Dual effects of the membrane-anchored MMP regulator RECK on chondrogenic differentiation of ATDC5 cells

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Summary

Extracellular matrix (ECM) undergoes continuous remodeling during mammalian development. Although involvement of matrix metalloproteinases (MMPs) in ECM degradation has been well documented, how this process is regulated to allow proper ECM accumulation remains unclear. We previously showed the involvement of a membrane-anchored MMP regulator, RECK (reversion-inducing cysteine-rich protein with Kazal motifs), in vascular development in mice. Here we report that *Reck* mRNA can be detected in developing cartilage in E13.5~16.5 mouse embryos and is progressively upregulated during differentiation of a chondrogenic cell line ATDC5 in vitro. In the early phase of ATDC5 differentiation, RECK expression stays low, multiple MMPs are upregulated, and there is ECM degradation at the sites of cellular condensation. In the later phase, RECK is upregulated inside the expanding cartilaginous nodules where type II collagen is accumulated while active ECM degradation persists along the rim of the nodules. Constitutive RECK expression suppressed initial cellular condensation, whereas RECK knockdown suppressed the later ECM accumulation in the cartilaginous nodules. These results suggest that RECK expression at the right place (in the core of the nodules) and at the right time (only in the later phase) is important for proper chondrogenesis and that RECK, together with MMPs, plays a crucial role in regulating dynamic processes of tissue morphogenesis.

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Key words: RECK, MMP, ECM remodeling, Tissue morphogenesis, Chondrogenesis

Introduction

The extracellular matrix (ECM) confers mechanical strength to the tissue and provides microenvironment and inductive cues essential for proper cell proliferation, migration and differentiation. The ECM undergoes continuous remodeling during animal development as well as during various pathological processes including wound healing, osteoporosis, rheumatoid arthritis and cancer. Proteases of the matrix metalloproteinase (MMP) family (more than 20 members) are believed to play major roles in the destructive aspects of ECM remodeling (Egeblad and Werb, 2002; Vu and Werb, 2000). The importance of several MMP family members in mouse development has been documented using gene-targeting techniques (Holmbeck et al., 1999; Itoh et al., 1997; Oh et al., 2004; Vu et al., 1998; Zhou et al., 2000). A group of endogenous, secreted MMP inhibitors, termed tissue inhibitors of metalloproteinases (TIMP1-TIMP4) have been identified, although their roles in development remain to be elucidated (Lambert et al., 2004).

RECK (reversion-inducing cysteine-rich protein with Kazal motifs) was first isolated as a transformation suppressor gene by cDNA expression cloning (Takahashi et al., 1998). It encodes a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that negatively regulates at least three members of the MMP family (MMP2, MMP9 and MT1-MMP) in vitro and in cultured cells (Oh et al., 2001; Takahashi et al., 1998). Although *RECK* is widely expressed in normal human organs and non-transformed cell lines, it is downregulated in various tumor tissues and transformed cells (Takahashi et al., 1998). Restored expression of *RECK* in tumor-derived cell lines results in strong suppression of their abilities to invade, metastasize, and induce angiogenesis (Oh et al., 2001;

Takahashi et al., 1998). Positive correlation between residual RECK expression in tumor tissues and survival of the patients have been documented in a variety of cancers (Furumoto et al., 2001; Masui et al., 2003; Span et al., 2003; Takenaka et al., 2004; Takeuchi et al., 2004). Mice lacking *Reck* die around embryonic day 10.5 (E10.5) with defects in angiogenesis (Oh et al., 2001). In these mutant mice, MMP activity is elevated, and the amount of type I collagen is greatly reduced. This phenotype is partially suppressed in mice lacking both *Reck* and *Mmp2*. These findings support the idea that RECK is an essential regulator of MMPs. Because of the embryonic lethality of *Reck*-deficient mice, however, the roles of RECK in mouse development after E10.5 remain to be elucidated.

In mammals, cartilage is one of the most ECM-rich tissues where type II collagen and aggrecan are particularly abundant. Formation of cartilage (or chondrogenesis) is a multi-step process involving (1) condensation of mesenchymal precursor cells at the future sites of skeletal elements; (2) differentiation of mesenchymal precursors in these dense cell masses along a chondrogenic pathway; (3) deposition of specialized ECM; and (4) cellular hypertrophy and calcification (Behonick and Werb, 2003; Cancedda et al., 1995; Hall and Miyake, 2000; Knudson and Toole, 1985). Expression of *Mmp2*, *Mmp9*, and *Mt1-mmp* in mesenchymal cells surrounding developing cartilage has been observed (Apte et al., 1997; Reponen et al., 1992; Reponen et al., 1994), and inhibition of cartilage formation by a synthetic MMP inhibitor in a mandibular explant culture system documented (Chin and Werb, 1997). Thus, MMPs seem

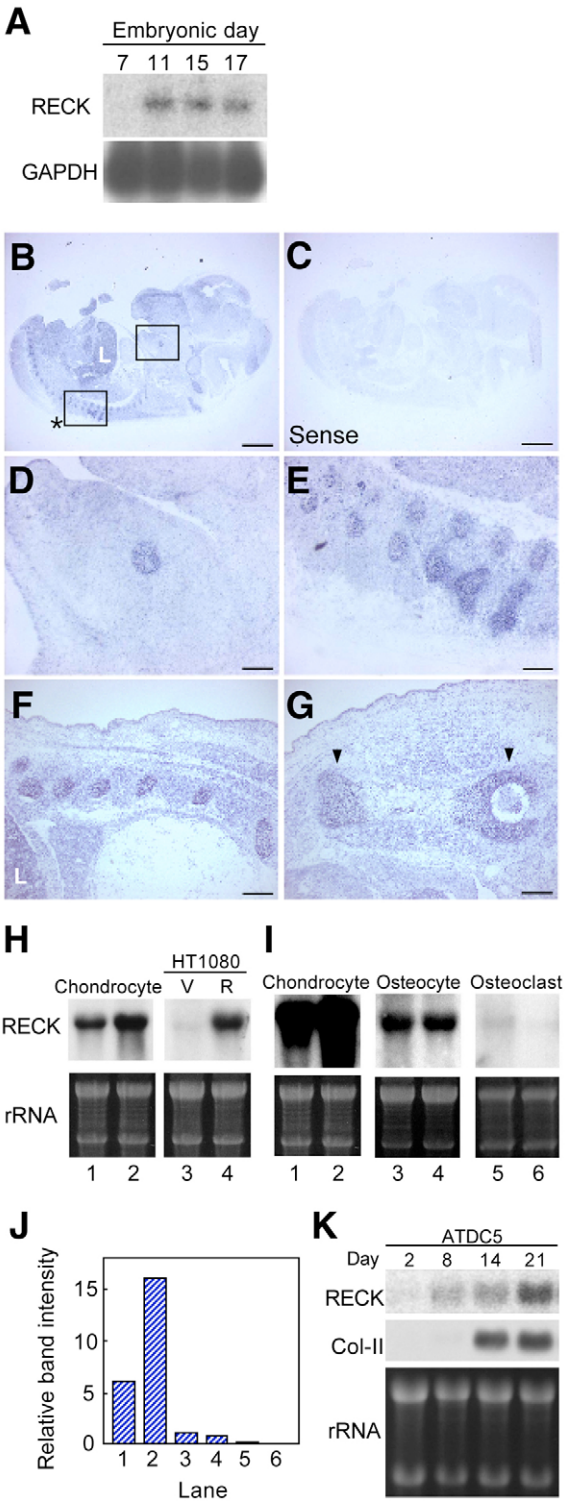

Fig. 1. Abundant expression of *Reck* in cartilage. (A) Mouse embryo multiple tissue northern blot (Clontech) was hybridized sequentially with cDNA probes for *Reck* and *Gapdh* (loading control). Each lane contained 2 μg of poly(A)$^+$ RNA from embryos at the indicated stage (days after gestation). (B-G) Detection of *Reck* mRNA in mouse embryos by in situ hybridization. Sagittal sections of an E13.5 embryo were hybridized with *Reck* antisense (B) or sense (C) riboprobe. Magnified views of the boxed areas in B are shown in D and E (*), respectively. The areas containing ribs (F) and a femur (G) in a section of E16.5 embryo hybridized with *Reck* anti-sense probe are also shown. Northern blot data (not shown) indicated that the apparently strong signals in the liver (labeled 'L' in panel B and F) are non-specific. Bars, 1.28 mm in B and C; 200 μm in D and E; 320 μm in F and G. (H-J) Detection of *Reck* mRNA in cultured cells by RNA blot hybridization. Total RNA extracted from the following cultured cells was analyzed by RNA blot hybridization using the *Reck* cDNA probe. (H) Lane 1: H4-1 (a primary culture derived from human bone marrow containing the cells capable of differentiating into chondrocytes) (Imabayashi et al., 2003; Mori et al., 2005). Lane 2: DEC (chondrocytes obtained from human cartilage) (Imabayashi et al., 2003). Lane 3: the human fibrosarcoma cell line HT1080-transfected with control vector (negative control). Lane 4: HT1080 transfected with RECK expression vector (positive control). (I) Lanes 1 and 2: longer exposure of lane 1 and 2 in H, (under the same conditions as lanes 3~6). Lane 3: KUM9 (multipotential progenitor cells derived from mouse bone marrow and capable of differentiating into osteocytes, adipocytes, myocytes and neurons) (Kohyama et al., 2001). Lane 4: KUSA-A1 (osteoblasts derived from mouse bone marrow) (Kohyama et al., 2001). Lanes 5 and 6: MDBM (osteoclast progenitor cells derived from mouse bone marrow) and RANKL-treated MDBM (mature osteoclasts), respectively (Takeshita et al., 2000). The amount of total RNA loaded was 10 μg for lanes 1-4 and 4 μg for lanes 5-6. Patterns of ribosomal RNA bands are also shown (bottom panels). (J) Relative intensity of the bands on the blot shown in I. The band intensity determined by densitometry and normalized against the RNA amount is presented as a bar graph for direct comparison. (K) Temporal expression pattern of *Reck* mRNA in ATDC5 cells during chondrogenic differentiation in vitro. Total RNA (20 μg) from ATDC5 cells that had been allowed to differentiate in culture for the indicated times (days) was subjected to RNA blot hybridization using a *Reck* cDNA probe (top panel). The same blot was re-probed with a type II collagen cDNA (middle panel; Col-II) to monitor chondrogenic differentiation.

to be important for ECM remodeling during chondrogenesis (Chin and Werb, 1997).

The mouse embryonal carcinoma-derived cell line ATDC5 has properties resembling those of undifferentiated mesenchymal cells and is able to recapitulate many aspects of chondrogenesis including cellular condensation, cartilaginous nodule formation, switching of collagen gene expression from type I to types II and X, accumulation of cartilage-type proteoglycans, etc. (Atsumi et al., 1990; Shukunami et al., 1996). Thus, ATDC5 cells have been widely used for studying temporal and spatial changes in morphology, gene expression, etc. during chondrocyte differentiation and for assessing the effects of various exogenous agents on these processes (Fujii et al., 1999; Fujita et al., 2004; James et al., 2005; Newman et al., 2001; Scheijen et al., 2003; Shen et al., 2002; Shukunami et al., 1997; Shukunami et al., 1998; Woods et al., 2005).

In the present study, we attempted to explore the functions of RECK after E10.5 using tissue slices prepared from wild-type mice at later developmental stages and cultured ATDC5 cells. Our data indicate that RECK is critically involved in the dynamic regulation of ECM remodeling and tissue morphogenesis during chondrogenesis.

Results

RECK is abundant in developing cartilage

RNA blot hybridization with poly(A)$^+$ RNA from whole mouse embryos at different stages indicated that although *Reck* mRNA is barely detectable at embryonic day 7 (E7), it is clearly detectable at E11 and thereafter (Fig. 1A). The observed onset of *Reck* expression is consistent with our previous finding that *Reck*-deficient mice died around E10.5 with prominent defects in vascular development (Oh et al., 2001). In an attempt to address the question of whether RECK plays any roles in events other than vascular development, we analyzed the expression of *Reck* in wild-type embryos at E13.5 and E16.5 by in situ hybridization. At E13.5, *Reck*-specific hybridization signals (blue signals in Fig. 1B) were found ubiquitously in the entire embryo and were especially intense in cartilaginous tissues, such as Meckel's cartilage (Fig. 1D), vertebrae and ribs (Fig. 1E). At E16.5, intense *Reck* signals were found in rib cartilage (Fig. 1F) and terminal regions of long bones (Fig. 1G, arrowheads).

To identify the cells expressing RECK, we analyzed the relative abundance of *Reck* mRNA in cultured cells of various origins by RNA blot hybridization (Fig. 1H-J). Strong *Reck* expression was detectable in the cells of the chondrocytic lineage

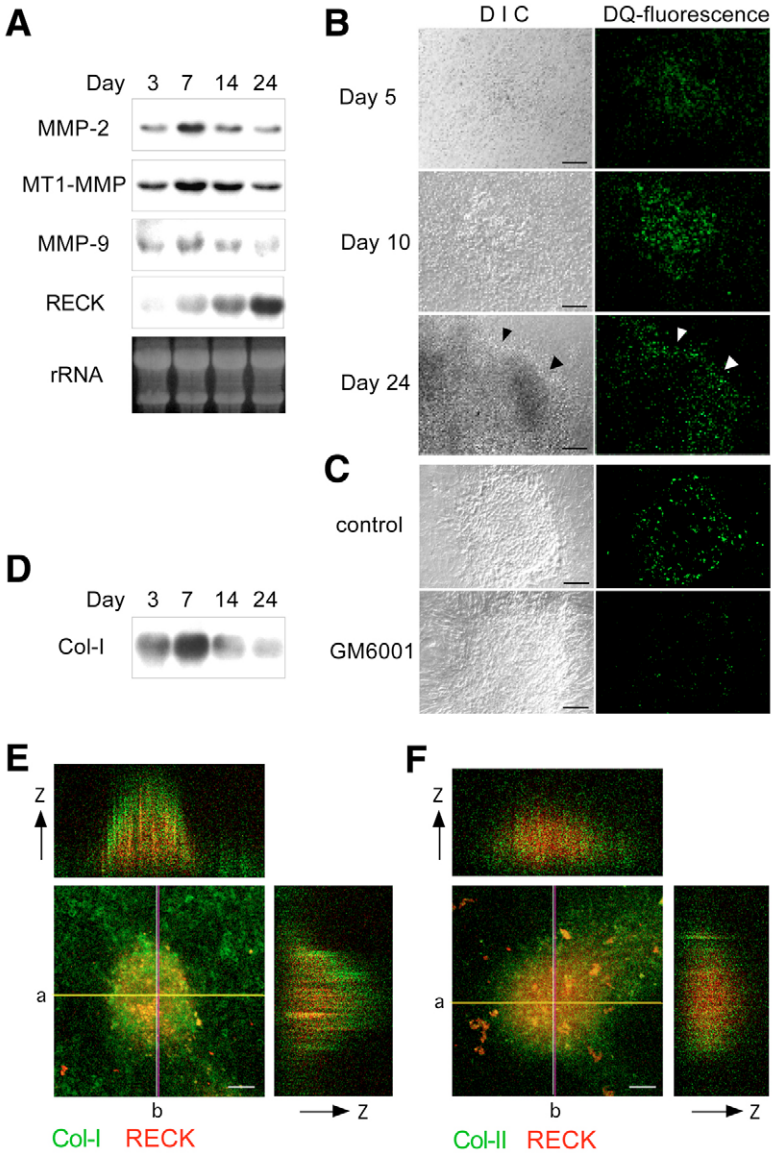

Fig. 2. Expression patterns of MMP, collagens and RECK during cartilaginous nodule formation by ATDC5 cells. (A) Temporal expression patterns of *Mmp*s and *Reck* in differentiating ATDC5 cells. Total RNA (20 μg) extracted from ATDC5 cells that had been incubated for the indicated times was analyzed by RNA blot hybridization using indicated probes. (B) Collagenase activity expressed by differentiating ATDC5 cells. ATDC5 cells were incubated for the indicated times and then overlaid with semi-solid medium containing reconstituted type I collagen and DQ collagen. After an additional 24 hours incubation, morphology (DIC) and collagenolytic activity (green DQ-fluorescence) were recorded with a confocal microscope. Bars, 100 μm in the day-5 and day-10 panels; 200 μm in the day-24 panel. Arrowheads indicate the rim of a large nodule. (C) Sensitivity of the DQ-fluorescent signals to an MMP inhibitor GM6001. The experimental conditions were the same as in B, day 24 except that the cells were exposed to GM6001 (100 μM; bottom panels) or an inactive analogue (100 μM; control) for the last 36 hours (i.e. 12 hours before overlay plus 24 hours after overlay). Bars, 100 μm. (D) Temporal expression pattern of type I collagen mRNA in differentiating ATDC5 cells. The same blot used in A was re-probed with a type I collagen cDNA. (E and F) Localization of type I and type II collagen at cartilaginous nodules formed by ATDC5 cells. ATDC5 cells incubated for 15 days were fixed and stained with anti-RECK (red) plus anti-type I collagen (green; E) or anti-RECK (red) plus anti-type II collagen (green; F). Reconstituted z-axis images along two cutting lines (a,b) are shown in the top and right panels, respectively. Bars, 100 μm in E,F.

(Fig. 1H-J, lanes 1 and 2). The level of *Reck* expression in these cells was much higher than that in the cells of the osteocytic (Fig. 1I-J, lanes 3 and 4) or osteoclastic lineage (Fig. 1I-J, lanes 5 and 6). Thus, RECK is abundantly expressed in chondrocytes.

To confirm and extend these findings, we examined the temporal expression patterns of *Reck* and type II collagen (a chondrocyte differentiation marker) mRNA in ATDC5 cells. Interestingly, the amount of *Reck* mRNA was relatively low in undifferentiated ATDC5 cells and was progressively increased as the chondrogenic differentiation proceeded (Fig. 1K).

Cartilaginous nodule formation is accompanied by differentially regulated MMP activity and RECK expression

We also examined the temporal expression patterns of three MMPs, *Mmp2*, *Mmp9*, and *Mt1-mmp*, in differentiating ATDC5 cells by RNA blot hybridization, since these MMPs are known to be regulated by RECK. Interestingly, all the genes were upregulated around day 7 and gradually declined thereafter, whereas *Reck* was progressively upregulated (Fig. 2A). Spatial distribution of MMP activity in differentiating ATDC5 cells was also monitored by in situ zymography using a cleavage-dependent fluorigenic substrate, DQ-collagen (Fig. 2B). The collagenolytic activity was detectable at sites of cellular condensation on day 5 until approx. day 10 and persisted in the rim of cartilaginous nodules at later stages (e.g. day 24, arrowheads). These fluorescent signals were barely detectable in the presence of a broad-spectrum MMP inhibitor, GM6001, suggesting that these signals most likely represent MMP activity (Fig. 2C).

Type I collagen is a major collagen species expressed in chondrogenic mesenchymal precursors. Expression of the type I collagen (α2 chain) gene was also upregulated at day 7 and declined thereafter (Fig. 2D). Immunofluorescent staining indicated that on day 15, type I collagen was prominent near the surface of the nodules (Fig. 2E, green signals; supplementary material Fig. S1B), whereas chondrocyte-specific type II collagen could be detected inside the nodules where RECK was also abundant (Fig. 2F, green and red signals, respectively; supplementary material Fig. S1, C and A).

These observations suggest that initial cellular condensation is accompanied by MMP upregulation and low RECK expression, i.e. a condition favorable for active ECM remodeling. By contrast, the later phase of ATDC5 differentiation is accompanied by increasing RECK expression inside the nodules and persistent collagenolysis along the rim of the nodules, a condition favorable for ECM accumulation inside the cartilaginous nodules and continuous enlargement of these nodules.

Forced expression of RECK affects cellular condensation

To address the question of whether progressive increase in *Reck* expression has any significance in chondrogenesis, we tried to manipulate RECK expression in ATDC5 cells. First, to study

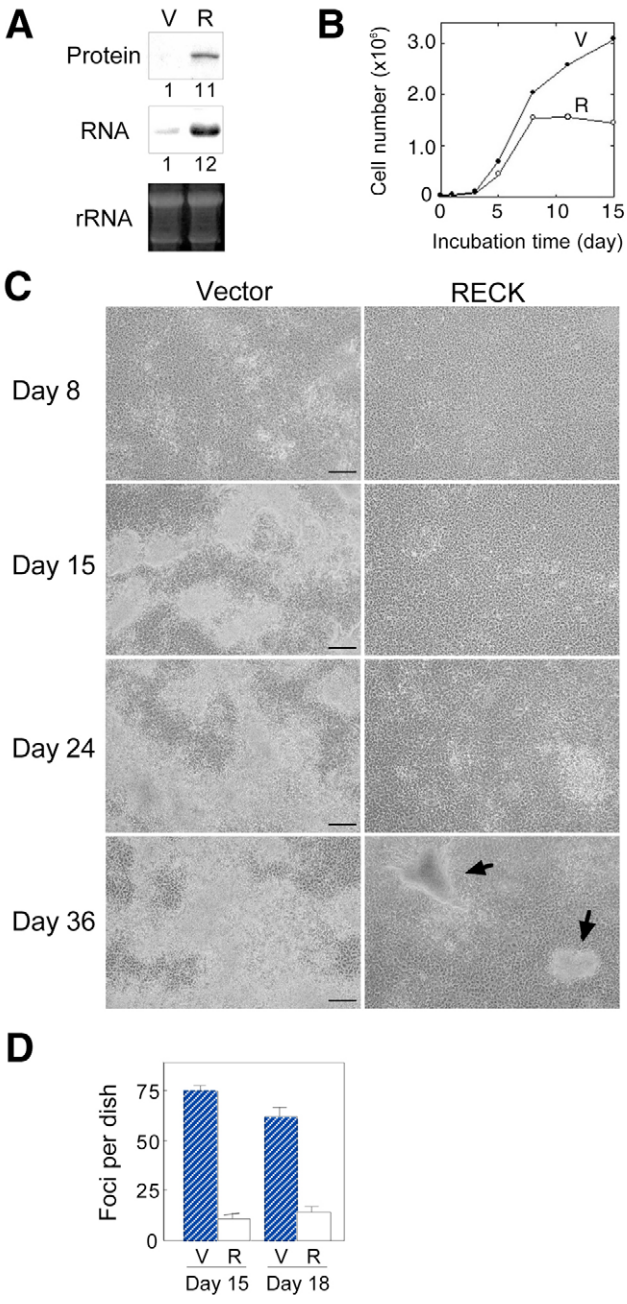

Fig. 3. Effects of forced RECK expression on nodule formation by ATDC5 cells. (A) ATDC5 cells were stably transfected with a mammalian expression vector pCXN2 (V) (Niwa et al., 1991) or the vector expressing human *RECK* (R). Expression of RECK protein (top panel) and *RECK* mRNA (middle panel) in the pooled transfectants harvested under the proliferative conditions was analyzed by immunoblot assay (30 μg protein per lane; top panel) and RNA blot hybridization (10 μg total RNA per lane; middle and bottom panels), respectively. Relative band intensity is shown under each lane. (B) Growth curves of the transfectants. The plating density at day 0 was 3×10^4 cells per 35-mm dish. The data are average values from duplicate dishes. (C) Phase-contrast micrographs of the vector-transfected cells (left panels) and *RECK*-transfected cells (right panels) incubated for the indicated times. Bars, 300 μm. Arrows indicate compact nodules. (D) Number of foci (areas of cell condensation) formed by the vector-transfected cells (hatched bars) and *RECK*-transfected cells (white bars) after incubation for the indicated times. The data are given as mean ± s.e.m. of quadrate dishes. Consistent results were obtained in two separate experiments using the same set of transfectants and in another experiment using retroviral vectors for gene transfer.

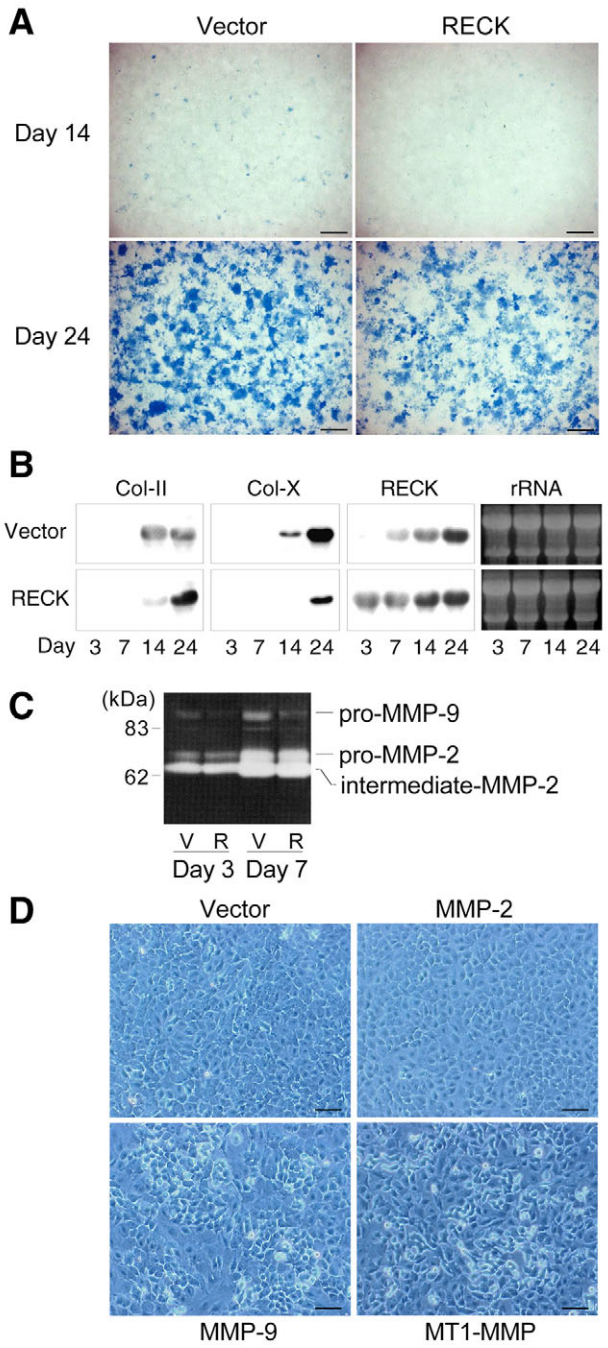

Fig. 4. Effects of forced expression of RECK or MMP on chondrogenic differentiation of ATDC5 cells. (A) The cells transfected with the control vector (left panels) or the vector expressing human *RECK* (right panels) were incubated for the indicated times and then stained with Alcian Blue. Bars, 2 mm. (B) Expression of chondrogenic differentiation markers in the transfectants. Total RNA (20 μg) extracted from the transfectants at the indicated times (bottom labels) was analyzed by RNA blot hybridization using the indicated probes (top labels). (C) Effects of forced *RECK* expression on the level of secreted gelatinases. Conditioned media (7 hours conditioned) prepared from vector- (V) or *RECK*-transfected ATDC5 cells (R) at the indicated times were analyzed by gelatin zymography. The positions of the pro-MMP9, pro-MMP2, and intermediate-MMP2 bands are indicated. The amounts of samples applied were adjusted between V and R so as to represent equal cell number. Day 7 samples were five times more concentrated than day 3 samples on a per-cell basis. (D) Effects of forced expression of *MMPs* on cellular condensation of ATDC5 cells. ATDC5 cells were stably transfected with a mammalian expression vector (pcDNA3.1/Hygro(+); Invitrogen) or the vector containing mouse *Mmp2*, mouse *Mmp9*, or human *MT1-MMP* cDNA. Phase-contrast micrographs of vector- or *MMP*-transfected ATDC5 cells incubated for 5 days are shown. Expression of MMP in each transfectant was confirmed by northern blot hybridization and gelatin zymography. Note the accelerated cellular condensation in *Mmp9*-transfected cells and *MT1-MMP*-transfected cells. Bars, 100 μm.

the effects of constitutive RECK expression, we generated ATDC5 cells stably transfected with a vacant expression vector or the vector containing human *RECK* cDNA and compared their behaviors (Fig. 3). Significant increase in RECK expression was detectable in the *RECK*-transfected cells (R) as compared to the control cells (V) under growing conditions (Fig. 3A). These pooled transfectants showed similar growth rates before reaching confluence (Fig. 3B, up to day 3). After this time point, however, *RECK*-transfected cells began to show lower cell density than the control (Fig. 3B, day 5 and thereafter). Between days 5 and 8, the control cells formed numerous foci, representing active cellular condensation (Fig. 3C, Vector, Day 8), and these foci continued to grow into cartilaginous nodules (Fig. 3C, Vector, Day 15~36). By contrast, the *RECK*-transfected cells formed markedly fewer foci than the control cells (Fig. 3C, RECK, day 8; Fig. 3D), and these foci grew into compact nodules (Fig. 3C, RECK, Day 36, arrows).

At day 14, the number of spots of Alcian Blue staining, representing cartilaginous ECM accumulation, was far fewer in *RECK*-transfected culture than the control (Fig. 4A, Day 14), although by day 24, smaller spots did appeared in the *RECK*-transfected culture (Fig. 4A, Day 24). Expression of two marker genes, type II collagen and type X collagen, was also significantly retarded in *RECK*-transfected cells around day 14 but was markedly increased by day 24 (Fig. 4B, Col-II and Col-X). Elevated baseline *RECK* expression in the *RECK*-transfected cells was evident up to day 14, but it was masked by the elevated endogenous *Reck* expression on day 24 (Fig. 4B, RECK). Thus, constitutive RECK expression lowers the efficiency of cellular condensation and delays the expression of cartilage-specific ECM components.

To understand how the over-expressed RECK suppressed cellular condensation, we analyzed the level of gelatinases (i.e. MMP2 and MMP9) present in the culture supernatant of the transfectants by gelatin zymography. Consistent with our previous findings (Takahashi et al., 1998), the level of secreted pro-MMP9 was significantly reduced in *RECK*-transfected cells (Fig. 4C, lanes R). We also found that the ATDC5 cells stably transfected with an *Mmp9*- or *MT1-MMP*-expression vector showed more rapid and extensive cellular condensation than the cells transfected with the empty vector or an *Mmp2*-expression vector (Fig. 4D). These findings suggest that some MMPs have a potential to promote cellular condensation of ATDC5 cells and that RECK may suppress cellular condensation by regulating such MMPs.

Formation of ECM-rich nodules is suppressed by RECK down-modulation

To assess the importance of *Reck* upregulation in the later

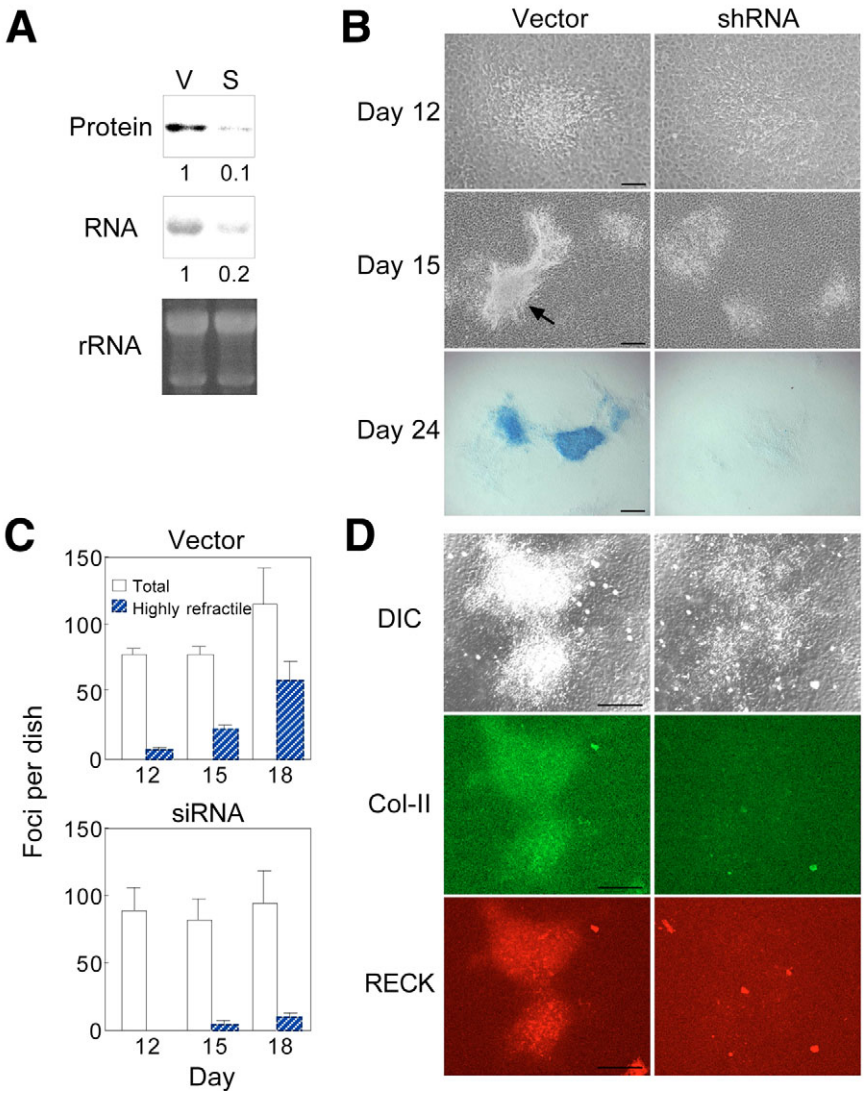

Fig. 5. Effects of *Reck* gene knockdown on cartilaginous nodule formation by ATDC5 cells. (A) ATDC5 cells were stably transfected with the control vector (V) or the vector expressing shRNA against *Reck* (S). Expression of RECK in these cells were analyzed by immunoblot assay (top panel) and RNA blot hybridization (middle panel). Total proteins (30 μg) harvested on day 7 and total RNA (20 μg) harvested on day 4 were used. The number under each lane indicates relative band intensity. (B) Phase-contrast micrographs (top 2 rows) and Alcian blue-stained foci (third row) of the vector-transfected cells (left panels) or RECK-siRNA-transfected cells (right panels) incubated for the indicated times. Bars, 100 μm (Day 12); 250 μm (Day 15) and 400 μm (Day 24). (C) The number and morphology of foci. After incubation for the indicated times, the number of all visible foci (Total foci) and the number of highly refractile foci (e.g. in B, Vector Day 15, arrow), which represent ECM-rich cartilaginous nodules (Shukunami et al., 1998), were scored under a microscope. Values are mean ± s.e.m. from quadruple dishes. (D) Immunofluorescent staining of foci at day 15. The cells were doubly stained with anti-type II collagen (green) and anti-RECK (red) antibodies. Morphology (DIC), type II collagen signals (Col-II), and RECK signals (RECK) around typical foci were recorded under the same microscopic conditions. Bars, 100 μm. The findings were consistent between two separate experiments using independently derived transfectants.

stages of ATDC5 differentiation, we generated ATDC5 cells stably transfected with a vacant expression vector or the vector containing small hairpin RNA (shRNA) designed to knockdown *Reck* mRNA (Fig. 5). Significant decrease in RECK expression was detectable in the shRNA-expressing cells (S) as compared to the control cells (V) (Fig. 5A). Under differentiating conditions, the shRNA-expressing cells did give rise to foci of cellular condensation (Fig. 5B, Days 12 and 15) as numerous as the control cells (Fig. 5C, open bars). These foci, however, were morphologically distinct from those of control cells: they were more flat and less refractile (Fig. 5B, Day 15; Fig. 5D, DIC; Fig. 5C, Day 15, hatched bars). These abnormal foci, which showed markedly reduced RECK immunoreactivity (Fig. 5D, RECK), were negative for Alcian Blue staining (Fig. 5B, Day 24) and type II collagen immunoreactivity (Fig. 5D, Col-II), indicating reduced ECM components in these structures. These results suggest that RECK is essential for the formation of mature, ECM-rich cartilaginous nodules.

Discussion

Our data indicate that *Reck* is abundantly expressed in developing cartilage in mouse embryos as well as in cultured cells of the chondrocytic lineage. We also found, however, that during the early phase of ATDC5 differentiation, *Reck* expression is low and multiple MMP family genes (*Mmp2*, *Mmp9* and *Mt1-Mmp*) are upregulated (Fig. 6A). The areas of elevated collagenolytic activity coincided with the foci of cellular condensation (Fig. 2B), suggesting that MMPs may play roles in cellular condensation. Our finding that forced expression of RECK, a membrane-anchored MMP regulator, reduced the efficiency of cellular condensation (Fig. 3) is consistent with the model that MMPs (e.g. MMP9, MT1-MMP) are required for this process (left part of Fig. 6B).

As differentiation proceeds, ATDC5 cultures become heterogeneous, and RECK becomes upregulated mainly inside the cartilaginous nodules where type II collagen is accumulated (Fig. 2F), whereas collagenolytic activity persists around the rim of the nodules (Fig. 2B, Day 24). This spatial arrangement agrees with the hypothesis that elevated RECK inside the nodule is important for ECM accumulation whereas persistent ECM degradation along the rim is required for nodule expansion (right panel of Fig. 6B). Our findings that RECK knockdown reduced the number of ECM-rich nodules (Fig. 5)

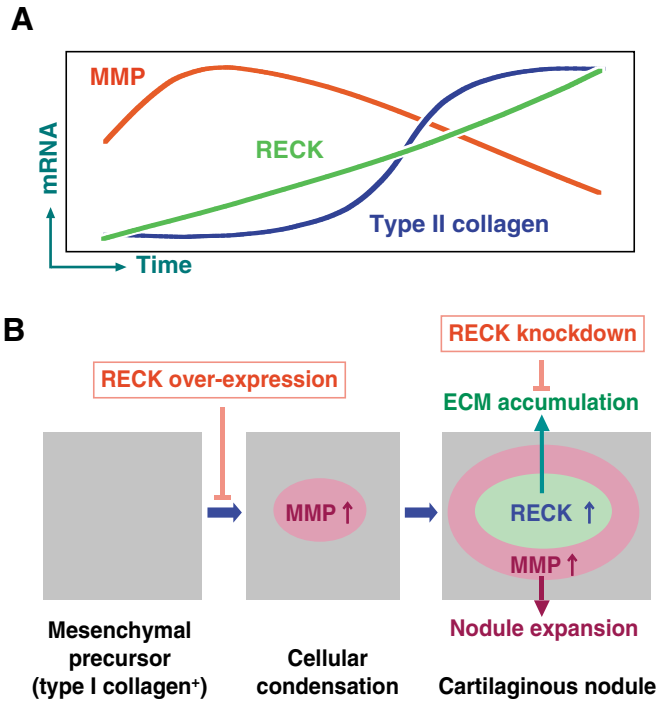

Fig. 6. Possible roles of RECK and MMPs during chondrogenic differentiation. (A) Schematic representation of the temporal changes in the amounts of mRNA encoding MMP (representing MMP2, MMP9 and MT1-MMP; red line), RECK (green line), and type II collagen (blue line) during ATDC5 differentiation. (B) A working hypothesis: elevated MMP activity is required for cellular condensation in the early phase and nodule expansion in the later phase in ATDC5 differentiation. Elevated RECK expression is required for ECM accumulation inside the nodules in the later phase. Constitutive RECK expression suppresses cellular condensation. RECK knockdown suppresses ECM accumulation and hence nodule maturation.

and that the forced expression of RECK gave rise to compact nodules (Fig. 3C) are consistent with this model.

Although RECK overexpression suppressed cellular condensation and delayed the onset of marker gene expression, it did not strongly suppress the upregulation of these markers in the later phase (Fig. 4A,B, Day 24). In fact, at day 24, the level of type II collagen in *RECK*-transfected cells exceeded the level in the control cells (Fig. 4B). This raises the possibility that RECK promotes differentiation in the later phase of chondrogenesis. Alternatively, RECK may activate the type II collagen gene specifically in certain cellular contexts. Whatever the molecular mechanism, this result seems to indicate the involvement of a positive feedback loop in this process where the inhibition of matrix proteases results in upregulation of their substrate gene, thereby accelerating ECM accumulation.

Parallels to our findings can be found in some previous reports describing studies using intact animals or tissue explants. Abundant expressions of *Mmp2*, *Mmp9*, and *Mt1-mmp* in mesenchymal cells surrounding cartilage have been reported (Apte et al., 1997; Reponen et al., 1992; Reponen et al., 1994). Non-overlapping expression patterns of type II collagen (cartilage) and type I collagen (perichondrium) have also been reported (Mizoguchi et al., 1990; Sandberg and Vuorio, 1987). The smaller cartilage formed after treatment of mandibular explants in culture with a synthetic MMP inhibitor (Chin and Werb, 1997) is reminiscent of the compact nodules formed by the ATDC5 cells constitutively expressing RECK (Fig. 3C). Such consistency strengthens our notion that ATDC5 is a good model system for studying the dynamic processes of chondrogenesis and that many findings in this system may have relevance to chondrogenesis in vivo.

There may be some other roles of RECK in chondrogenesis, however, which would be hard to explore in the ATDC5 system. For instance, blood vessel invasion into avascular cartilage is a critical step in endochondral bone formation, and active roles for MMP9 and MT1-MMP in this process have been documented (Holmbeck et al., 1999; Vu et al., 1998; Zhou et al., 2000). Given its activity in regulating angiogenesis in vivo (Oh et al., 2001) and its abundance in cartilage, RECK is likely to be involved in the regulation of endochondral bone formation. Perhaps, more elaborate experimental systems in vivo, such as conditional knockout or conditional transgenic mice, need to be employed to test such a hypothesis.

In our previous study using an immunohistochemical approach, little RECK expression could be detected in developing cartilage (Echizenya et al., 2005). In this study, however, in situ hybridization as well as experiments with cultured cells revealed abundant expression of *Reck* in cartilage and/or chondrocytes. Cartilage is notoriously difficult to analyze in immunohistochemical studies because of its abundant ECM. In fact, pre-treatment with hyaluronidase was necessary to stain RECK protein in ATDC5 cartilaginous nodules in the present study (Fig. 2E,F, Fig. 5D). Moreover, our in situ hybridization data did indicate moderate levels of *Reck* expression in muscles (e.g. intercostals regions in Fig. 1F and the areas surrounding developing femur in Fig. 1G). It is, therefore, likely that we failed to detect the RECK protein in cartilage for some technical reasons in the previous immunohistochemical studies where we focused on developing muscles.

Our previous study using *Reck*-deficient mice indicated that RECK is essential for vascular development around E10.5 and that this is probably due to the activity of RECK in regulating MMPs and to protect fibrillar collagen (Oh et al., 2001). More recent studies (Echizenya et al., 2005) (also this study) have revealed that RECK is expressed in mouse embryos beyond E10.5. This, together with our previous observation that RECK is expressed in various human adult tissues (Takahashi et al., 1998), suggest that RECK probably continues to play important roles in the later stages of development and in adult life. Interestingly, RECK expression is low during the early phase of muscle development where myoblast proliferation, migration and fusion are actively taking place, but RECK expression is upregulated in the later phase where individual myofibers become ensheathed by basement membranes (Echizenya et al., 2005). Hence, progressive upregulation of RECK occurs in two different tissues (skeletal muscle and cartilage) during development; in both cases, low RECK expression is associated with early morphogenetic steps whereas elevated RECK expression is associated with later, consolidation steps or ECM accumulation. The GPI-mediated membrane anchoring makes RECK unique among endogenous MMP regulators and particularly suitable for a task that requires delicate and dynamic regulation of pericellular ECM remodeling.

Cartilage is known to have very limited potential of self-repair (Buckwalter, 2002). Cell transplantation experiments to repair injured cartilage has been unsuccessful, a major obstacle being the difficulty in inducing proper morphogenesis (Buckwalter, 2002). Destruction of joint ECM is one of the major symptoms of rheumatoid arthritis (RA); involvement of MMPs in this process has been documented in a number of studies (Jackson et al., 2001; Martel-Pelletier et al., 2001). Interestingly, reduced expression of RECK in RA synovial membrane has recently been reported (van Lent et al., 2005). Thus, further studies on RECK in the context of cartilage development and maintenance may also yield information useful for clinical application.

In conclusion, our data points to the importance of regulated expression of RECK during cartilage morphogenesis. Its ubiquitous expression in embryonic tissues and the previously demonstrated roles in developing blood vessels (Noda et al., 2003; Oh et al., 2001) and skeletal muscles (Echizenya et al., 2005) support the idea that RECK serves as an essential and versatile regulator of tissue morphogenesis.

Materials and Methods

RNA blot hybridization

Total RNA extracted from cultured cells was separated by electrophoresis in 1% agarose gels, transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech), and hybridized with a ^{32}P-labeled probe. The following cDNA fragments were used as probes: 4.1 kb mouse *Reck* (Takahashi et al., 1998), 4.4 kb human *RECK* (Takahashi et al., 1998), 2.58 kb human *MT1-MMP* (Sato et al., 1994), 2.3 kb mouse *Mmp2* (Reponen et al., 1992), 3.2 kb mouse *Mmp9* (Tanaka et al., 1993), 0.85 kb mouse type I collagen (α2) (pAZ1002; kindly provided by Benoir de Crombrugghe, M. D. Anderson Cancer Center, Houston, TX), 1.4 kb rat type II collagen (α1) (Kimura et al., 1989), 0.65 kb mouse type X collagen (α1) (Apte et al., 1992), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH Control Amplifier Set; Clontech). The quantity and quality of RNA applied to each lane were assessed by the patterns of ribosomal RNAs visualized by ethidium bromide or SYBR Green (Biowhittaker Molecular Applications). Densitometric analyses of X-ray film images were performed using the NIH Image software.

In situ hybridization

The 0.68 kb 3′-UTR fragment of mouse RECK cDNA (GenBank AB006960, nucleotides 3412-4094) was amplified by PCR and used as a template for generating riboprobes. Digoxigenin-labeled antisense and sense (control) riboprobes were prepared using the DIG RNA Labeling Kit (Roche Diagnostics). Mouse embryos were embedded in OCT (Tissue-Tek), frozen on liquid nitrogen, sectioned at 12 μm thickness with a cryostat, and fixed briefly in 4% paraformaldehyde at room temperature. Hybridization was performed at 58°C for 16 hours as described by Braissant et al. (Braissant et al., 1996), and after washing under high stringency conditions, signals were visualized using DIG Nucleic Acid Detection Kit (Roche Diagnostics).

Cell culture

ATDC5 cells (Atsumi et al., 1990; Shukunami et al., 1996) were cultured in growth medium consisting of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (Invitrogen) supplemented with 5% fetal bovine serum (Equitech-Bio Inc., Ingram, TX, USA), 10 μg/ml bovine insulin, 10 μg/ml human transferrin and 3×10^{-8} M sodium selenite. Inoculum density for induction of chondrogenesis was 9×10^4 cells/35-mm dish, 2.3×10^5 cells/60-mm dish or 6.5×10^5 cells/100-mm dish, unless noted otherwise. Transfection was performed by the calcium-phosphate method using 2 μg plasmid DNA mixed with 1 μg calf thymus DNA (carrier). After selection in growth medium containing 1 mg/ml G418 or 208 U/ml hygromycin-B, transfectant colonies were pooled and expanded. Preparation of conditioned media and gelatin zymography were performed as described previously (Takahashi et al., 1998) except that plating density, medium and pre-incubation time were adapted to the cell line (see above).

Indirect immunofluorescent staining

Cells cultured on Lab-Tek Chamber Slides (Nunc) were fixed with 4% paraformaldehyde in phosphate-buffered saline containing divalent cations [PBS (+)], treated with 2.5 % hyaluronidase in PBS (+), and permeabilized with 0.1% Triton X-100 in PBS (+), followed by pre-treatment to block non-specific reactions with 5% skim milk and 1.5% non-immune goat serum in PBS (+). For collagen and RECK double staining, the primary immunoreaction was carried out using rabbit antiserum against either type I collagen (Rockland, #600-401-103-0.5; 1:100 dilution) or type II collagen (Rockland, #600-401-104-0.5; 1:400 dilution) mixed with mouse monoclonal antibodies against RECK (5B11D12; a gift from Amgen; 4.5 μg/ml). To avoid cross reaction, the secondary immunoreaction was carried out in two steps: (1) incubation with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoReserch Laboratories, #211-095-109; 1:500 dilution) followed by rinsing and blocking with PBS (+) containing skim milk and 1.5% non-immune rabbit serum, and (2) incubation with TRITC-conjugated rabbit anti-mouse IgG (DAKO, #R0270; 1:30 dilution) followed by rinsing. The fluorescence images were recorded with a confocal laser microscope (FV300, Olympus) using the sequential scan mode to avoid leakage from different wavelengths. We also performed two types of control experiments: (1) single staining, using antibodies against either RECK, type I collagen or type II collagen, and (2) omission of primary antibodies (see supplementary material Fig. S1). The results indicate that the signals detected in the double-labeling experiments shown in Fig. 2E,F reflect the specific immunoreactivity of respective primary antibodies. Specificity of the anti-RECK antibodies (5B) has been confirmed in a number of previous experiments, which include comparison between the following sample pairs: (1) vector-transfected and *RECK*-transfected HT1080 cells (low endogenous RECK) in immunoblot assay (Takahashi et al., 1998), (2) embryo fibroblasts derived from wild-type and *Reck*-null mice in immunoblot assay (Oh et al., 2001), and (3) wild-type and *Reck*-null mouse embryos after immunohistochemical staining (Oh et al., 2001).

Immunoblot assay

To extract proteins, cultured cells were scraped in cell extraction buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% deoxycholate, 0.1% SDS, protease inhibitors (Complete™; Roche Diagnostics)] at 4°C. After sonication, the lysates were cleared by centrifugation, and the concentration of soluble proteins was determined. The proteins (30 μg) were separated by SDS-PAGE (10% acrylamide), blotted onto PVDF membranes (Millipore), and detected using the anti-RECK antibodies (5B11D12) and ECL System (Amersham Pharmacia Biotech). Densitometric analyses were performed using NIH Image.

Alcian Blue staining

To visualize accumulation of sulfated glycosaminoglycans (a marker for chondrogenic differentiation), cells were rinsed with PBS, fixed with 95% methanol for 20 minutes, and stained with 0.1% Alcian Blue 8GS (Fluka, Buchs, Switzerland) in 0.1 M HCl overnight at room temperature.

In situ zymography

Cells grown on 35 mm glass-bottomed dishes (Iwaki, Chiba, Japan) were washed with PBS and overlaid with 1 ml medium containing freshly neutralized Cellmatrix type I collagen (Nitta Gelatin, Osaka, Japan) and 10 μg/ml FITC-labeled DQ™-type I collagen (Molecular Probes). After solidification at 37°C for 30 minutes, 1 ml growth medium was gently added, and the dishes were incubated for an additional 24 hours. FITC signals, representing digested DQ™-type I collagen, were recorded using the confocal laser microscope.

Gene silencing

Three small hairpin RNA (shRNA) sequences (19 mers) against the mouse *Reck* mRNA were designed and inserted into the mammalian expression vector pRNA-U6.1./Neo (GeneScript). The vector yielding highest activity of gene silencing (target sequence: ACGCCTGCAAGAGAATTCT) was selected and used in this study.

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