LAB. INVESTIGATION-HUMAN/ANIMAL TISSUE

Regulation of cell invasion and signalling pathways in the pituitary adenoma cell line, HP-75, by reversion-inducing cysteine-rich protein with kazal motifs (RECK)

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Abstract Degradation and remodelling of the extracellular matrix has been investigated, with the main focus on the balance between matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP). Recent reports disclose the presence of a novel MMP-inhibiting cell membrane-anchored glycoprotein designated 'reversion-inducing cysteine-rich protein with Kazal motifs' (RECK). Our main aim in this study was to elucidate the role of RECK in cell invasion of pituitary adenomas and its contribution to signal transduction. The function of RECK in cell invasion was investigated by comparing data obtained from full-length RECK clone transfection and gene silencing with RECK mRNA-targeting siRNA. RECK expression was confirmed using real-time RT-PCR and Western blotting. Levels of matrix metalloproteinases (MMP-2 and -9) and TIMP-1 were measured by zymography and reverse zymography, respectively. Cell invasion was examined with a 3-D invasion assay. The signal cascade was investigated by cDNA microarray analysis. As expected, expression of RECK was elevated upon cDNA transfection, and diminished using siRNA. We observed elevation of MMP-2 and -9 expression and consequent 3-D cell invasion in cells under-expressing RECK. However, TIMP expression was not affected by RECK. Analysis with cDNA microarray revealed that RECK additionally upregulates growth hormone-releasing hormone receptor (GHRHR) and latrophilin 2 at the transcriptional level. Our findings collectively suggest that RECK regulates the cell signalling pathway, playing a critical neuroendocrinological role in the pituitary adenoma cell line.

Introduction

Extracellular matrix (ECM) is a complex of macromolecule materials surrounding the cell body, of which collagen is the major ingredient. Recent studies, along with repeat remodelling analyses, show that ECM is not simply a filler between neighbouring non-active cells, but significantly influences proliferation, differentiation, motility and cell death [1]. In fact, mutation of a specific component of ECM causes various deformities and diseases [2].

The matrix metalloprotease (MMP) family is a major driving force in regulating ECM remodelling, and more than 20 subtypes have been identified to date [3]. An artificial MMP inhibitor that may disrupt permeation/metastasis/ blood vessel instructions in cancer has been developed [4–6], but knowledge about functions of MMPs in pituitary adenomas is currently limited. Thus, several studies have analyzed the degradation and remodelling of ECM, mainly focusing on the balance between matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP). Recently, reversion-inducing cysteine-rich protein with Kazal motifs (RECK), a cell membrane-anchored glycoprotein, was identified as a novel endogenous MMP inhibitor [7]. This protein downregulates MMPs, and suppresses the invasive and metastatic potential of several non-tumoural cell lines, as well as some types of tumours [8–10]. To our knowledge, RECK expression and function in tumour invasiveness have never been studied in pituitary adenomas. We hypothesize that RECK not only inhibits MMP activity

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in the extracellular space, but also potentially regulates intracellular signal transduction, in view of its cellular membrane domain [11].

In this study, we elucidated the biological functions of RECK in pituitary adenomas by comparing data obtained from cell transfection with full-length RECK cDNA and gene silencing with RECK mRNA-targeting siRNA. Furthermore, we sought to establish the intracellular signalling pathway by cDNA microarray analysis. Our findings collectively show that RECK suppresses MMP-2, and -9 activities, consequently inhibiting cell invasion, and alternatively, plays an important role in signal transduction for tumour growth in the pituitary adenoma cell line.

Materials and methods

Cell culture

The human HP-75 non-functioning pituitary adenoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's minimum essential (DMEM) medium (BioWhittaker, Cambrex Corp., Nottingham, UK) supplemented with 15% horse serum (TCS Cellworks, Buckingham, UK), 2.5% foetal calf serum (Life Technologies, Paisley, UK), 0.05% (w/v) glutamine, 50 µg/ml streptomycin, and 50 IU/ml penicillin (hereafter denoted 'culture medium'), and maintained at 37°C in humidified air under 5% carbon dioxide. Cells were grown in T-75 flasks (Nalge Nunc International, Rochester, NY, USA), the culture medium exchanged twice weekly, and subconfluent cells passaged using 0.05% trypsin/0.02% ethylenediamine tetra acetic acid (EDTA). Each cell line was passaged three times prior to use in experiments.

Generation of cell lines over- or under-expressing RECK

For generation of HP-75 cells overexpressing RECK, full-length human RECK cDNA (IOH46822; Ultimate ORF Clone; Invitrogen, Tokyo, Japan) was purified from glycerol stock solution, and subsequently cloned into pcDNA3.2/V5-DEST vector (Gateway LR reaction system, Invitrogen, Tokyo, Japan). The viral packaging *E. coli* system (One Shot OmniMAX Chemically Competent *E. coli*; Invitrogen, Tokyo, Japan) was transformed with the CMV viral construct using the standard heat-shock method.

For subcloning, transformed *E. coli* cells were incubated overnight on LB agar containing ampicillin, single colonies were picked, and inocula grown overnight in a shaking incubator at 37°C. The vector was purified using the PureLink HQ Mini Plasmid Purification Kit (Invitrogen, Tokyo, Japan).

For generation of HP-75 cells expressing low levels of RECK, we designed specific short interfering ribonucleic acids (siRNA) according to an on-line detection system (http://www.ambion.com/jp/catalog/sirna_search.php), and purchased double-stranded RNA targeting the human RECK gene (Accession number; NM_021111) and a scramble oligonucleotide duplex (non-specific sequence confirmed with BLAST), which was used as the negative control (siRNA targeting scramble; 5′-GCGCGCTTTGTAGGATTCG). The final siRNA concentration used was 50 nM. Transfection into HP-75 cells was performed using the lipofection reagent, Lipofectamine 2000 (Invitrogen, Tokyo, Japan).

For subsequent assays, experiments with siRNA, full-length cDNA, control, mock, scramble oligonucleotide and empty vector were performed.

Real-time RT-PCR

Cells (5×10^6 in 10 ml culture medium) were seeded onto wells of a 6-well plate (Nalge Nunc International K.K. Rochester, NY, USA) and allowed to attach overnight. Transfection was performed as specified above. Total RNA was prepared using the TRIZOL reagent (Gibco-BRL, Tokyo, Japan), and cDNA synthesized with pd(N)6 Random Hexamer primers (Amersham Bioscience, Piscataway, NJ, USA). The mRNA levels were assayed quantitatively by real-time reverse transcription-polymerase chain reaction (RT-PCR) performed using the appropriate reagents (TaqMan® One-Step RT-PCR Master Mix Reagent Kit; Applied Biosystems, Tokyo, Japan) and a Gene Amp 5700 thermal cycler (Applied Biosystems, Tokyo, Japan). Amplification was performed using primers that spanned at least one intron, and monitored with FAM-labelled probes to detect RECK mRNA designed according to the on-line detection system (http://myscience.appliedbiosystems.com/index.jsp). Each PCR amplification utilized cDNA derived from 20 µl mRNA solution (10 ng/µl), and the relative amounts of PCR products were calculated as the threshold cycle (CT) value of the sample divided by that of human b-actin (TaqMan® Endogenous Controls, Applied Biosystems, Tokyo, Japan).

Western blotting

The level of RECK protein following transfection of siRNA (10, 20, 50 nM), full-length cDNA, control, mock, scramble oligonucleotide or empty vector was detected using Western blotting, as described previously [12]. Briefly, cells were lysed in 2 ml of RIPA buffer containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 2 µg/ml leupeptin (Cosmo Bio Co., Tokyo, Japan). Lysates (10 µg protein) were loaded on a 5–20% gradient SDS gel (60 min at 100 V), and transferred to a PVDF membrane (Amersham International, Buckinghamshire,

UK) for 60 min at 100 mA. The membrane was incubated for 2 h with the primary antibody (0.2 µg/ml rabbit anti-RECK or 0.2 µg/ml rabbit anti-β-actin as the internal standard; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Blots were incubated for 1 h in secondary antibody (peroxidase-conjugated swine anti-rabbit immunoglobulin, 10 ng/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and visualized with an ECL detection system (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Gelatin zymography and reverse zymography

HP-75 (1×10^6) cells cultured on 60 mm plastic dishes (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) were incubated in medium devoid of serum for 24 h immediately after transfection, as described above. The supernatant fractions were separated by 7.5% SDS-PAGE (BioRad, Hemel Hempstead, UK) impregnated with gelatin (0.1 mg/ml) under non-reducing conditions, according to earlier reports [13, 14]. Gels were incubated in zymography digestion buffer (200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02% (v/v) Brij-35, pH 7.6; Sigma Chemical Co, St. Louis, MO, USA) for 18 h at 37°C. Subsequently, gels were stained by immersion in staining solution (0.5% Coomassie Blue R250 in 30% methanol/10% glacial acetic acid in H_2O) for 3 h, followed by destaining to reveal discrete areas where the gelatin substrate was hydrolyzed by gelatinase.

Tissue TIMPs were detected by reverse zymography. The cell supernatant fractions were separated as described above. Gels were incubated in reverse zymography digestion buffer (Calbiochem, Cambridge, MA, USA) for 4 h. Gels were counterstained (as for zymography) with staining buffer, and stained for the incorporated gelatin. TIMP activity was visualized as a dark band on the lighter background.

3-D cell invasion assay on a PuraMatrix™ gel

PuraMatrix™ gel was kindly supplied by 3-D Matrix Japan Ltd. (Tokyo, Japan). HP-75 cells were pre-stained immediately prior to this assay using the Q-Tracker 525Cell Labeling Kit (Quantum Dot Corp., Hayward, CA, USA) to allow observation of cell plating efficiency or invasion in 3-D cultures as previously reported [15]. HP-75 cells (5×104) were collected, and suspended in 10 µl of culture medium. A cell suspension (1 µl) was injected and cultured in a PuraMatrix gel (0.5% PuraMatrix, 5% sucrose in ddH_2O) impregnated with collagen type I (50 ng/ml) as 3-D culture. Cell invasion was observed with an inverted fluorescence microscope at $2 \times$ magnification (IX71-ARCHEVA, Olympus Co., Tokyo, Japan), comparing maximal horizontal expansion by time-lapse analysis with an microscope incubation system (INU-WDS-O-F1, Tokai Hit Co., Ltd, Sizuoka, Japan) for 3 days.

Complementary DNA microarray analysis for the gene expression profile

HP-75 (1×10^7) cells were plated onto 60 mm plastic dishes (Sumitomo Bakelite Co., Ltd, Tokyo, Japan), and incubated with normal culture medium for 24 h immediately after transfection with siRNA, full-length cDNA, or lipofectant reagent (Lipofectamine™2000, Invitrogen Corp., Carisbad, CA, USA) alone as the control. Total RNA from each sample was isolated immediately using TRIZOL (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's protocol. The concentration was calculated spectrophotometrically, and quality control of RNA was analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Tokyo, Japan). Messenger RNA/cDNA hybrids were generated via T7 oligo dT primers, followed by addition of DNA polymerase and ligase (Filgen Co., Tokyo, Japan) to obtain double-stranded cDNA. The sample tagged with chemiluminescent substrate, Cy3, for the full-length clone, siRNA for RECK, or Cy5 for the control was hybridized on a microarray chip (Filgen Array Human35 K, Filgen, Tokyo, Japan). We employed a mixture of total RNAs from the two cultures for labelling reactions.

Fluorescence-labelled cDNA was synthesized by direct incorporation of Cy3-dUTP or Cy5-dUTP (Amersham Bioscience, NJ, USA) during random-primed reverse transcription, using 5.9 µg total RNA and an RNA fluorescence labelling core kit (M-MLV version 2.0, TaKaRa, Japan). We prehybridized the microarray for 1 h at 42°C in solution containing $5\times$ SSC ($1\times$ SSC comprises 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium lauryl sulfate (SDS), and 10 mg/ml bovine serum albumin. The microarray was washed at room temperature in distilled water three times for 1 min, rinsed in 2-propanol, and dried by centrifugation at 150 g for 2 min. We performed hybridization for 16 h at 42°C in 12 µl solution containing $5\times$ SSC, 0.1% SDS, 30% formamide, and heat-denatured the labelled cDNA. The microarray was performed at room temperature with $2\times$ SSC containing 0.1% SDS for 4 min, $0.1\times$ SSC containing 0.1% SDS for 4 min, and three times with $0.1\times$ SSC for 1 min. Fluorescence images of Cy3 and Cy5 dye channels were obtained using a GenePix 4000B scanner (Axon Instruments, CA, USA), and the image analyzing software, Array-Pro Analyzer (Filgen, Tokyo, Japan), was used to quantify signal intensities using Microarray Data Analysis Tool Ver.1.5, Filgen, Tokyo, Japan). Elevation and reduction of pixel expression, observed as intensity changes greater than 1.6-fold (increase) and lower than 0.66-fold (decrease), respectively, were classified as 'a significant change in mRNA expression'. Data were analyzed according to the online system (GenMAPP: http://www.genmapp.org/, Cytoscape: http://www.cytoscape.org/), based on gene annotation data (NCBI: http://www.ncbi.nlm.nih.gov/).

Statistical analysis and imaging

Data were expressed as means ± SD (standard deviation). Statistical analysis was performed with the GraphPad Prism Ver. 5.0 (GraphPad Software Inc., San Diego, CA, USA). The Mann–Whitney U-test was employed for data analysis. For imaging analysis of the 3-D cell invasion assay, Image-Pro Plus version 4.5 (Media Cybernetics, Division of Nippon Roper, Tokyo, Japan) was used to evaluate fluorescence microscopy data. Statistical significance was defined as $P < 0.05$. All data are representative of at least three independent experiments.

Results

Expression of RECK in pituitary adenoma and HP-75 cell

We initially confirmed the expression of RECK in pituitary adenoma tissue (Fig. 1a) by performing immunohistochemistry with laser confocal microscopy, and as a ~102 kDa band in HP-75 cells by Western blotting (Fig. 1b).

MMP and TIMP activities modulated by RECK

Full-length RECK (Invitrogen, IOH46822) cloned into pcDNA3.2/V5-DEST vector was confirmed by Northern blotting at 8.3 kb (data not shown). RECK mRNA interference by targeting siRNA (NM_021111) was evaluated at concentrations of 10, 20 and 50 nM. Western blotting disclosed suppression of the RECK protein level in a dose-dependent manner by siRNA targeting RECK mRNA (Fig. 2). A final concentration of 50 nM siRNA was employed for all subsequent experiments. Real-time RT-PCR confirmed that transfection with full-length RECK cDNA induced significantly elevated RECK mRNA expression, while the siRNA method led to marked attenuation, compared to the control (means of expression relative to b-actin, standard deviation (P value); full-length clone, 1.59 ($P = 0.022$), 0.14; siRNA, 0.14, 0.06 ($P = 0.0022$); control, 0.79, 0.11). Meanwhile, transfection with mock, scramble oligonucleotide and empty vector did not influence the expression of RECK mRNA (Fig. 3). Concomitantly, Western blotting revealed equivalent findings at the protein level (Fig. 4a).

Conversely, activities of MMP-2 and -9 examined by zymography in cells treated with RECK siRNA were elevated, and diminished in those transfected with full-length RECK DNA. Again, transfection with mock, scramble oligonucleotide and empty vector did not affect MMP activity. No effects on the activity of tissue inhibitor of metalloproteinase (TIMP)-1 were evident in reverse zymography experiments (Fig. 4b).

Effect of RECK in 3-D invasion

Following culture of the cell suspension in PuraMatrix™ gel impregnated with collagen type I, cell invasion was observed with inverted fluorescence microscopy using a comparison of maximal horizontal expansion by time-lapse analysis as a 3-D invasion system (Fig. 5a). In comparison with the control, cell invasion was clearly inhibited upon transfection of full-length RECK, but elevated by gene

Fig. 1 Expression of reversion-inducing cysteine-rich protein with kazal motifs (RECK). (a) Fluorescence immunohistochemistry confirms RECK expression in the parenchyma of pituitary adenoma tissue (DAPI, nucleus: Texas Red, RECK) (PRL-oma, 44 y/o male). (b) Western blotting reveals a 102 kDa band in the pituitary adenoma cell line, HP-75
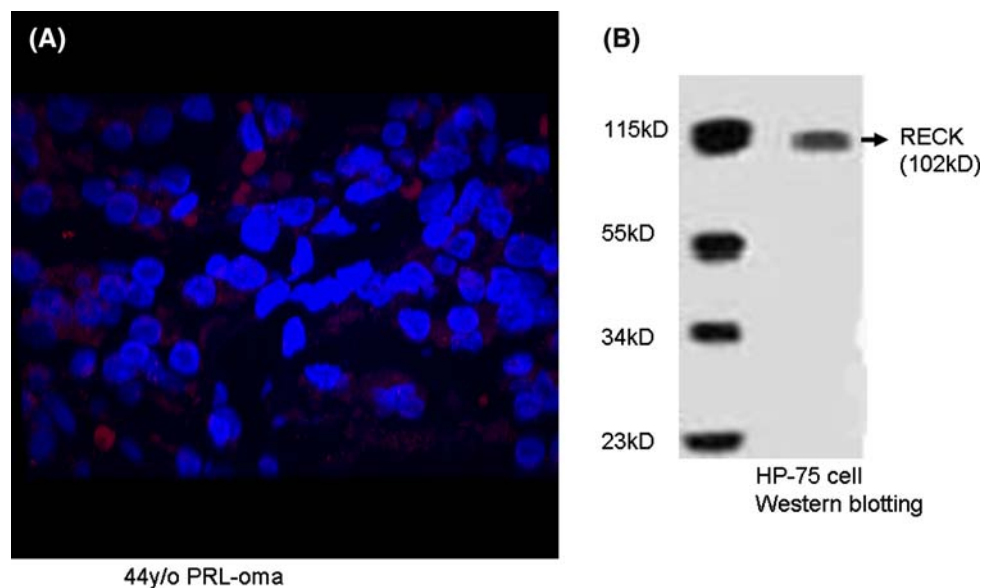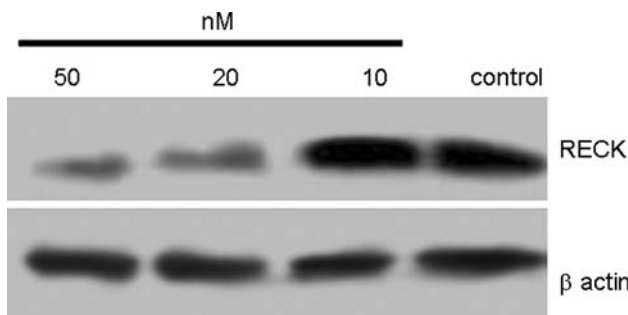
Fig. 2 Western blotting after transfection of siRNA targeting RECK (NM_021111) at 10, 20, 50 nM, compared with control (0 nM). The siRNA-treated cells display suppressed RECK expression in a dose-dependent manner. Accordingly, a concentration of 50 nM is employed throughout the study
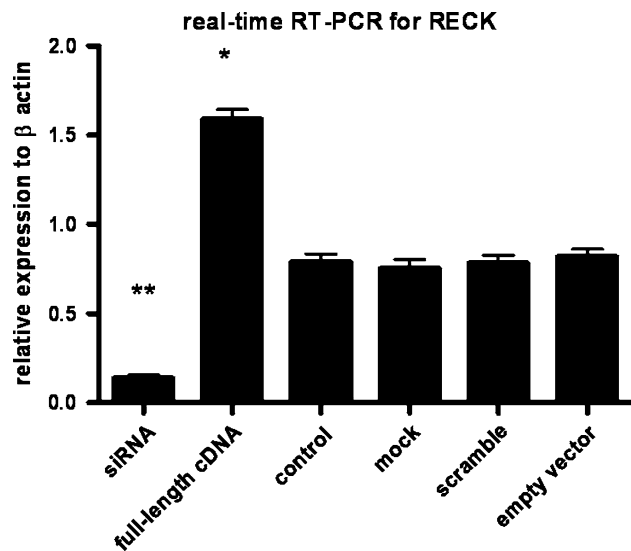

Fig. 3 Real-time RT-PCR demonstrates that full-length RECK cDNA transfection leads to significantly elevated expression of RECK mRNA. Conversely, the siRNA method results in marked attenuation of RECK expression (means of expression relative to β-actin). Mock, scramble oligonucleotide and empty vector do not influence the expression of RECK mRNA. *Full-length clone; $P = 0.0022$; **siRNA; $P = 0.0022$

Fig. 4 (**a**) Western blotting demonstrates an equivalent protein level. (**b**) Zymography & reverse zymography. Zymography data reveal that activities of MMP-2 and -9 in cells treated with siRNA are elevated, and diminished in cells transfected with full-length RECK (*upper picture*). However, mock, scramble oligonucleotide and empty vector do not affect activity. Tissue inhibitor of metalloproteinase (TIMP)-1 activity is not affected by RECK, as shown using reverse zymography (*lower picture*)

cDNA microarray analysis

We evaluated the quality of total RNA by cDNA microarray analysis. Values of raw siRNA or full-length clone (Cy3)/control (Cy5) were assessed as normalized values in dot-blot analysis, and hierarchical cluster analysis was performed with the above software (Fig. 7). The cDNA microarray analysis disclosed that full-length RECK enhanced the expression of latrophilin 2, resistin like beta, zinc finger protein B-14 and elastin microfibril interfacer 3. Moreover, siRNA targeting RECK mRNA suppressed the levels of basic helix-loop-helix domain containing, class B, nuclear factor I/A and growth hormone releasing hormone receptor (GHRHR) (Table 1). We obtained no significant data suggesting gene downregulation by RECK (data not shown).

Discussion

The present study yielded the following results: (1) RECK is expressed in pituitary adenoma tissue and the HP-75 cell line; (2) cell lines over- and under-expressing RECK are successfully generated by transfecting full-length RECK cDNA and siRNA, respectively, as confirmed using

silencing with siRNA at 72 h (Fig. 5b). Maximum horizontal expansion was measured every 12 h. Cells transfected with full-length RECK displayed a lower invasion rate (% of maximum horizontal expansion versus 0 h) after 24 h, and those treated with siRNA exhibited a higher invasion rate after 36 h. At 72 h, the invasion rates of cells transfected with the full-length clone and siRNA were 132.56% and 689.56%, respectively, while the control displayed 212.96% invasion, compared with values measured at 0 h (Fig. 6).
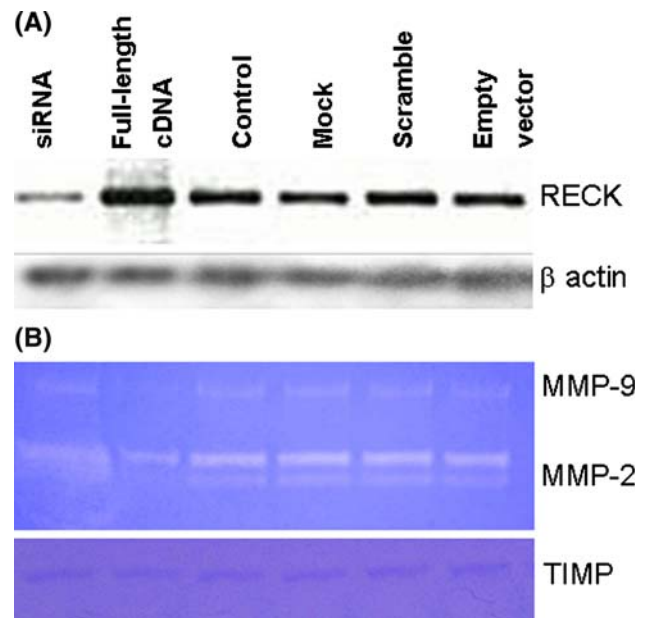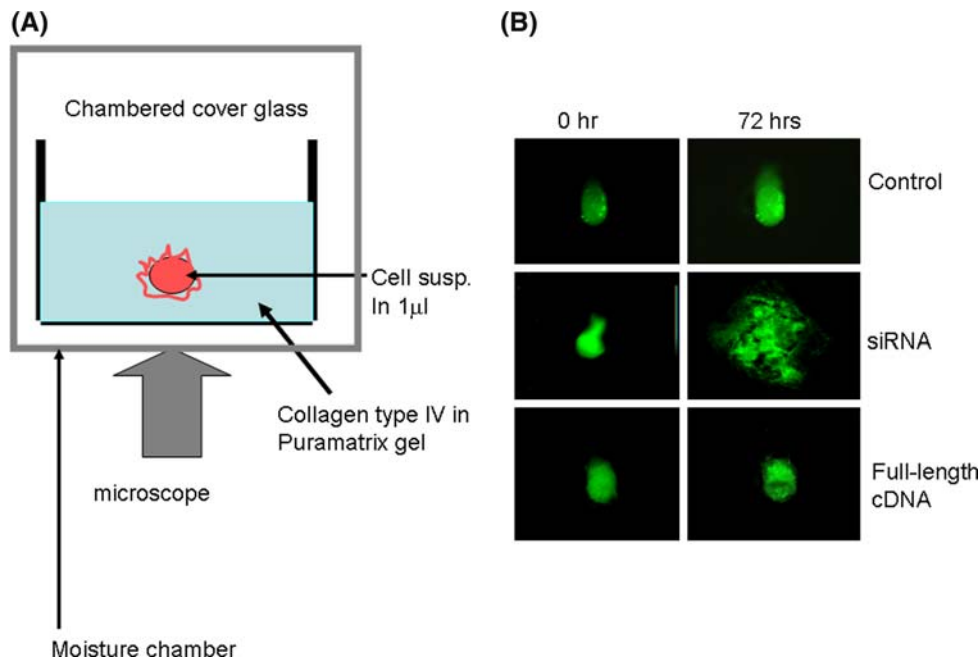
Fig. 5 Real-time 3-D invasion assay. (**a**) In a PuraMatrix™ gel impregnated with collagen type 1, cell invasion is observed by inverted fluorescence microscopy using a maximal horizontal comparison by time-lapse analysis per 12 h for 72 h. (**b**) Images with fluorescence microscopy. At 72 h, the invasion of cells transfected with siRNA is dramatically elevated, while the invasion of cells transfected with full-length clone is inhibited

real-time RT-PCR; (3) zymography experiments show that cellular activities of MMP-2 and -9 are elevated by gene silencing with siRNA, and diminished in those transfected with the full-length clone; (4) a 3-D invasion assay of PuraMatrix™ gel impregnated with collagen type I with time-lapse analysis discloses that cell invasion is inhibited

Fig. 6 In 3-D invasion assay, the invasion rates of cells transfected with the full-length clone and siRNA are 132.56% and 689.56%, respectively, while the control displays 212.96% invasion at 72 h, compared with values measured at 0 h

after transfection of the full-length clone, but considerably elevated upon gene silencing with siRNA; (5) compensatory DNA microarray analysis demonstrates that RECK regulates several additional signals, including latrophilin 2 and GHRHR. However, transcriptional regulation of the MMP and TIMP family proteins by RECK was not observed.
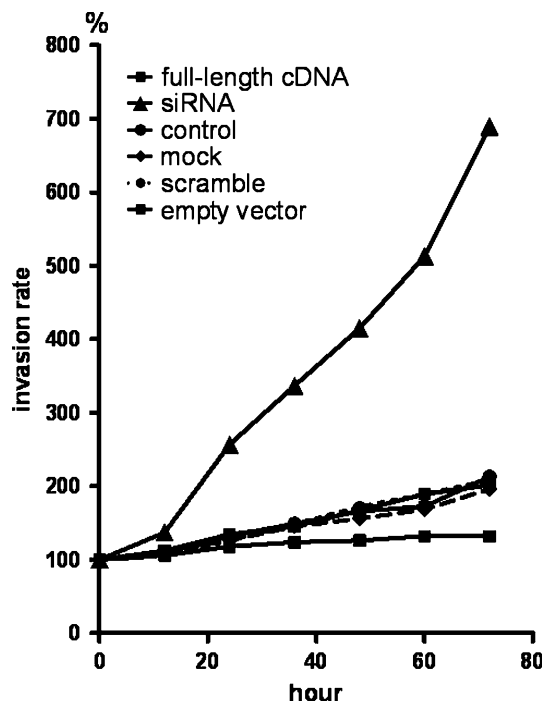
Tumour invasion by pituitary adenoma

While pituitary adenomas belong to the benign tumour family, approximately 15–25% of cases display tumour invasion into the hypophysis or cavernous sinus at initial diagnosis [16, 17]. Invasive behaviour, the pathological hallmark of pituitary adenomas, is responsible for the failure of surgery as therapy [18]. *Invasiveness of pituitary adenoma can be observed especially in PRL-oma and ACTH-oma*. Thus, tumour invasion is integral to the severity of pituitary adenomas. To date, few studies have examined the molecular mechanisms that regulate these invasive phenotypes with a view to improving the prognosis of pituitary adenoma patients [19–22]. Gong et al. showed that MMP-9 expression can be consistently used to distinguish invasive from non-invasive pituitary tumours, and reflect the extent of invasiveness in pituitary tumours according to tumour subtype, size, extension, primary and secondary surgery, even at the early stages of invasiveness. MMP-9 is thus a confirmed biomarker used to predict the invasive nature of pituitary tumours. Liu and colleagues suggested that MMP-2 is associated with aggressiveness and invasion in pituitary adenomas, but not tumour size or secretory function. They concluded that MMP-2 is a useful

Fig. 7 We evaluate the quality of total RNA in cDNA microarray analysis. Values of raw siRNA or full-length clone (Cy3)/control (Cy5) are assessed as the normalized value in dot blot analysis, and hierarchical cluster analysis performed according to specific software (GenMAPP: http://www.genmapp.org/, Cytoscape: http://www.cytoscape.org/) based on gene annotation data (NCBI: http://www.ncbi.nlm.nih.gov/)

Quality of total RNA

Control vs. full-length clone

Hierarchical cluster analysis
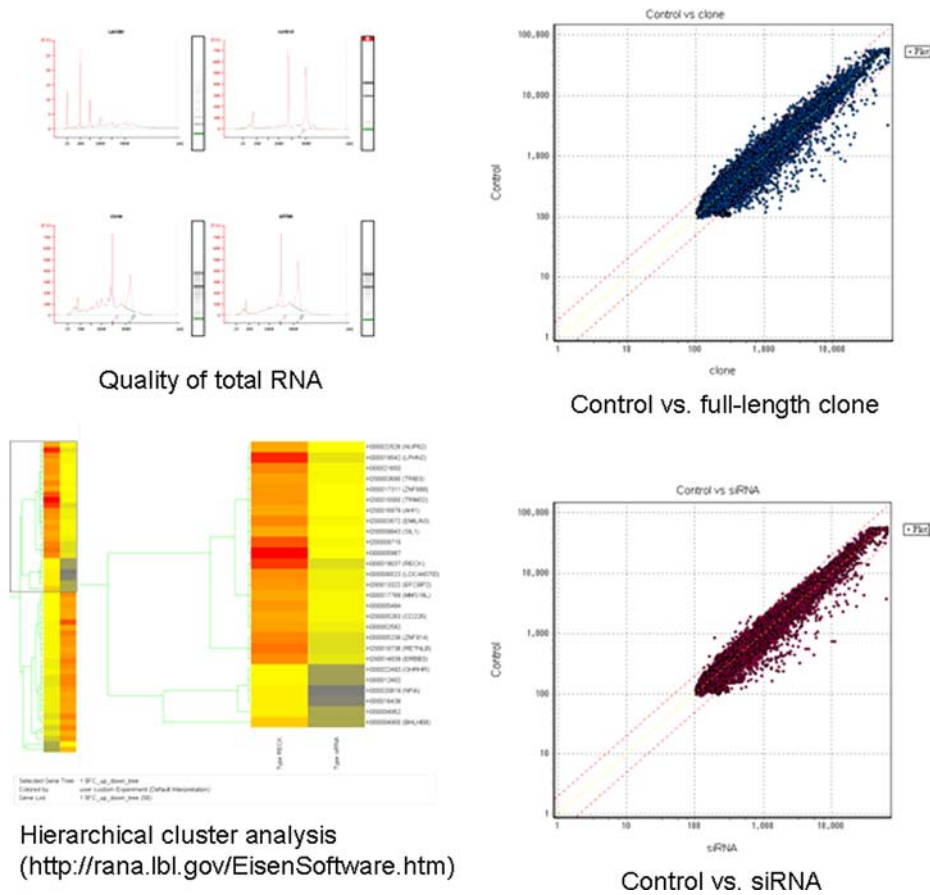(http://rana.lbl.gov/EisenSoftware.htm)

Control vs. siRNA

Table 1 Gene regulation by RECK. Elevation and reduction of pixel expression with intensity changes greater than 1.60-fold (increase) and lower than 0.66-fold (decrease) are considered significant in terms of changes in mRNA expression

Latrophilin 2 and growth hormone releasing hormone receptor (GHRHR) are regulated by RECK

RECK cDNA	RECK siRNA	Key word	Description
4.312	0.202	Proliferation	Lctophilin 2
2.436	0.358	Anti-apoptosis	Resistin-like beta
2.134	0.562		Zinc finger protein 814
2.134	0.425		Elastin microfobril interfacer 3
1.785	0.624		Basic helix-loop-helix domain containing, class B, 8
1.624	0.526		Nuclear factor 1/A
1.621	0.424 (folds)		GHRHR

tool for assessing invasive potential. The MMP subtype that predominantly regulates the invasion of pituitary adenomas remains to be established. Molecular regulation of MMPs is essential for proper ECM remodelling and invasion, and MMPs are promising targets for tumour therapy [23–25]. We previously reported that discoidin domain receptor-1 mediates cell invasion-related signalling between collagen type I and MMP-2 and -9 in pituitary adenoma cells [25]. However, limited information is available on the molecular mechanisms regulating MMP activity in pituitary adenomas.

RECK and the anti-MMP function

The glycoprotein RECK (~ 102 kDa) is an endogenous MMP inhibitor anchored to the cell membrane via glyco-sylphostidylinositol [26]). The protein contains multiple *epidermal growth factor-like* repeats and serine protease inhibitor-like domains. RECK was isolated by cDNA expression cloning as a gene that induces flat morphology upon expression in the v-Ki-ras-transformed NIH 3T3 cell line. Moreover, studies have shown that RECK antagonizes multiple MMPs [7] and the function of membrane type 1

matrix metalloproteinase (MT1-MMP) to promote proMMP-2 and -9 maturation [11]. Clinically, in many tumour types, expression of RECK is highly correlated with lower invasiveness, and patients with high RECK expression in tumour tissues show better prognosis [9]. The RECK gene is expressed in various normal tissue and cell lines [27], but is remarkably reduced and sometimes not even expressed in tumour-derived cell lines [11, 28–33]. To our knowledge, this is the first study examining RECK expression in a pituitary adenoma cell line. Previous reports show that the amounts of extracellular pro-MMP and active forms are reduced in RECK-overexpressing stable cells and their invasive potential is suppressed in glioma or osteosarcoma cells [31, 34]. In our experiments, the levels of pro-MMP-9 and active MMP-2 in the supernatant were decreased after RECK expression was induced in HP-75 pituitary adenoma cells. Conversely, MMP protein levels were elevated after silencing of RECK mRNA with siRNA. The in vitro invasive ability of HP-75 cells in the 3-D invasion assay was considerably enhanced after RECK siRNA transfection, which may be attributed to the abolished RECK expression. Consistent with our previous findings, incorporation of cells into PuraMatrix™, an artificial peptide impregnated with collagen type I, provided a more permissive environment to analyze the role of ECM in intracellular signal interactions [15]. Here, we employ an optimal clinically relevant invasion model that is attenuated by RECK. Our findings collectively suggest that RECK plays a critical role in the regulation of MMPs and tumour invasion in pituitary adenoma cells.

Signals regulated by RECK

While both RECK and TIMP proteins are MMP inhibitors, they may play distinct roles in different processes. RECK knock-out is lethal in mice at the embryonic stage, while lack of TIMP-1 and -2 has little effect on development, indicating that RECK shares little functional redundancy with TIMPs. Mice lacking RECK died in utero with reduced integrity of blood vessels, neural tube and mesenchymal tissues. In these mice, MMP activity was elevated, and the amount of collagen type I was greatly reduced [35]. The phenotypes exhibited by RECK-deficient mice may be a consequence of aberrant metabolism of types I and IV collagen. Furthermore, our data show that RECK upregulates latrophilin 2, a component of epithelial-mesenchymal transition within the atrioventricular canal of the embryo [36], and GHRHR that mediates GHRH to produce GH. Among the other factors affected by RECK, nuclear factor 1/A binds to silencer elements in the glutathione transferase P (GST-P) gene, and the carboxy terminal region of NF1-A represses the transcriptional activity of human metallothionein IIA (hMTIIA) promoter

[37], while resistin-like beta stimulates naive bone marrow-derived macrophages to secrete significant amounts of TNF-alpha and IL-6 [38]. Therefore, the abolishment of RECK expression in knock-out mice is lethal, since lack of this gene can lead to dysfunction of downstream genes that are closely related to development. In pituitary adenoma cells, expression of GHRHR via RECK may contribute to tumour development [39–41].

RECK does not alter the transcriptional levels of MMPs in the HT-1080 fibrosarcoma model [42], but interacts directly with MMP-2 and -9 as well as *MT1-MMP* to inhibit their activities [11]. Our current zymography experiments confirm that the activities of MMP-2 and -9 are regulated by RECK. However, reverse zymography data reveal that TIMP activity is not influenced by RECK. The distribution of RECK in pituitary adenoma subtypes requires further investigation.

Conclusion

We have demonstrated that RECK not only interacts directly with MMP-2, and -9, but also regulates the intracellular signalling of latrophilin 2 and GHRHR which, in turn, modulate the tumour growth of pituitary adenomas. These results support the theory that RECK is a promising target for the treatment of pituitary adenoma.

References

1. Kim YY, Chae SY, Kim S, Byun Y, Bae YH (2005) Improved phenotype of rat islets in a macrocapsule by co-encapsulation with cross-linked Hb. J Biomater Sci Polym Ed 16:1521–1535
2. Tischer T, Vogt S, Aryee S, Steinhauser E, Adamczyk C, Milz S, Martinek V, Imhoff AB (2007) Tissue engineering of the anterior cruciate ligament: a new method using acellularized tendon allografts and autologous fibroblasts. Arch Orthop Trauma Surg 127:735–741
3. Tunuguntla R, Ripley D, Sang QX, Chegini N (2003) Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinases TIMP-3 and -4 in benign endometrium and endometrial cancer. Gynecol Oncol 89:453–459
4. Bendardaf R, Buhmeida A, Ristamaki R, Syrjanen K, Pyrhonen S (2007) MMP-1 (collagenase-1) expression in primary colorectal cancer and its metastases. Scand J Gastroenterol 1–6 (Epub ahead of print)
5. Bister V, Skoog T, Virolainen S, Kiviluoto T, Puolakkainen P, Saarialho-Kere U (2007) Increased expression of matrix metalloproteinases-21 and -26 and TIMP-4 in pancreatic adenocarcinoma. Mod Pathol 20:1128–1140
6. Delgado-Enciso I, Cepeda-Lopez FR, Monrroy-Guizar EA, Bautista-Lam JR, Andrade-Soto M, Jonguitud-Olguin G, Rodriguez-Hernandez A, Anaya-Ventura A, Baltazar-Rodriguez LM, Orozco-Ruiz M, Soriano-Hernandez AD, Rodriguez-Sanchez IP,

Lugo-Trampe A, Espinoza-Gomez F, Michel-Peregrina ML (2007) Matrix metalloproteinase-2 promoter polymorphism is associated with breast cancer in a Mexican population. Gynecol Obstet Invest 65:68–72

7. Takagi S, Kato Y, Asano K, Ohsaki T, Bosnakovski D, Hoshino Y, Okumura M, Kadosawa T, Fujinaga T (2005) Matrix metalloproteinase inhibitor RECK expression in canine tumors. J Veter Med Sci/Jpn Soc Veter Sci 67:761–767

8. Accorsi-Mendonca T, Paiva KB, Zambuzzi WF, Cestari TM, Lara VS, Sogayar MC, Taga R, Granjeiro JM (2007) Expression of matrix metalloproteinases-2 and -9 and RECK during alveolar bone regeneration in rat. J Mol Histol 39(2):201–208

9. Clark JC, Thomas DM, Choong PF, Dass CR (2007) RECK-a newly discovered inhibitor of metastasis with prognostic significance in multiple forms of cancer. Cancer Metastasis Rev 26:675–683

10. Kawashima S, Imamura Y, Chandana EP, Noda T, Takahashi R, Adachi E, Takahashi C, Noda M (2007) Localization of the membrane-anchored MMP-regulator RECK at the neuromuscular junctions. J Neurochem 104(2):376–385

11. Takagi S, Hoshino Y, Osaki T, Okumura M, Fuginaga T (2007) Expression of membrane-anchored matrix metalloproteinase inhibitor reversion inducing cysteine rich protein with Kazal motifs in murine cell lines. Exp Oncol 29:30–34

12. Ong EK, Suphioglu C, Singh MB, Knox RB (1990) Immunodetection methods for grass pollen allergens on western blots. Int Arch Allergy Appl Immunol 93:338–343

13. Le QT, Ohashi A, Hirose S, Katunuma N (2005) Reverse zymography using fluorogenic substrates for protease inhibitor detection. Electrophoresis 26:1038–1045

14. Patel BP, Shah PM, Rawal UM, Desai AA, Shah SV, Rawal RM, Patel PS (2005) Activation of MMP-2 and MMP-9 in patients with oral squamous cell carcinoma. J Surg Oncol 90:81–88

15. Yoshida D, Teramoto A (2007) The use of 3-D culture in peptide hydoegel for analysis of discoidin domain receptor 1-collagen interaction. Cell Adhesion Migrat 1:92–98

16. Esposito V, Santoro A, Minniti G, Salvati M, Innocenzi G, Lanzetta G, Cantore G (2004) Transsphenoidal adenomectomy for GH-, PRL- and ACTH-secreting pituitary tumours: outcome analysis in a series of 125 patients. Neurol Sci 25:251–256

17. Karavitaki N, Collison K, Halliday J, Byrne JV, Price P, Cudlip S, Wass JA (2007) What is the natural history of nonoperated nonfunctioning pituitary adenomas? Clin Endocrinol (Oxf) 67:938–943

18. Chang CZ, Huang YH, Howng SL (2000) Follow-up of invasive pituitary macroadenoma in 56 patients within a duration of 5 years. Kaohsiung J Med Sci 16:339–344

19. Gong J, Zhao Y, Abdel-Fattah R, Amos S, Xiao A, Lopes MB, Hussaini IM, Laws ER (2008) Matrix metalloproteinase-9, a potential biological marker in invasive pituitary adenomas. Pituitary 11:37–48

20. Hussaini IM, Trotter C, Zhao Y, Abdel-Fattah R, Amos S, Xiao A, Agi CU, Redpath GT, Fang Z, Leung GK, Lopes MB, Laws ER Jr (2007) Matrix metalloproteinase-9 is differentially expressed in nonfunctioning invasive and noninvasive pituitary adenomas and increases invasion in human pituitary adenoma cell line. Am J Pathol 170:356–365

21. Knappe UJ, Hagel C, Lisboa BW, Wilczak W, Ludecke DK, Saeger W (2003) Expression of serine proteases and metalloproteinases in human pituitary adenomas and anterior pituitary lobe tissue. Acta Neuropathol 106:471–478

22. Liu W, Kunishio K, Matsumoto Y, Okada M, Nagao S (2005) Matrix metalloproteinase-2 expression correlates with cavernous sinus invasion in pituitary adenomas. J Clin Neurosci 12:791–794

23. Liu W, Matsumoto Y, Okada M, Miyake K, Kunishio K, Kawai N, Tamiya T, Nagao S (2005) Matrix metalloproteinase 2 and 9 expression correlated with cavernous sinus invasion of pituitary adenomas. J Med Invest 52:151–158

24. Turner HE, Nagy Z, Esiri MM, Harris AL, Wass JA (2000) Role of matrix metalloproteinase 9 in pituitary tumor behavior. J Clin Endocrinol Metabol 85:2931–2935

25. Yoshida D, Teramoto A (2007) Enhancement of pituitary adenoma cell invasion and adhesion is mediated by discoidin domain receptor-1. J Neuro-oncol 82:29–40

26. Takagi S, Kitamura T, Hosaka Y, Ohsaki T, Bosnakovski D, Kadosawa T, Okumura M, Fujinaga T (2005) Molecular cloning of canine membrane-anchored inhibitor of matrix metalloproteinase, RECK. J Veter Med Sci/Jpn Soc Veter Sci 67:385–391

27. Welm B, Mott J, Werb Z (2002) Developmental biology: vasculogenesis is a wreck without RECK. Curr Biol 12:R209–211

28. Chang HC, Cho CY, Hung WC (2007) Downregulation of RECK by promoter methylation correlates with lymph node metastasis in non-small cell lung cancer. Cancer Sci 98:169–173

29. Cho CY, Wang JH, Chang HC, Chang CK, Hung WC (2007) Epigenetic inactivation of the metastasis suppressor RECK enhances invasion of human colon cancer cells. J Cell Physiol 213:65–69

30. Cho YB, Lee WY, Song SY, Shin HJ, Yun SH, Chun HK (2007) Matrix metalloproteinase-9 activity is associated with poor prognosis in T3–T4 node-negative colorectal cancer. Hum Pathol 38:1603–1610

31. Kang HG, Kim HS, Kim KJ, Oh JH, Lee MR, Seol SM, Han I (2007) RECK expression in osteosarcoma: correlation with matrix metalloproteinases activation and tumor invasiveness. J Orthop Res 25:696–702

32. Ohta S, Lai EW, Morris JC, Pang AL, Watanabe M, Yazawa H, Zhang R, Green JE, Chan WY, Sirajuddin P, Taniguchi S, Powers JF, Tischler AS, Pacak K (2007) Metastasis-associated gene expression profile of liver and subcutaneous lesions derived from mouse pheochromocytoma cells. Mol Carcinog 47:245–251

33. Takemoto N, Tada M, Hida Y, Asano T, Cheng S, Kuramae T, Hamada JI, Miyamoto M, Kondo S, Moriuchi T (2007) Low expression of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) indicates a shorter survival after resection in patients with adenocarcinoma of the lung. Lung Cancer 58:376–383

34. Correa TC, Brohem CA, Winnischofer SM, da Silva Cardeal LB, Sasahara RM, Taboga SR, Sogayar MC, Maria-Engler SS (2006) Downregulation of the RECK-tumor and metastasis suppressor gene in glioma invasiveness. J Cell Biochem 99:156–167

35. Noda M, Oh J, Takahashi R, Kondo S, Kitayama H, Takahashi C (2003) RECK: a novel suppressor of malignancy linking oncogenic signaling to extracellular matrix remodeling. Cancer Metastasis Rev 22:167–175

36. Doyle SE, Scholz MJ, Greer KA, Hubbard AD, Darnell DK, Antin PB, Klewer SE, Runyan RB (2006) Latrophilin-2 is a novel component of the epithelial–mesenchymal transition within the atrioventricular canal of the embryonic chicken heart. Dev Dyn 235:3213–3221

37. Osada S, Ikeda T, Xu M, Nishihara T, Imagawa M (1997) Identification of the transcriptional repression domain of nuclear factor 1-A. Biochem Biophys Res Commun 238:744–747

38. Barnes SL, Vidrich A, Wang ML, Wu GD, Cominelli F, Rivera-Nieves J, Bamias G, Cohn SM (2007) Resistin-like molecule beta (RELMbeta/FIZZ2) is highly expressed in the ileum of SAMP1/YitFc mice and is associated with initiation of ileitis. J Immunol 179:7012–7020

39. Fainstein Day P, Frohman L, Garcia Rivello H, Reubi JC, Sevlever G, Glerean M, Fernandez Gianotti T, Pietrani M, Rabadan A, Racioppi S, Bidlingmaier M (2007) Ectopic growth hormone-releasing hormone secretion by a metastatic bronchial carcinoid tumor: a case with a non hypophysial intracranial tumor that shrank during long acting octreotide treatment. Pituitary 10:311–319

40. Horvath E, Kovacs K (2006) Pathology of acromegaly. Neuro-endocrinology 83:161–165

41. Roh SG, Doconto M, Feng DD, Chen C (2006) Differential regulation of GHRH-receptor and GHS-receptor expression by long-term in vitro treatment of ovine pituitary cells with GHRP-2 and GHRH. Endocrine 30:55–62

42. Annabi B, Bouzeghrane M, Currie JC, Hawkins R, Dulude H, Daigneault L, Ruiz M, Wisniewski J, Garde S, Rabbani SA, Panchal C, Wu JJ, Beliveau R (2005) A PSP94-derived peptide PCK3145 inhibits MMP-9 secretion and triggers CD44 cell surface shedding: implication in tumor metastasis. Clin Exp Metastasis 22:429–439