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Reversion inducing cysteine rich protein with Kazal motifs and cardiovascular diseases: The RECKlessness of adverse remodeling

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Abstract

The Reversion Inducing Cysteine Rich Protein With Kazal Motifs (RECK) is a glycosylphosphatidylinositol (GPI) anchored membrane-bound regulator of matrix metalloproteinases (MMPs). It is expressed throughout the body and plays a role in extracellular matrix (ECM) homeostasis and inflammation. In initial studies, RECK expression was found to be downregulated in various invasive cancers and associated with poor prognostic outcome. Restoring RECK, however, has been shown to reverse the metastatic phenotype. Downregulation of RECK expression is also reported in non-malignant diseases, such as periodontal disease, renal fibrosis, and myocardial fibrosis. As such, RECK induction has therapeutic potential in several chronic diseases. Mechanistically, RECK negatively regulates various matrixins involved in cell migration, proliferation, and adverse remodeling by targeting the expression and/or activation of multiple MMPs, A Disintegrin And Metalloproteinase Domain-Containing Proteins (ADAMs), and A Disintegrin And Metalloproteinase With Thrombospondin Motifs (ADAMTS). Outside of its role in remodeling, RECK has also been reported to exert anti-inflammatory effects. In cardiac diseases, for example, it has been shown to counteract several downstream effectors of Angiotensin II (Ang-II) that play a role in adverse cardiac and vascular remodeling, such as Interleukin-6 (IL-6)/IL-6 receptor (IL-6R)/glycoprotein 130 (IL-6 signal transducer) signaling and

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Credit Author Statement

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Epidermal Growth Factor Receptor (EGFR) transactivation. This review article focuses on the current understanding of the multifunctional effects of RECK and how its downregulation may contribute to adverse cardiovascular remodeling.

Keywords

Adverse Remodeling; Fibrosis; Metallopeptidases; RECK; EGFR; Inflammation

1. Introduction

The processes governing extracellular matrix (ECM) structure and composition are tightly regulated, involving a variety of cell types and signaling intermediates. Under physiological conditions, the matrix adapts to allow proper organ development and function, cellular migration, vascularization, and tissue remodeling in response to injury. However, persistent remodeling disrupts matrix homeostasis and leads to excess deposition of ECM proteins, leading to fibrosis and adverse remodeling [1]. Fibroblasts, a major contributor to fibrosis, undergo a process of activation characterized by differentiation and α-smooth muscle actin expression followed by proliferation. The differentiated fibroblasts (often referred to as myofibroblasts) then express and deposit ECM proteins in excess, leading to adverse remodeling and disease. For example, adverse remodeling occurs prior to hypertension-associated end-organ damage and atherosclerosis [2]. Furthermore, increased load on the heart resulting from hypertension, aortic stiffening, or valvular stenosis initially promotes compensatory remodeling to handle the increased wall stress, characterized by myocardial hypertrophy and fibrosis [3]. However, sustained remodeling eventually leads to myocardial dysfunction and heart failure development [3]. In the case of vascular remodeling, vascular smooth muscle cells de-differentiate from a contractile to a synthetic phenotype and undergo hypertrophy, hyperplasia, and migration [4].

Cardiovascular remodeling occurs in response to a wide range of stimuli, such as inflammation, growth hormone release, activation of the Renin-Angiotensin-Aldosterone System (RAAS), hypoxia, and increased wall stress. Diverse enzymes and signaling molecules regulate this remodeling process, including matrix metalloproteinases (MMPs), A Disintegrin And Metalloproteinase Domain-Containing Proteins (ADAMs), A Disintegrin And Metalloproteinase With Thrombospondin Motifs (ADAMTS), inflammatory mediators, growth factors and the Reversion Inducing Cysteine Rich Protein With Kazal Motifs (RECK). During the remodeling process, the extracellular matrix is degraded by increased release and activation of various MMPs, such as MMPs-1,2,3,7,8,9,13,14, which contribute to cell migration and growth hormone release [5]. A more detailed overview of MMPs, ADAMs, ADAMTS in cardiovascular diseases has been previously reviewed [6–9]. This article focuses on RECK and its potential mechanistic contribution to fibrosis and pathologic cardiovascular remodeling.

2. RECK Structure

RECK was first cloned and characterized in 1998 by Noda and colleagues [10]. The primary structure of the protein is shown in Fig. 1. The human *RECK* gene spans 87 kb located on

chromosome region 9p13 [10]. The gene encodes a 971 amino acid protein with cysteine accounting for 9% of residues. At the nucleotide level, human RECK shows ~93% homology with mouse, ~86% with rat, ~94% with bovine, and ~98% with monkey. RECK expression was found to be critical for proper development as constitutive *RECK* deletion leads to lethality in mice due to defective blood vessel maturation at approximately embryonic day 10.5 [11]. The NH_2- and COOH-terminals of RECK contain hydrophobic regions. The hydrophobic portion of the NH_2-terminal serves as a secretory signal peptide while the COOH-terminal contributes to RECK's membrane anchoring via a Glycosylphosphatidylinositol (GPI). This GPI anchor contributes to RECK's ability to regulate various membrane bound proteases such as MT1-MMP, ADAM10, and ADAM17 [8, 12, 13]. It has been shown that Glycerophosphodiester Phosphodiesterase 2 (GDE2), an enzyme that cleaves GPI anchors, releases RECK from the cell surface [14]. Upon release, RECK appears to lose its ability to inhibit protease activity [14]. Interestingly, RECK also contains two epidermal growth factor (EGF)-like domains similar to that described in ADAMs, TGF-β, fibrillin, and EGFR ligands [10, 15]. EGF-like domains have been implicated in ligand shedding, calcium binding, and blood coagulation [15, 16]. Currently, the function of EGF-like domains in RECK is not known, and warrants further investigation. In addition, RECK contains three Kazal motifs. Kazal motifs belong to the I1 family of serine protease inhibitors and typically contain three disulfide bonds between 6 cysteine residues [10, 17]. In the canonical RECK protein, residues 635–654 match a Kazal motif, while the other two domains (716–735 and 754–772) resemble incomplete Kazal motifs [10].

The NH_2-terminal of RECK contains five cysteine knot motifs and five asparagine (Asn) residues [10]. The cysteine knot motifs are shown to promote proper development of the blood-brain barrier by facilitating Wingless-Type MMTV Integration Site Family Member 7a/b (Wnt7A/B) signaling in endothelial cells [18, 19]. The first cysteine knot motif (CC-1) has been shown to interact with Adhesion G Protein-Coupled Receptor A2 (Gpr124, an endothelial cell receptor involved in angiogenesis), whereas the fourth cysteine knot motif (CC-4) interacts directly with Wnt7a/Wnt7b [19–21]. Together, these interactions help facilitate the formation of a protein complex that increases Wnt7a/Wnt7b signaling and central nervous system angiogenesis [19]. RECK also has asparagine residues that act as glycosylation sites to regulate RECK's function. It has been shown that glycosylation at Asn^{297} contributes to suppression of MMP-9 secretion, and glycosylation at Asn^{352} is required for RECK's ability to impair MMP-2 activation [10, 22]. Importantly, blocking glycosylation at Asn^{86}, Asn^{297}, and Asn^{352} prevents RECK's ability to suppress tumor cell invasion [22].

The role of RECK in tumor suppression has been widely described, and while a wide range of human tissues express RECK, including the heart, it's expression is low or undetectable in transformed cancer cell lines [10]. Restoring RECK expression in these transformed cancer cells suppressed their invasive ability by reducing MMP-9 activation. Taken together, the protein was named RECK for its ability to **re**verse the malignant phenotype, the high percentage of **c**ysteine residues, and the presence of **K**azal motifs [10].

Similar to many other genes, several isoforms of RECK have been described [23]. Specifically, two shorter isoforms of RECK have been shown to counteract some of the anti-migratory and anti-growth effects of the canonical isoform [24, 25]. The first 212 amino acid residues at the NH2-terminal are identical between the isoforms, and they all possess the first three cysteine knot motifs (Fig. 1). However, the shorter isoforms lack several domains of canonical RECK, including the three Kazal motifs involved in protease regulation, the two EGF-like domains and the GPI anchor. The lack of a GPI anchor on the shorter isoforms may indicate that these are secreted proteins, and may interact with and regulate canonical RECK and other extracellular proteins away from the cell surface. In fact, it was demonstrated that RECKVar5 can interact with canonical RECK's Kazal motif, preventing the 110 kDa isoform from inhibiting MMP-9, but not MMP-2 [24]. Interestingly, the expression of the 25 kDa isoform (RECKVar5) is upregulated during proliferation and after Transforming Growth Factor- β1 (TGF-β1) treatment [24]. The ratio of RECKVar5 to canonical RECK has also been shown elevated in more aggressive breast cancers. RECKVar3, another RECK isoform, has been shown to promote glioma cell growth by increasing anchorage-independent growth [23, 25]. Furthermore, a higher ratio of RECKVar3 to canonical RECK has been shown to correlate with lower survival rates in melanoma patients [25]. Interestingly, increased expression of RECKVar3 leads to elevated MMP-14 and MMP-9 mRNA expression, but decreased canonical RECK induction [25]. Expression and regulation of these alternative RECK isoforms in the heart and vasculature during health and disease, however, is currently not known, and warrants investigation.

3. RECK Regulation

RECK is a highly regulated gene. Its expression is regulated at both transcriptional and post-transcriptional levels, including regulation by histone acetylation, DNA methylation, and modulation by microRNAs [24–27]. There are two Specificity Protein 1 (SP1)-binding sites in its proximal promoter region, and activation of SP1 by extracellular signal-regulated kinase (ERK) downregulates RECK in cancer cells [26, 28]. We have previously demonstrated that Ang-II suppresses RECK expression through an ERK/SP1-dependent pathway, and that forced expression of canonical RECK inhibits Ang-II-induced cardiac fibroblast migration [29]. Proinflammatory cytokines negatively regulate RECK expression; we previously reported that IL-18, whose increased expression contributes to adverse cardiac remodeling, suppresses RECK expression in cardiac fibroblasts [30]. Estrogen has been shown to reduce RECK expression in mouse uterine epithelial cells [31] via mechanisms not fully known. Whether RECK expression is differently regulated in the heart and vessels in females is not known, and warrants further investigation. While many pathways have been shown to downregulate RECK, some of which are described above, it can be upregulated by activation of the farnesoid X receptor (FXR) response element in the first intron of the gene [32].

Promoter methylation also regulates RECK expression. Specifically, hypermethylation of the *RECK* promoter in oral and hepatic cancer cells is associated with reduced RECK mRNA and protein expression [33, 34]. In addition, *RECK* transcription is regulated by histone acetylation, and increased histone deacetylase (HDAC) activity is associated with reduced RECK expression [33]. In fact, the HDAC inhibitor apicifin is shown to upregulate RECK

expression by blocking HDAC4 interaction with SP1 binding elements in the *RECK* promoter [35]. We have previously demonstrated that the HDAC inhibitors Trichostatin A and mocetinostat reverse Ang-II-induced RECK suppression in cardiac fibroblasts by blocking SP1 binding to its promoter [36].

As shown in Fig. 2, multiple miRNAs have been experimentally demonstrated to affect the post-transcriptional regulation of RECK. Of note, while many of these non-coding RNAs suppress RECK expression, some are shown to promote its induction [37–59]. For example, miR-342 has been shown to restore RECK expression in colorectal cancer cells by reducing *RECK* promoter methylation by inhibiting DNA methyltransferase 1 [55].

In addition to microRNAs, a long non-coding RNA (lncRNA) known as Growth Arrest Specific 5 (GAS5) has been shown to increase RECK protein expression by binding to miR-21, and reducing miR-21-mediated RECK degradation [60]. Recently, transfection of cells with double-stranded RNA complementary to the *RECK* promoter region was shown to induce its protein expression, while suppressing MMP expression and activity [61]. However, the translational significance of double stranded RNA in a clinical setting is currently not known.

4. RECK and Tissue Inhibitors of Metalloproteinases

Even though RECK shares structural and functional characteristics with other MMP inhibitors, such as the tissue inhibitors of metalloproteinases (TIMPs), it possesses unique characteristics and features in regulating tissue remodeling as summarized in Table 1. Whereas Kazal motifs contribute to RECK's regulation of proteases, TIMPs contain a conserved netrin (NTR) module involved in chelating the Zn^{2+} cofactor from the MMP active site, leading to MMP inhibition [62, 63]. While both Kazal motifs and NTR modules possess three disulfide bonds between six cysteine residues, only the NTR module chelates Zn^{2+} and inhibits MMP activation with high potency [62–64]. Furthermore, TIMPs are smaller proteins, with less than 200 residues [65, 66]. Interestingly, RECK and TIMP-3 are not soluble, whereas TIMPs 1, 2, and 4 can be secreted to act distally [67]. RECK is bound to the cell surface through a GPI anchor, whereas TIMP-3 is bound to the ECM [68].

While RECK and all four TIMPS work to limit excess angiogenesis, they target different substrates [67]. TIMP-1 possesses fewer substrates compared to other TIMPs and RECK, weakly targeting membrane-type MMPs 14, 16, 19 and 24. Both TIMP-2 and TIMP-3 can target a majority of MMPs, but TIMP-3 can also inhibit various members of the ADAM/ADAMTS family. RECK most closely resembles TIMP-3, regulating a broad spectrum of MMPs, ADAM10/17, and ADAMTS. Further, RECK and TIMP-3 are both able to suppress proliferation and migration of vascular smooth muscle cells and promote apoptosis [67, 69–71]. Constitutive knockout of TIMP-1 leads to structural changes in the heart, including exacerbated remodeling following infarction, whereas TIMP-2 KO leads to motor defects [67]. TIMP-3 KO leads to increased cardiac fibrosis and elevated cardiac TGF-β1 and Tumor Necrosis Factor-α (TNF-α) expression in aged mice, and increased myocardial hypertrophy and fibrosis in aortic banded mice [72, 73]. On the other hand, RECK deletion is embryonically lethal, implicating an irreplaceable role for RECK in vascular development

[11]. Despite several similarities with TIMPs, RECK's membrane localization and requirement for proper development suggest a unique role in tissue homeostasis that requires further characterization.

5. RECK and Matrix Metalloproteinases

The anchorage of RECK to the membrane allows it to interact with and regulate the activity of other membrane associated proteins, as well as secreted proteins, including those responsible for activating matrixins and various pathophysiological signaling pathways [11]. RECK's extracellular portion contains protease inhibitor-like domains that sequester pro-MMP-9 and prevent its activation. Elevated expression and persistent activation of the gelatinase MMP-9 contributes to adverse cardiac remodeling, in part through MMP-9-mediated degradation of the ECM and activation of latent growth factors. RECK has also been shown to inhibit *MMP9* transcription in cultured cells [11, 74] by blocking binding of the Activator Protein (AP)-1 subunits Fra-1 and c-Jun to the TRE (12-O-tetradecanoylphorbol-13-acetate-responsive element) site in its promoter region [74]. However, these studies did not find RECK in the nucleus, suggesting that RECK-mediated suppression of *MMP9* transcription is indirect. Outside of MMP-9, RECK has also been shown to inhibit the catalytic activity of MMPs 2, 7, and 14 [11, 75]. Chang et al. tested full length RECK and several shorter constructs spanning the cysteine knot motif (residues 285–368), all three Kazal motifs (K123, residues 605–799), and a third construct containing the last two Kazal motifs (K23, residues 676–799) to identify the critical region responsible for RECK's MMP inhibitory activity. Their results suggest that the K23 domain of RECK impaired MMP-9 secretion and activity in lung cancer cells. In fact, immunoprecipitation assays demonstrated that the K23 domain binds and inhibits active MMP-9 [76].

Interestingly, a recent study proposed that earlier results demonstrating the direct inhibition of MMP-9 catalytic activity by RECK stemmed from contamination with a serine protease during purification of RECK protein [77]. By adding a serine inhibitor, the study found that neither RECK nor the two constructs containing the Kazal motifs (residues 621–797, 697–797) are able to significantly influence MMP activity [77]. However, these authors indicated that RECK could still influence MMP activity *in vivo* outside of direct inhibition, potentially via downregulation of *MMP* transcription, reducing MMP secretion, or by binding/sequestering MMPs at the cell surface [77]. It is also of note that the constructs in this recent study spanned slightly different residues compared to those from the study by Chang et al. While RECK may not have direct inhibitory action on the catalytic domain of MMP-9, it still appears to negatively regulate MMP-9 activity. This suggests that part of RECK's role is to slow down MMP-9-mediated remodeling.

In addition to MMP-9, RECK has also been shown to inhibit pro-MMP-2 secretion and activation in human fibrosarcoma-derived HT1080 cells [11] by physically interacting with MT1-MMP (also known as MMP-14) [12]. Recently, Noda et al. reported that RECK also promotes pro-MT1-MMP processing to mature MT1-MMP, and that RECK combined with ADAMTS10 could influence the gelatinolytic and collagenolytic activity of both pro-MT1-MMP and mature MT1-MMP [64]. Interestingly, RECK and ADAMTS10 interaction led to increased proteolytic activity of pro-MT1-MMP, but decreased proteolytic activity of mature

MT1-MMP. Furthermore, they also found that RECK, in association with MT1-MMP, enhanced fibrillin and fibronectin deposition [64]. Taken together, these data indicate that RECK may influence MT1-MMP function in a context-dependent manner, it inhibits the proteolytic activity of mature MT1-MMP and MMP-2, but promotes fibrillin and fibronectin deposition in association with MT1-MMP [11, 64].

More recently, RECK has been shown to inhibit the activation of MMP-7, a matrilysin involved in adverse cardiac remodeling [75]. It also inhibits activation of MMP-17, a GPI-anchored MMP and reduces MMP-17-dependent neural crest cell migration [78]. It is however not known whether RECK regulates the activation of MMP3, a stromelysin, whose increased expression contributes to fibrosis [79]. Overall, RECK appears to negatively regulate multiple matrixins involved in migration and remodeling. Therefore, inducing or sustaining RECK expression has therapeutic potential in inhibiting adverse cardiac and vascular remodeling.

Besides targeting multiple MMPs, RECK also targets ADAMs 10 and 17 [80]. In fact, RECK has been shown to inhibit Notch ligand shedding and signaling by targeting ADAM10 [13, 80]. ADAM17, which is also known as TNF-α Converting Enzyme (TACE), acts as a sheddase and releases TNF-α and other transmembrane proteins that play a role in inflammation, cardiac hypertrophy and fibrosis [81]. Together, these reports indicate that RECK is a membrane anchored multi-functional protein that can target activation and/or expression of MMPs and ADAMs.

6. RECK and TGF-β1

TGF-β1, which plays a role in cardiac hypertrophy and fibrosis, induces the expression of MMPs 2 and 9 and TIMP2 in breast cancer cells, but suppresses RECK [82], indicating that TGF-β1 is a negative regulator of RECK in these cells. Further, inhibition of ERK½ prevented TGF-β1-induced increases in MMP-9 and TIMP-2, but not MMP-2, and reversed RECK suppression. Moreover, targeting p38 MAPK inhibited TGF-β1-induced MMP-2 expression, but not MMP-9 or RECK [82] (Fig. 3). In endothelial cells, exposure to TGF-β1 increases miR-21 expression and promotes endothelial to mesenchymal transition (EndMT) [83]. The process of EndMT increases the number of fibroblasts and accounts for a third of fibroblasts in fibrotic regions of the diseased heart [84]. TGF-β1 also suppresses RECK protein expression in hepatic oval cells, while upregulating miR-21 (Fig. 3), and targeting miR-21 prevents TGF-β1-induced EndMT and renal fibrosis in diabetic nephropathy [85].

Activation of TGF-β1 is a multistep process, including removal of the Latency TGF- β1 Binding Protein (LTBP) [86, 87]. The LTBP contains a Collagen Binding Domain (CBD) that sequesters latent TGF-β1 to collagen fibers, allowing it to be activated by changes in protease activity or physical strain on the ECM [88]. Since both MMPs 2 and 9 play a role in TGF-β1 release, suggesting a potential role for RECK in excess TGF-β1 activation [89, 90] (Fig. 4). Though anti-TGF-β1 therapies have shown promise, questions remain regarding their efficacy and safety. Therefore, identifying RECK inducers may provide beneficial effects in tissue fibrosis and adverse remodeling by inhibiting excess TGF-β1 activation and

signaling. However, unlike in cancer cells, it is not known whether TGF-β1 suppresses RECK expression in cardiac fibroblasts or vascular cells, and needs further investigation.

7. RECK and Urokinase-Type Plasminogen Activator

Another target of RECK involved in extracellular matrix degradation is the plasminogen activator (PA) system [91]. The Urokinase-Type Plasminogen Activator (UPA) is a key serine protease involved in transforming plasminogen into active plasmin. This allows active plasmin to initiate a proteolytic cascade to degrade components of the ECM and promote cell migration (288). Both human and animal studies report that macrophage accumulation and increased PA activity contribute to the pathogenesis of cardiac fibrosis. In fact, elevated PA activity was detected in failing human hearts [92, 93]. Consistent with this observation, UPA knockout mice are resistant to developing cardiac fibrosis [94]. The UPA receptor (UPAR) is bound to the cell membrane by a GPI anchor, and UPA ligand binding increases the ability of UPA to convert plasminogen to plasmin [95, 96]. It has been shown that RECK can physically associate with the UPAR and interfere with UPA activity. RECK knockdown increases UPA secretion and invasion of breast cancer cells, and is rescued by concomitant knockdown of UPA [91] or RECK induction [91]. Therefore, further investigations are required to determine whether RECK induction inhibits UPA secretion, ECM remodeling, cell migration, macrophage accumulation, and fibrosis in a diseased or injured heart.

8. Potential Targets of RECK: IL-6R and EGFR

Chronic inflammation, mild or severe, contributes to adverse cardiac and vascular remodeling. It has been previously reported that monocyte recruitment following myocardial infarction promotes myocardial wound healing by recruiting myofibroblasts to the injured area [97], and that these myofibroblasts express pattern recognition receptors that can respond to pathogen-associated molecules to induce the secretion of various proinflammatory cytokines and chemokines [98, 99]. Upon stimulation with lipopolysaccharide (LPS), human cardiac fibroblasts have been shown to secrete IL-1β, TNF-α, IL-6, and macrophage chemoattractant protein-1 (MCP-1) [98]. Cardiac fibroblasts are also shown to express increased levels of IL-1β and IL-18 following myocardial infarction [99]. Inflammatory mediators contribute to remodeling and changes in cell behavior. For instance, IL-17A promotes the migration and proliferation of vascular smooth muscle cells (VSMC), whereas IL-18 induces cardiac fibroblast migration [30, 100]. In both instances, these proinflammatory cytokines suppressed RECK expression, and restoring RECK reversed their migratory and proliferative responses.

Increased circulating IL-6 is a known risk factor in chronic cardiac diseases, including hypertension, cardiac hypertrophy, fibrosis, congestive heart failure, and atherosclerosis [101–109]. Circulating IL-6 levels serve as a marker of vascular inflammation and are released by vessels in response to vascular injury, Ang-II, and inflammatory mediators such as TNF-α and IL-1 [107, 110–114]. In addition, circulating IL-6 levels positively corelate with blood pressure, plasma Ang-II, and vascular hypertrophy [115]. IL-6 signals by binding to IL-6 receptor α (IL-6Rα) on the cell surface [116]. This association leads to oligomerization of the IL-6/IL-6Rα complex with gp130, the signal transducing subunit of

the heterodimer receptor, resulting in IL-6/IL-6Rα/gp130 complex [117], and activation of the JAK/STAT pathway that contribute adversely to cardiac and vascular remodeling. Interestingly, using immunoprecipitation and immunoblotting, RECK has been shown to physical associate with IL-6R and gp130 in breast cancer cells [91]. However, in that study, the biological consequence of their interaction has not been investigated.

To investigate the role of RECK in IL-6 signaling, we performed a preliminary set of experiments to examine whether RECK inhibits IL-6-mediated VSMC proliferation and determined whether RECK physically interacts with IL-6R and gp130. We found that treatment with IL-6 induced SMC proliferation, and pretreatment with IL-6R neutralizing antibody and exposure to gp130 inhibitor SC144 each inhibited IL-6-mediated STAT3 phosphorylation and SMC proliferation (Fig. 5). For the first time, we also show that IL-6 suppresses RECK expression in a STAT3-dependent manner. Moreover, forced expression of RECK by adenoviral transduction suppressed IL-6-induced SMC proliferation. Confirming earlier results, immunoprecipitation and immunoblotting showed that RECK physically associates with IL-6R and gp130, suggesting that RECK induction blunts IL-6's pro-mitogenic effects by binding to IL-6R and gp130. Taken together, these results suggest that RECK may inhibit migration and proliferation of VSMCs in response to IL-6 and may help protect against vascular inflammation and remodeling.

9. RECK and EGFR Transactivation

Another potential target of RECK that warrants further investigation is the transactivation of the Epidermal Growth Factor Receptor (EGFR). Increased activation of EGFR has been linked to vascular remodeling, and suppression of its activity could serve as a therapeutic target in adverse remodeling [118]. Transactivation of EGFR also contributes to hypertrophy, migration and proliferation of VSMCs [119–122]. Knockdown of ADAM10/17 in VSMCs reduces Ang-II-mediated EGFR transactivation and hypertrophy, suggesting that ADAM10/17 play a critical intermediate step in Ang-II/AT1R-mediated EGFR transactivation [123, 124]. Blocking EGFR also reduces Ang-II-induced cardiomyocyte hypertrophy and upregulation of fibronectin in cardiac fibroblasts [125, 126]. Of note, VSMC and endothelial cells are known to express some of the EGFR ligands, including EGF, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HBEGF), betacellulin (BTC), and epiregulin (EREG) [127]. These ligands are produced as transmembrane precursors that must be cleaved by sheddases, such as ADAMs10/17 or MMPs 3 and 7, to become solubilized and bind to EGFR. ADAM10/17 are responsible for the shedding of TGF-α, while ADAM17 facilitates the release of soluble/active EGF [128–130]. Importantly, RECK is a negative regulator of ADAM10/17. Therefore, inducing RECK may indirectly suppress EGFR transactivation by targeting ADAM10/17-mediated ligand release, thereby providing an avenue to put the brakes on vascular and cardiac adverse remodeling. In Fig. 6, we show that Ang-II induces aortic SMC proliferation through transactivation of EGFR, and that adenoviral RECK transduction attenuated Ang-II-induced EGFR transactivation and aortic SMC proliferation.

10. RECK Inducers

Since its discovery, decreased RECK expression has been associated with malignant transformation and progression of many types of cancer [131]. Previously, we reported that ectopic expression of RECK by adenoviral transduction inhibits Ang-II-induced cardiac fibroblast proliferation and migration by targeting MMPs 2, 9, and MMP-14 [29]. Adenoviral transduction of RECK also suppresses Ang-II- and inflammatory cytokine-induced cardiac fibroblast and SMC migration [29]. However, the use of viral vectors in clinical practice has been met with challenges. Therefore, it is important to identify RECK inducers from existing drugs (repurposing) or by developing newer small molecule inducers. Of note, Noda and colleagues used a reporter assay to test 880 bioactive compounds for their ability to induce activation of the *RECK* promoter, and identified 34 compounds that induced RECK expression [132]. Among these compounds, 12 were anticancer drugs, such as doxorubicin. However, several of these anticancer drugs are cardiotoxic thereby limiting their clinical utility. This screening process also identified minocycline as a RECK inducer, increasing *RECK* promoter activity by 2.6-fold. Minocycline, an FDA-approved second-generation semisynthetic tetracycline, exerts antioxidant, anti-apoptotic, and anti-inflammatory effects independent of its antimicrobial properties. We recently reported that minocycline inhibits Platelet Derived Growth Factor-BB (PDGF-BB)-induced human aortic smooth muscle cell proliferation and migration in vitro by reversing miR-221- and −222-mediated RECK suppression [133].

We have also previously reported that acetylsalicylic acid (aspirin) and docosahexanoic acid (DHA; an omega 3 lipid) induce RECK expression in cultured cells [30, 134]. In those studies, aspirin and DHA upregulated RECK expression by reducing the expression of oxidative stress-responsive miRNAs such as miR-21. These reports indicate that induction of RECK by minocycline, aspirin, and DHA are not direct. Though double-stranded RNA complimentary to the *RECK* promoter region induces RECK expression in vitro [61], their therapeutic potential in vivo needs investigation. Lastly, extracts from some natural products have shown to induce RECK in cancer cells [135, 136]. However, their effect on RECK induction also appears to be not specific or direct. Thus, identifying an effective inducer of RECK will have therapeutic benefit not only in cancer, but also in cardiovascular diseases.

11. RECK and Cardiovascular Diseases

While RECK has been shown to attenuate the fibrotic phenotype of cardiac fibroblasts and VSMC, the role of RECK in myocardial hypertrophy, ischemia, doxorubicin toxicity, myocardial cell death, and cardiac dysfunction has not been elucidated. Interestingly, Ang-II has been shown to induce cardiac hypertrophy through transactivation of the EGFR, which was attenuated by EGFR inhibition [137]. Furthermore, TGF-β1 deficient mice did not show increased left ventricular hypertrophy following Ang-II treatment [138]. This raises the possibility that RECK induction may help counteract myocardial hypertrophy by reducing EGFR transactivation and latent TGF-β1 activation. With respect to ischemia, it has been shown in human embryonic kidney epithelial cells that hypoxia reduces RECK expression via HDAC1 and Hypoxia Inducible Factor-1α (HIF-1α), and that in colorectal cancer cells hypoxia induced miR-590–5p expression, leading to RECK suppression [139, 140]. While

the role and expression of RECK has not been investigated in a model of myocardial infarction/reperfusion, it is tempting to speculate that RECK expression would be downregulated allowing progression of compensatory remodeling to pathological remodeling and contractile dysfunction. As previously mentioned, doxorubicin increases *RECK* promoter activity in the HT-1080 fibrosarcoma cell line. Further studies are needed to determine if this induction also occurs in the heart or vasculature, and whether RECK plays a protective/detrimental role in doxorubicin cardiotoxicity.

12. Conclusions and future directions

Independent of underlying cause, the pathogenesis of cardiovascular diseases is associated with enhanced expression and/or activation of MMPs, ADAMs and UPA, proinflammatory mediators (*e.g.*, gp130 ligands), growth factors (*e.g.*, EGFR ligands: heparin-binding EGF [HBEGF], TGF-α), cytokines (*e.g.*, TGF-β), cell surface receptors (*e.g.*, β2-adrenergic receptors, vascular endothelial growth factor receptor-2), and altered ECM components. When overactivated, these diverse processes work in tandem to drive remodeling, and over time impair cardiovascular function. In this review article, we examined how RECK regulates critical steps of each pathway (summarized in Fig. 7), and how it may act as a brake to slow remodeling. As mentioned above, while RECK is expressed widely in various organs under physiological conditions, its expression is markedly suppressed in many diseases that promote remodeling, migration, and proliferation. In these RECKless conditions, the overactivation of proteases leads to the release of both membrane-bound and ECM-sequestered growth factors, as well as the breakdown of collagen, ultimately leading to adverse cardiovascular remodeling. Therefore, a RECK-centered strategy could inhibit the function of multiple pro-hypertrophic, pro-fibrotic and proinflammatory mediators to blunt adverse cardiac and vascular remodeling.

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Abbreviation

ADAM	Disintegrin And Metalloproteinase Domain-Containing Protein
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospodin Motifs
Ang-II	Angiotensin II
Asn	Asparagine
AT1R	Angiotensin II Receptor Type 1
BTC	Betacellulin

CBD	Collagen Binding Domain
CC	Cysteine Knot Motif
CT-1	Cardiotrophin-1
DHA	Docosahexanoic Acid
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EndMT	Endothelial to Mesenchymal Transition
ErbB	Epidermal Growth Factor Receptor Family
EREG	Epiregulin
ERK	Extracellular Signal-Regulated Kinase
FXR	Farnesoid X Receptor
GAS5	Growth Arrest Specific 5
GDE2	Glycerophosphodiester Phosphodiesterase 2
gp130	Interleukin 6 Signal Transducer
GPCR	G-Protein Coupled Receptor
GPR124	Adhesion G Protein-Coupled Receptor A2
GPI	Glycosylphosphatidylinositol
HB-EGF	Heparin Binding EGF-Like Growth Factor
HDAC	Histone Deacetylase
HF	Heart Failure
HIF-1α	Hypoxia Inducible Factor 1 Subunit α
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-6Rα	Interleukin-6 Receptor Subunit α
JAK	Janus Kinase
LAP	Latency Associated Peptide
lncRNAs	Long non-coding RNAs
LPS	Lipopolysaccharide
LTBP	Latent Transforming Growth Factor β Binding Protein

LV	Left Ventricle
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
MT1-MMP	Membrane Type 1 Matrix Metalloproteinase
NTR	Netrin
PA	Plasminogen Activator
PDGF-BB	Platelet Derived Growth Factor-BB
RAAS	Renin-Angiotensin-Aldosterone System
RECK	Reversion Inducing Cysteine Rich Protein With Kazal Motifs
SC144	Small-molecule gp130 inhibitor
sIL-6Rα	Soluble Interleukin-6 Receptor Subunit α
SP1	Specificity Protein 1
STAT	Signal Transducer And Activator Of Transcription
TACE	TNF-α Converting Enzyme/ADAM17
TGF-α	Transforming Growth Factor α
TGF-β	Transforming Growth Factor β
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumor Necrosis Factor-α
UPA	Urokinase-Type Plasminogen Activator
UPAR	Urokinase-Type Plasminogen Activator Receptor
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
VSMCs	Vascular Smooth Muscle Cells
Wnt7	Wingless-Type MMTV Integration Site Family Member 7
α1-AR	α1-adrenoceptor
β2-AR	β2-adrenoreceptor

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Highlights

1. RECK is a membrane-anchored matrix metalloproteinase regulator
2. RECK inhibits inflammation
3. RECK is antifibrotic
4. RECK reduces adverse cardiac remodeling
5. RECK induction is cardioprotective

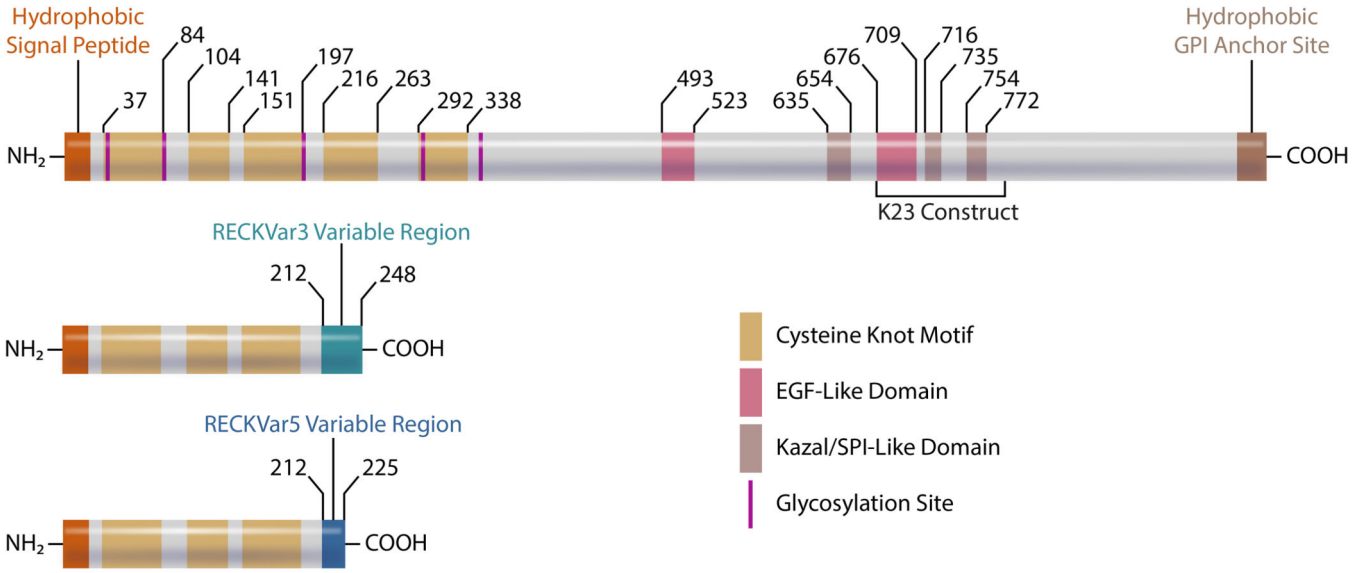

Fig. 1:

Domains/motifs of canonical RECK (top) and RECK isoforms, including corresponding amino acid residues. RECKVar3 and RECKVar5 share the first 212 amino acids with canonical RECK but differ in the COOH-terminal. Illustration made in Adobe Illustrator.

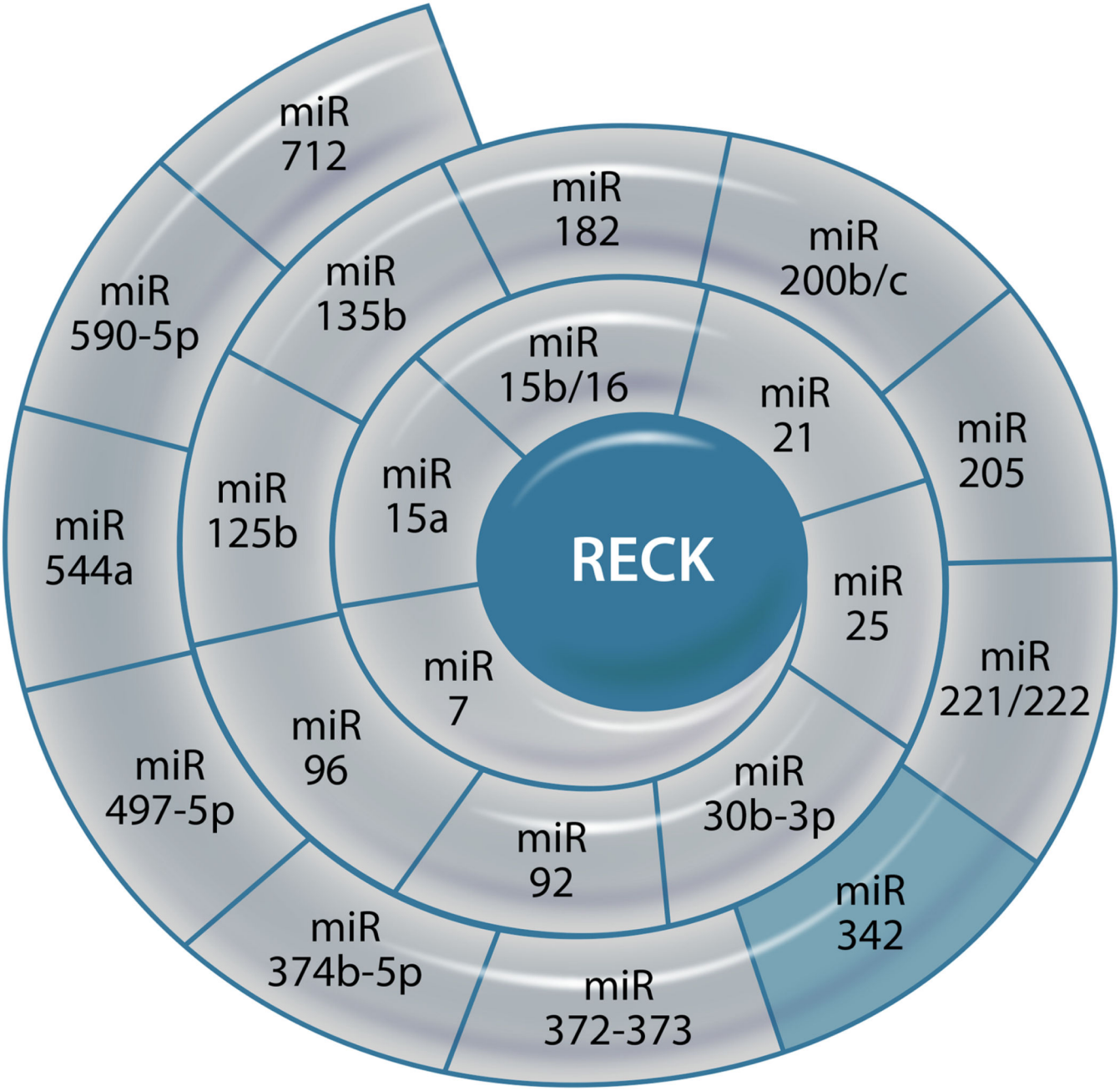

Fig. 2.

Post-transcriptional regulation of RECK by microRNAs. Those in light grey suppress RECK expression, while the one in light blue promotes its expression. Illustration made in Adobe Illustrator.

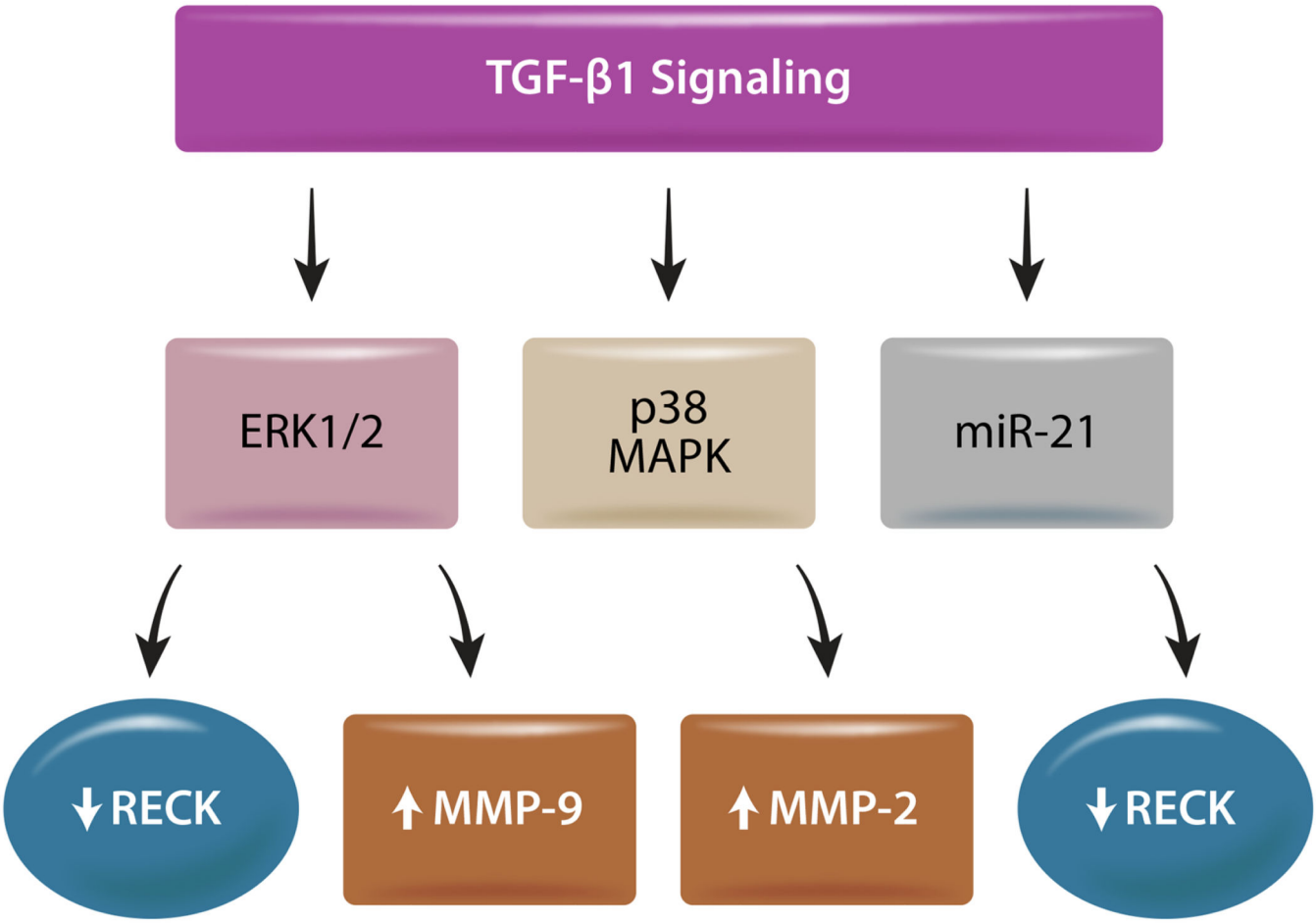

Fig. 3:

Downstream effects of TGF-β1 signaling. TGF-β1 increases MMP-9 expression but suppresses RECK through ERK½ activation in cancer cells. It also upregulates MMP-2 through p38 MAPK activation. TGF-β1 has also been shown to suppress RECK expression in part via miR-21 in hepatic oval cells. Illustration made in Adobe Illustrator.

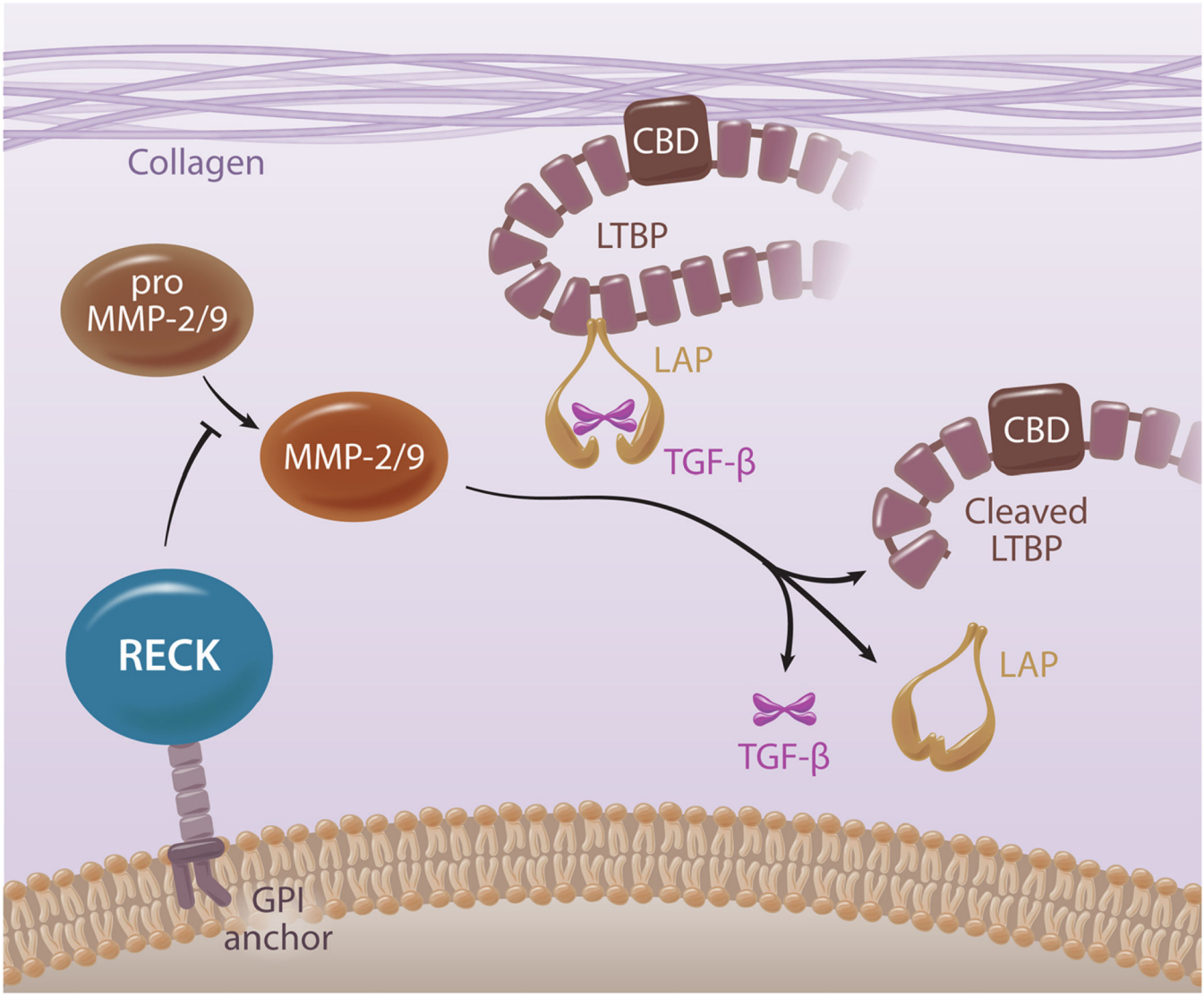

Fig. 4:

Schematic for protease mediated activation of latent TGF-β1, and the potential role RECK plays in regulating this process. Collagen Binding Domain (CBD), Latency Associated Peptide (LAP), Latent TGF-β1 Binding Protein (LTBP). Illustration made in Adobe Illustrator.

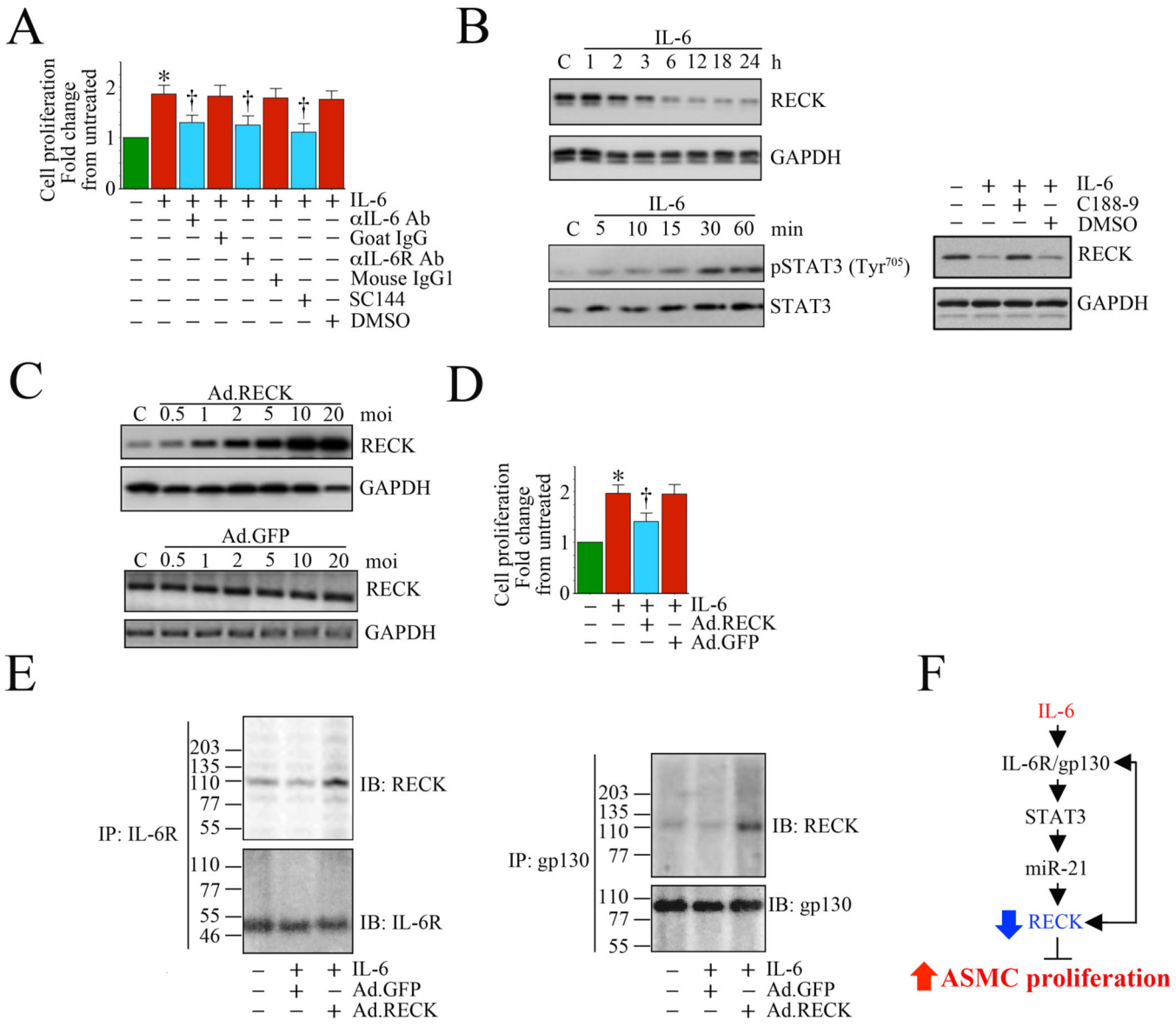

Fig. 5: Ectopic expression of RECK suppresses IL-6 mediated aortic smooth muscle cell (ASMC) proliferation. RECK physically associates with IL-6R and gp130.

A, Recombinant human IL-6 induces human ASMC (SMC) proliferation. SMC were grown in SmGM-2 medium, and at 70% confluency, the culture medium was replaced with basal medium containing 0.5% BSA (conditioning medium). After 48 h incubation, the quiescent SMC were incubated with IL-6 (10 ng/ml) for 48 h and analyzed for proliferation by CyQuant assay (n=6). Specificity of IL-6 was verified by incubating quiescent SMC with neutralizing IL-6 or IL-6R antibody (10 mg/ml for 1 h) or the gp130-specific inhibitor SC144 (5 μM in DMSO for 24 h) prior to IL-6 addition (n=6). **B**, IL-6 suppresses RECK via STAT3 activation. Quiescent SMC were incubated with IL-6 (10 ng/ml) for the indicated time periods. In a subset of experiments, SMC were incubated with the STAT3 inhibitor C188–9 (10 mM in DMSO for 15 min) prior to IL-6 addition (10 ng/ml for 6 h). RECK expression (upper panel) and STAT3 phosphorylation (lower panel) were analyzed in cleared

whole cell homogenates (20 μg) by Western blotting. GAPDH and total STAT3 served as loading controls. **C**, Adenoviral transduction of RECK (upper panel), but not control GFP (lower panel), increases RECK expression in a dose-dependent manner. Quiescent SMC were transduced with Ad.RECK (upper panel) or Ad.GFP (lower panel) at the indicated multiplicity of infection (moi) for 24 h. RECK protein expression was analyzed by Western blotting. **D**, Forced expression of RECK inhibits IL-6-induced SMC proliferation. SMC were transduced with Ad.RECK or Ad.GFP (moi10 for 24 h) were treated with IL-6 (10 ng/ml for 48h) and analyzed for proliferation as in A (n=6). **E**, RECK physically associates with IL-6R and gp130. SMC transduced with Ad.RECK or Ad.GFP and then treated with IL-6 were analyzed for IL-6R/RECK and gp30/RECK association by immunoprecipitation (IP) and immunnoblotting (IB) using soluble membrane fractions. **F**, Schematic showing the signaling pathway involved in IL-6/IL-6R/gp130-mediated STAT3 activation, RECK suppression and SMC proliferation. Importantly, forced expression of RECK suppressed IL-6-mediated SMC proliferation. Double head arrow: Physical association of RECK with IL-6R or gp130. *P<0.05 vs. untreated, †P<0.05 vs. IL-6.

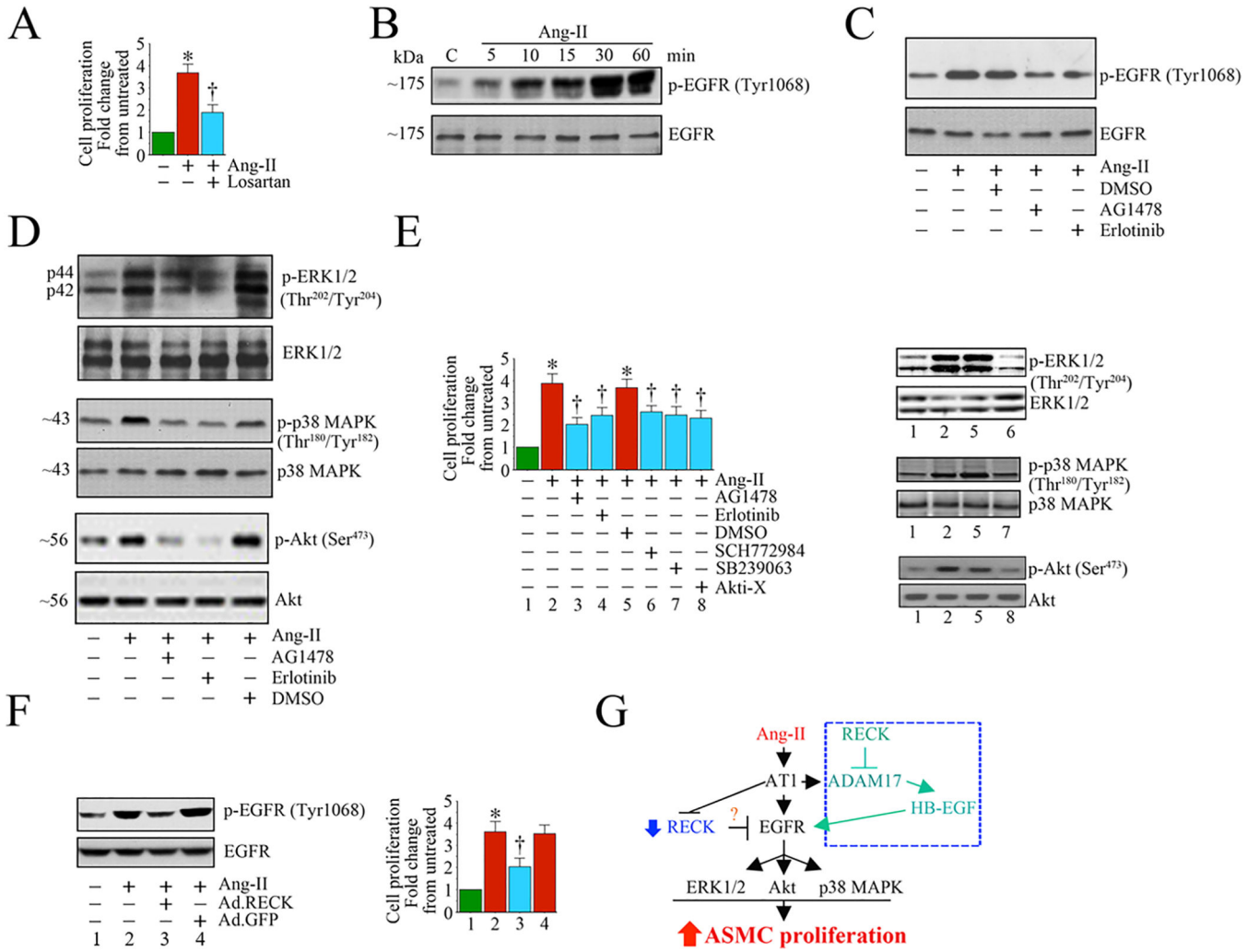

Fig. 6:

Ectopic expression of RECK suppresses Angiotensin (Ang)-II-induced human aortic smooth muscle cell (SMC) proliferation by inhibiting EGFR activation. **A,** Angiotensin (Ang)-II stimulates SMC proliferation via AT1. Quiescent SMC were incubated with the AT1 antagonist Losartan potassium (10 μM) for 1 h prior to Ang-II addition (100 nM for 48 h). Cell proliferation was analyzed by CyQuant assay (n=6). **B**, **C**, Ang-II induces EGFR activation in a time-dependent manner and is inhibited by AG1478 and erlotinib. Quiescent SMC treated with Ang-II were analyzed for EGFR activation by Western blotting using cleared whole cell lysates and activation-specific antibodies. Total EGFR served as a loading control. In a subset of experiments (**C**), quiescent SMC were treated with the EGFR-specific inhibitors AG1478 (100 μM in DMSO for 30 min) or erlotinib (1 μM in DMSO for 1h) prior to Ang-II (100 nM for 30 min). **D, E,** Ang-II induces SMC proliferation via EGFR-dependent ERK½, p38 MAPK and Akt activation. Quiescent SMC were treated with AG1478 or erlotinib prior to Ang-II addition (100 nM for 1 h). Activation of ERK½, p38 MAPK, and Akt were analyzed by Western blotting using cleared whole cell lysates and activation-specific antibodies (**D**). In a subset of experiments, quiescent SMC incubated with EGFR inhibitors AG1478 or erlotinib, the ERK½ inhibitor SCH772984 (10 μM in DMSO

for 1h), p38 MAPK inhibitor SB239063 (10 μM in DMSO for 1h) or the Akt inhibitor Akti-X (1 μM in DMSO for 1h) prior to Ang-II addition (100 nM for 48 h) were analyzed for proliferation as In A (**E**). The efficacy of inhibitors on respective target proteins was analyzed by Western blotting as shown on the right. **F**, Ectopic expression of RECK inhibits Ang-II-induced EGFR activation. SMC transduced with Ad.RECK or control GFP were incubated with Ang-II (100 nM for 30 min) were analyzed for EGFR activation by Western blotting (left hand panel). In a subset of experiments, SMC transduced with Ad.RECK or Ad.GFP were made quiescent, treated with Ang-II (100 nM for 48 h) and then analyzed for proliferation (right hand panel). **G,** Schema showing possible signaling pathways involved in Ang-II/AT1-mediated EGFR activation, RECK suppression and SMC proliferation. While Ang-II induced EGFR activation, it suppressed RECK expression. Further, targeting EGFR inhibits Ang-II-induced ERK½, Akt and p38 MAPK activation, and ASMC proliferation. Importantly, ectopic expression of RECK suppresses EGFR activation and inhibits Ang-II-induced SMC proliferation. RECK suppresses EGFR activation without physical association (data not shown), suggesting that RECK-mediated suppression of EGFR activation is indirect, and may involve (hypothesis) RECK inhibition of Ang-II/AT1/ADAM17-mediated cleavage and release of EGFR ligands such as HB-EGF from the cell surface, and binding to EGFR (blue box at the top right). *P<0.05 vs. untreated, †P<0.05

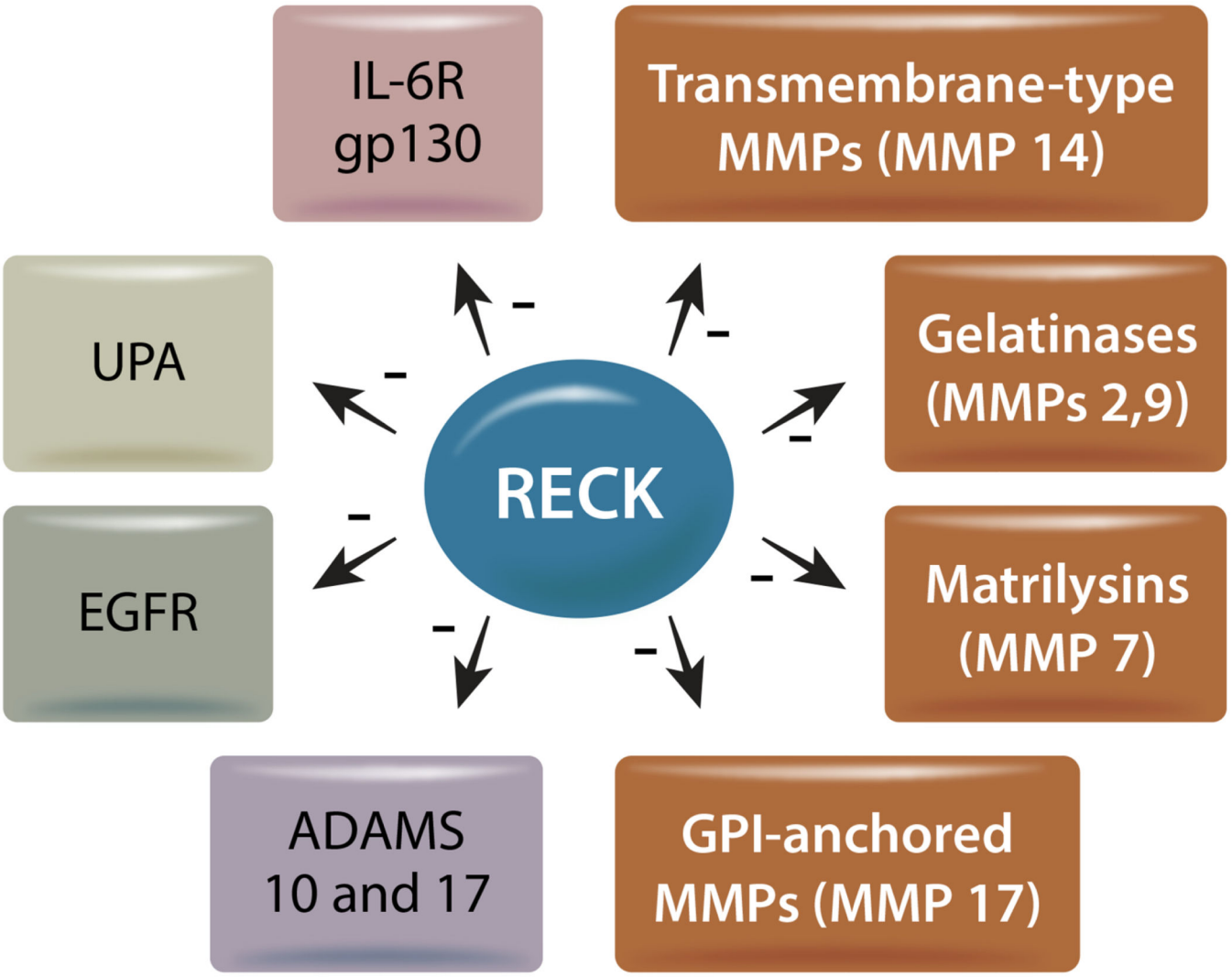

Fig. 7:
Regulation of RECK substrates involved in extracellular remodeling, inflammation, migration and proliferation. Illustration made in Adobe Illustrator.

Table 1:

Similarities and differences between RECK and TIMPs 1–4, focused on their structure, substrates and functions, as well as their knockout phenotype

Enzyme	RECK	TIMP1	TIMP2	TIMP3	TIMP4
Substrates	MMP-(2,7,9,14,17), ADAMTS, ADAM10/17, EGFR, IL-6R, uPA	MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13 and MMP16 Low affinity for membrane type MMPs	pro-MMP2, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13, MMP-14, MMP-15, MMP-16 and MMP-19.	MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, MMP-14 and MMP-15. Broadest inhibition spectrum, inhibits several members of the ADAM and ADAMTS families	MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9.
KO phenotype	• Embryonic lethality due to improper blood vessel maturation	• Increased ventricular remodeling • Altered ventricularstructure and function • Accelerated hepatocyte cell cycle • Increased resistance to corneal and pulmonary infection • Decreased adiposetissue weight on HFD • Impaired learning and memory	• Motor defects and deficiency in prepulse inhibitor of the startle reflex	• Enlargement of airspace in lungs • Enhanced apoptosis during mammary gland involution • Excessive cardiac fibrosis • Increased TGFβ1and TNFα. • Increased risk of endotoxin shock • Unaltered tumorigenesis and angiogenesis	• Reduced adipose tissue hypertrophy and fibrosis on a high fat diet. • Lower metabolic rate and energy expenditure.
Role in Angiogenesis	• Negatively Regulates	• Negatively Regulates	• Negatively Regulates • Enhances expression of RECK by interacting with α3β1 integrin switching signaling from Rac 1 to Rap 1	• NegativelyRegulates • Binds VEGFR2 to block VEGF signaling on endothelial cells. • Binds AT2R	• Negatively Regulates
Role in Cell Migration	• Inhibitsendothelial cell migration • SuppressesSMC proliferation and migration	• Increases lungcancer cell migration • Increases hepatoma cell migration • Increases cancer associated fibroblasts migration • Decreases microvascular endothelial cell migration	• Inhibits cancercell migration • Inhibitsmacrophage migration to atherosclerotic plaques	• Suppresses SMC proliferation and migration	• Inhibits SMCs migration
Role in Proliferation	• Anti-proliferative	• Promotes growth ofkeratinocytes and fibroblasts • Increases Ras-GTP • Inhibits caspase mediated apoptosis	• Potentiates erythroid activity and cell growth in metanephric mesenchyme cells • Increases amount of Ras-GTP • Reduces apoptosis	• Promotes apoptosis in a number of cancer cell lines and rat vascular smooth muscle cells	• Induces aortic SMC apoptosis
Structure	• Rich incysteine residues • 971 residues • 110kDa • 6 disulfidebonds (Kazal motif) • 5 asparagine glycosylation sites	• Conserved cysteineresidues • 207 residues • 25kDa • 6 disulfide bonds (NTR module) • 2 asparagine glycosylation sites	• Conserved cysteine residues • 220 residues • 25kDa • 6 disulfide bonds (NTR module)	• Conserved cysteineresidues • 211 residues • 25kDa • 6 disulfide bonds (NTR module)	• Conserved cysteine residues • 224 residues • 25kDa • 6 disulfidebonds (NTR module)