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ORIGINAL ARTICLE

Estrogen suppresses expression of the matrix metalloproteinase inhibitor reversion-inducing cysteine-rich protein with Kazal motifs (RECK) within the mouse uterus

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Abstract RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a membrane-anchored glycoprotein which regulates MMP2 and MMP9 activity and has been proposed to play a role in embryo implantation while misexpression of RECK has been associated with a variety of carcinomas. Unfortunately, understanding on the steroidal regulation of uterine RECK is lacking. To address this gap in our knowledge, we examined steroidal regulation and cellular expression of *Reck* mRNA and protein within the mouse uterus in vivo. Uterine *Reck* mRNA and protein were decreased by estrogen, while progesterone alone had no effect. The estrogen-induced down regulation could be partially blocked by progesterone. RECK was localized primarily to luminal and glandular epithelial cells and the level of expression was regulated in a similar fashion as in whole tissue by the steroids. Knock-down of endogenous RECK in human endometrial epithelial and stromal cells resulted in a significant increase in active MMP9 expression but not that of pro-MMP9 or MMP2. These studies demonstrate that RECK expression in the mouse uterus is steroidally regulated and that within endometrial epithelial and stromal cells, RECK regulates MMP9, but not MMP2 activity.

Keywords Uterus · RECK · MMP9 · Estrogen · Progesterone

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Introduction

Matrix metalloproteinase-2 (MMP2; also known as gelatinase-A) and MMP9 (also referred to as gelatinase-B) belong to the family of MMPs which are vital to a variety of biological functions ranging from tooth eruption to embryo implantation. Both MMP2 and MMP9 have been implicated to play a role in both normal uterine physiology [1] as well as uterine pathologies such as dysfunctional uterine bleeding or breakthrough bleeding [2–5], endometriosis [6–9], infertility [10], leiomyoma [11], and endometrial cancer [12–16].

Under normal physiological conditions, the fine-tuned and tightly regulated activity of these MMPs at the tissue level is critical. Disregulation or an imbalance in the ability to regulate MMP activity is associated with the aforementioned pathologies. It is well documented that the proteolytic activity of both MMP2 and MMP9 is regulated at the tissue level by tissue inhibitors of metalloproteinases (TIMPs; reviewed in 1). Four distinct TIMP family members have been described to date: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 [17–19], all of which, except TIMP-4 are expressed within the uterus of mice [20], humans [21–25] and nonhuman primates [26, 27]. MMP2 is primarily regulated by TIMP2, while MMP9 is regulated by TIMP1 and TIMP3 [1, 18].

Less well understood is a reversion-inducing gene which encodes an extracellular protein with protease inhibitor-like domains termed RECK or reversion-inducing cysteine-rich protein with Kazal motifs [28]. RECK is a 110 kDa membrane-anchored glycoprotein which shows preferential inhibition of MMP2 and MMP9 [1]. RECK is widely expressed throughout the human body [28] and proposed to play a vital role in development as Reck deficient mice die at embryonic day 10.5 [29]. RECK expression can be

down-regulated by oncogenes and reduced levels or absence of RECK is characteristic of carcinoma of various organs [30]. From a functional standpoint, RECK has been demonstrated to inhibit MMP2 and MMP9 both at the level of protease secretion and activity [28]. Not surprising, RECK, MMP2, and MMP9 have been postulated to play a role in a wide variety of tumor malignancies [31] with repressed RECK and elevated MMP2 and/or MMP9 expression/activity correlated with the degree of tumor invasion.

Information on the in vivo expression and regulation of uterine RECK is minimal. Nuttall et al. [32] first reported *Reck* mRNA expression in the post-coital developing mouse uterus. More recently, *Reck* expression has been described within the mouse uterus during embryo implantation [33] and has been shown to functionally impact the angiogenesis and vascular remodeling which occurs during embryo implantation [34]. Other than these reports on RECK expression within the uterus, a thorough assessment of the mechanisms which control RECK expression within the uterus is lacking. As such, the objective of this study was to examine the steroidal regulation of uterine RECK using the ovariectomized, steroid-reconstituted mouse model.

Materials and methods

Animals and treatments

Fourteen days after ovariectomy, mice were injected s.c. with vehicle (0.1 ml sesame oil), estradiol-17β (E_2, 10 μg/kg BW), progesterone (P_4, 100 mg/kg BW) or $E_2 + P_4$ (previous doses). Mice were then killed at 0 h (no treatment), 4, 8, and 24 h after injection to evaluate early, intermediate, and late effects of each steroid, respectively. Mice were killed by cervical dislocation and uteri were removed, trimmed of fat and connexion, and then snap-frozen in liquid nitrogen until utilized for protein or total RNA extraction. In addition, the middle ½ of the uterine horn from each animal was prepared for immunohistochemical localization or in situ hybridization (ISH).

To examine whether the steroidal regulation of RECK expression is mediated via their cognate nuclear receptors, mice were injected s.c. with estrogen receptor antagonist ICI-182,780 (ICI; Tocris Cookson Inc., Ellisville, MO; 20 mg/kg dissolved in 100% ethanol and resuspended in sesame oil) or the progesterone receptor antagonist RU486 (Mifepristone; Dr. A. F. Parlow, NIDDK's National Hormone and Pituitary Program, Torrance, CA; 20 mg/kg dissolved in 100% ethanol and resuspended in sesame oil) 30 min before steroid administration.

In situ hybridization

To assess cellular localization of *Reck* mRNA, ISH was performed as previously described [35] with minor modification. Briefly, the middle ½ of the uterine horns were excised and embedded in O.C.T. tissue freezing medium (Sakura Fineteck U.S.A., Inc. Torrance, CA, USA) and snap-frozen on dry ice. 10 μm cryostat sections were placed on Fisherbrand superfrost slides and stored at −80°C until utilized. cRNA probes for *Reck* were synthesized using PCR method and digoxigenin labeling as previously described [35]. Primers were designed with Beacon primer design 5.0 incorporating T7 and T3 RNA polymerase promoters in the 5′ end of the primers. Digoxigenin-labeled antisense and sense cRNA probes were made by in vitro transcription using the T7/T3 transcription kit (Ambion). Prehybridization was performed at 68°C for 1 h in the hybridization mixture (50% formamide, 5× SSC, 120 μg/ml salmon sperm DNA). After denaturing the probes for 5 min at 80°C, hybridization was performed at 68°C for 1 h and then cooled to 42°C for 18 h. Hybridization signal was detected by antidigoxigenin-alkaline phosphatase antibody (Roche, USA) according to manufacturer's instructions. Color was developed at room temperature with 0.0175% 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche), 0.045% nitroblue tetrazolium chloride (NBT; Roche), 100 mM NaCl, and 50 mM $MgCl_2$ (pH 9.5), 0.1 M levamisole for 24 h in dark.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qPCR) was performed as previously described [36]. cDNA was synthesized from 2 μg total RNA isolated with Trizol reagent using Maloney murine leukemia virus reverse transcriptase and random hexamer primer. The resulting cDNAs were diluted 1:10 in sterile water, and 1 μl aliquots was used in the quantitative real-time PCR. Primers for *Reck* and 18S mRNA were designed using Primer Express 3.0 software and synthesized by Integrated DNA Technology (IDT, Coralville, IA, USA). Resulting material was then used for independent qRT-PCR which was carried out on an Applied Biosystems HT7900 Sequence Detector. In order to account for differences in starting material, human 18S primers and probe reagents were used. A standard curve was run in each assay, with an arbitrary value assigned to the highest standard and corresponding values to the subsequent dilutions. Each cDNA sample was run in triplicate and the relative abundance of each target divided by the relative abundance of 18S to normalize for the starting quantity of cDNA. Each primer set included a minus RT control. The delta–delta CT method was used to calculate the fold change values among samples. A no-template reaction was

included during each experiment to control for DNA contamination in the reagents.

Western analysis

Total protein was extracted from frozen uteri using RIPA buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing a protease/phosphatase inhibitor cocktail (0.1 mg/ml PMSF, 30 µl/ml aprotinin, 5 µg/ml leupeptin, 1 mM sodium orthovanadate; Sigma). Protein concentration in each sample was determined using the *DC* Protein Assay (Bio-Rad Laboratories, Richmond, CA). The same amount of protein (50 µg) was subjected to 4–12% Bis–Tris (Invitrogen, Carlsbad, CA, USA) gel electrophoresis and electroblotted onto PVDF membranes (Invitrogen). Rabbit anti-RECK (1:300, Santa Cruz) and Goat anti-rabbit secondary antibody (1:5000, Jackson) were used. Stripping and reprobing for β-actin (Santa Cruz) was conducted to normalize RECK protein expression levels. Immunodetection was carried out using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry

Immunohistochemistry (IHC) was performed following the protocol supplied by Vector Laboratories Inc. (Burlingame, CA) and using the same RECK antibody described above for Western analysis. Briefly, uterine specimens were fixed in 10% neutral-buffered formalin (NBF) and embedded in paraffin wax (5 µm sections) followed by deparaffinization with xylene and rehydration with a graded series of ethanol. Antigen retrieval was conducted using antigen-unmasking solution (H-3300, Vector Laboratories Inc.). Sections were incubated in 3% H_2O_2 in water for 10 min for quenching of endogenous peroxidase activity. IHC analysis was carried out using the VECTASTAIN elite ABC Kits from Vector Laboratories Inc. for rabbit (PK6101) primary antibody according to the recommendations of the manufacturer. Slides were then stained with DAB substrate (SK-4100, Vector Laboratories, Inc.), and briefly counterstained with hematoxylin.

Human endometrial epithelial and stromal cell line culture and transfection

To determine if RECK regulates human endometrial epithelial cell MMP9 activity, human endometrial epithelial and stromal cells were separately transfected with siRNA for human RECK. Briefly, the well-characterized human endometrial epithelial cell line HES-1 [37] and human endometrial stromal cell line T-HESC (obtained from the ATCC, Manasses, VA, and described in [38]) were separately cultured in phenol red-free DMEM:F12 supplemented with 10% charcoal-stripped FBS, 1 mM sodium pyruvate, penicillin, and streptomycin. Cells were switched to media lacking antibiotics and FBS at 70% confluency. Cells were transfected with siRNAs specific for human *RECK* transcript (Dharmacon RNAi Technologies, Lafayette, CO) or control sequences (Dharmacon). Additional controls consisted of media containing transfection reagent alone (Transfection reagent 1; Dharmacon). Forty-eight hours after transfection, *RECK* transcript expression was examined by qRT-PCR while MMP9 activity was assessed in the corresponding media by gelatin zymography using standard techniques. Transfection efficiency was assessed by co-transfection with and evaluation of BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo (Invitrogen).

Statistical analysis

All data were analyzed using GraphPad Instat 3 (GraphPad Software, Inc., La Jolla, CA). ANOVA was used for comparison across treatment regimes. When an F test indicated statistical significance, post-hoc analysis was made using the Tukey HSD procedure. Significance was set at $P < 0.05$ for all comparisons.

Results

Steroidal regulation of uterine Reck expression

Both Reck transcript and protein were detected in murine uterine tissue. Estrogen induced a significant reduction in *Reck* mRNA levels at 4, 8, and 24 h post-steroid administration as detected by qPCR (Fig. 1a). A similar effect of estrogen was detected for Reck protein expression ($\sim$110 kDa). Reck protein levels were highest at 0 h and were significantly reduced at 4, 8, and 24 h after estrogen administration (Fig. 1b). Progesterone alone had minimal effect on either *Reck* transcript (Fig. 2a) or Reck protein (Fig. 2b) with no statistical significance detected among the means for either mRNA or protein. In contrast, progesterone dampened the estrogen effect on both *Reck* mRNA and Reck protein expression. Compared to estrogen alone, progesterone plus estrogen resulted in a delayed decrease in *Reck* transcript expression which occurred at 8 h post-steroid treatment (Fig. 3a). Twenty-four hours after steroid treatment, *Reck* transcript levels were not significantly different compared to control levels (Fig. 3a). Progesterone dampened the estrogen decrease in Reck protein expression (Fig. 3b). Reck protein expression was not affected at 4 or 8 h after administration of both steroids and was significantly reduced only at 24 h after administration of progesterone plus estrogen.

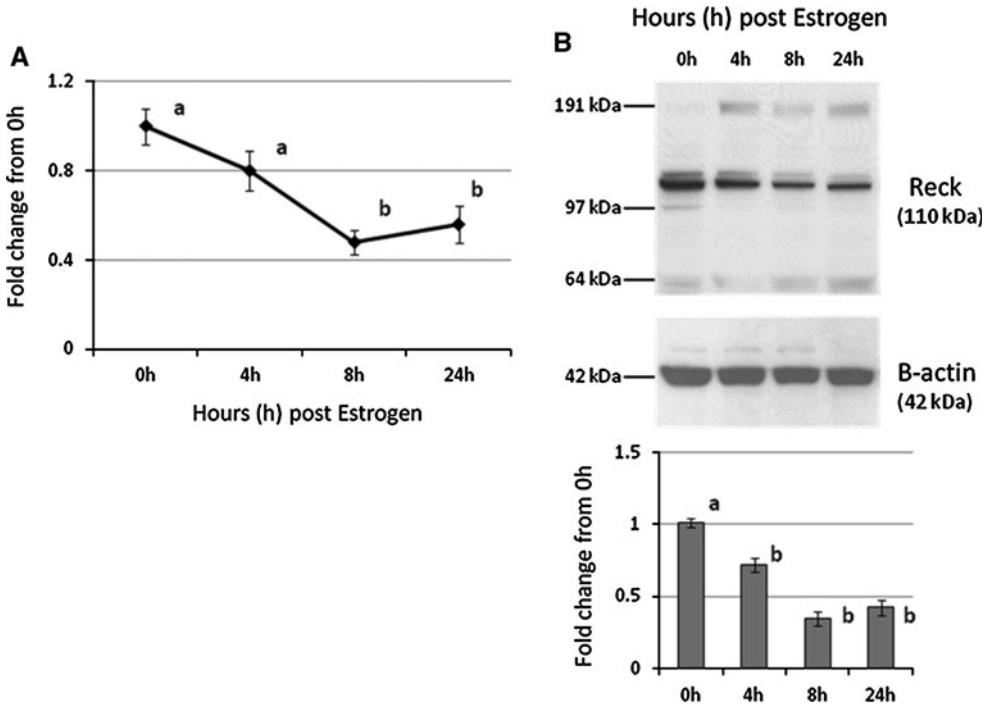

Fig. 1 Estrogen regulation of uterine *Reck* mRNA and protein expression. Mice were ovariectomized and treated with estrogen as described in "Materials and methods." **a** *Reck* mRNA was quantitated by real-time quantitative PCR, while **b** Reck protein was assessed by Western blot analysis. *Reck* mRNA levels were normalized to 18S levels and are displayed as the fold change from 0 h control values (±SEM). The level of Reck protein (110 kDa) was normalized to the level of beta-actin (42 kDa) and Reck protein was expressed as the fold change from 0 h controls (±SEM; *bar graph* below Western blot). Data are representative of four separate analysis per time point ($N = 4$). *Different letters* (a vs. b) indicate statistical significance ($P < 0.05$) among each mean by one-way ANOVA

Steroidal regulation of uterine Reck expression occurs via cognate steroid receptors

To demonstrate that estrogen down regulation of Reck expression was mediated through the cognate estrogen nuclear receptors, we examined the impact of the estrogen receptor antagonist ICI 182,780 on *Reck* transcript and Reck protein expression at 8 h post-estrogen administration (based on the significant reduction at this time point from our earlier study). Similar to results obtained in Fig. 1, estrogen significantly reduced *Reck* transcript (Fig. 4a) and Reck protein expression (Fig. 4b). Pretreatment with ICI blocked the estrogen-induced decrease in *Reck* mRNA (Fig. 4a) and protein expression (Fig. 4b).

Reck localizes primarily to uterine epithelial cells in vivo

To determine the cell types which express *Reck* mRNA and protein, ISH and immunohistochemical localization were performed, respectively. *Reck* mRNA was localized primarily to uterine luminal and glandular epithelial cells. Expression was highest at 0 h and was decreased in response to estrogen treatment at 8 and 24 h (Fig. 5). Assessment of Reck protein indicated a similar pattern of expression to that of *Reck* transcript with expression primarily localized to luminal and glandular epithelial cells. Estrogen decreased expression by 8 h post-steroid administration (Fig. 5). It was also noted that at 24 h post-estrogen expression, stromal cell expression became more apparent compared to that at 0 h (Fig. 5). Progesterone could block the estrogen decrease in Reck expression but did not alter the pattern of cellular localization, while progesterone alone had no affect on either Reck protein localization or level of expression compared to controls (data not shown).

RECK modulates epithelial and stromal cell MMP9 activity

To verify that RECK functionally regulates uterine cell MMP9 activity, T-HESC (immortalized human endometrial stromal) cells and HES-1 cells (human endometrial epithelial) were transfected with siRNA for RECK. As displayed in Fig. 6, the pro-form of MMP9 was expressed in all cells regardless of treatment, albeit at low levels. Knockdown of endogenous *RECK* transcript in HES-1 cells was confirmed by qPCR (Fig. 6a) and resulted in a marked increase in the active form of MMP9 which was not detected in any of the other treatment groups (Fig. 6b). We

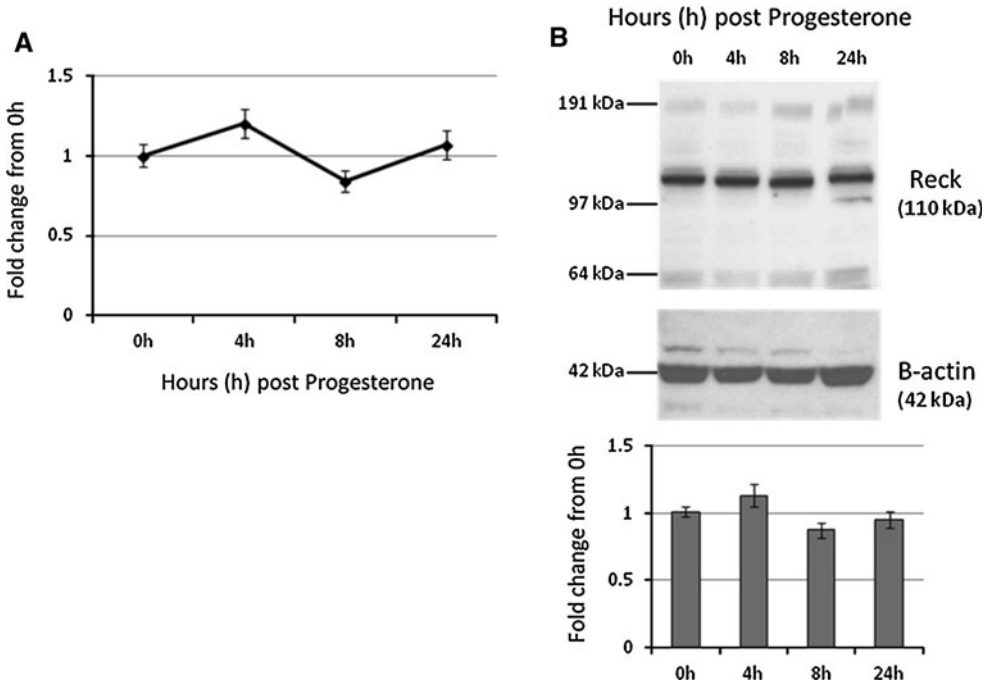

Fig. 2 Progesterone regulation of estrogen modulation of uterine *Reck* mRNA and protein expression. Mice were ovariectomized and treated with estrogen and progesterone as described in "Materials and methods." **a** *Reck* mRNA was quantitated by real-time quantitative PCR, while **b** Reck protein was assessed by Western blot analysis. *Reck* mRNA levels were normalized to 18S levels and are displayed as the fold change from 0 h control values (±SEM). The level of Reck protein (110 kDa) was normalized to the level of beta-actin (42 kDa) and Reck protein was expressed as the fold change from 0 h controls (±SEM; *bar graph* below Western blot). Data are representative of four separate analysis per time point ($N = 4$). No statistical significance ($P > 0.05$) was detected among the means by one-way ANOVA

also noted that MMP2 was expressed in all cells regardless of treatment and transfection with siRNA for RECK did not affect MMP2 activity.

Compared to HES-1 cells, T-HESC cells expressed higher levels of pro-MMP9 and MMP2 in all treatment groups and this expression was unaffected by knockdown of RECK transcript (Fig. 6c, d). Knockdown of endogenous RECK (confirmed by qPCR in Fig. 6c) resulted in the appearance of active MMP9 which was not detected in cells which were transfected with control siRNA or mock transfected (Fig. 6d). Collectively, these studies demonstrate that RECK regulates MMP9 activity in both T-HESC and HES-1 cells.

Discussion

RECK was first isolated by an expression cloning strategy designed to isolate human cDNAs inducing flat reversion in a v-Ki-ras transformed NIH3T3 cell line [28]. RECK is expressed in the human lung, kidney, heart, small intestines, and prostate as well as the ovary and testis. In addition to characterization in the human, *Reck* mRNA expression has been characterized in developing organs of the mouse [39]. In the mouse, *Reck* transcript expression has been shown to be the highest in the developing lung with moderate levels of transcript also detected in the heart and kidney as well as reproductive organs such as the ovary, uterus, placenta, and term deciduas [39]. Takagi et al. [40] have examined canine tissue expression. In agreement with data obtained in the human, Reck is expressed in canine heart, skeletal muscle, liver, spleen, kidney, lung and testis, with the later two organs expressing peak levels.

Despite the fact that RECK appears to be wildly expressed throughout the body, the function of RECK is less clear. Difficulty in assessing RECK function stems from the fact that Reck deficient mice are embryonic lethal with death occurring at embryonic day 10.5 [29]. While this observation suggests that Reck plays a vital role in development of the mouse, this lethality also prevents assessment of Reck function beyond this time point in vivo. Due to this difficulty, the majority of the studies which have examined RECK function have had to rely on descriptive experiments utilizing immunohistochemical localization or in vitro cell culture systems. These studies have provided important insight into the potential function of RECK in a variety of biological processes. For example, investigators have proposed that RECK may regulate muscle development [41], angiogenesis [29, 42], neuromuscular junction formation [43], bone regeneration [44],

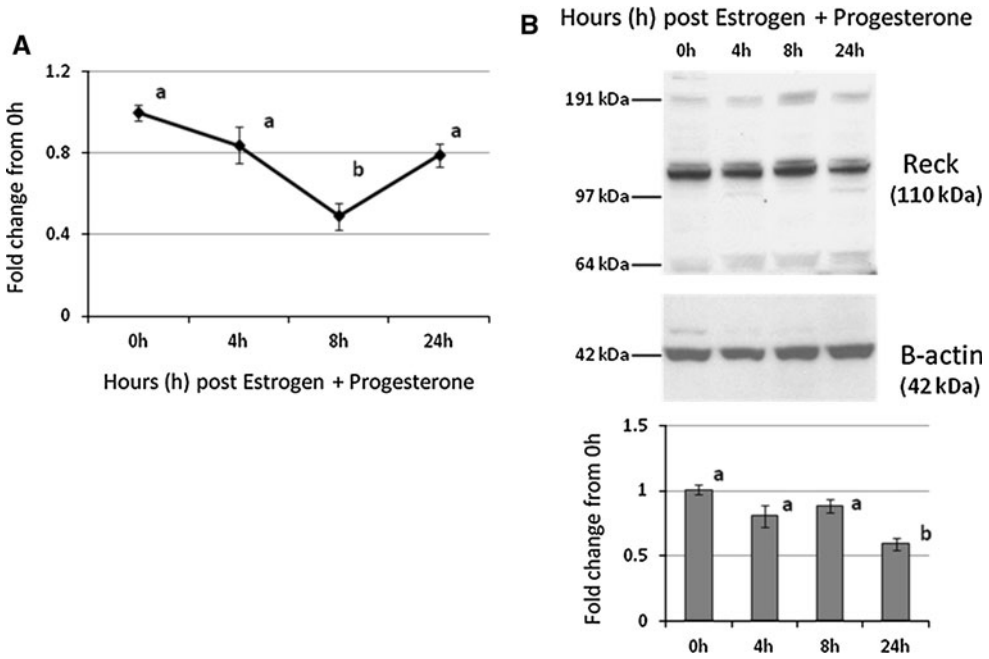

Fig. 3 Effect of chronic exposure to estrogen and estrogen plus progesterone on uterine *Reck* mRNA and protein expression. Mice were ovariectomized and treated with estrogen as described in "Materials and methods." **a** *Reck* mRNA was quantitated by real-time quantitative PCR, while **b** Reck protein was assessed by Western blot analysis. *Reck* mRNA levels were normalized to 18S levels and are displayed as the fold change from 0 h control values (±SEM). The level of Reck protein (110 kDa) was normalized to the level of beta-actin (42 kDa) and Reck protein was expressed as the fold change from 0 h controls (±SEM; *bar graph* below Western blot). Data are representative of four separate analysis per time point ($N = 4$). *Different letters* (a vs. b) indicate statistical significance ($P < 0.05$) by one-way ANOVA among each mean

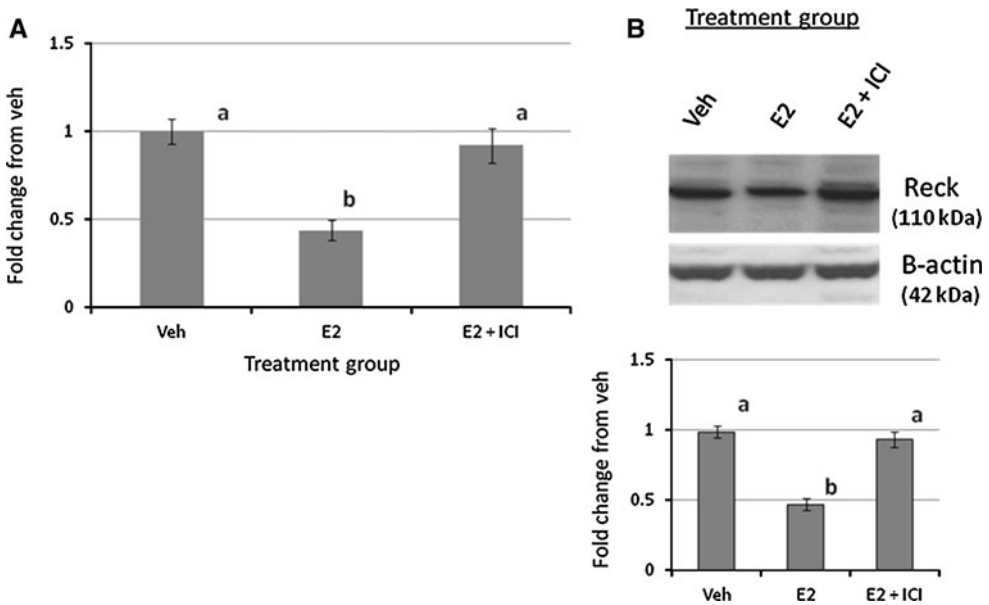

Fig. 4 Specificity of estrogen regulation of uterine *Reck* mRNA and protein expression. Mice were ovariectomized and pre-treated with the estrogen receptor antagonist, ICI 182,780 (ICI) followed by treatment with estrogen (E2) as described in "Materials and methods." **a** *Reck* mRNA was quantitated by real-time quantitative PCR, while Reck protein **b** was assessed by Western blot analysis. *Reck* mRNA levels were normalized to 18S levels and are displayed as the fold change from 0 h control values (±SEM). The level of Reck protein (110 kDa) was normalized to the level of beta-actin (42 kDa) and Reck protein was expressed as the fold change from Veh controls (±SEM; *bar graph* below Western blot). Data are representative of four separate analysis per time point ($N = 4$). *Different letters* (a vs. b) indicate statistical significance ($P < 0.05$) by one-way ANOVA

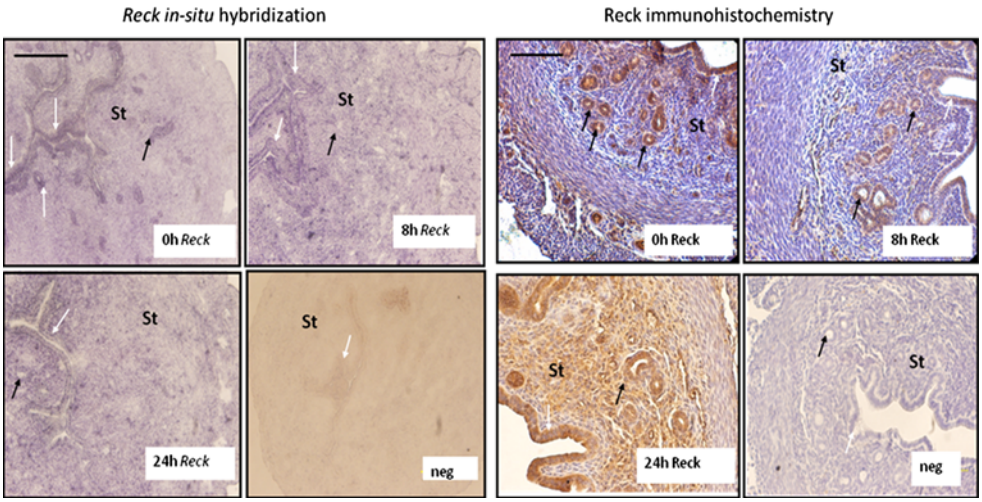

Fig. 5 Localization of uterine *Reck* mRNA and protein expression. Mice were ovariectomized, treated with estrogen (E2) and killed at the indicated time points. *Reck* mRNA was localized by in situ hybridization (*left panels*) while Reck protein was localization by immunohistochemistry (*right panels*) following methods as described under "General Methodology." *White arrows* indicate the localization of *Reck* transcript and protein to primarily luminal epithelial cells, while *yellow arrows* highlight expression in the glandular epithelial cells with minimal signal of the stroma (St) and myometrium at 0 and 8 h time points. Data are representative of four separate experiments ($N = 4$ animals/time point/treatment). *Scale bar* 100 μm for all sections. Negative controls (neg) represents sections in which sense probe was used and demonstrates lack of signal for in situ hybridization while for immunohistochemistry the negative control (neg) represents substitution of primary antibody with isotype control. In all figures *white arrows* point to luminal epithelial while *black arrows* point to glandular epithelium. St identifies the stromal compartment of the uterus

chondrogenesis [45], trophoblast invasion in early pregnancy [33, 34, 46], and brain development [47]. In many of these studies, an inverse pattern of expression between RECK and MMP2 and/or MMP9 was detected. This has lead many investigators to postulate that the mechanisms by which RECK plays a pivotal role in these processes occurs via regulation of MMP activity. In fact, in vitro studies have demonstrated that RECK inhibits MMP2 and/or MMP9 both at the level of protease secretion and activity. As such, the general consensus is that elevated levels of RECK inhibit MMP2 and/or MMP9 activity and modulate extracellular matrix (ECM) turnover and/or integrity. Reduced expression of RECK then allows controlled increase of MMP2 and/or MMP9 activity and the necessary tissue remodeling/ECM turnover needed for these biological processes to occur. In pathological processes, a further reduction or down regulation of RECK and elevated MMP2 and/or MMP9 activity can then lead to ECM breakdown as is thought to occur in tumor development [30].

Within the context of reproductive biology, Hu and colleagues [33] first reported that *Reck* is expressed in the uteri of pregnant mice. Further, these investigators demonstrated that *Reck* expression is decreased at implantation sites and shows an inverse correlation with *Mmp2* and *Mmp9* expression. This study also demonstrated that Reck functionally regulates Mmp9, but not Mmp2 activity within murine stromal cells. This observation is similar to that of our study where we show that estrogen increases Mmp9 but not Mmp2 activity and that there is an inverse relationship between Reck levels and those of Mmp9 activity in uterine tissue. Further, we demonstrate using human endometrial epithelial (HES-1) and stromal (T-HESC) cell lines that knockdown of RECK expression results in elevated MMP9, but not MMP2 activity thereby demonstrating a functional role for the MMP inhibitor within uterine cells.

In the mouse, uterine Reck appears to be steroid-regulated. Our data clearly demonstrate that estrogen decreases *Reck* transcript and protein expression and that this occurs via the cognate estrogen receptor pathway. Further, progesterone can at least partially block this estrogen down regulation of Reck expression. The mechanism by which estrogen decreases Reck expression is currently unknown but may involve modulation via the Ras pathway. Ras has been shown to repress RECK expression [31]. This observation coupled with the fact that estrogen induces mouse uterine *Ras* expression (Nothnick, unpublished observation) may suggest that estrogen down regulation of *Reck* may occur via induction of Ras. The mechanism by which estrogen regulates Reck expression is currently under investigation at the cellular level in our laboratory.

While to date, data on the regulation and function of RECK in vivo is lacking, there is a considerable amount of data on the regulation and function of RECK obtained from in vitro studies. The majority of these studies have come from the field of cancer biology (see [30] for review). In the human, RECK expression has been examined in cancer of the liver [48], pancreas [49], breast [50], lung [51], colon/

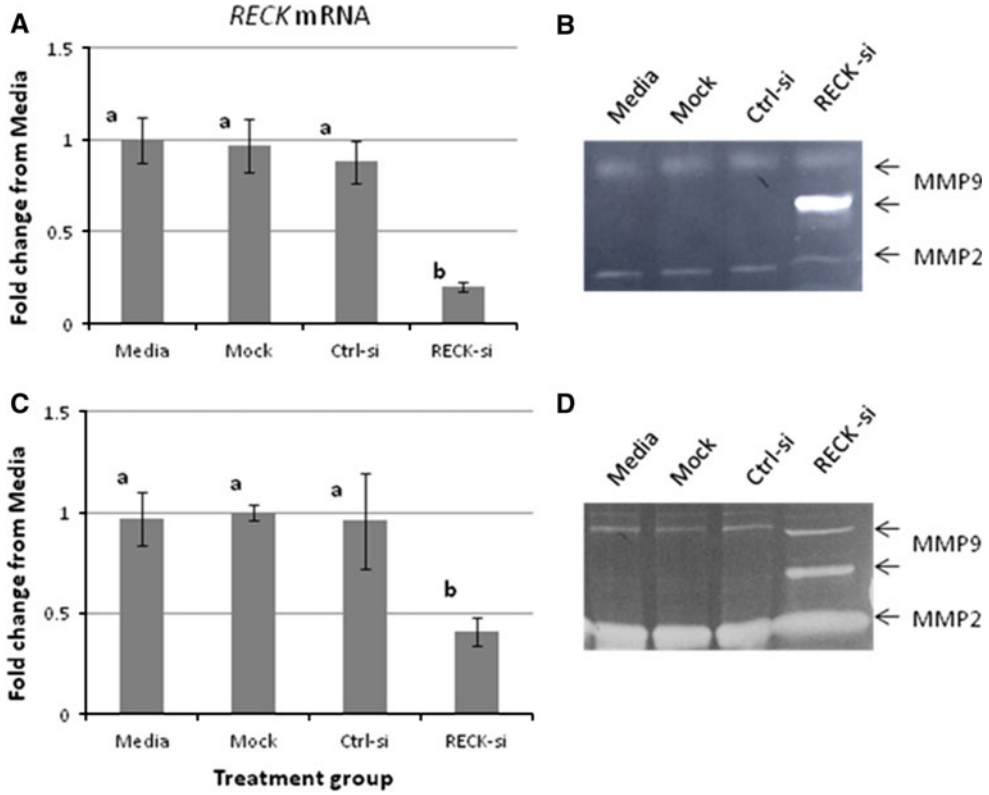

Fig. 6 RECK knockdown confirms functional regulation of endometrial stromal cell MMP9 activity. HES-1 (**a**, **b**) and T-HESC (**c**, **d**) cells were transfected with siRNA for RECK (RECK-si) or scrambled siRNA (Ctrl-si) as well as cultured with media containing transfection reagent alone (Mock) or just media (Media). Cell lysates were then analyzed for RECK mRNA (**a**, **c**) while conditioned media was assessed for MMP9 activity by gelatin zymography (**b**, **d**). *RECK* mRNA levels were normalized to 18S levels and are displayed as the fold change from Media control values ± SEM. *Arrows* indicate the pro- and active form of MMP9 (*top* and *middle arrow*, respectively). Data are representative of six separate analysis per treatment group ($N = 6$) for *RECK* mRNA analysis and four separate analysis for zymography ($N = 4$). *Different letters* (a vs. b) indicate statistical significance ($P < 0.05$) among the means as determined by one-way ANOVA

rectum [52–54], esophagus [55], and prostate [56–58] as well as in hilar cholangiocarcinomas [59], gastric cancer [60], and osteosarcomas [61]. For the most part, these studies have been limited to assessing RECK and MMP2 and/or MMP9 expression in tissue specimens using immunohistochemical analysis. From these observations, correlations among RECK expression, MMP2/9 expression, and when possible, patient prognosis/survival were formulated. The overwhelming consensus from these studies as it pertains to RECK and cancer is that RECK expression is inversely correlated with MMP2/9 expression and positively correlated with cancer prognosis/survival rate. Thus, it is viewed that RECK may be a promising target molecule or therapeutic tool for cancer therapy. Our observation that RECK is expressed in the human endometrium and its expression is markedly decreased in endometrial adenocarcinoma suggest that this protease inhibitor may play a similar role in the pathophysiology within the endometrium.

As stated earlier, functional studies on the role of RECK have been limited primarily to in vitro models with the exception of a few studies conducted using animal models. Okamura and colleagues [62] demonstrated that RECK expression in carcinogen-induced tumors was reduced compared to control tissues which did not develop tumors. The bulk of our knowledge on the function of RECK has come from in vitro studies. Takahashi et al. [28] initially demonstrated that the level of RECK expression was undetectable in a variety of malignant cancer cell lines including 4 rodent and 19 human cell lines obtained from various types of tumors. Further, it was demonstrated that restored RECK gene expression in these tumor-derived cell lines could block tumor invasion in vitro and that this was associated with reduced MMP9 activity. Additional functional studies on RECK have demonstrated that glycosylation of the RECK protein mediates its suppression of tumor cell invasion by multiple mechanisms which include suppressing MMP2 activation and MMP9 secretion/activity [63]. Collectively, data obtained from human specimens, animal models, and in vitro functional studies strongly suggest that RECK expression is down-regulated in a variety of solid tumors and the extent of down regulation often correlates with poor prognosis. Further, the

level of RECK expression is inversely correlated with elevated MMP9/2 activity. While these studies have provided invaluable information with respect to the possible role on RECK in tumor cell biology, in vivo studies are lacking not only for studies relevant to cancer biology but also for understanding the potential role of this MMP inhibitor in many normal biological processes in which regulation of MMP2 and/or MMP9 is critical such as embryo implantation.

In summary, we show for the first time that murine uterine Reck is decreased by estrogen treatment and that this pattern of expression is inversely correlated with that of Mmp9 activity. Further, we demonstrate that Reck is localized primarily to uterine epithelial cells with localization in both epithelial and stromal cells becoming apparent with estrogen treatment. Finally, we demonstrate that RECK regulates MMP9 activity in endometrial cells having little if any effect on MMP2 activity.

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Standards The authors declare that all reported experiments were performed in accord with all applicable laws of the United States.

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