Review Article

Recklessness as a hallmark of aggressive cancer

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Cancer recurrence after surgical treatment is a major concern for patients and doctors. Understanding what makes tumors prone to recurrence would be an important step toward its prevention. Accumulating evidence indicates that the level of membrane-associated protease regulator reversion-inducing cysteine-rich protein with Kazal motifs (RECK) expressed in tumor tissue is a good prognostic indicator in several common cancers. Certain members of the matrix metalloproteinase family are often upregulated in advanced cancers and are known to play important roles in tumor angiogenesis, invasion and metastasis. RECK negatively regulates several matrix metalloproteinases. Therefore, RECK itself may well be considered a promising tool or target molecule to be activated in cancer therapy. Here we review the recent advances in RECK research and discuss some of the important issues to be addressed in future studies. (*Cancer Sci* 2007; 98: 1659–1665)

What is reversion-inducing cysteine-rich protein with Kazal motifs? Early findings

Reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*) was first identified as a cDNA clone inducing morphological reversion ('flat reversion') in NIH3T3 cells transformed by the *v*-K-*ras* oncogene.[1] *RECK* encodes a glycosylphosphatidylinositol (GPI)-anchored glycoprotein of approximately 110 kDa, containing multiple serine protease inhibitor-like motifs. Despite this structural feature, RECK negatively regulates multiple matrix metalloproteinase (MMP) family members (e.g. MMP-2, MM-9 and MT1-MMP) (Fig. 1). *RECK* is downregulated upon cell transformation by a variety of oncogenes.[1–4] *RECK* mRNA is expressed ubiquitously in normal human tissues, but it is undetectable in a number of tumor-derived cell lines. These cell lines showed reduced tumor angiogenesis, invasion and metastasis when stably transfected with a RECK expression vector.[5] *RECK*-deficient mice die at approximately embryonic day 10.5 (E10.5) with reduced extracellular matrix (ECM) integrity (e.g. reduced type I collagen, disrupted basement membranes, cellular disarray, increased tissue fragility) and halted vascular development; this phenotype is partially rescued in the *Mmp-2-null* background,[6] demonstrating that RECK is essential for development and that MMP-2 is a critical target of RECK *in vivo*. In contrast, mice lacking the expression of individual MMP family members tend to show little or no obvious developmental abnormalities during embryogenesis, probably due to functional redundancy.[7] Mice lacking RECK, the negative regulator of multiple MMP, may therefore provide an important opportunity for finding clues to understanding the roles of MMP, ECM and ECM remodeling during mammalian development. The implications of these early studies have been discussed in several previous reviews.[8–12] Here we focus on more recent work on RECK.

The more RECK, the better the prognosis

Hepatoma and pancreatic cancer. In their pioneering study, Furumoto *et al*. compared the levels of RECK mRNA in resected hepatocellular carcinomas and surrounding non-tumorous tissues ($n = 64$) by RNA blot hybridization. They noticed that tumors expressing *RECK* at higher levels showed better prognosis ($P = 0.02$).[13] They also detected a weak correlation between *RECK* expression and MMP-9 immunoreactivity. Subsequently, Masui *et al*. detected a positive correlation between RECK immunoreactivity in pancreatic carcinomas and survival of the patients ($n = 50$; $P = 0.0463$).[14] Consistent with the earlier findings in experimental systems using cultured cells and nude mice,[13] inverse correlations between *RECK* expression and the extent of MMP-2 activation as well as the invasive properties of the tumors were also noted.[14]

Breast cancer. Span *et al*. determined the level of RECK mRNA in mammary tumor tissues and surrounding normal tissues ($n = 10$) by quantitative reverse transcription–polymerase chain reaction (qRT-PCR), and found a significant reduction of *RECK* mRNA in tumors (tumor : normal = 1.7 : 3.9; $P = 0.028$). Their larger study focusing on tumor specimens ($n = 278$) indicated a significant correlation between the level of RECK expression and survival of the patients ($P = 0.037$). They also found that the level of RECK expression differed depending on whether samples were taken from patients before menopause or after menopause (7.2 : 5.6; $P = 0.045$). No correlation was detected between the level of RECK expression and age.[15] These findings are interesting in terms of how RECK is regulated physiologically.

Lung cancer. Takenaka *et al*. analyzed RECK expression in resected non-small cell lung cancers (NSCLC) of various cell types and various stages ($n = 171$) by immunohistochemistry. They detected a significant correlation ($P = 0.016$) between high RECK expression in tumors and survival of the patients, especially in the advanced-stage adenocarcinoma cases. They also found an inverse correlation between RECK expression and intratumoral microvessel density, particularly in tumors positive for vascular endothelial growth factor (VEGF).[5] In a subsequent study focusing on stage IIIA N2 cases with lymph node metastasis ($n = 118$), they found that a significant correlation between RECK expression and survival was seen only in patients with single N2 node metastasis and not with multiple N2 node metastases. Interestingly, RECK expression was significantly higher in metastatic lesions than in the original tumors.[16]

Colorectal cancer. Takeuchi *et al*. studied another common cancer, colorectal carcinoma ($n = 53$), and found downregulation of RECK mRNA in tumors. They also detected correlations between higher RECK immunoreactivity in tumors and several

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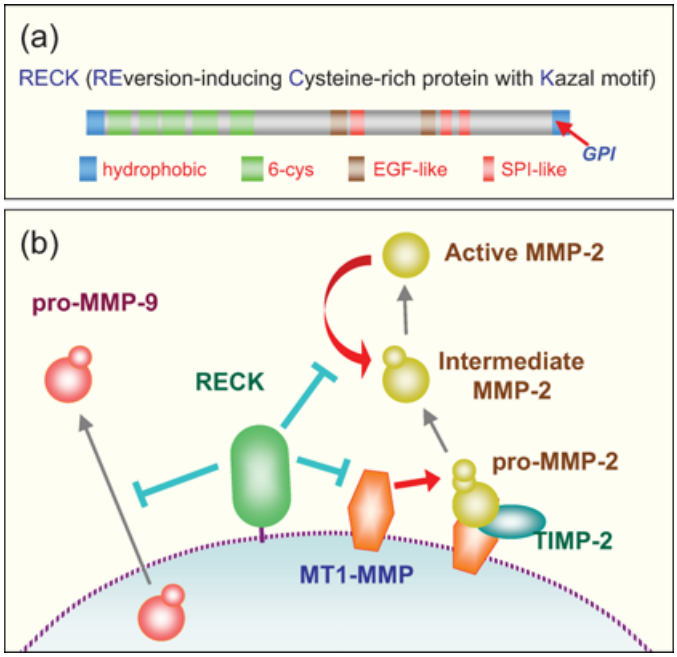

Fig. 1. Primary structure and proposed actions of reversion-inducing cysteine-rich protein with Kazal motifs (RECK). (a) RECK contains several motifs found commonly in extracellular proteins, such as an N-terminal signal peptide, C-terminal glycosylphosphatidylinositol (GPI)-anchoring signal, 6-cysteine repeats and epidermal growth factor (EGF)-like repeats. In addition, RECK contains three Kazal-type serine protease inhibitor (SPI)-like motifs. (b) Biochemical studies indicate that RECK regulates matrix metalloproteinases (MMP). Recent evidence suggests that RECK regulates some membrane-bound proteases, such as MT1-MMP and CD13, by recruiting them to a specific endocytic pathway (GEEC) and accelerating their degradation.

other properties including higher differentiation, lower lymph node metastasis, lower Duke's stage, and higher 5-year survival ($P = 0.011$). A weak correlation between lower MMP-9 expression and better prognosis could be detected among these patients ($P = 0.053$). Importantly, a good correlation was detected when both parameters were considered in combination in the data analysis: patients with tumors expressing low levels of MMP-9 and high levels of RECK showed a remarkably higher 5-year survival rate than the high-MMP-9/low-RECK group (89 vs 41%; $P = 0.0003$). Thus, in this tumor type, RECK and MMP-9 seem to be regulated independently. In addition, they found correlations between high RECK expression and low VEGF expression as well as low vascular density.[17] van der Jagt *et al*. also studied *RECK* expression in resected colon cancers ($n = 63$) by qRT-PCR and confirmed: (1) downregulation of *RECK* in tumors; (2) a correlation between the level of RECK mRNA and survival of the patients; and (3) an inverse correlation between *RECK* expression and MMP-2 activation. In addition, they found differential expression of *RECK* among tumors originating from different areas of the colon (high in sigmoid and rectal, low in descending and ascending colon) and suggested that the ratio of *RECK* expression between tumors and their surrounding normal tissues, rather than the absolute level of *RECK*, may serve as an accurate prognostic indicator (i.e. prognosis was better when RECK was high in tumors and low in normal tissues; prognosis was poor when RECK was low in tumors and high in normal tissues).[18]

Prostate cancer. Ohl *et al*. compared RECK mRNA expression between prostate carcinomas, benign prostatic hyperplasia from adenomectomy specimens and normal adjacent tissue of prostate carcinomas by qRT-PCR. Interestingly, *RECK* expression was increased in hyperplasia and decreased in carcinoma; the extent of *RECK* downregulation in carcinoma was correlated with recurrence.[19,20] Riddick *et al*. systematically analyzed the expression profiles of matrix proteases and their inhibitors in prostate tumors, both benign ($n = 26$) and malignant ($n = 44$), by qRT-PCR and detected a correlation between downregulation of *RECK* and malignancy.[21]

Other cancers. A correlation between RECK downregulation and poor prognosis has also been found in other tumor types including hilar cholangiocarcinomas,[22] gastric cancer,[23] ameloblastic tumors[24] and osteosarcomas.[25] However, Nabeshima *et al*. examined the expression of six MMP, three inhibitors and emmprin, an MMP inducer, in tumors of the peripheral nervous system (14 schwannomas, 14 neurofibromas and 12 malignant peripheral nerve sheath tumors) by immunostaining and found no alteration of RECK expression in these tumors.[26]

RECK expression in tumors has also been studied in animal models. Okamura *et al*. compared gene expression profiles between cancinogen-induced tumors and non-treated tissues from mice carrying the wild-type *HRAS* transgene under the control of its own promoter. They found consistent downregulation (~1/3) of RECK in tumors induced in three different experiments (7,12-dimethylbenz[a]anthracene [DMBA]-induced squamous cell carcinoma, N-ethyl-N-nitrosourea [ENU]-induced squamous cell carcinoma and urethane-induced adenoma).[27] Takagi *et al*. found downregulation of RECK in metastatic osteosarcomas in dogs.[28]

Summary. In summary, RECK is downregulated in many types of solid tumors (e.g. those in the pancreas, breast, lung non-small cell, colorectum, prostate, stomach, bone), and the extent of downregulation often correlates with poor prognosis (Fig. 2a). Correlations between RECK expression and several other parameters have also been reported within certain types of tumors. The generality of such findings, both inside the reported tumor type and among several different tumor types, needs to be confirmed in future studies. These findings may also provide important clues in understanding how RECK regulates tumor formation and progression.

Regulation of RECK expression and activity

Transcription. Chang *et al*. studied the mechanism of *RECK* promoter repression by *ras* or *HER-2/neu* oncogene signaling.[29,30] They confirmed the earlier finding by Sasahara *et al*.[2] that the Sp1-binding sites (GC boxes) located downstream of the transcription start site, termed Sp1(B), is a *cis*-element involved in this repression. Furthermore, they demonstrated that phosphorylated Sp1 (Thr^{453}/Thr^{739} catalyzed by external signal-regulated kinase [ERK]) and histone deacetylase 1 were recruited to the Sp1(B) site in this process.

Certain histone deacetylase [HDAC] inhibitors are know to induce flat reversion and suppress tumor invasion.[31,32] Liu *et al*. reported that trichostatin A, the first discovered HDAC inhibitor, activated the *RECK* promoter three-fold and reduced the level of secreted MMP-2 in a RECK-dependent manner.[33] Chang *et al*. also proposed another mechanism of *RECK* silencing in response to Ras/ERK signaling. Based on the experiments using a DNA methylation inhibitor (5′-azacytidine) and small interfering RNA (siRNA), they proposed the involvement of DNA methyltransferase 3b in this process.[34]

To extend this finding, Cho *et al*. determined the levels of RECK mRNA, RECK protein, and methylation of the *RECK* promoter in resected colon cancers ($n = 25$) and found a significant correlation between *RECK* downregulation and *RECK* promoter methylation ($P = 0.028$). In addition, treatment of colon cancer-derived cell lines (SW480 and SW620) with 5′-azacytidine resulted in the suppression of invasive activity, and upregulation of *RECK* was required for this suppression.[35] In their study on non-small cell lung cancers ($n = 55$), they

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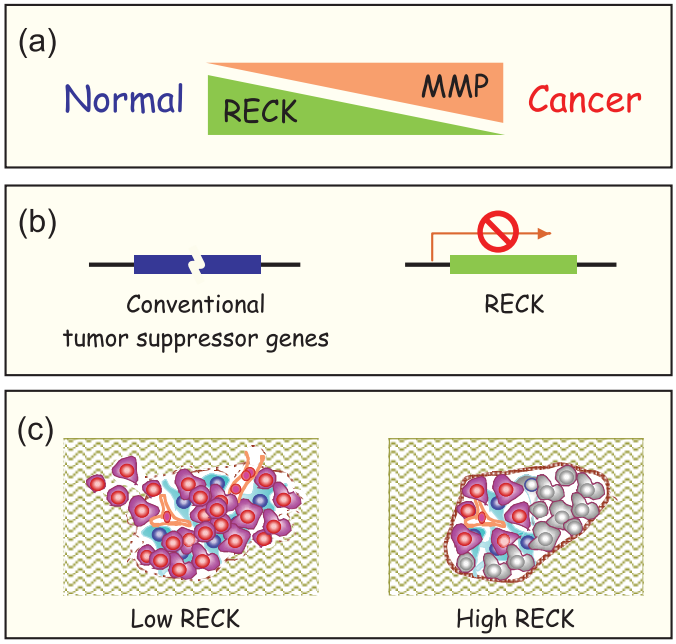

Fig. 2. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) in carcinogenesis. (a) Certain matrix metalloproteinases (MMP) are often upregulated in advanced or aggressive tumors. In contrast, RECK is downregulated in tumors of poor prognosis. (b) Conventional tumor suppressor genes are often inactivated or altered by mutations or chromosomal rearrangements. The *RECK* gene is probably intact in tumor cells but its expression is frequently downregulated. (c) Forced RECK expression in tumor cells improves basement membrane integrity (thick brown capsule) and inhibits vascular invasion and branching (orange), resulting in the death of tumor cells far from blood vessels (gray). Prevention of basement breakdown and angiogenesis may contribute to the observed suppression of tumor invasion and metastasis.

detected significant correlations between *RECK* downregulation and *RECK* promoter methylation ($P = 5 \times 10^{-6}$), between *RECK* downregulation and lymph node metastasis ($P = 0.038$), and between *KRAS* mutation (condon 12) and promoter methylation ($P = 0.047$) as well as *RECK* downregulation ($P = 0.023$). In cultured cells (H520, H358, A549), they found *RECK* upregulation and invasion suppression caused by 5′-azacytidine treatment.[36]

Upstream pathways. Liu *et al*. identified a couple of other *RECK* expression modulators. Epstein–Bar virus latent membrane protein 1, known as a tumor metastasis inducer, was found to repress the *RECK* promoter via the ERK–Sp1 pathway.[37] Non-steroidal anti-inflammatory drugs, NS398 and aspirin, were found to upregulate RECK mRNA and downregulate MMP-2 in a lung cancer cell line; this effect seems to be independent of cycloxygenase-2 (COX-2) inhibition, as prostaglandin E_2 and COX-2 overexpression failed to reverse the effect.[38]

Oh *et al*. investigated the up-stream signaling that regulates RECK expression and found an interesting link between a classical MMP regulator, tissue inhibitor of metalloproteinases-2 (TIMP-2), and RECK.[39] TIMP-2 inhibits migration of human microvascular endothelial cells, and previously identified mechanisms include direct inhibition of MMP and of VEGF signaling.[40] Their new study indicates the involvement of *RECK* induction via the Crk–C3G–Rap1 pathway in this process.[39] They also reported some evidence supporting the model that TIMP-2 stimulates the phosphorylation of Src at Tyr-527 by C-terminal SRC kinase (CSK), leading to underphosphorylation of paxillin at Tyr-31/118; this reduces the level of active Rac1 due to dissociation of the paxillin–Crk–DOCK180 complexes, and the concomitant increase in paxillin–Crk–C3G complexes leads to an increase in the level of active Rap1.[41] Subsequent studies using adenoviral vectors indicated that TIMP-2 promotes Rap1 expression and its association with actin.[42]

Correa *et al*. reported on the upregulation of RECK and gelatinases by type I collagen in glioma cells.[43] In contrast, Takagi *et al*. observed downregulation of RECK by Matrigel, a reconstituted basement membrane preparation from the murine Englebreth–Holm–Swarm sarcoma, probably due to the upregulation of gelatinases.[44] ECM components seem to have activities to induce both RECK and MMP, and the final outcome may depend on the balance between the two. Data obtained using cultured cells should therefore be interpreted with caution, taking into account the possibility that a slight shift in balance between RECK and MMP (and other targets; see below) may lead to robust changes in cell behavior.

Post-translational regulation. Shimizu *et al*. reported another mode of RECK regulation: glycosylation. They introduced point mutations at five putative N-glycosylation sites in RECK and found that glycosylation at three of the sites (Asn^{86}, Asn^{297}, Asn^{352}) was required for its biological activities. They also found functional segregations among these mutants: the glycosylation at Asn^{297} is required for suppression of MMP-9-secretion, whereas glycosylation at Asn^{352} is required for inhibition of pro-MMP-2-processing.[45]

Mori *et al*. isolated a cDNA exhibiting transforming activity in NIH3T3 cells from an adult T-cell leukemia cDNA library. Its product, named Tgat, contains a Rho guanine nucleotide exchange factor (RhoGEF) domain and a unique C-terminal 15 amino acids. They used this C-terminal fragment as bait in a yeast two-hybrid screening and cloned a fragment of RECK cDNA. They showed that RECK partially suppresses Tgat-mediated NIH3T3 transformation and that full-length Tgat, but not its mutant lacking the C-terminal portion, reversed the RECK-mediated inhibition of Matrigel invasion of HT1080 (fibrosarcoma) cells. Based on these findings, they proposed that Tgat may inhibit the activity of RECK through its C terminus.[46] Although the evidence suggests an interesting link between cytoskeletal dynamics and RECK, the important questions as to when and where this cytoplasmic protein with a RhoGEF domain can interact with the GPI-anchored external protein RECK need to be clarified.

Summary. As RECK is probably inactivated in tumors through epigenetic mechanisms in most cases (Fig. 2b), artificial activation of the dormant *RECK* gene is likely to have beneficial effects (Fig. 2c). It is therefore important to continue our efforts to elucidate how RECK is regulated *in vivo* and to find reagents that upregulate RECK but not other unwanted molecules.

Functions and mechanisms of action

Bioactivity. Suppression of tumor invasion and MMP activation in cultured cells have been used widely to assess RECK bioactivity. Kang *et al*. observed an inverse correlation between the level of RECK mRNA (determined by qRT-PCR) and pro-MMP-2 activation (gelatin zymography) among osteosarcoma samples ($n = 23$; $P = 0.037$). They also observed reduced MMP-2 activity, morphological changes and suppression of invasion *in vitro* in an osteosarcoma-derived cell line (HOS) after *RECK* transfection.[25] This study not only supports earlier findings with the HT1080 fibrosarcoma cell line but also demonstrates their clinical relevance.[25] Takagi *et al*. cloned dog *RECK* cDNA, measured the expression of RECK mRNA by qRT-PCR, and found its high expression in normal lung and testis. They also found low expression of *RECK* in various dog tumors ($n = 36$), including mast cell tumors, and relatively high expression in osteosarcoma. They could also detect the ability of RECK to suppress invasion when transfected into transitional cell carcinoma.[47] Such studies in animals may be important in both scientific and practical terms; comparative studies among different species may yield important scientific insights, whereas dogs

may be useful in testing the effectiveness and safety of RECK-targeted therapies in future.

Tissue distribution. Precise knowledge of the temporal and spatial patterns of RECK expression in our body will provide an important foundation for future studies aiming to understand RECK's physiological functions and its roles in various disorders. Nuttall *et al*. carried out a systematic survey on the expression patterns of a whole series of molecules (i.e. 23 MMP family members, seven a disintegrin and metalloproteinase (ADAM) family members, five endogenous metalloproteinase inhibitors) in various organs from mice at several time points (E11.5–P28) during development by qRT-PCR.[48] The data indicate that at the organ level, *RECK* is widely (most abundant in lung) expressed throughout the developmental stages.

Guo and Zou collected placental tissue from 52 normal pregnant women (27 in early pregnancy, 25 in term pregnancy) and determined RECK protein level, its localization and MMP-2 activation. They detected a significant increase in RECK expression and a decrease in MMP-2 activation in the term pregnancy group compared with the early pregnancy group. Immunohistochemistry showed that RECK expression was found in villous tissues of both the early pregnancy and term pregnancy, localized mainly in the cell membrane and cytoplasm of cytotrophoblasts and syncytiotrophoblasts, increasing with gestational time, and significantly lower in cellular column with invasion ability.[49] Thus, interplay between MMP and RECK may play a role in the regulation of trophoblast invasion. This study also demonstrates the dynamic nature of RECK expression inside an organ, which is likely to be undetectable by RNA blot hybridization or qRT-PCR using RNA extracted from whole organs.

Physiological functions. Srivastava *et al*. used *Drosophila* genetics to explore the molecular pathways regulating MMP-mediated basement membrane degradation during developmental invasion (i.e. imaginal disc evasion) and tumor invasion. They found that c-Jun N-terminal kinase (JNK) mediates MMP upregulation in both processes. Interestingly, although ectopic expression of TIMP was sufficient to inhibit developmental invasion, both TIMP and RECK were required to stop tumor invasion.[50] Insects are evolutionally the most remote organisms known to carry *RECK* orthologues; no *RECK* is found in *Caenorhabditis elegans*. *RECK* is a single gene both in *Drosophila* and mammals. *Drosophila* would therefore serve as a good model system to explore conserved functions of *RECK*, including invasion suppression.

As mentioned above, *RECK*-deficient mice die at mid-gestation around E10.5,[5] making it difficult to elucidate the functions of RECK in later stages of development using these mice. To address this issue, we analyzed the expression patterns of RECK protein and mRNA in mouse embryos at later stages. When immunohistochemistry was used, abundant RECK expression was found in skeletal muscles in E13.5–E14.5 embryos, and the staining pattern was reminiscent of that of a myogenic transcription factor, MRF4, but differed from that of MyoD. Experiments using cultured myogenic cells indicated that ectopic expression of RECK inhibits myotube formation and that the *RECK* gene promoter was inhibited by MyoD and activated by MRF4 in luciferase reporter assays. These data are consistent with the model (Fig. 3a) that RECK is downregulated by MyoD at the early stage of skeletal muscle development where myoblasts actively migrate and fuse to form myotubes, whereas RECK is upregulated by MRF4 at the stage when myotubes are sheathed by basement membranes to form myofibers and around the sites where mechanical strength is required (e.g. myo-tendinous junctions).[51]

When *in situ* hybridization was used, however, abundant RECK mRNA was detected in cartilage in E13.5–E16.5 embryos. The mouse embryonal carcinoma-derived cell line ATDC5 is known to be able to recapitulate cartilage development in culture. At the initial stage, ATDC5 cells actively migrate and form small aggregates or foci, which we call 'cellular condensation'. At the later stage, these foci continue to grow and form larger ridges called cartilaginous nodules. We found that during cellular condensation, RECK expression is repressed and MMP expression is elevated especially at the center of the foci. At the later stage, RECK becomes upregulated in cartilaginous nodules where chondrocyte-specific type II collagen is accumulated; MMP remain active in the periphery of the expanding nodules. Ectopic expression of RECK suppresses cellular condensation, whereas RECK siRNA inhibits ECM accumulation and nodule consolidation[52] (Fig. 3b). Thus, in both skeletal muscle and cartilage, the level of RECK is low in the early phase of tissue morphogenesis in areas where cell migration and dynamic cell–cell interaction

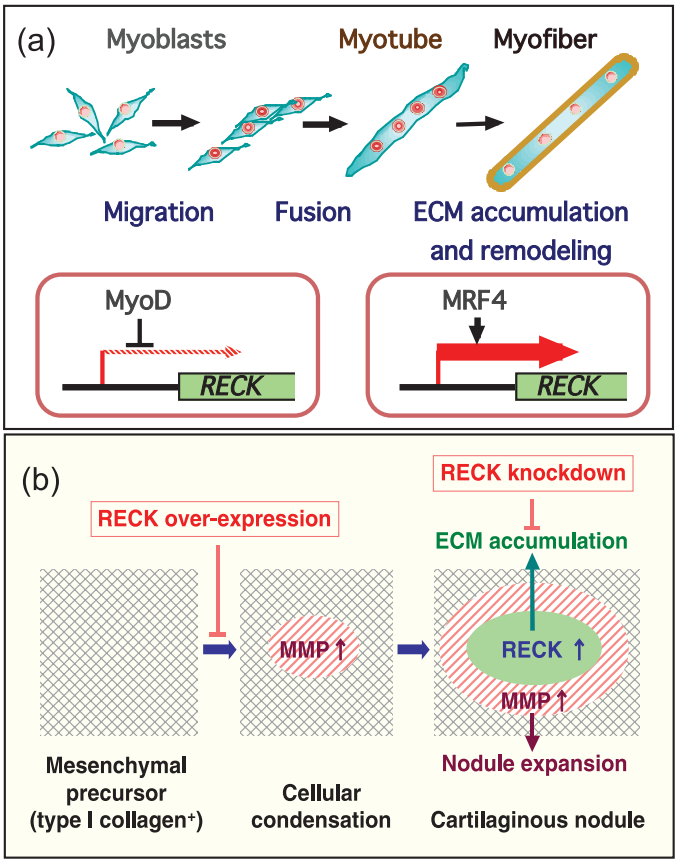

Fig. 3. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) in tissue morphogenesis. (a) RECK in skeletal muscle development. The *RECK* gene promoter is inhibited by MyoD and stimulated by MRF4. Indeed, MRF4, but not MyoD, shares similar expression patterns with RECK in mouse embryos. RECK overexpression suppresses myotube formation, and RECK-deficient embryonic cells show enhanced myogenic activity. These observations are consistent with the model that RECK is downregulated by MyoD during the early phase of muscle development in areas where cells actively migrate and fuse to form myotubes, whereas RECK is activated in the later phase in areas where myotubes become sheathed by basement membrane and acquire mechanical strength. (b) RECK as a key player in cartilage differentiation. Matrix metalloproteinase (MMP) expression is high and RECK expression is low in the initial stage of chondrogenic differentiation of ATDC5 cells. At this stage, cells migrate and form aggregates (cellular condensation). In the later stage, RECK expression is activated inside the large cell aggregates (now called cartilaginous nodules) where type IV collagen also accumulates, whereas MMP stay active in the periphery of the expanding nodules. RECK overexpression suppresses initial cellular condensation, whereas RECK knockdown inhibits extracellular matrix (ECM) accumulation and consolidation of the nodules. In both systems, RECK stays low in the initial phase and is upregulated in the later phase, suggesting that RECK plays a general role in helping to accumulate ECM accumulation and stabilizing tissue architecture.

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take place, whereas the level of RECK becomes elevated in the later phase in areas where ECM accumulation is required.

We recently identified a developmental abnormality, including precocious neuronal differentiation and reduction in the number of nestin-positive neural precursor cells, in the central nervous system of *RECK*-deficient mice. This and the vascular phenotype[6] of *RECK*-deficient mice is reminiscent of the phenotype seen in mice deficient in Notch signaling. Indeed, remarkable downregulation of Notch signaling was observed, and the mechanism probably involves excessive ectodomain shedding of Notch ligands mediated by ADAM10.[53] Thus, RECK regulates an ADAM family member and supports cell proliferation in this system. The Notch pathway is conserved across species and is involved in various biological events. It would be interesting to address whether RECK plays any roles in other species or in other biological events.

We also characterized the effects of RECK on other molecules on the cell surface. RECK was found to form a complex with MT1-MMP and inhibit its proteolytic activity. When expressed in HT1080 cells, RECK increased the amount of MT1-MMP that associated with the membrane microdomain corresponding to the 'lipid raft' during sucrose density gradient ultracentrifugation, and this effect was abolished when membrane cholesterol was perturbed. Thus, RECK may regulate MT1-MMP function by modulating its behavior on the cell surface as well as inhibiting its enzymatic activity. A subsequent study indicated that RECK interacts with CD13/aminopeptidase N and inhibits the proteolytic activity of CD13 in a cholesterol perturbation-sensitive manner. Moreover, RECK was found to recruit these membrane proteases to the specific endocytic pathway known as GPI-anchored protein-enriched early endosomal compartment (GEEC), resulting in accelerated degradation.[54]

These new findings have widened our view on the biological functions of RECK and expanded the repertoire of RECK targets as well as substrates protected by RECK.

Future directions

Overview. As summarized above, compelling evidence indicates a good correlation between the extent of RECK downregulation and poor prognosis in a wide variety of solid tumors. Knowledge on the transcriptional regulation of *RECK* is also accumulating. However, the number of reports on the mechanisms of action of RECK has been limited. One of the important issues at the molecular level is to clarify the spectrum of RECK targets. We also need to find molecules that interact physically with RECK, to determine the 3-dimensional structure of RECK, and to understand the nature of interactions between RECK and its targets at the atomic level. At the single-cell level, the role of RECK in cell differentiation and migration needs to be clarified. Signaling pathways and drugs regulating RECK expression should also be explored. At the system level, involvement of RECK in animal development and various diseases has to be studied. Various model systems including simple organisms (e.g. fruit fly) and genetically engineered mice will be valuable in such studies.

Human genetics. Studies on the possible involvement of RECK in various diseases may yield information of both clinical and biological importance. Single nucleotide polymorphism (SNP) markers may serve as powerful tools for such studies. For instance, Lei *et al*. analyzed SNP in the RECK promoter region in healthy control ($n = 952$) and mammary tumor patients ($n = 959$) and found a correlation between C/T heterozygosity at the –420 position and better prognosis.[55] Eisenberg *et al*. established the entire genomic structure of the human *RECK* gene (mapped at 9p13→p12) which spans more than 87 kb and consists of 21 exons and 20 introns. They identified 13 SNP: four in the coding region (exons 1, 9, 13 and 15) and nine in introns (introns 5, 8, 10, 12, 15 and 17).[56] These and other SNP markers will be valuable in future studies.

Other disorders. Involvement of MMP in the pathology of rheumatoid arthritis has been well documented. van Lent *et al*. analyzed RECK expression in the synovial membrane by both qRT-PCR and immunohistochemistry; they found that RECK expression is reduced in patients with rheumatoid arthritis whereas MMP-14 shows no significant difference.[57] Milner *et al*. used small pieces of bovine nasal septum cartilage in culture to analyze the effects of inflammatory cytokines, interleukin-1 and oncostatin M, on the expression of several MMP and MMP inhibitors to understand their roles in cartilage resorption. In this system, no significant alteration in the level of RECK expression was detected.[58] In light of the suggested roles of RECK in chondrogenesis[52] it should be important to clarify the roles of RECK in rheumatoid arthritis and other disorders affecting cartilaginous tissues.

Some respiratory disorders are accompanied by tissue destruction and might involve an imbalance between RECK and MMP. Indeed, Paulissen *et al*. reported that the level of RECK mRNA was significantly decreased in the cells in the sputum from patients with asthma ($n = 21$) compared with healthy controls ($n = 17$), and that RECK expression was positively correlated with forced expiratory volume in the first second (FEV_1), an indicator of maximal exercise capacity ($r = 0.45$, $P = 0.01$).[59]

Recent findings indicate that MMP act on pro-inflammatory cytokines, chemokines and other proteins to regulate various aspects of inflammation and immunity.[60] RECK may well be involved in the regulation of these events, but we have no evidence for or against this possibility at the moment. Expression of RECK in macrophages has been reported.[57] A reduction in the phagocytotic activity of macrophages has been found in endometriosis. Wu *et al*. reported that this phenomenon involves prostaglandin E_2 (PGE_2)-mediated downregulation of MMP-9 but not changes in the expression of TIMP or RECK.[61] Thus, the functions of RECK in macrophages and its role in endometriosis remain unclear.

Conclusions. Here we have cited most, if not all, of the papers on RECK published since the announcement of its discovery in 1998.[1] These limited number of studies already illuminate the importance of RECK (or its absence) in some physiological processes and diseases, especially those involving ECM remodeling. Further studies on various aspects of RECK will lead to a better understanding of how this molecule works to prevent tumor angiogenesis, invasion and metastasis and how we can selectively manipulate these processes.

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