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REVIEW

RGC-32 and diseases: the first 20 years

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Abstract

The response gene to complement (RGC)-32 acts as a cell cycle regulator and mediator of TGF-β effects. However, recent studies have revealed other functions for RGC-32 in diverse processes such as cellular migration, differentiation, and fibrosis. In addition to its induction by complement activation and the C5b-9 terminal complement complex, RGC-32 expression is also stimulated by growth factors, hormones, and cytokines. RGC-32 is induced by TGF-β through Smad3 and RhoA signaling and plays an important role in cell differentiation. In particular, RGC-32 is essential for the differentiation of Th17 cells. RGC-32$^{-/-}$ mice display an attenuated experimental autoimmune encephalomyelitis phenotype that is accompanied by decreased central nervous system inflammation and reductions in IL-17- and GM-CSF-producing CD4^{+} T cells. Accumulating evidence has drawn attention to the deregulated expression of RGC-32 in human cancers, atherogenesis, metabolic disorders, and autoimmune disease. Furthermore, RGC-32 is a potential therapeutic target in multiple sclerosis and other Th17-mediated autoimmune diseases. A better understanding of the mechanism(s) by which RGC-32 contributes to the pathogenesis of all these diseases will provide new insights into its therapeutic potential.

Keywords RGC-32 · Atherosclerosis · Cancer · Autoimmunity · Cell cycle

Introduction

Twenty years ago, in October 1998, a research team led by Horea Rus first reported the cloning of the RGC-32 gene in rat oligodendrocytes using differential display, in a quest to describe the genes that are differentially expressed in response to complement activation [1]. The cloning of human and mouse RGC-32 genes by the same group followed shortly [2]. The human RGC-32 gene, encoding a 137-amino acid protein with a high similarity (92%) to the corresponding rat and mouse proteins, is found on chromosome 13 in humans [2]. RGC-32 is abundantly expressed in the placenta, skeletal muscle, kidney, pancreas, liver, and aortic endothelial cells and weakly expressed in the heart and brain; in contrast, lung tissue lacks mRNA for RGC-32 [2]. In addition to its induction by complement activation, a large number of diverse stimuli are able to modulate RGC-32 expression, including serum, growth factors [3, 4], steroid hormones [5], and precursors of thyroid hormones [5]. Since the initial report describing RGC-32 as a gene induced by complement activation and involved in cell cycle regulation [2], an impressive body of experimental work has detailed the functional role of RGC-32 in fundamental biological processes such as cell proliferation, differentiation, tumorigenesis, and immunity and its possible role in cardiovascular, oncologic, neurologic, and immune-mediated diseases. This

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review will highlight the recent advances in RGC-32 research and discuss its association with a wide range of diseases.

RGC-32 and cell proliferation

Sublytic C5b-9 induces cell cycle activation and increases in the levels of cyclin-dependent kinase (CDK) 4, CDK2, cell division cycle protein 2 (CDC2) [6, 7], and RGC-32 expression [2]. RGC-32 functions as a substrate and regulator of CDC2 activity [2]. Phosphorylation of RGC-32 at Thr-91 is required for RGC-32 to stimulate CDC2 kinase activity [2]. We have shown that RGC-32 is present in the cytoplasm of smooth muscle cells (SMC) and translocates to the nucleus when SMC are exposed to activated complement [2]. Overexpression of this protein in human aortic SMC is followed by entry into S-phase and G2/M in unstimulated cells, and exposure to C5b-9 further accelerates the G2/M progression [2]. Likewise, overexpressing RGC-32 in the OLGxC6 glioma cell line also results in an increase in DNA synthesis in response to serum growth factors [1].

On the other hand, silencing RGC-32 in these cells using specific siRNA abolishes DNA synthesis induced by C5b-9 and serum growth factors. SiRNA-induced RGC-32 knockdown also significantly reduces C5b-9-induced CDC2 activation and Akt phosphorylation in aortic endothelial cells (AEC). RGC-32 has been found to physically associate with Akt and to be phosphorylated by Akt in vitro. These results suggest that the C5b-9-induced cell cycle in AEC is RGC-32-dependent, and the induction is accomplished in part through the mediation of Akt phosphorylation and growth factor release [8].

We have shown that SP600125, a c-Jun N-terminal kinase (JNK) inhibitor, and PD98059, an mitogen activated protein kinase kinase 1 (MEK1) inhibitor, both effectively inhibit RGC-32 expression induced by C5b-9 in human aortic smooth muscle cells [5]. The involvement of p38 mitogen-associated protein kinase (MAPK) has also been reported, since the p38 inhibitor SB202190 has been shown to block the basal expression of RGC-32 in human endothelial cells [9]. RGC-32 has been documented to regulate the G2/M transition in SW480 colon cancer cells [10], C6 glioma cells [11], and Epstein–Barr virus (EBV)-immortalized B cells [12] as well as during renal tubular epithelial cell repair secondary to TNF-α-induced acute injury [13].

RGC-32 has also been found to negatively affect T cell proliferation. Our group has shown that $CD4^+$ and $CD8^+$ T cells purified from the spleens of RGC-32 knockout (KO) mice show greater proliferation than do those from wild-type (WT) mice after anti-CD3/CD28 co-stimulation. $CD4^+$ cells from RGC-32 KO mice show an increased IL-2 expression and an increased activation of the phosphatidylinositol-3-kinase (PI3K) pathway. These results suggest that RGC-32 negatively modulates T lymphocyte proliferation by inhibiting the T cells' cell cycle [14].

A significant contribution of RGC-32 to the neuronal cell cycle has also been documented. Aberrant neuronal cell cycle re-entry characterizes the onset of Alzheimer's disease (AD), and RGC-32 mRNA and protein levels have been found to be upregulated by ~ 50–60% in frontal cortex samples of patients with mild cognitive impairment or AD when compared to samples from individuals with no cognitive impairment. Moreover, RGC-32 protein levels have been seen to correlate with poorer antemortem global cognitive performance. Experimental inhibition of RGC-32 expression leads to an increased survival of PC12 cells and precludes cyclin B1 up-regulation [15].

In conclusion, these data together indicate an important role for RGC-32 in the cell cycle in a variety of cell types under both physiological and pathological conditions.

RGC-32 and cell differentiation

RGC-32 has proved critical for the TGF-β-induced differentiation of vascular SMC from neural crest cells [4]. RGC-32 expression is upregulated after TGF-β stimulation of Monc-1 cells, an immortalized neural crest cell line; the authors of this study found that TGF-β activates Smad and RhoA signaling, which stimulates RGC-32. RGC-32, in turn, switches on the transcription of smooth muscle genes, including α-smooth muscle actin (α-SMA), SM22α, and calponin, thereby initiating the differentiation of vascular SMC [4]. In addition Smad2 regulation of RGC-32 transcription is essential for SMC differentiation from neural crest cells and polyomavirus enhancer activator 3 (PEA3) appears to enhance Smad2 activity [16].

Other studies have shown that RGC-32 is also important for macrophage differentiation. RGC-32 is expressed in macrophages [17] and has been shown to favor polarization toward the M2 macrophage phenotype and to suppress the M1 phenotype [18, 19]. RGC-32 has proved essential for in vitro macrophage phagocytosis, since it has been found to regulate particle internalization through activation of protein kinase C-induced F-actin assembly and to promote phagosomal cup formation [18]. Zhao et al. have reported that RGC-32 expression is increased during IL-4-induced polarization of THP-1 cells and monocyte-derived macrophages toward the M2 phenotype and decreased in LPS-polarized M1 macrophages. Increased RGC-32 levels in M2 macrophages are associated with a reduction in the expression of IL-6 and an increase in the expression of TGF-β, thus promoting their anti-inflammatory phenotype [19]. Tumor-associated macrophages, a subtype of M2 macrophages present in the tumor microenvironment and promoting tumor progression, also

express high levels of RGC-32 [19]. Because they regulate the balance between the M1 and M2 macrophage polarization in the tumor microenvironment, RGC-32 may be a possible target in cancer therapy [20].

Our group has shown that RGC-32 plays an important role in the differentiation of Th17 cells [21]. RGC-32 is upregulated under conditions favorable for Th17 differentiation, in the presence of IL-6 and TGF-β. $CD4^+$ lymphocytes isolated from RGC-32 KO mice show an impaired differentiation toward Th17, with lower expression of IL-17 and the key transcription factors for the Th17 lineage, such as RORγt, interferon regulatory factor 4 (IRF4), and basic leucine zipper transcription factor (BATF) [21]. Interestingly, RGC-32 KO $CD4^+$ cells normally differentiate toward the Th1, Th2, and Treg subsets, suggesting a preferential role for RGC-32 in Th17 differentiation in vitro. Moreover, RGC-32 deficiency has been shown to attenuate the production of $IL\text{-}17^+$ T cells isolated from the intestinal lamina of mice, implying that RGC-32 also plays an important role in the generation of Th17 cells in vivo [21]. The role of RGC-32 in the differentiation of astrocytes will be discussed below when we consider RGC-32's involvement in multiple sclerosis (MS).

RGC-32 in cardiovascular diseases

Atherosclerosis

Both innate and adaptive immune responses are essential during the initiation and progression of atherosclerosis. As part of innate immunity, the complement system has been shown to be involved in the induction and progression of atherosclerosis [22]. Since RGC-32 is a mediator of C5b-9 effects that regulate cellular proliferation, its role in the initiation and progression of atherosclerosis has recently been investigated [22, 23].

RGC-32 and ECs

We have demonstrated that RGC-32 silencing in primary human AEC abrogates the ability of sublytic C5b-9 to induce the cell cycle, migration, and CDC2 activation, while also suppressing the activation of Akt [8, 22]. We also found that a significant number of genes regulated by RGC-32 possess critical functions related to endothelial cell proliferation, migration, cytoskeleton reorganization, and extracellular matrix processes. These differentially regulated genes include cyclin D1, cyclin D3, Akt, rho-associated coiled-coil-containing protein kinase 1 (ROCK1), Rho GDP dissociation inhibitor alpha (Rho-GDI), and profilin [22]. RGC-32 silencing also alters the C5b-9-induced expression profile of growth factors pertinent to atherogenesis, diminishing the release of leptin, placental growth factor (PIGF), and regulated upon activation normal T cells expressed and secreted (RANTES) and increasing the release of IL-8, tissue inhibitors of metalloproteinases 1 (TIMP-1), and vascular endothelial growth factor D (VEGF-D) [8]. Our data suggest that C5b-9 stimulation prompts Akt activation; Akt, in turn, activates RGC-32, which then can cause cell cycle activation (via ROCK–Ras–MAPK–Cyclin D1), migration, and release of growth factors or focal adhesion of EC (through induction of profilin1 and actin polymerization) in AEC [8, 22].

We found that RGC-32 is expressed by EC and in the media of the human atherosclerotic aortic wall, where it co-localizes with SMC, immune-inflammatory cells, and the C5b-9/membrane attack complex. Foam cells were also found to express RGC-32 in their cytoplasm. RGC-32 expression increases with the progression of atherosclerosis in both the intima and media of the aortic lesions [8]. A recent report from Cui et al., however, shows the presence of RGC-32 in the EC, with no significant expression in the SMC in either human coronary arteries with atherosclerotic lesions or aortic roots in $ApoE^{-/-}$ mice fed a high-fat diet. In this mouse model, RGC-32 expression has virtually no effect on collagen or α-SMA expression, nor upon the thickness of the fibrous cap in the atherosclerotic lesions [23]; furthermore, $ApoE^{-/-}$ $RGC32^{-/-}$ mice show diminished formation of atherosclerotic lesions when compared to $ApoE^{-/-}$ control mice [23]. These data indicate a pro-atherogenic role for RGC-32, a function accomplished in this experimental model by a monocyte-endothelial cell interaction occurring as a result of the induction of endothelial intercellular adhesion molecules 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression via NF-κB signaling [23] (Fig. 1).

RGC-32 and SMCs

RGC-32 contributes to vascular lesion formation in a rat carotid balloon-injury model and enhances both the proliferation and migration of rat aortic SMC. RGC-32 promoted SMC migration by generating enhanced ROKα activity, which in turn induced focal adhesion contact and stress fiber formation [24]. These data are consistent with our data showing that RGC-32 is involved in SMC proliferation [2] (Fig. 1).

In addition, our immunohistochemistry data have shown the expression of RGC-32, as well as collagens I, IV, and V, in aortic SMC in human atherosclerotic fibrous plaques and intimal thickenings. In cultured aortic SMCs, blocking RGC-32 mRNA expression leads to a significant reduction in TGF-β-induced expression of collagens I, IV, and V and fibronectin. Thus, RGC-32 appears to function as a mediator of TGF-β-induced production of ECM in atherosclerotic plaques [25].

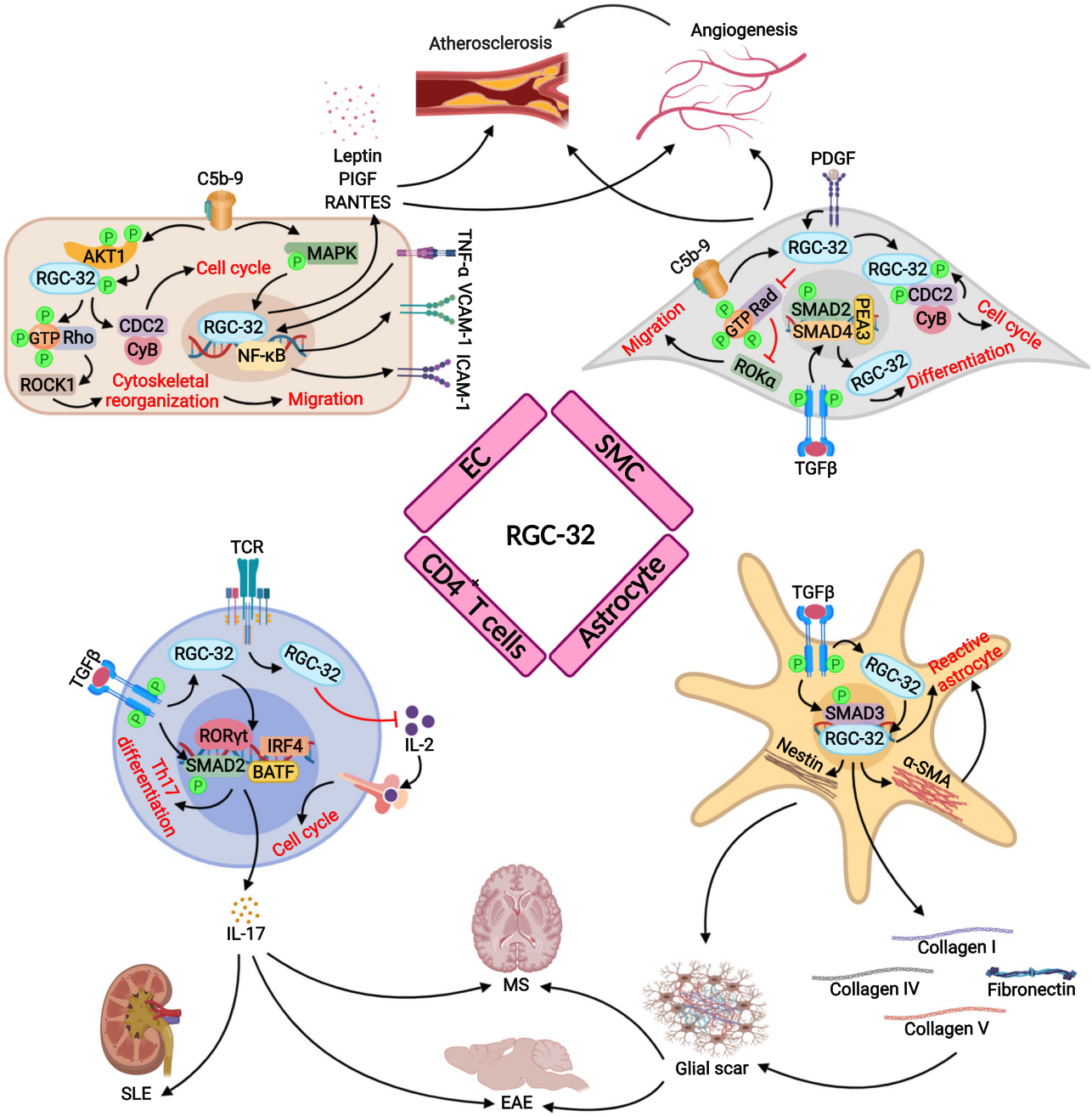

Fig. 1 RGC-32 role in cellular processes and its link to diseases. The role of RGC-32 in regulating various biological processes has been extensively studied in four cell types: (1) In endothelial cells (EC), RGC-32 expression is induced by sublytic C5b-9 and other cytokines (e.g., TNF-α) and stimulates cellular proliferation, cytoskeletal reorganization, migration, and focal adhesion by enhancing the activity of CDC2/cyclin B1 (CDC2/CyB) complexes, regulating the Rho-ROCK axis, inducing the expression of adhesion molecules such as ICAM-1 and VCAM-1, and promoting growth factor release (PIGF, Leptin, RANTES). (2) In smooth muscle cells (SMCs), RGC-32 is crucial for TGF-β-induced SMC differentiation in that it promotes the expression of genes associated with differentiation. C5b-9-induced RGC-32 expression is also important for cell proliferation, migration, focal adhesion, and stress fiber formation through CDC2/CyB activity enhancement and Rad-ROKα pathway regulation. In the pathological setting, all these pathways described in EC and SMC eventually contribute to atherogenesis and angiogenesis. (3) In $CD4^+$ T cells, RGC-32 expression is upregulated by T cell receptor (TCR)-CD28 costimulation and inhibits cell cycle and proliferation by suppressing IL-2 release; moreover, RGC-32 is induced under conditions of Th17 differentiation and stimulates the expression of key transcription factors for the Th17 lineage, e.g., RORγt, BATF, and IRF4, suggesting a role for RGC-32 in pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), and its experimental model experimental autoimmune encephalomyelitis (EAE). (4) In astrocytes, RGC-32 is induced by TGF-β and translocated into the nucleus in association with SMAD3. The induction of RGC-32 results in expression of the reactive astrocytic markers α-SMA and nestin and the synthesis of the ECM component collagen type I, type IV, type V, and fibronectin, thereby modulating astrocytic reactivity and the formation of the glial scar

Hypertension and pre-eclampsia

The involvement of RGC-32 in regulating blood pressure was recently demonstrated in a study showing that RGC-32 KO mice exhibit higher blood pressure than do WT mice. The small-resistance arteries isolated from RGC-32 KO mice showed an increased vascular tone and a decreased distensibility, mainly as the result of an increased expression of angiotensin II type 1 receptor (AT1R) and α1 adrenergic receptor (α1-AdR). In vitro data showed that RGC-32 regulates the expression of AT1R in conjunction with the transcription factor Sp1 [26], regulating AT1R gene transcription by interacting with Sp1 and blocking its binding to the AT1R promoter, leading to suppression of AT1R expression. This reduction in AT1R leads to a reduction in α1-AdR expression, which is critical for achieving the proper sympathetic-parasympathetic balance in the control of vascular tone. It is important to mention that a downregulation of RGC-32 in arterial SMC is associated with low birth weight and hypertension in humans [26].

There are several lines of evidence supporting a proangiogenic role for RGC-32 in pre-eclampsia. An absence of RGC-32 during pregnancy in mice has been shown to result in fetal growth restriction produced by impaired angiogenesis and defective placental development [27]. In addition, the RGC-32 levels are significantly downregulated in pre-eclamptic placentas when compared with normal controls. RGC-32 silencing significantly inhibits cell migration and invasion. These effects are all associated with decreased activity and expression of matrix metalloproteinases (MMP)-2 and 9 and with a reduced level of Akt phosphorylation [28]. In a mouse model of pre-eclampsia, RGC-32 expression, along with that of proangiogenic factors PIGF and VEGF, is downregulated in the placenta; meanwhile, the expression of sFLT-1 is increased, and the complement system is activated. These changes form the basis for a compromised post-implantation decidual angiogenesis, with concurrent excess complement deposition around the placental vasculature [29].

Cardiac diseases

In line with our observation of a role for RGC-32 in Th17 differentiation [21], Li et al. have shown that RGC-32 levels are directly correlated with those of Th17 cells and inversely correlated with those of Tregs in the plasma from patients with dilated cardiomyopathy. Overexpression of RGC-32 in the peripheral blood mononuclear cells (PBMCs) obtained from these patients results in an increased frequency of Th17 cells and a decreased frequency of Tregs [30].

RGC-32 in metabolic disorders

Obesity

Low-grade inflammation in the endothelium, central obesity, and insulin resistance are all intimately connected through visceral fat-derived metabolic products, hormones, and cytokines [31].

A high-fat diet (HFD) has been shown to induce RGC-32 upregulation in murine adipose and liver tissue [32, 33]. The absence of RGC-32 from HFD-fed mice translates into a leaner body phenotype, increased energy expenditure (thanks to enhanced browning of adipose tissue), improved lipid homeostasis and insulin sensitivity, along with a lessening of adipose tissue and systemic inflammation (local or circulating levels of adiponectin, leptin, IL-6, and TNFα) [32]. An RGC-32 deficiency results in improvements in HFD-induced dyslipidemia and insulin resistance as well as systemic inflammation. Moreover, an RGC32 deficiency induces the browning of adipose tissues and increases energy expenditure. In murine adipose tissue, RGC-32 has also been observed to inhibit the expression of vital thermogenic genes such as uncoupling protein-1 (UCP-1) and peroxisome proliferator-activated receptor co-activator 1α (PGC-1α); this inhibition appears to be mediated through the activation of PI3K/Akt signaling and hindered expression of β3-adrenergic receptor, which in turn restrains mTORC1/S6K1 activity [34]. An extremely relevant finding in this study concerning the atherosclerotic process and vascular inflammation is that the perivascular adipose tissue (PVAT) in RGC-$32^{-/-}$ mice contains more beige cells that stain positive for UCP-1 and also express elevated mRNA levels of UCP-1 and PGC-1α than does the PVAT in control mice [34]. These results imply that an RGC-32 deficiency can drive the browning of the PVAT, thus hampering inflammation in the vascular milieu.

Hepatic steatosis

Since non-alcoholic fatty liver is considered by some to represent the hepatic aspect of metabolic syndrome [35], given the effects exerted by RGC-32 in the inflamed arterial wall and in adipose tissue, one might anticipate that RGC-32 would be an active contributor to the development of hepatic steatosis. Indeed, RGC-32 deficiency has been shown to preclude HFD-induced hepatic steatosis in mice, with a decreased liver triglyceride content and improved insulin sensitivity documented in RGC-$32^{-/-}$ mice. In mouse hepatic tissue, RGC-32 supports de novo lipogenesis by inducing the sterol regulatory element binding protein-1c transcription factor (SREBP-1c) and its target lipogenic gene product stearoyl-CoA desaturase 1 (SCD1), via activation of the liver X receptor [36]. Interestingly, RGC-32 also belongs to a list of

potential biomarkers that have been shown to discriminate between healthy subjects and those with non-alcoholic steatohepatitis [37].

DM

RGC-32 has deleterious effects on glucose metabolism, since RGC-32 KO mice have been reported to be protected against HFD-induced dyslipidemia and insulin resistance [32]. RGC-32 has been reported to contribute to the endothelium-related effects of glucose regulation. RGC-32 has been found to improve the glucose excursion after carbohydrate stress, seemingly by regulating several genes related to glucose metabolism (GFPT1, GLUT12, and GLP2R) [33].

Recently, RGC-32 was found to be induced in the diabetic kidney, possibly via activation of pregnane X receptors (PXR). These data suggest that PXR-RCG-32 is part of a candidate pathway that modulates profibrotic and/or metabolic changes in the diabetic kidney [38].

In addition, the role of RGC-32 has been investigated in the development of diabetic retinopathy (DR). No significant changes in histopathology or RGC-32 expression have been found between the retinas of type 2 diabetes (T2D) mice and control mice at 16 or 24 weeks of age. However, RGC-32 expression is significantly decreased in T2D mouse retinas when compared to those of the control group at 32 weeks of age, when T2D shows features of the early clinical stages of DR (reduced retinal thickness and increased ganglion cell death) [39]. RGC-32 is predominantly expressed in the photoreceptor inner segments in control mice, but its expression is dramatically lowered in the retinas of the T2D mice. In addition, the level of anti-apoptotic protein Bcl-2 is decreased, with a concomitant increase in cleaved caspase-3 in the T2D retinas when compared to control retinas. These data suggest that RGC-32 plays a role in DR pathogenesis [39].

All these data indicate that RGC-32 plays an important role in diet-induced obesity, insulin resistance, and diabetes mellitus; thus, RGC-32 may serve as a potential novel drug target for treating obesity and metabolic disorders.

RGC-32 in renal diseases

Renal fibrosis

Fibrosis is characterized by excessive extracellular matrix (ECM) deposition in various organs and is primarily the result of chronic inflammation that leads to an accumulation of inflammatory cells in the target organ. The infiltrating cells produce a wide range of pro-inflammatory cytokines that stimulate the differentiation of myofibroblasts, and these myofibroblasts in turn secrete large quantities of ECM components [40]. The myofibroblasts can be derived from a variety of other cells, including fibroblasts, epithelial cells, or endothelial cells.

An important mechanism governing fibrosis is the epithelial–mesenchymal transition (EMT), which refers to the dedifferentiation of the epithelial cells toward a mesenchymal phenotype. This process is largely driven by TGF-β and is associated with an increase in the expression of mesenchymal markers, such as α-SMA and vimentin and a decrease in the expression of epithelial markers such as E-cadherin and cytokeratin [41] (Fig. 2). The EMT also plays an important role in cancer progression and metastasis, because the cancerous cells tend to lose their adhesiveness and gain migratory capacity, both of which are features of dedifferentiated cells [42–44].

A role for RGC-32 in TGF-β-induced EMT and renal fibrosis has been shown in several studies [42–44]. For example, it has been demonstrated that RGC-32 expression is upregulated in renal interstitial cells in a mouse model of kidney fibrosis [43]. In vitro, RGC-32 expression is induced by TGF-β stimulation and makes an important contribution to fibroblast activation and the EMT of renal proximal tubular cells [43]. TGF-β-induced EMT of renal proximal cells depends on the physical interaction between RGC-32 and Smad3; interestingly, Smad2 does not seem to interact with RGC-32 [42]. The RGC-32-Smad3 interaction is critical for the expression of the EMT transcription factors Snail and Slug and the upregulation of α-SMA, as well as the extracellular matrix components fibronectin and collagen I [42–44] (Fig. 2).

IgA nephropathy

Niu et al. have investigated the expression of RGC-32 in the human kidneys, in both healthy children and children with IgA nephropathy [45]. They found that RGC-32 protein is present in the cells of the proximal and distal tubules and collecting ducts, but not in the interstitium, glomeruli, or renal blood vessels. The same authors found that RGC-32 co-localizes with TGF-β1 and α-SMA in tubular epithelial cells in children with IgA nephropathy, and their expression is positively correlated with an increasing severity of the renal lesions, suggesting a possible participation of RGC-32 in EMT-related tubulointerstitial fibrosis in IgA nephropathy [45].

AKI

Other studies have assessed the expression of RGC-32 during acute kidney injury (AKI). As compared to control cells, RGC-32 is decreased in the tubular cells of a rat model of AKI 24 to 72 h after injury, but the levels return to normal after 1 week [46]. Liu et al. have found higher levels of serum RGC-32 in patients with AKI who underwent cardiopulmonary bypass (CPB) than in those with no AKI; these authors have suggested that serum RGC-32 could be a better biomarker than serum creatinine or serum cystatin C for detecting AKI

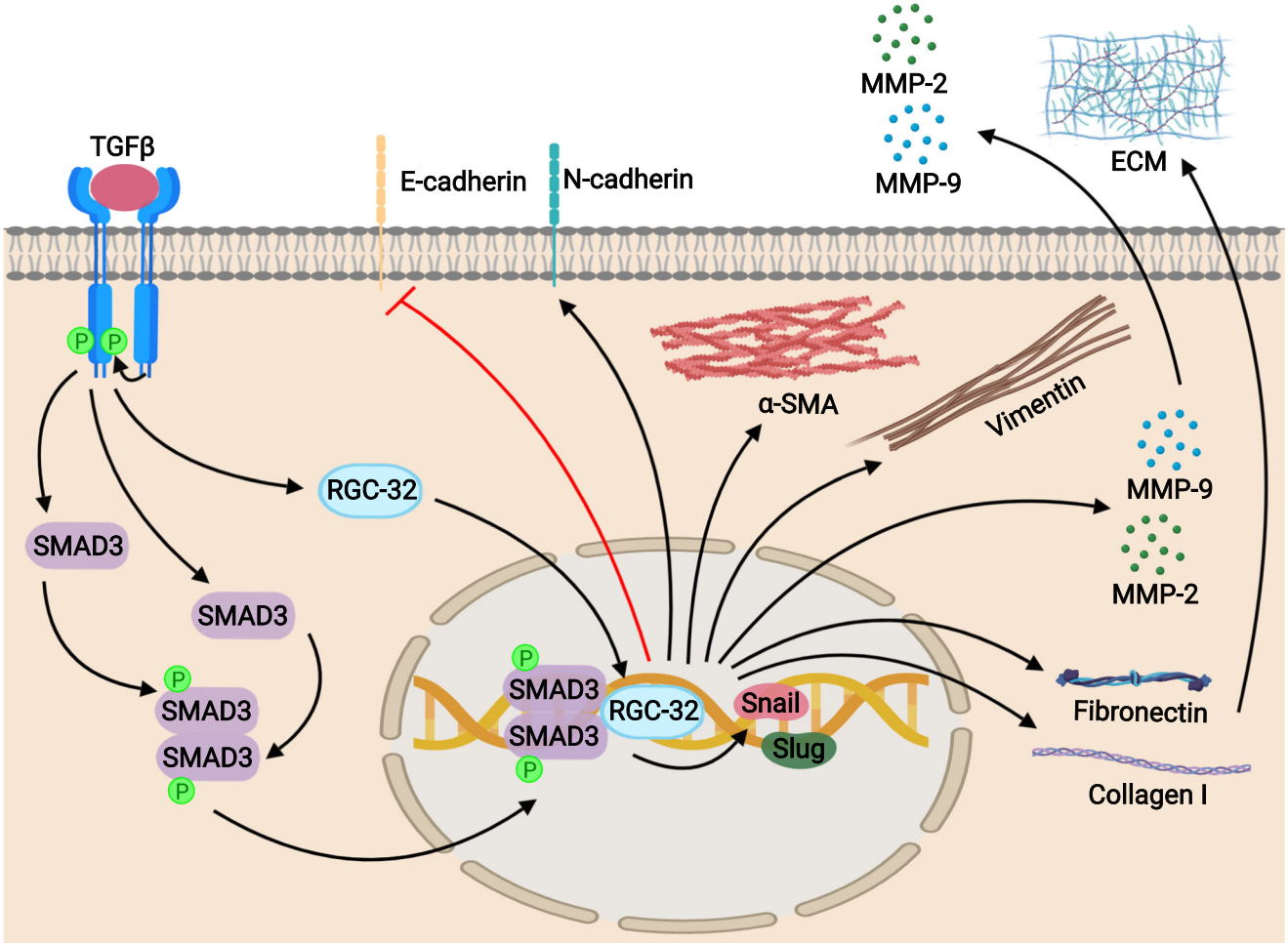

Fig. 2 Role of RGC-32 in the TGF-β-induced epithelial-to-mesenchymal transition (EMT) and fibrosis. During EMT, TGF-β stimulation results in an increased expression of RGC-32, which, in turn, interacts with the SMAD3 complexes in the nuclei of epithelial cells. This process results in the augmented expression of the key EMT-related transcription factors Snail and Slug, which inhibit the expression of the epithelial marker E-cadherin and stimulate the expression of N-cadherin, vimentin, and α-SMA, all markers of mesenchymal cells. In addition, TGF-β-induced RGC-32 expression is associated with the synthesis of MMP2 and 9, fibronectin, and collagen I. The RGC-32-regulated EMT is associated with the development of renal fibrosis after kidney injury and with cancer invasion and metastasis in lung, pancreatic, and colon adenocarcinomas

in patients with CPB [47]. In vitro data have also noted the substantial impact of RGC-32 on the repair of renal tubular epithelial cells after AKI [13, 46]. Using cells of the rat kidney cell line NRK-52E that have been acutely injured with TNF-α, they have found that RGC-32 regulates the G2/M-phase cell cycle checkpoint as well as cell fibrosis and cell adhesion [13, 46].

RGC-32 in cancer

RGC-32 expression in tumors

RGC-32 is intimately involved in cell cycle control, and for that reason, data concerning the aberrant RGC-32 expression in human cancers is abundant. RGC-32 expression has been documented as being upregulated in colon [3, 48], ovarian [49, 50], breast [3, 51, 52], and prostate [3] cancers, and in cutaneous T cell lymphomas [53], as well as in Burkitt's lymphoma group III and lymphoblastoid cell lines [54]. Other groups have reported downregulation of RGC-32 mRNA expression in advanced stages of primary astrocytomas [11], high-grade prostate intraepithelial neoplasia and invasive prostate cancer [55], adrenocortical carcinoma [56], multiple myeloma [57], and drug-resistant glioblastoma [58].

Evidence with regard to RGC-32 expression in metastatic cancer is sometimes conflicting: It is upregulated in breast cancer with osteolytic metastasis but downregulated in metastatic liver carcinoma and androgen-resistant metastatic prostate cancer [5, 52, 59, 60]. Our group was the first to characterize RGC-32's expression in colon carcinogenesis [10]. Unlike normal colonic tissues, we found that a considerable

number of adenocarcinoma and adenoma samples were positively stained for RGC-32 protein, and the intensity of the RGC-32 staining tended to correlate with the TNM staging of the malignant tumors [10]. Data from Wang et al. later confirmed that the RGC-32 expression in colorectal cancer is higher in colon adenocarcinoma tissue samples than in normal colorectal mucosa and, furthermore, that higher RGC-32 expression levels are correlated with shorter patient survival [48]. Immunostaining data from pancreatic cancer tissue samples echo our immunohistochemistry findings in colon cancer: the expression of RGC-32 is upregulated in pancreatic malignancy and is correlated with both lymph node metastasis and TNM staging [61].

Epigenetic modifications induced by RGC-32

Gene profiling experiments on SW480 human colon adenocarcinoma cells have identified the functional groups of genes upon which RGC-32 silencing has the most significant impact [10]: genes involved in chromatin assembly, the cell cycle, and RNA processing. Enhanced lysine acetylation at multiple sites on histones H2B, H3, and H4, along with a diminished expression of the histone deacetylase SIRT1, are seen in response to the silencing of RGC-32 expression in SW480 cells [10]. Moreover, silencing RGC-32 induces DNA synthesis and mitosis in SW480 colon cancer cells [10]. Likewise, others have found that overexpression of RGC-32 in several cancer cell lines causes a delay in G2/M progression [11]. Thus, our data suggest that epigenetic alterations controlled by RGC-32 can contribute to cell cycle regulation in tumor cells.

Recent in vitro work contradicts the notion that RGC-32 acts solely as a negative cell cycle regulator in malignant tissues, in that RGC-32 has been reported to promote malignant cell proliferation in SW480 colon adenocarcinoma cells and LTE lung adenocarcinoma cells [48, 62]. Overexpression of RGC-32 protein in EBV-immortalized B cells results in disruption of the G2/M checkpoint via CDC2 activation, suggesting that deregulated RGC-32 expression may be instrumental in tumor development [12, 54]. RGC-32 has proved indispensable for the growth and survival of EBV-immortalized lymphoblastoid cells. The intimate mechanism through which RGC-32 protein expression becomes activated in these cells involves relief of translational repression regulated by Pumilio proteins (evolutionarily conserved RNA-binding proteins) [63].

In terms of identifying the pathway(s) through which RGC-32 expression is regulated during tumorigenesis, many studies have identified RGC-32 as the molecular target of either relevant oncogenes or tumor suppressor genes.

As a verified transcriptional target of p53, RGC-32 has been observed to localize to the cytoplasm of tumor cells during interphase and become concentrated in the centrosomes and spindle poles during prometaphase and metaphase [11]. Consistent with its proposed role as a cell cycle regulator, RGC-32 forms a complex with polo-like kinase 1 (Plk1) during mitosis and is phosphorylated by Plk1 [11].

In human breast carcinoma cell lines, complement protein C5a has been shown to induce malignant cell proliferation through Akt-activated expression of the RGC-32 gene [64].

In murine metastatic prostate cancer cells, RGC-32 has been identified as a potential molecular target for the metastasis suppressor N-myc downstream regulated gene-1 (Ndrg-1) [65], whereas in the human epithelial ovarian cancer TOV112 cell line, RGC-32 expression is augmented following the overexpression of downstream of tyrosine kinase 1 (DOK1). DOK1 is one of the novel hypermethylated genes in epithelial ovarian cancer that apparently exerts tumor-suppressive activity in both hematopoietic and nonhematopoietic malignancies [66].

Reporter assays carried out in DG75 human B-lymphoblastoid cells have certified that RUNX1 activates RGC-32 expression in these cells [54].

Role of RGC-32 in invasion and metastasis

Faced with signals indicating a microenvironmental energy crisis, tumor cells reprogram their metabolic and epigenetic phenotypes; a major part of this complex reprogramming involves cellular dedifferentiation, during which more differentiated epithelial cancer cells re-enter the undifferentiated state, expressing stem cell markers and exhibiting stem cell traits [67]. Provocative findings have illustrated cooperation between RGC-32 and SMAD3 in acting as TGF-β downstream effectors in order to regulate the EMT (see above); this cooperation sets the stage for a putative role for RGC-32 in invasion and metastasis.

Overexpression of RGC-32 in various carcinoma cells has been shown to promote the EMT and induce metastasis. For instance, excessive RGC-32 expression results in the upregulation of vimentin, N-cadherin, Snail, and Slug expression and downregulation of E-cadherin expression in the BxPC-3 human pancreatic cancer cell line [61] and in SW480 colon adenocarcinoma cells [48]. Furthermore, overexpression of RGC-32 in a colon cancer cell line causes reorganization of the cytoskeleton, thus promoting cell migration [68].

Transwell cell migration assays in the BxPC-3 human pancreatic cancer cell line have shown that TGF-β treatment enhances BxPC-3 cell migration, whereas knocking down RGC-32 counteracts this effect [61]. In addition, several in vitro experiments have exposed RGC-32's ability to induce the EMT and enrich the migration and invasion arsenal of lung adenocarcinoma cells via diminished protein levels and activity of the matrix metalloproteinases MMP2 and MMP9 [62, 69]. Induction of the EMT by RGC-32 has been achieved in

A549 lung adenocarcinoma cells through the activation of the nuclear factor (NF)-κB signaling pathway [69], whereas in colon adenocarcinoma cells, it relies on the activation of the Smad/Sip1 pathway [48].

Thus, RGC-32 seemingly functions in a pleiotropic manner in distinct malignant settings, dependent on the cellular lineage, the potential ligands involved, and the protein level and activity of RGC-32, either impelling cells toward accelerated proliferation or toward mitotic arrest. Intriguingly, two of the RGC-32 regulator molecules, RUNX1 and TGF-β [4, 12], both display these paradoxical dual features, acting either as tumor suppressors or as oncogenes, depending on the cellular context [70, 71].

RGC-32 and autoimmunity

The first evidence linking RGC-32 with the immune system came from a study by Tanaka et al., who showed that, as compared to healthy controls, RGC-32 is the most upregulated gene in $CD4^+$ cells isolated from patients with hyper-IgE syndrome [72]. Later, RGC-32 was found to be involved in controlling the cell cycle of T cells in vivo, and this effect was shown to be mediated by IL-2 in a PI3K-dependent manner [14]. Since these first findings, RGC-32 expression and function have been explored in a number of autoimmune diseases.

MS

We have analyzed RGC-32 expression PBMCs isolated from MS patients and found that RGC-32 mRNA levels are significantly decreased during the active periods of relapsing–remitting MS and in patients who do not respond to glatiramer acetate therapy [73].

RGC-32 mRNA levels are positively correlated with those of Fas ligand (FasL) mRNA and inversely correlated with those for IL-21. In fact, receiver operating curve (ROC) analysis has shown that RGC-32 may be a reliable biomarker for detecting relapse and response to glatiramer acetate [73]. By using lentiviral-mediated RGC-32 knockdown in PBMCs, we have demonstrated that RGC-32 downregulation leads to a decrease in FasL expression [17]. Since the Fas-FasL system induces apoptosis of T cells [74], these data suggest that RGC-32 is a modulator of Tcell apoptosis in MS, and low levels of RGC-32 and FasL may predispose to a defect in apoptosis in autoreactive T lymphocytes.

We have also found that RGC-32 silencing leads to a decrease in the expression of SIRT1 in PBMCs. SIRT1 is a histone deacetylase involved in regulating metabolism, gene transcription, DNA repair, and cell survival. Since SIRT1 can be phosphorylated by cdc2-cyclin B1 complexes, which are activated by RGC-32, it is possible that the low levels of SIRT1 seen during relapses may be, at least in part, a result of the decreased RGC-32 expression; this RGC-32 effect may represent another mechanism by which RGC-32 modulates T cell survival [75].

Our in vivo studies have shown that RGC-32's ability to influence Th17 differentiation can also affect the degree of neuroinflammation. RGC-32 KO mice with experimental autoimmune encephalomyelitis (EAE) display a more attenuated phenotype of the disease than do WT mice, with less severe spinal cord demyelination and a smaller number of $IL\text{-}17^+$ infiltrating T cells isolated from spinal cord inflammatory lesions [21]. A lack of RGC-32 also affects the number of $GM\text{-}CSF^+$ T cells, a highly pathogenic subtype. Transfer of WT $CD4^+$ T cells into WT or RGC-$32^{-/-}$ mice before MOG immunization results in EAE of similar severity, suggesting that the attenuated EAE phenotype in RGC-$32^{-/-}$ mice is T cell-mediated [21]. These data indicate that RGC-32 exerts a pro-inflammatory role in EAE by promoting Th17 immune responses and the generation of GM-CSF and that RGC-32 blockade may be a novel therapeutic strategy in MS.

Compelling evidence suggests that RGC-32 may also play a role in astrogliosis, a process in which astrocytes respond to central nervous system injury with molecular, morphological, and functional changes; this process can lead to the deposition of ECM, forming glial scars [76–78]. We have shown that astrocytes express high levels of RGC-32 in chronic plaques from MS patients, and RGC-32 expression is augmented by TGF-β stimulation in cultured rat astrocytes [17]. By silencing RGC-32 expression with siRNA, we have been able to demonstrate a significant reduction in the TGF-β-induced expression of ECM components (collagen type I, type IV, V, and fibronectin) as well as nestin and α-SMA, proteins expressed by reactive astrocytes (Fig. 1). These results have also been confirmed in RGC-32 KO astrocytes in mice [79]. As in renal cells, RGC-32 forms complexes with Smad3 and is translocated into the astrocyte nucleus, in a process that depends on Smad3 phosphorylation and ROCK activation. Moreover, the absence of RGC-32 seems to influence astrocytic morphology in an inflammatory setting. Immunohistochemical detection of glial fibrillary acidic protein (GFAP), a marker of astrocytes, indicates that astrocytes from RGC-32 KO mice with EAE have an elongated shape, similar to that of radial progenitor neural cells, as opposed to those from WT diseased mice, which show hypertrophy [79]. These results indicate that RGC-32 is an important link in the TGF-β signaling pathways that mediate extracellular matrix deposition and astrocytic differentiation and reactivity in neuroinflammation.

SLE

The role of RGC-32 in Th17 differentiation has also been explored in conjunction with SLE pathology. Increased expression of IL-17 in the kidneys of SLE

patients and lupus-prone mice is critical for the development of lupus nephritis (LN) [80]. We have previously shown that RGC-32 expression is increased in T cells from SLE patients and in the tubules and glomerulus-infiltrating cells in kidney biopsies of patients with LN. In vitro, RGC-32 promotes the differentiation of human Th17 cells, suggesting that RGC-32 signaling may enhance disease expression in SLE by promoting abnormalities in the Th17 pathway [80].

To directly assess whether RGC-32 plays a local role in LN downstream of antibody production, we used the anti-glomerular basement membrane antibody-induced glomerulonephritis (AIGN) model to compare parameters of disease severity in RGC-32 KO and WT mice. RGC-32 mRNA was significantly upregulated in the kidneys of WT mice with AIGN when compared to controls. RGC-32$^{-/-}$ mice displayed an attenuated nephrotoxic injury, as demonstrated by significantly decreased proteinuria and decreased histopathological glomerular scores. Tubulointerstitial damage did not differ between RGC-32$^{+/+}$ and KO mice. IL-17A mRNA expression was upregulated in kidneys of WT mice with AIGN and downregulated in RGC-32$^{-/-}$ mice. These results suggest that RGC-32 contributes to the pathogenesis of immune complex-mediated glomerulonephritis by promoting local IL-17A production and the subsequent development of end-organ damage [81].

Systemic sclerosis

RGC-32's role in macrophage differentiation has been linked to systemic fibrosis, an autoimmune condition characterized by widespread deposition of ECM in various organs, such as the skin, joints, and lungs. Using a bleomycin-induced fibrosis model in mice, Sun et al. have shown that RGC-32 deficiency attenuates lung and skin fibrosis in bleomycin-treated mice and impairs LPS- and IFN-γ-induced macrophage polarization toward the M1 phenotype, resulting in a decreased production of inducible nitric oxide synthase (iNOS) and IL-1β, two pro-inflammatory factors that promote fibrosis [82]. More recently, Atamas et al. have investigated the role of RGC-32 in a chronic bleomycin exposure model of pulmonary fibrosis. However, they found that not only were RGC-32$^{-/-}$ mice protected from fibrosis, but they also accumulated significantly more collagen in response to bleomycin challenge, indicating that RGC-32 acts protectively against fibrosis in the lungs. Further supporting this notion, overexpression of RGC-32 has been shown to attenuate TGF-β-induced upregulation of collagen in primary human lung fibroblast cultures [83].

RA

A recent study has examined the differentially expressed genes that are potentially implicated in the dysregulated cartilage homeostasis seen in the joint destruction of rheumatoid arthritis (RA). RGC-32 was among the top dysregulated genes identified as being potentially affected in the RA joint microenvironment. Similarly, downregulated expression of RGC-32 was observed in RA chondrocytes and in gene arrays from clinical RA synovial tissues. These data suggest an important role for RGC-32 in regulating the cell cycle progression of chondrocytes in the context of the hypoxic joint microenvironment in RA. The downregulated RGC-32 may potentially induce cell cycle activation in RA chondrocytes, leading to further cartilage tissue damage [84].

Psoriasis

A study by Kim et al. has found significantly lower levels of RGC-32 mRNA in psoriatic lesions than in the skin of healthy individuals, and this trend was also noticed in psoriasiform lesions from the skin of mice treated topically with imiquimod. RGC-32 levels were correlated with a decreased number of M2 macrophages in the inflammatory lesions, and this observation was corroborated in vitro by a decreased expression of RGC-32 in M1-polarized THP-1 cells [85].

In conclusion, RGC-32 plays an important role in the pathogenesis of many diseases. More work needs to be done, and we foresee a bright future for RGC-32 as an important molecule that is situated at the crossroads of many important processes and pathways and is emerging as an important therapeutic target.

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Compliance with ethical standards

Conflict of interest Horea Rus has received a grant from TEVA Neuroscience (CNS-2014-174). All other authors declare that they have no conflict of interest.

Abbreviations α-SMA, alpha smooth muscle actin; AEC, aortic endothelial cells; BATF, Basic leucine zipper transcription factor; CDC2, cell division cycle protein 2 homolog; EBV, Epstein–Barr virus; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; HFD, high-fat diet; ICAM-1, intercellular adhesion molecules 1; IRF4, interferon regulatory factor 4; KO, knockout; MAPK, mitogen-associated protein kinase; MMP, matrix metalloproteinases; MS, multiple sclerosis; PI3K, phosphatidylinositol-3-kinase; PIGF, placental growth factor; RGC-32, response gene to complement 32; ROCK, rho-associated coiled-coil-containing protein kinase; SLE, systemic lupus erythematosus; SMC, smooth muscle cells; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; WT, wild type

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