASMC induces cell cycle activation and proliferation; requirement for extracellular signal regulated kinase 1 (ERK1)

To determine whether overexpression of RGC-32 induces the cell cycle in primary human ASMC, we transfected these cells with RGC-32-containing vectors [12]. ASMC were transfected with pVP22-RGC-32 or the empty vector pVP22 (Fig. 1), and then the cells were exposed to sublytic C5b-9, and cell cycle activation was assessed by thymidine incorporation. Sublytic C5b-9 in VP22-RGC-32-transfected cells was able to induce a significant increase in thymidine incorporation as compared to the unstimulated cells ($p < 0.001$) or cells transfected with the pVP22 control ($p < 0.01$). Since overexpression of RGC-32 in serum- and growth factor-free medium was able to induce cell cycle activation, we wanted to see if RGC-32 might be able to circumvent signaling pathways such as the ERK1 pathway, or whether it was still dependent upon them. Therefore, ASMC overexpressing RGC-32 were exposed to MEK1 inhibitors, and cell cycle induction was assessed by thymidine incorporation. Pre-treatment with PD98059 significantly reduced the sublytic C5b-9-induced thymidine incorporation into unstimulated cells levels, indicating a role for the MEK-ERK1 pathway in RGC-32-mediated cell cycle activation (Fig. 1).

The increase in cell number as a result of RGC-32 overexpression

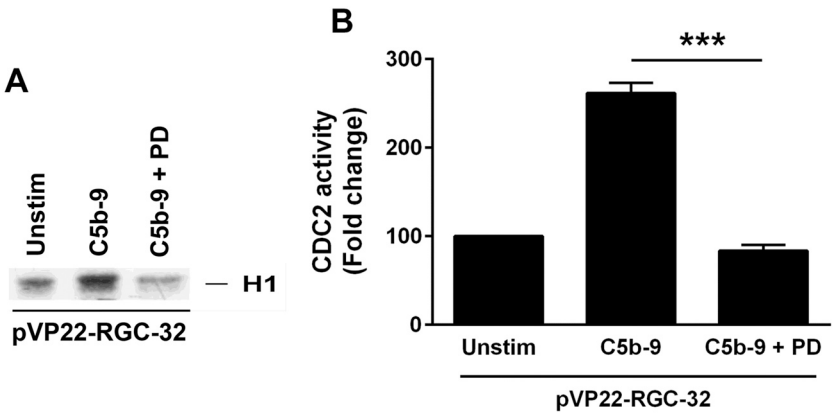

Fig. 3. Overexpression of RGC-32 enhances CDC2 activity induced by sublytic C5b-9. **A.** ASMC were transfected with pVP22-RGC-32 or the empty vector pVP22. The cells were cultured in serum- and growth factor-free medium for 18 h. The transfected cells were pretreated with 50 μM PD98059 (PD) to inhibit MEK1 and then treated with anti-HLA I antibody and sublytic C5b-9 for 24 h. CDC2 activity was assayed by *in vitro* kinase assay by immunoprecipitating CDC2 from 100 μg of total protein lysate. Immunoprecipitated CDC2 was incubated with [γ-^{32}P]ATP and histone H1 as the substrate. Phosphorylated histone H1 was detected by autoradiography. **B.** Densitometric scanning was performed to quantify CDC2 activity. RGC-32 overexpression led to a roughly 2.5-fold increase in sublytic C5b-9-induced CDC2 activity when compared to cells transfected with empty vector. PD98059 abolished RGC-32-induced CDC2 activity, indicating that ERK1 activation is required for CDC2 phosphorylation by RGC-32. CDC activity in unstimulated cells was considered to be 100, and the results are shown as -fold change. Data are expressed as mean ± SEM ($n = 3$). *** = $p < 0.001$.

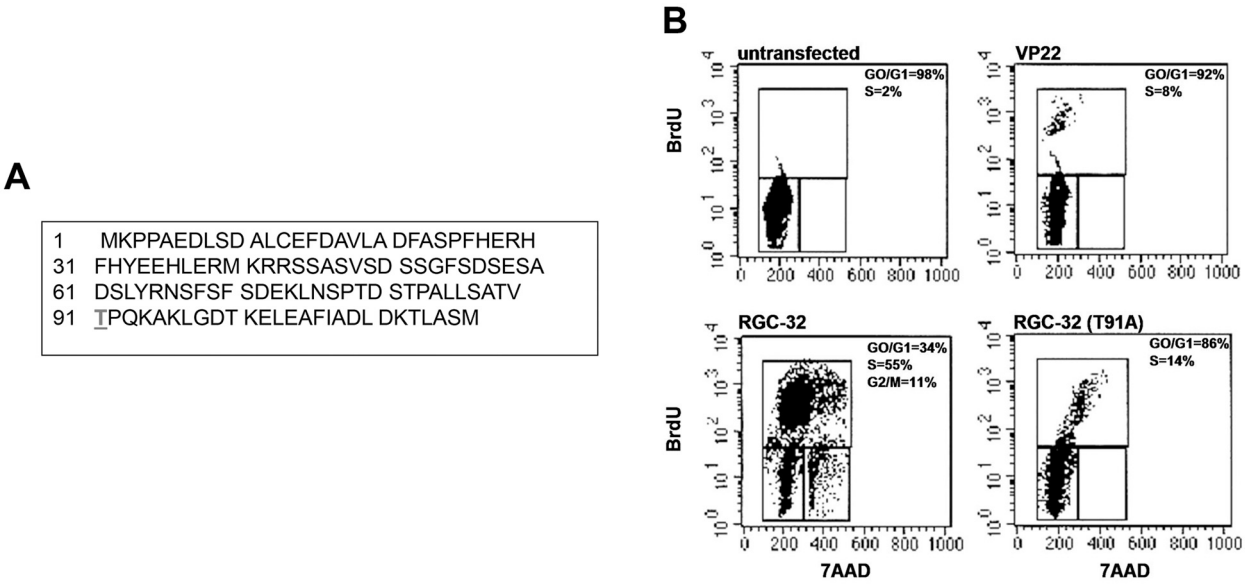

Fig. 4. Phosphorylation of RGC-32 at Thr-91 by CDC2 is required for cell cycle activation in ASMC. ASMC were transfected with wild-type RGC-32 (RGC-32), the mutant-RGC-32 (T91A), or the empty vector pVP22 for 18 h, and the medium was replaced with fresh growth medium. The cells were serum- and growth factor-starved for 18 h, and the medium was replaced with fresh basal medium. After 18 h, the cells were pulse-labeled with BrdU, and the cell cycle status was determined by FACS analysis. Mutation of Thr-91, which lies within a CDC2 consensus phosphorylation site (TPQK) (**A**), to alanine [RGC-32 (T91A)] abolished S-phase entry, which was seen when wild-type RGC-32 was overexpressed (**B**). These data suggest that Thr-91 is a critical residue for RGC-32 activation of the cell cycle.

after stimulation with C5b-9 (Fig. 2; $p = 0.002$) may be explained by RGC-32 increasing the cell cycle transition so that the cell division requires less time than normal, and therefore the doubling time is decreased. These data suggest that RGC-32 is an integral component of the cell cycle machinery and an important factor in the induction of the cell cycle.

3.2. Role of ERK1 in CDC2 activation by C5b-9/RGC-32

RGC-32 has been shown to enhance CDC2 (also known as cyclin-dependent kinase 1 - CDK1) activity in a cell-free assay [12]. To determine whether RGC-32 affects CDC2 activity *in vivo*, we transfected ASMC with pVP22-RGC-32, and assessed CDC2 kinase activity. In cells overexpressing RGC-32, sublytic C5b-9 induced a 2.4-fold increase in CDC2 kinase activity over unstimulated cells (Fig. 3). In addition, pretreatment with PD98059, the MEK1 inhibitor, abolished C5b-9/RGC-32-mediated CDC2/CDK1 activation (Fig. 3, $p < 0.001$). These data suggest that ERK1 is required for the C5b-9/RGC-32-mediated activation of CDC2/CDK1.

3.3. RGC-32 phosphorylation by CDC2 is required for ASMC cell cycle activation

RGC-32 contains a consensus CDC2 sequence TPQK, with a threonine at 91, a proline at +1, and a basic residue at the +3 position (Fig. 4A). The phosphorylation site at Thr-91 was found to be the target for CDC2 kinase [12]. This site was mutated to alanine (T91A), and the RGC-32 mutant was transfected into ASMC. After culturing in basal medium to synchronize the cells in G_1/G_0-phase, the cells were pulsed with BrdU, and the cell cycle was analyzed by fluorescence-activated cell sorting (FACS) (Fig. 4B). Overexpression of wild-type RGC-32 (RGC-32) induced cell cycle activation in unstimulated cells (S-phase-55%, G2M phase −11%). Mutated RGC-32 (T91A) was able to induce a modest increase in S-phase cells (14%) as compared to the control vector VP22 (S-phase cells: 8%). Mutated RGC-32 (T91A) did not lead to any movement of ASMC cells into G2/M phase. These data suggest that Thr-

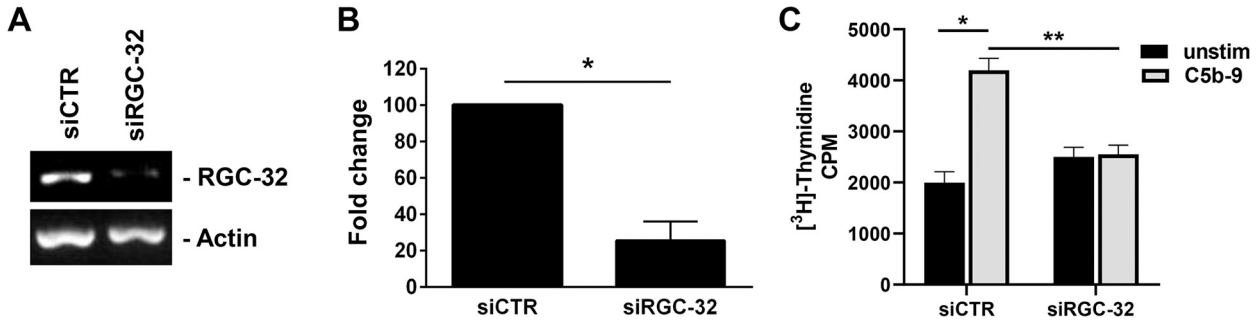

Fig. 5. RGC-32 is required for cell cycle activation by sublytic C5b-9 in T/G HA-VSMC. **A.** T/G HA-VSMC were transfected with siRGC-32 or siCTR complexed with Lipofectamine 2000. The cells were changed into fresh growth medium and further cultured for 24 h. RNA was extracted, and RGC-32 expression was measured by RT-PCR. **B.** RGC-32 and actin levels were quantitated by densitometric scanning. RGC-32 silencing led to a significant reduction in RGC-32 expression when compared to siCTR. The expression of the mRNA in siCTR was considered to be 100, and the results are shown as -fold change. Data are expressed as mean ± SEM ($n = 3$). * = $p < 0.05$. **C.** T/G HA-VSMC were transfected as shown in (**A**). They were then starved in serum-free medium for 18 h and stimulated with sublytic C5b-9 for 24 h. Cell cycle activation was assessed by thymidine incorporation. RGC-32 silencing significantly decreased the thymidine incorporation induced by sublytic C5b-9. Data are expressed as mean ± SEM ($n = 3$). * = $p < 0.05$; ** = $p < 0.01$.

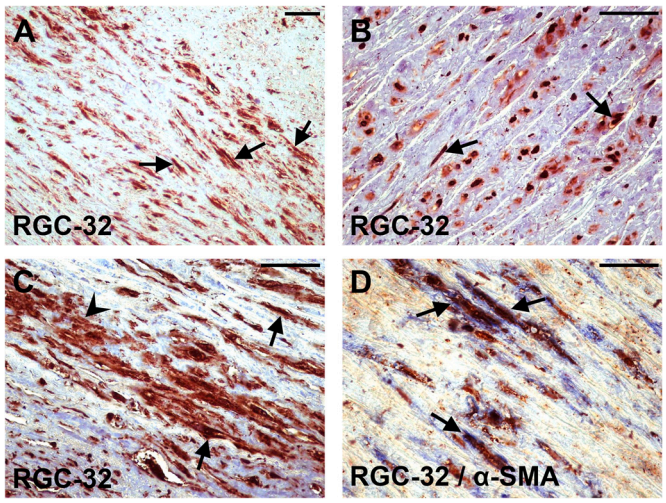

Fig. 6. Immunohistochemical localization of RGC-32 in the human aortic atherosclerotic wall. Paraffin-fixed sections of human aortas ($n = 9$) were stained with IgG anti-RGC-32 antibody and then counterstained with hematoxylin (A-C) or were double-stained with anti-RGC-32 (red) and anti- α-SMA (blue) antibodies (D). RGC-32 was found to be expressed by SMC in the media of intimal fibrous plaques (A, C) and thickenings (B). Significant extracellular deposits could be found around the SMC in the media of the fibrous plaques (C, arrowhead). Double staining shows co-localization of RGC-32 with α-SMA (D, arrows). Original magnification: A: x20; B-D: x40. Scale bars: A: 40 μm; B-D: 20 μm. The microphotographs in the figure are representative for 5 intimal thickenings ($n = 5$), and for 4 fibrous plaques ($n = 4$).

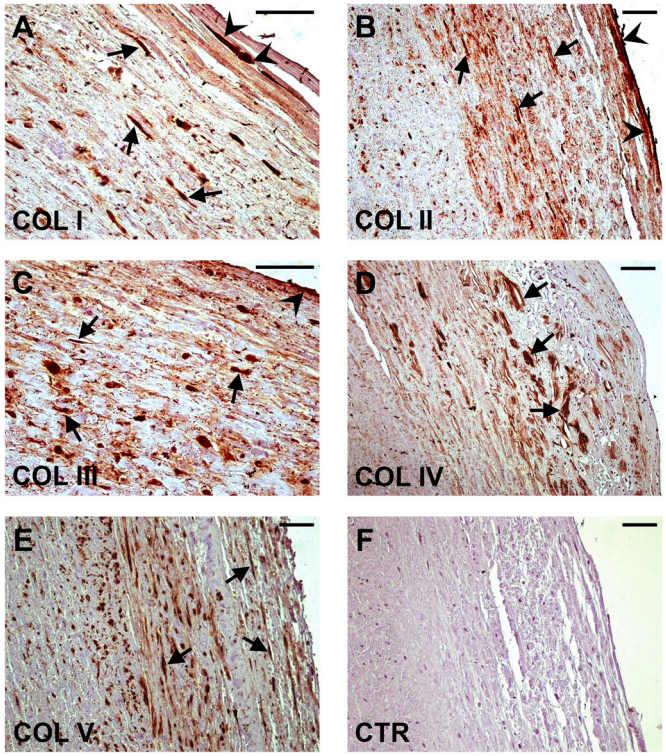

Fig. 7. Immunohistochemical localization of collagen types I-V in human aortic intimal thickenings and fibrous plaques. Human abdominal aortas with atherosclerotic lesions ($n = 9$) were immunostained with rabbit IgG anti-collagen I (A), collagen II (B), collagen III (C), collagen IV (D), or collagen V (E), then counterstained with Harris hematoxylin. All the types of collagens investigated were found in the fibrous plaques and IT (A-E, arrows). Collagen types I-III are shown also to be present in endothelial cells in the current fig. (A-C, arrowheads). Some of the cells had a typical aspect of "pancake-like cells" (A-C, E, arrows). The control for the immunoperoxidase reaction was negative (F). Original magnification: A, C: x40; B, D, E, F: x20. Scale bars: A, C: 20 μm; B, D, E, F: 40 μm. The microphotographs in the figure are representative for 5 intimal thickenings ($n = 5$), and for 4 fibrous plaques ($n = 4$).

91 is a critical residue for RGC-32 activation of the cell cycle G2/M phase.

3.4. RGC-32 is required for cell cycle activation by sublytic C5b-9 in ASMC cell line

We then went on to establish whether RGC-32 was indeed required for the activation of the cell cycle in an ASMC cell line. siRGC-32 complexed with Lipopfectamine 2000 was transfected into T/G HA-VSMC (a smooth muscle cell line derived from the normal aorta of an 11-month-old child; ATCC® CRL-1999™). After 48 h, RGC-32 expression was reduced by 78% when compared to cells transfected with siCTR (Fig. 5A, B, $p < 0.05$). We then examined the T/G HA-VSMC cells to determine whether they required RGC-32 for cell cycle activation (Fig. 5C). We transfected T/G HA-VSMC with siRGC-32 or siCTR and then starved them of serum and growth factors for 18 h, then stimulated the cells with sublytic C5b-9 for 24 h and measured their thymidine incorporation. In control siRNA-transfected cells, sublytic C5b-9 was able to induce a 2.1-fold increase in thymidine incorporation as compared to unstimulated cells ($p < 0.05$). In RGC-32 siRNA-transfected cells, the sublytic C5b-9

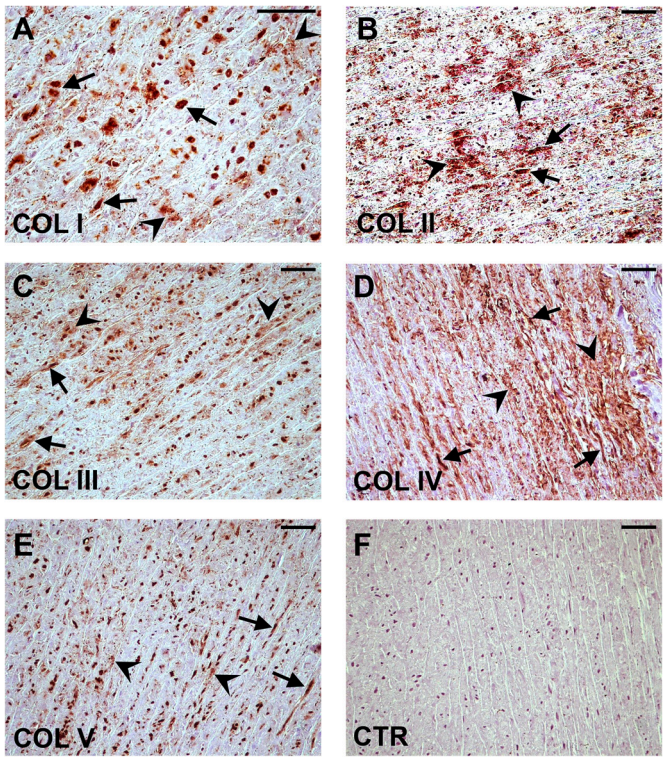

Fig. 8. Immunohistochemical localization of collagen I -V in the aortic atherosclerotic media. Human aorta paraffin sections from individuals with atherosclerosis ($n = 9$) were immunostained with rabbit IgG recognizing collagen I (A), collagen II (B), collagen III (C), collagen IV (D), or collagen V (E), then counterstained with Harris hematoxylin. All collagen types were present in the SMC (A–E, arrows), as well as in extracellular deposits around the SMC (A–E, arrowheads). The control for the immunoperoxidase reaction was negative (F). Original magnification: A: x40; B–F: x20. Scale bars: A: 20 μm; B–F: 40 μm. The microphotographs in the figure are representative for 5 intimal thickenings ($n = 5$), and for 4 fibrous plaques ($n = 4$).

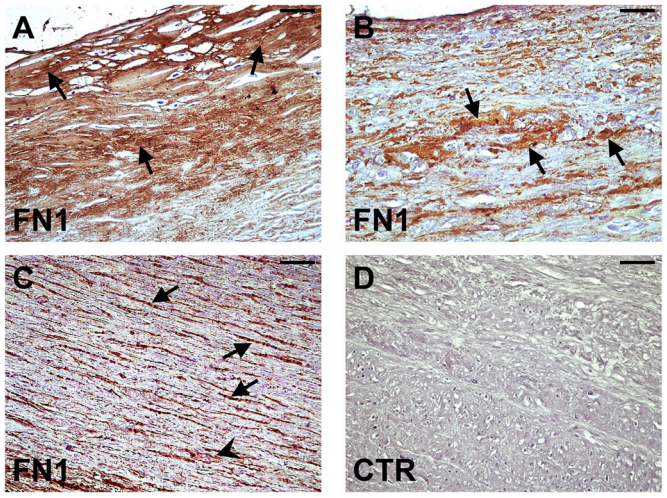

Fig. 9. Localization of FN in the human aortic atherosclerotic wall. Paraffin sections from abdominal aortas with atherosclerotic lesions ($n = 9$) were immunostained with rabbit IgG anti-fibronectin and then counterstained with Harris hematoxylin. FN was found to be localized in both fibrous plaques (A, arrows) and intimal thickenings mostly as extracellular deposits (B, arrows), as well as in the media, in both SMC (C, arrows) and around SMC (C, arrowhead). Control of immunoperoxidase reaction was negative (D). Original magnification: ×20. Scale bars: 40 μm. The microphotographs in the figure are representative for 5 intimal thickenings ($n = 5$), and for 4 fibrous plaques ($n = 4$).

effect was abolished ($p < 0.01$).

Taken together, these data in SMC suggest that RGC-32 is a critical gene involved in cell cycle activation. It is important to mention that RGC-32 can also inhibit cell cycle activation in T cells, as we have shown previously [25].

3.5. Expression of RGC-32 in the human aortic atherosclerotic wall

RGC-32 was found to be expressed by ASMC in the human atherosclerotic wall, and its expression increased with the progression of atherosclerosis (Table 2) as previously reported [17]. We observed substantial deposits of RGC-32 on SMC in the media of fibrous plaques (FP) (Fig. 6A, C, arrows) and fewer deposits of RGC-32 on the cells in the media of intimal thickenings (IT) (Fig. 6B, arrows). In the media of both aortic intimal thickenings and fibrous plaques, RGC-32 was co-localized with SMC (Fig. 6D, arrows). The controls for the immunoperoxidase reaction were all negative (data not shown). Extracellular deposits were also noted, particularly in the media, surrounding SMC expressing RGC-32 (Fig. 6C, arrowhead), suggesting that SMC secrete RGC-32 into the surrounding environment.

3.6. Expression of collagens and fibronectin in the human aortic atherosclerotic wall

Since RGC-32 was found to mediate the expression of ECM components [15], we further investigated the expression of collagens and fibronectin in the human atherosclerotic wall. The expression of collagens I-V and fibronectin was found to be increased in fibrous plaques when compared to intimal thickenings (Table 2). Collagens I-V were found to be deposited in the intima, necrotic core (Fig. 7, arrows), and media of the aortic atherosclerotic wall (Fig. 8, arrows). We also identified the presence of collagens I-V in the cells and around the cells in fibrous cap. Some of the cells had a typical aspect of "pancake-like cells" (Fig. 7A–C, E, arrows). In addition, extracellular deposits of collagens I-V were also found around the SMC, suggesting that the collagens were secreted by these cells (Fig. 8A–E, arrowheads). Controls for the immunoperoxidase reaction were all negative (Figs. 7F, 8F). Furthermore, we found that fibronectin was also expressed in the intimal thickenings and fibrous plaques (Fig. 9, arrows). In the fibrous cap, we found mostly extracellular deposits (Fig. 9A–B, arrows), which were also present in the necrotic core around the cholesterol and lipid deposits (data not shown). In the media, significant deposits were found in SMC (Fig. 9C, arrows), which were associated with extracellular deposits (Fig. 9C, arrowhead). The control for the immunoperoxidase reaction was negative (Fig. 9D).

These *in vivo* data suggest that RGC-32 is present in SMC from atherosclerotic lesions and might be associated with the increased ECM accumulation that occurs during the progression of atherosclerotic lesions.

3.7. Role of RGC-32 in ECM production

3.7.1. RGC-32 mediates TGF-β-dependent ECM production in ASMC

The abovementioned findings prompted us to investigate whether RGC-32 regulates ASMC expression of ECM components *in vitro*. Thus, to investigate the role of RGC-32 in ECM production by ASMC, we transfected ASMC with either siRGC-32 or siCTR and then stimulated them with TGF-β. We found that silencing RGC-32 leads to the reduction of the RGC-32 mRNA expression by 80% (Fig. 10A) and this led to a significant decrease in the mRNA expression of pro-collagens I ($p < 0.05$), IV ($p < 0.05$), and V ($p < 0.05$) when compared with siCTR (Fig. 10B–D).

3.7.2. RGC-32 mediates TGF-β induction of myocardin and differentiation markers in ASMC

We next investigated whether RGC-32 might participate in the

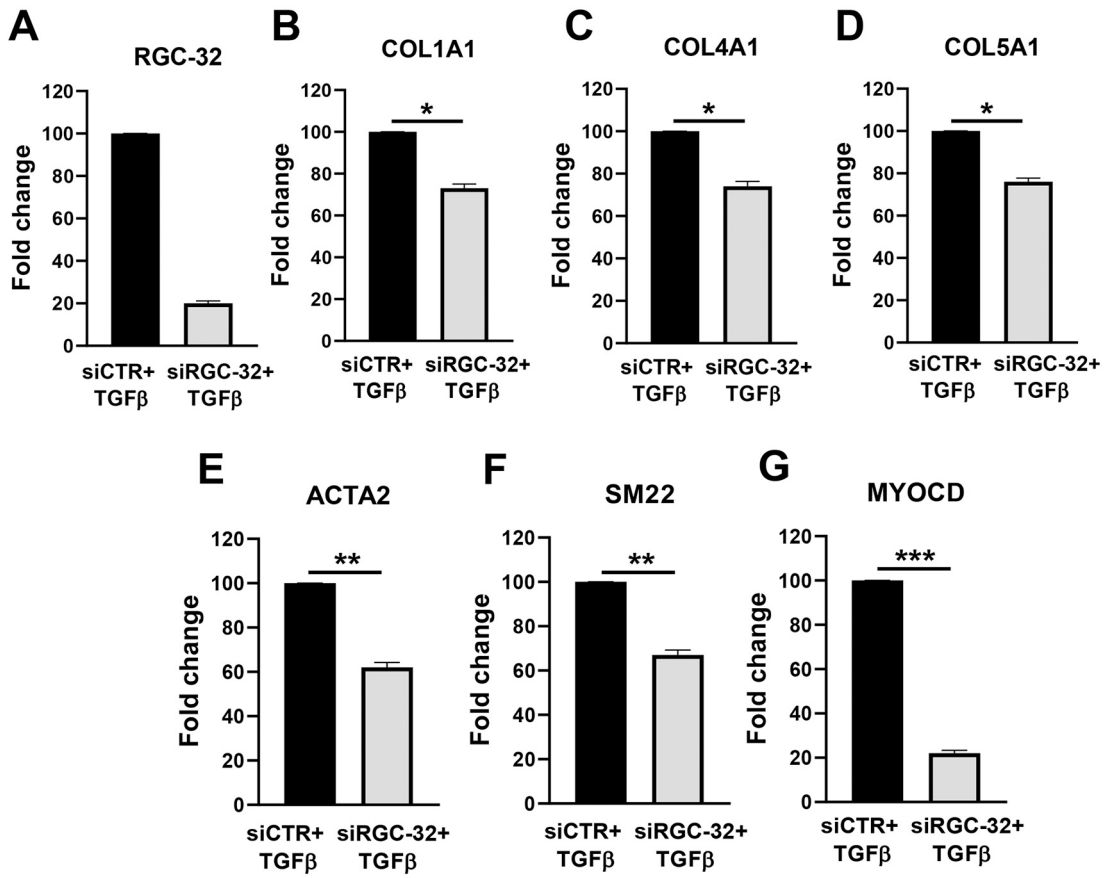

Fig. 10. Effect of RGC-32 silencing on mRNA expression of pro-collagens I, IV, V and ASMC differentiation markers. ASMC were transfected with either siCTR or siRGC-32 and then treated with 10 ng/ml TGF-β for 18 h, then the mRNA expressions of RGC-32 (**A**), pro-collagen type I (COL1A1, **B**), type IV (COL4A1, **C**), type V (COL5A1, **D**), α-smooth muscle actin (ACTA2, **E**), SM22, (**F**), and myocardin (MYOCD, **G**) were measured by Real-Time PCR. SiRGC-32 reduced the mRNA expression of RGC-32 by 80% and significantly decreased the levels of TGF-β-induced expression of all markers analyzed when compared to siCTR. The expression of mRNA in the siCTR-transfected cells was considered to be 100, and the results are shown as -fold change. Data are expressed as mean ± SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

regulation of TGF-β-induced ASMC differentiation markers. VSMC in the normal arterial media express a range of specific SMC differentiation markers, such as SM22 (or transgelin), MYOCD, and α-SMA [5]. Silencing of RGC-32 in ASMC was followed by a significant reduction in TGF-β-induced expression of MYOCD ($p < 0.001$), SM22 ($p < 0.01$), and α-SMA ($p < 0.01$) (Fig. 10E–G).

4. Discussion

Complement activation and C5b-9 assembly was found to contribute to several critical events during atherogenesis: EC activation, SMC migration, platelets adhesion and aggregation, and plaque rupture [26,27]. RGC-32 was first cloned from rat oligodendrocytes by differential display in a search for genes that are differentially expressed in response to complement activation [13]. Sublytic C5b-9 was found to induce the expression of RGC-32 in human ASMC and to induce its nuclear translocation [12]. We report here that RGC-32 has a dual role in SMC, promoting proliferation in cells exposed to sublytic C5b-9 and production of ECM components after exposure to TGF-ß. Sublytic C5b-9 induces the expression of RGC-32 in ASMC [12], we observed here that RGC-32 co-localized with SMC (Fig. 8) and with C5b-9 deposits in the media of both intimal thickenings and fibrous plaques, as we have reported [17].

Our data also show that that silencing of RGC-32 results in the inhibition of cell cycle activation and that RGC-32 overexpression induces cell proliferation in ASMC. We also show for the first time that RGC-32 overexpression results in cell proliferation that is abolished when its Threonine 91 residue is mutated to alanine. These data are in agreement with our previous observation that mutation of the RGC-32 protein at Thr-91 prevents CDC2-mediated phosphorylation of RGC-32 and results in the loss of CDC2 kinase-enhancing activity [12]. In addition, we show here that CDC2 activation is also dependent on MEK1-ERK1 pathway activation and ASMC cell cycle activation. Our data clearly demonstrate a role for RGC-32 in cell proliferation and establish important signaling pathways that are required for cell cycle activation by C5b-9/RGC-32 pathway in ASMC (Fig. 11).

Our immunohistochemical staining of human aortic lesions has also shown that RGC-32 is expressed by AEC and in the media of the atherosclerotic aortic wall, where it co-localizes with SMC, immune-inflammatory cells, and C5b-9 neoantigens [17]. RGC-32 expression increases with the progression of atherosclerosis in both intima and media of the aortic lesions [17]. RGC-32 also contributes to vascular lesion formation in murine models of atherosclerosis by stimulating the proliferation as well as the migration of SMC [14]. In addition, experimental evidence suggests that RGC-32 mediates atherogenesis by facilitating monocyte-EC interactions *via* the induction of endothelial intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression, at least partially through the nuclear factor kappa B (NF-κB) signaling pathway [28]. These data indicate that RGC-32 plays a proatherogenic role and that its deficiency has a protective role.

In addition, our data suggest a role for RGC-32 as a mediator of TGF-β-dependent extracellular matrix ECM production in atherosclerotic lesions, since silencing of RGC-32 significantly reduced the TGF-β-induced

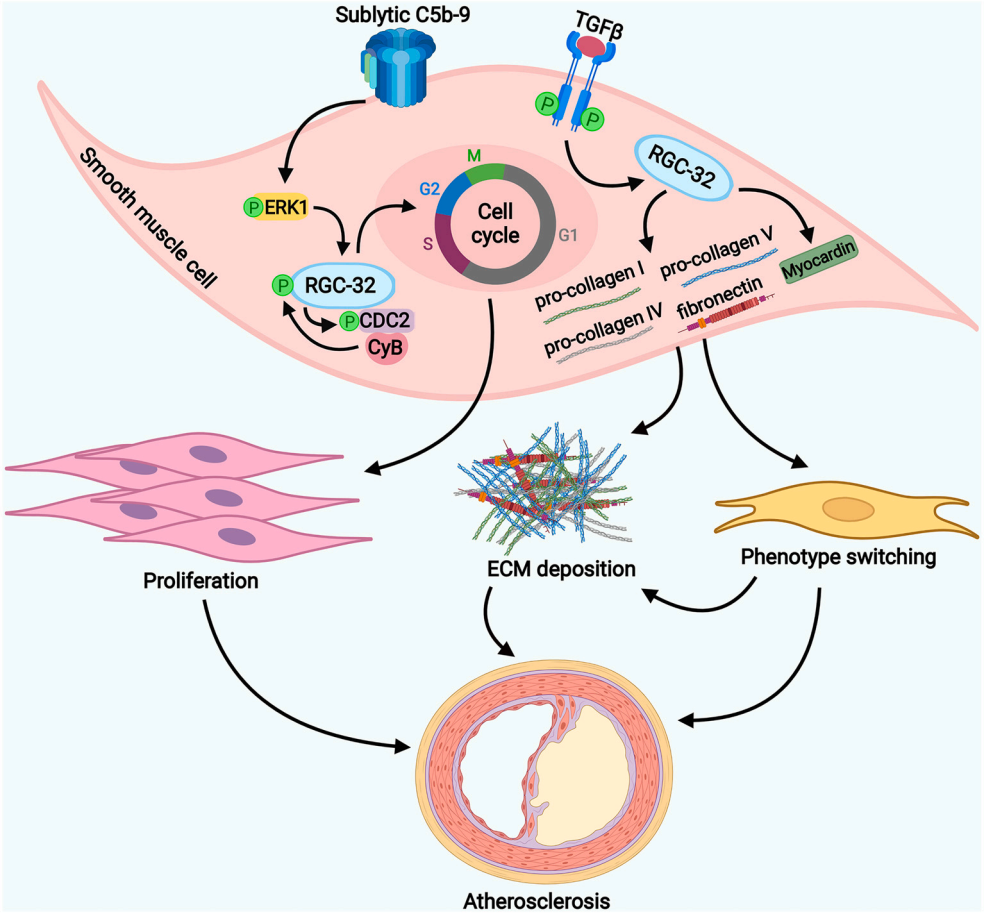

Fig. 11. Dual role of RGC-32 in SMC. RGC-32 has a dual role in both the C5b-9-induced proliferation of ASMC and the TGF-β-dependent production of ECM. Our results show that RGC-32 mediates C5b-9 induced cell cycle activation in SMC through a process dependent upon RGC-32 phosphorylation by CDC2 kinase. Moreover, RGC-32 regulates the TGF-β-induced levels of ECM components collagen type I, type IV, type V and fibronectin, as well as SMC differentiation markers such as myocardin. RGC-32 expression result in a phenotype modulation in SMC which can favor ECM deposition and a synthetic phenotype.

expression of pro-collagens I, IV, and V in ASMC *in vitro*. This role of RGC-32 in ASMC mirrors the effects of RGC-32 that we have previously reported in cultured rat astrocytes, where silencing RGC-32 leads to a significant reduction in the TGF-β-induced expression of ECM components (collagen type I, IV, and V and fibronectin) as well as the differentiation markers nestin and α-SMA [29]. Likewise, others have documented the contribution of RGC-32 to TGF-β-induced ECM in renal proximal tubular cells, where RGC-32 has been found to be critical for TGF-β-induced epithelial-mesenchymal transition [30]. Liu et al. have previously shown that collagen I stimulates SMC proliferation, whereas collagens I and IV, fibronectin, and laminin stimulate SMC migration *via* PI-3 K signaling [31]. Several matrikines (fragments of type I and type IV collagens, elastin, fibronectin, laminins, and hyaluronan) have been shown to exhibit chemotactic activity to attract inflammatory cells [32]. Of major interest, the accumulation of collagen in atherosclerotic plaques has been seen in association with a shift in SMCs from a contractile phenotype to a synthetic phenotype [33,34]. Taken together, these data suggest that ASMC that express C5b-9-induced RGC-32 may also be involved in TGF-β-mediated ECM production, suggesting a role in the phenotypic switch in ASMC during atherosclerosis (Fig. 11).

These data raise the following dilemma: does the ability to stimulate ECM deposition in atherosclerotic lesions automatically confer pro-atherogenic properties on RGC-32? The answer is a rather nuanced one. The experiments linking excessive ECM to accelerating the progression of the atherosclerotic plaques, mainly through the abundant retention of apolipoproteins by proteoglycans produced by the synthetic SMC [5,35,36], coupled with evidence of RGC-32-induced SMC proliferation and migration [14] would argue for a definite pro-atherogenic role for RGC-32. Our data are also in agreement with experiments that indicate that RGC-32 has a proatherogenic role and that its deficiency has a protective role [28]. However, an increased fibrous cap size consistent with stable plaques derived from enhanced ECM production, along with a reduced lesion size and reduction in necrotic zones, has been observed by upregulation of miR-29 which targets *Col1A1* and *Col3A1* in an atherosclerotic mouse model [37]. Similarly, experimental work by Rohwedder et al. has shown that fibronectin, an essential ECM component, promotes the formation of the protective fibrous cap in atherosclerotic lesions [19]. These data argue for a possible protective/plaque-stabilizing role for RGC-32-driven ECM production by SMC in the atherosclerotic wall.

Of note, we also report here that in ASMC, RGC-32 modulates the TGF-β-dependent expression of several relevant differentiation markers: SM22, myocardin, and α-SMA. These data suggest that the synthetic phenotype of ASMC maintains some of the markers that are characteristic of the contractile phenotype, at least *in vitro* [38]. The inflammation characterizing atherosclerotic lesions is contingent on SMC plasticity and their ability to switch between different phenotypes. As such, during the atherogenic process, apart from their basic contractile phenotype, SMC may acquire a synthetic phenotype [39]. Synthetic SMC appear to be a major source of ECM in early atherosclerotic lesions, which translates to increased intimal thickness and augmented lipid retention, but also a more abundant content of foam cells [40,41]. When we used immunohistochemical staining to examine human aortic fibrous plaques and intimal thickenings, the SMC clearly showed a predominantly synthetic phenotype. Jeon and collab have demonstrated the vital contribution of the Rho/actin/myocardin-related transcription factor A signaling axis in controlling the SMC phenotype [42]. We found that activation of RGC-32 through the C5b-9/Akt pathway resulted in cell cycle activation (*via* ROCK – Ras – MAPK - cyclin D1) and cellular migration in AEC [17]. These observations raise the possibility that

RGC-32 is involved in SMC phenotype regulation through the Rho/actin/myocardin signaling pathway.

RGC-32 has a dual role in both the C5b-9-induced proliferation of ASMC and the TGF-β-dependent production of ECM and the cells' differentiation into a pro-atherogenic synthetic phenotype. Further investigations are needed to identify future RGC-32-targeted treatments to prevent or mitigate atherosclerotic disease.

Abbreviations

α-SMA	alpha smooth muscle actin
AEC	aortic endothelial cells
ASMC	aortic smooth muscle cells
CDC2	cell division control 2
EC	endothelial cells
ECM	extracellular matrix
ERK1	extracellular signal-regulated kinase 1
FP	fibrous plaques
IT	intimal thickenings
LDL	low-density lipoprotein
MYOCD	myocardin
NF-κB	nuclear factor *kappa B*
NHS	*normal human serum*
RGC-32	Response Gene to Complement-32
SM22	Smooth Muscle Protein 22
TGF-β	*transforming growth factor*-β
VSMC	vascular smooth muscle cells

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