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Research Article

MicroRNA-31 controls phenotypic modulation of human vascular smooth muscle cells by regulating its target gene cellular repressor of E1A-stimulated genes

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ABSTRACT

Phenotypic modulation of vascular smooth muscle cells (VSMCs) plays a critical role in the pathogenesis of a variety of proliferative vascular diseases. The cellular repressor of E1A-stimulated genes (CREG) has been shown to play an important role in phenotypic modulation of VSMCs. However, the mechanism regulating CREG upstream signaling remains unclear. MicroRNAs (miRNAs) have recently been found to play a critical role in cell differentiation via target-gene regulation. This study aimed to identify a miRNA that binds directly to CREG, and may thus be involved in CREG-mediated VSMC phenotypic modulation. Computational analysis indicated that miR-31 bound to the CREG mRNA 3′ untranslated region (3′-UTR). miR-31 was upregulated in quiescent differentiated VSMCs and downregulated in proliferative cells stimulated by platelet-derived growth factor and serum starvation, demonstrating a negative relationship with the VSMC differentiation marker genes, smooth muscle α-actin, calponin and CREG. Using gain-of-function and loss-of-function approaches, CREG and VSMC differentiation marker gene expression levels were shown to be suppressed by a miR-31 mimic, but increased by a miR-31 inhibitor at both protein and mRNA levels. Notably, miR-31 overexpression or inhibition affected luciferase expression driven by the CREG 3′-UTR containing the miR-31 binding site. Furthermore, miR-31-mediated VSMC phenotypic modulation was inhibited in CREG-knockdown human VSMCs. We also determined miR-31 levels in the serum of patients with coronary artery disease (CAD), with or without in stent restenosis and in healthy controls. miR-31 levels were higher in the serum of CAD patients with restenosis compared to CAD patients without restenosis and in healthy controls. In summary, these data demonstrate that miR-31 not only directly binds to its target gene CREG and modulates the VSMC phenotype through this interaction, but also can be an important biomarker in diseases involving VSMC phenotypic modulation. These novel findings may have extensive implications for the diagnosis and therapy of a variety of proliferative vascular diseases.

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Introduction

Vascular smooth muscle cells (VSMCs) are not terminally differentiated and possess the ability to regulate their phenotype in response to changing local environmental cues [1]. The transition of VSMCs from a differentiated and non-proliferative phenotype to a synthetic, proliferative state is known to play a critical role in physiologic vasculature remodeling, restenosis after angioplasty, and in disease states such as atherosclerosis [2]. The phenotypic transition of VSMCs reacquires the abilities to migrate, proliferate, and produce extracellular matrix components [3]. Identifying the mechanisms regulating VSMC phenotypic switching are thus currently an important research topic in the area of proliferative cardiovascular diseases [4].

Cellular repressor of E1A-stimulated genes (CREG) is a recently-identified glycoprotein that antagonizes cellular transformation and transcription activation induced by the adenovirus E1A oncoprotein [5]. Accumulating evidence suggests that CREG may play an important role in modulating cell differentiation and proliferation, thus antagonizing malignant proliferation. We and other investigators found that both CREG mRNA and protein levels were significantly increased during phenotypic switching of proliferative VSMCs to differentiated cells *in vitro* [6–9]. Moreover, recent studies demonstrated that CREG levels were significantly decreased in the vascular media of rat carotid artery tissue after balloon injury [10], whereas adenovirus-mediated CREG overexpression inhibited VSMC proliferation and attenuated neointimal hyperplasia in balloon-injured rabbit carotid arteries [11]. However, the mechanisms responsible for CREG upstream signaling remain unclear.

The most important recent breakthrough regarding gene expression regulation has been the discovery of microRNAs (miRNAs) [12]. miRNAs comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate gene expression via degradation or translational inhibition of their target mRNAs [13–16]. As a group, miRNAs may directly regulate at least 30% of the genes in a cell [17]. Moreover, one miRNA can regulate the expression of multiple genes, because it is able to bind to its mRNA targets as either a perfect or imperfect complement [18]. Thus, miRNAs are functionally important transcription factors and miRNA analysis is an important aspect of cell biology. Many miRNAs have recently been found to play critical roles in cell differentiation [19–22]. Several studies have shown that knockdown of miR-31 substantially repressed lung cancer cell growth and tumorigenicity in a dose-dependent manner [23] and antagonized the morphological changes, and migratory and invasive properties of carcinoma cells [24]. Other studies have revealed the involvement of miR-31 in cancer cell proliferation, migration, and invasion [25–27]. These results suggest that the level of miR-31 may be a critical factor controlling cell proliferation, differentiation and phenotype. The biological function of each individual miRNA is cell-specific, and the same miRNA may have different effects in different cells; for instance, the effects of miR-21 differ between glioblastoma and Hela cells [28,29]. Importantly, computational analysis suggests that miR-31 is able to bind to the CREG mRNA 3′-UTR. However, the role of miR-31 in VSMCs and its involvement in CREG-mediated VSMC phenotypic modulation is currently unknown.

The present study aimed to determine the role of miR-31 in regulating CREG expression, and its potential regulatory effects on CREG-mediated VSMC phenotypic modulation.

Materials and methods

Cell culture

Human VSMCs were isolated from unused segments of human internal thoracic arteries retrieved during coronary bypass surgery. Primary cultures were maintained in medium 199 (M199) supplemented with 10% fetal bovine serum. VSMC identity was confirmed morphologically and by positive immunostaining for smooth muscle (SM) α-actin (1A4, Dako, Denmark). Cloning of human VSMCs was performed using a modified cloning-ring approach. Clones were deemed to be uniquely informative with respect to differentiation of human VSMCs. One clone was characterized in this context, and forms the focus of this report. The use of human tissues was approved by Shenyang Northern Hospital Ethics Committees and all patients gave their informed consent.

Oligo transfection, target protector transfection, miR-31 knockdown and overexpression in cultured VSMCs

VSMCs were isolated as described above. Oligo transfection was performed according to the manufacturer's instructions. Briefly, cells were transfected using a transfection reagent (Qiagen, CA, USA) 24 h after seeding. Transfection complexes were prepared according to the manufacturer's instructions. For the miR-31 knockdown, a miR-31 inhibitor (Qiagen) was added to the complexes at a final oligonucleotide concentration of 100 nmol/L. A miR-31 mimic (Qiagen) was added to the complexes at a final oligonucleotide concentration of 25 nmol/L to induce miR-31 overexpression. A target protector (Qiagen), which protects the miR-31-binding site of the CREG mRNA 3′-UTR, was added to the complexes at a final oligonucleotide concentration of 500 nmol/L, to protect the miR-31 target on the CREG mRNA 3′-UTR. Vehicle and oligo controls (Qiagen) were applied. The transfection medium was replaced with regular culture medium at 12 h post-transfection.

Generation of CREG-knockdown human VSMC lines

CREG-knockdown VSMCs were generated by infecting cells with retrovirus-expressing CREG short hairpin RNAs (sh-CREG), followed by selection with 6 μg/mL puromycin for 2 weeks. Cell lysates were harvested and CREG expression was evaluated by western blot analysis with an anti-CREG antibody. β-actin served as control. Three retroviral vectors containing short hairpin RNAs (shRNA) targeting the open reading frame of human CREG were purchased from Open Biosystems (Huntsville, AL).

Protein extraction and western blotting

For western analysis, cell lysates were prepared in lysis buffer containing 10% sodium dodecyl sulfate (SDS). After centrifugation at $15{,}000 \times g$ for 10 min, supernatants were used for western blotting. Total cell protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, CA, USA). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with appropriate primary antibodies. CREG