

Human DEAD-box/RNA unwindase rck/p54 contributes to maintenance of cell growth by affecting cell cycle in cultured cells

YUKIHIRO AKAO¹, KENJI MATSUMOTO¹, KENJI OHGUCHI¹,
YOSHIHITO NAKAGAWA¹ and HITOSHI YOSHIDA²

¹Gifu International Institute of Biotechnology, Kakamigahara, Gifu 504-0838; ²Molecular Oncology Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-Chome, Chuo-Ku, Tokyo 104-0045, Japan

Received January 12, 2006; Accepted March 8, 2006

Abstract. Understanding the control of gene expression in cancer cells requires defining the molecular and cellular basis of RNA metabolism compared with that in steady-state normal cells. Previously, we reported evidence that human RNA structure-modifying unwindase rck/p54, a member of the DEAD-box family, was highly expressed in most of the malignant cell lines tested and that this expression was linked to malignant transformation. Here, we show that rck/p54 positively affects cell growth, probably by modulating the gene expression at the translational level in cultured cells. In cell growth and differentiation induced by external stimuli, the level of rck/p54 expression was up-regulated during cell proliferation and down-regulated during differentiation. The down-regulation of rck/p54 in HeLa cells by RNAi induced cell growth inhibition through cell cycle arrest at S phase. Immunoprecipitation using anti-rck/p54 antibody in HeLa cells demonstrated the co-precipitation of rck/p54 with eIF4E, which is well-known to bind to the 5'cap-structure, resulting in initiation of translation. These data suggest that rck/p54 contributes to cell growth possibly by modulating translation-initiation control of the genes involved in the cell proliferation, which is a newly defined mechanism leading to carcinogenesis.

Introduction

RNA is a biologically important molecule that is required by all living organisms. It is involved in all aspects of gene expression, and RNA plays important roles to ensure the fidelity of biological reactions. Recently, it has become increasingly clear that the structure of RNA molecules is important for RNA-RNA, RNA-DNA and RNA-protein interactions, leading directly to modification of gene expression. In such interactions, RNA molecules can require helper molecules to assist in the folding or refolding of polypeptide chains. The ability to unwind folded RNAs or to modify RNA-RNA interactions has been attributed to RNA unwindase.

RNA unwindase is an enzyme that is thought to unwind double-stranded RNAs (dsRNAs) in an energy-dependent fashion through the hydrolysis of NTP. They are present in almost all organisms, ranging from bacteria and viruses to humans. Historically, such enzymes are members of the so-called DEAD-box family, which was named according to one of its conserved motifs (1). After the characterization of the DEAD box family, other related families were defined based on divergent sequence motifs (2). At that time, the typical eukaryotic translation initiation factor, eIF4A, which forms part of the cap-binding complex including eIF4E, was shown to unwind or rearrange RNA-duplex structures at the 5'-end of eukaryote mRNA to prepare it for scanning by the small ribosome subunit (3), and the genetic characterization of other proteins of the DEAD-box family was in agreement with such an RNA helicase function. These findings were further strengthened by sequence analysis of the RNA unwindases, which revealed conserved motifs that were shared with the well-characterized DNA helicases (4).

Most human genetic disorders are caused by mutations affecting the protein-coding regions of genes, e.g. missense or frame-shift mutations within exons or introns that disrupt pre-messenger RNA (pre-mRNA) processing. Dys-regulated gene expression at the level of transcription is another well-known, although less frequently encountered, mechanism in human disease (5). Recently, mutations that cause disease through increased or decreased efficiency of mRNA translation have been discovered as a novel mechanism of human disease (6-8). This discovery indicates that deregulation at the translational level through abnormal interaction with the proteins associated with RNAs might cause human diseases.

Correspondence to: Dr Yukihiro Akao, Gifu International Institute of Biotechnology, Kakamigahara, Gifu 504-0838, Japan
E-mail: yakao@giib.or.jp

Abbreviations: DEAD, single letter code of Asp-Glu-Ala-Asp; ATP, adenosine triphosphate; RACE, rapid amplification of cDNA ends; RT, reverse transcription; PBS, phosphate-buffered saline; FBS, fetal bovine serum; TPA, 12-O-tetradecanoyl phorbol-13-acetate; SDS, sodium dodecyl sulphate; FACS, fluorescence-activated flow cytometry; CD, cell differentiation; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; RNAi, ribonucleic acid interference; siRNA, small interfering RNA; UTR, untranslated region; RNP, ribonucleoprotein

Key words: DEAD-box/RNA unwindase, rck/p54, translation initiation, cell growth, cell cycle

In higher eukaryotes, the expression of approximately 1 gene in 10 is strongly regulated at this level. Translational control enables a cell to increase the concentration of a protein very rapidly and, therefore, appears to be especially suited to regulating genes implicated in cell proliferation and damage prevention (9). The presence of equal amounts of different mRNA species does not necessarily ensure synthesis of equivalent quantities of the corresponding proteins. In fact, some mRNAs that reach the cytoplasm are not translated at all. Thus, negative or positive control can influence the rate of translation (9). These controls can be exerted either by global machinery that simultaneously affects all cellular mRNAs or by more specific mechanisms that influence individual mRNAs or subsets of mRNAs.

The *RCK* gene at 11q23 is juxtaposed to an immunoglobulin heavy chain gene upon t(11;14) chromosome translocation, which is observed in approximately 5% of patients with B-cell lymphoma carrying 14q32 translocations. This gene was decapitated at its first intron, and its protein product (rck/p54) was found to be overexpressed in RC-K8 cells (10-12), as was *c-myc* of t(8;14) in Burkitt's lymphomas and *bcl-2* of t(14;18) in follicular B-cell lymphomas (13). *RCK* gene transcripts of 7.5 kb were found to be expressed ubiquitously in mouse and human tissues (12). rck/p54 exhibits ATP-dependent RNA unwinding activity and participates in mRNA masking and 5'-3' RNA decay (14-16).

Previously, we reported that rck/p54 was highly expressed in the majority of malignant cell lines and human colorectal tumors and HCV-related hepatocellular tumors (12,17,18). Also, high expression of rck/p54 frequently accompanied the *c-myc* overexpression seen in colon tumor cells; and evidence was obtained by the use of rck/p54-overexpressing SW480 cells, supporting the contribution of rck/p54 to the stabilization of *c-myc* mRNA and efficiency of *c-myc* translation (19). However, the enforced expression of rck/p54 in guinea pig fibrosarcoma cell line 104CI resulted in severe growth inhibition and morphological change (20). Such inverse findings on rck/p54 expression have remained an unsolved problem for many years.

In the current study, we demonstrated that rck/p54 contributes positively to cell growth, mostly by modulating the expression of genes involved in cell proliferation at a translational level.

Materials and methods

Identification of the transcription start sites of *RCK* mRNA. The 5'-end of human *RCK* mRNA was identified by using the conventional 5'RACE method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, total RNA of various cell lines was extracted by a standard procedure, and then *RCK*-specific antisense primer (5'-ATCACCAGGT TTAATAGTGGT-3') was used to prime reverse transcription. After the first-strand cDNA had been tailed with dATP by terminal deoxynucleotide transferase, 2 rounds of PCR were performed by using the nested *RCK*-specific primer (5'-CTG GCCGTGCTCATGCTGTT-3'). Products were cloned into the TA cloning vector 2.1 (Invitrogen) and sequenced.

Semi-quantitative RT-PCR. Total RNA was isolated by using an RNAqueous-4PCR kit (Ambion, Austin, TX, USA) according

to the manufacturer's instructions. RNA samples were reverse-transcribed by using SuperScript II RNase H-reverse transcriptase (Invitrogen) and oligo(dT) primer (Invitrogen). Prepared cDNA samples were purified by use of a PCR Purification kit (Qiagen, Hilden, Germany) and used for PCR. PCR was performed according to the manufacturer's instructions. β -actin was used as an internal control. Primers for *RCK* and *c-myc* were as follows: T-*RCK* forward, 5'-GG CTGGGAAAAGCCATCT-3'; T-*RCK* reverse, 5'-ACCTGA TCTTCCAATACG-3'; TSS-1 forward, 5'-AGAGGAGCCG AGTTAGCTCA-3'; TSS-1 reverse, 5'-AGCTGGTTCATCT GTTGCTG-3'; TSS-2 forward, 5'-CCATTTTGGGAGCTTC GAGT-3'; TSS-2 reverse, 5'-AGCTGGTTCATCTGTTGC TG-3'; *c-myc* forward, 5'-ACATCATCATCCAGGACTG-3'; *c-myc* reverse, 5'-TTTAGCTCGTTCCTCTCTG-3'. The PCR products were evaluated by agarose electrophoresis. The intensity of bands for PCR products was measured by densitometry.

Cell culture, treatment for differentiation, FACS, morphological study and cell viability. Human malignant cell lines HL60, K562 and NB4 (myeloid leukemias), U937 (monocytic leukemia), HeLa (uterine cervical cancer), PC3 and LNCaP (prostate cancer), and SW480 (colon cancer) were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS (Sigma, St. Louis, MO, USA) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO₂ at 37°C. Human peripheral blood lymphocytes from healthy donors were collected by gravity centrifugation using Ficoll-paque (Amersham Biosciences AB, Uppsala, Sweden) and incubated in RPMI-1640 culture medium for 8 h. Then the cells were stimulated with concanavalin-A (Con-A, 15 μ g/ml; Sigma) for 48 h. TPA was also purchased from Sigma. Protein kinase C inhibitors, bisindolylmaleimide I (Bis I) and Ro-32-0432 (Ro), were purchased from Calbiochem (San Diego, CA, USA). These inhibitors were dissolved in DMSO. FACS analysis was performed as described previously (21). Fluorescein isothiocyanate (FITC)-anti-human CD38 antibody (eBioscience, San Diego, CA, USA) was also purchased. Briefly, the cells (5×10^5) were incubated with antibodies for 30 min on ice, and then washed with PBS. They were subsequently diluted with PBS, and subjected to FACS using a FACScalibur and CellQuest software (Benton-Dickson, San Jose, CA, USA). The number of viable cells was determined by the trypan blue dye exclusion test.

Antibodies. Rabbit polyclonal anti-rck/p54 antibody against N-terminal amino acids Nos.1-10 (anti-rck/p54N)(22) and C-terminal Nos. 369-385 (anti-rck/p54C) were developed in our laboratory (Fig. 1E). Recognition of rck/p54 protein by the antibody was confirmed by performing an enzyme-linked immunosorbent assay (data not shown) and Western blotting (data not shown). Human monoclonal anti- β -actin antibody was purchased from Sigma. Antibodies specific for Erk and Akt and their phosphorylated forms (CellSignaling, Beverly, MA, USA), *c-myc* (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and eIF4E (BD Bioscience, Palo Alto, CA, USA) were also purchased. HRP-conjugated sheep anti-mouse immunoglobulin (Ig) and donkey anti-rabbit Ig antibodies were obtained from Amersham Biosciences (Piscataway, NJ, USA).

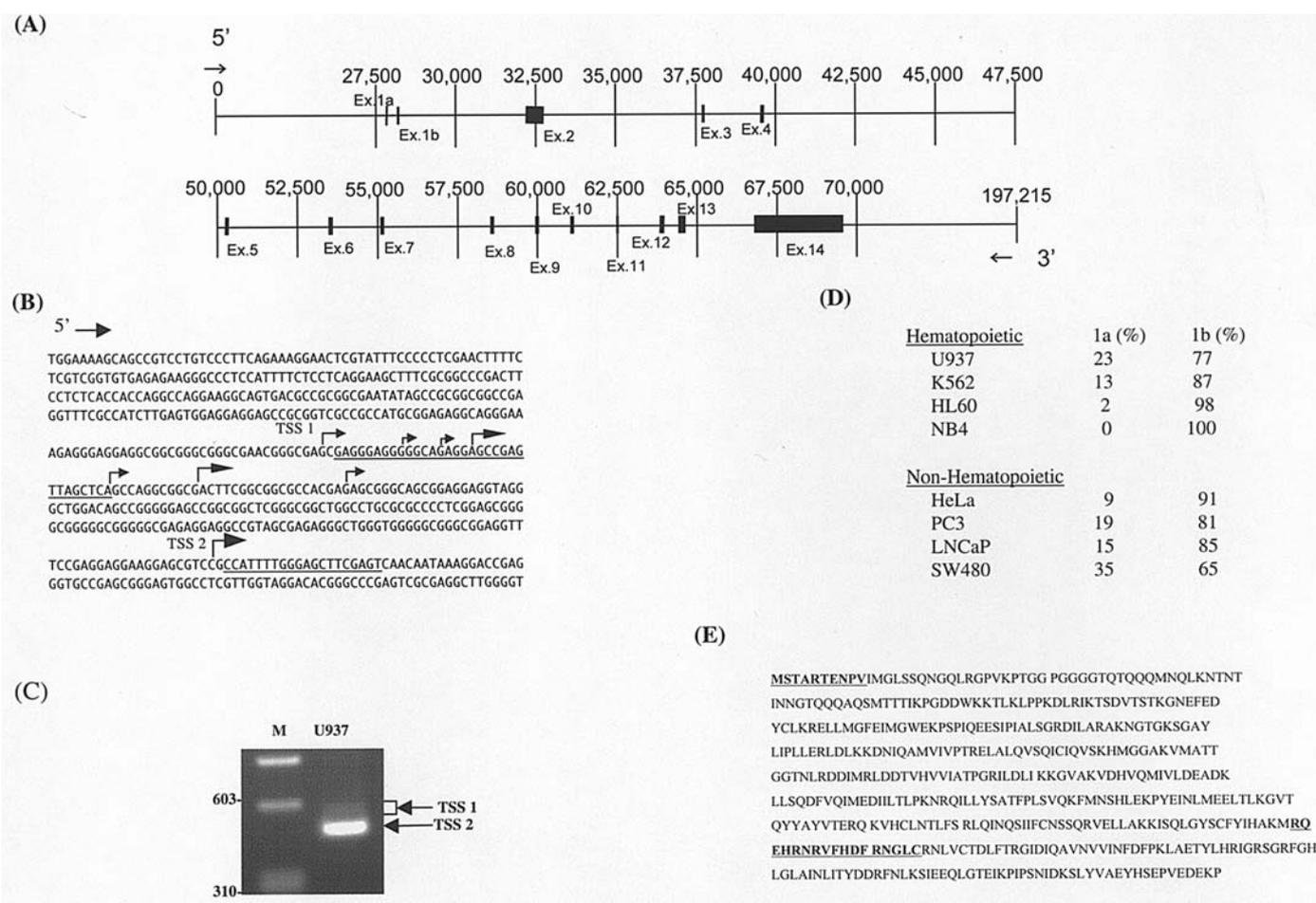


Figure 1. *RCK* genomic structure and identification of transcription start sites. (A) Schematic representation of the *RCK* genomic structure of exon 1 to exon 14. The structure spanning exons 2-14 was determined by survey of the sequences shown in NCBI GenBank AP002954 (197215 bp). Exons (Ex) were indicated as closed boxes. (B) Identification of multiple transcription start sites. Arrows indicate the transcription start sites. The sequences of sense primers for TSS1 (transcripts from exon 1a) and TSS2 (transcripts from exon 1b) are underlined. (C) The *RCK* gene has 2 major transcription start sites in U937 cells. PCR products from exons 1a (552 bp) and 1b (492 bp) are shown by 2% gel electrophoresis. (D) Relative usage of the 2 alternative exons in the cell lines tested. The usage was evaluated by the intensity of the bands of the PCR products. (E) Targeted amino-acid sequences used for generating rabbit polyclonal anti-rck/p54 antibodies are underlined.

Western blotting. The cells were homogenized in chilled lysis buffer comprising 10 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA and 1% Protease Inhibitor Cocktail (Sigma) and stood for 30 min on ice. After centrifugation at 14,000 rpm for 20 min at 4°C, the supernatants were collected as protein samples. Protein contents were measured with a DC Protein assay kit (BioRad, Hercules, CA, USA). One microgram of lysate protein for Western blot of rck/p54 or eIF4E was separated by SDS-PAGE using a 12% polyacrylamide gel and electroblotted onto a PVDF membrane (Du Pont, Boston, MA). After blockage of non-specific binding sites for 1 h with 5% non-fat milk in PBS containing 0.1% Tween-20, the membrane was incubated overnight at 4°C with anti-human rck/p54N antibody. For Western blotting of c-myc, Akt, Erk and their phosphorylated forms, 10 µg of lysate protein was used. The membranes were then washed 3 times with PBS containing 0.1% Tween-20, incubated further with HRP-conjugated sheep anti-mouse or donkey anti-rabbit Ig antibody (Amersham) at room temperature, and then washed 3 times with PBS containing 0.1% Tween-20. The immunoblots were visualized by use of

an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, USA).

Knockdown of *RCK* in HeLa cells by RNAi. HeLa cells were seeded in a 6-well plate at a concentration of 1-2x10⁵/well (30-40% confluence) on the day before transfection. The potential siRNA target sequences were found on *RCK* by use of a siRNA Designer computer system (Invitrogen). An siRNA for targeting *RCK* mRNAs was obtained from Invitrogen. The target sequence for siRNA was 5'-AACAGATGAACCAGC TGAAA-3'. The siRNAs were used to transfect the cells (80 nM in a well) by using cationic liposomes TransIT-TKO (Mirus Bio Co., Madison, WI, USA) according to the manufacturer's Lipofection protocol.

The transfection efficiency was evaluated by the transfection of HeLa cells with a duplex siRNA-FITC and the expression of interferon-induced OAS-1 mRNA was examined by RT-PCR using 5'-CGATGTGCTGCCTGCCTTTGAT GC-3' (sense) and 5'-GTCTCCACCACCAAGTTTCTGT-3' (antisense) as primers. Non-specific control duplex (NS, 57% GC content; Dharmacon, Lafayette, CO, USA) was used as

a control for non-specific effects. The evaluation of RNAi for RCK was assayed at 36 h after transfection by Western blot and RT-PCR analyses.

Cell-cycle analysis. After culture for 36 h, cells were trypsinized and washed twice in ice-cold PBS. The cell pellets were resuspended in 300 μ l of 1% FBS/PBS and fixed for 15 min at 4°C by the addition of 700 μ l of 100% ethanol. The fixed cells were washed and resuspended in 500 μ l of PBS containing 2.5 μ l of RNase. The cells were incubated at 37°C for 15 min, and DNA content was determined by the addition of 50 μ l propidium iodide (Sigma). Cell-cycle analysis was performed by FACS using a FACScalibur and CellQuest software (Becton-Dickson).

Immunoprecipitation. The experiments were performed by using non-treated HeLa cells and rabbit anti-rck/p54C and mouse anti-eIF4E antibody (BD Bioscience). Cell lysates were incubated for 4 h at 4°C, with gentle mixing, with protein A-Sepharose beads (50 μ l) and anti-rabbit or anti-mouse immunoglobulin heavy chain G (IgG) antibodies (Sigma). The cell lysates were centrifuged, and the supernatant was incubated with 0.5 μ l of anti-rck/p54C or anti-eIF4E antibody for 2 h at 4°C, prior to addition of 50 μ l of the anti-IgG-coated protein A-Sepharose beads. Incubation was then continued for 4 h at 4°C. The beads were washed in 3x1 ml of buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Nonidet P40, 0.5% sodium deoxycholate). A 10-20 μ l aliquot of each sample was separated SDS-PAGE, and the proteins that had bound to the beads were detected by Western blot analysis.

Results

RCK genomic structure and identification of the transcription start sites. We characterized the RCK promoter region in detail by the use of 5'RACE (Fig. 1A and B) and constructed the partial genomic structure (Fig. 1A). The nucleotide sequence of the promoter region and possible multiple transcription start sites were determined as shown in Fig. 1B and C. The RCK gene showed 2 major transcription start sites, in exons 1a and 1b, in HL60 and U937 cells. We designated the long transcription product from exon 1a as TSS1 and the shorter one from exon 1b as TSS2. The relative usage of the 2 alternative transcription start sites is shown in Fig. 1D. Deviation of usage of these 2 major transcription sites existed and depended on the cell lines tested. However, the major transcripts in most of the cell lines were those from exon 1b.

The gene structure of exons 1-14 corresponding to the 5' terminal 4.1 kb of RCK cDNA was determined by referring to NCBI GenBank AP002954 (Fig. 1A). Exons 1-14 spanned ~43 kb of the genome (12).

rck/p54 expression levels during cell growth and differentiation. To examine the expression of rck/p54 in cells, we developed 2 kinds of polyclonal antibodies against rck/p54 protein. The amino-acid sequences used as immunogens are shown in Fig. 1E. The antibodies reacted with recombinant rck/p54 and the bands for rck/p54 protein detected by the antibodies on Western blots were almost totally missing when antisera were preincubated with the respective immunogens (data not shown) (14).

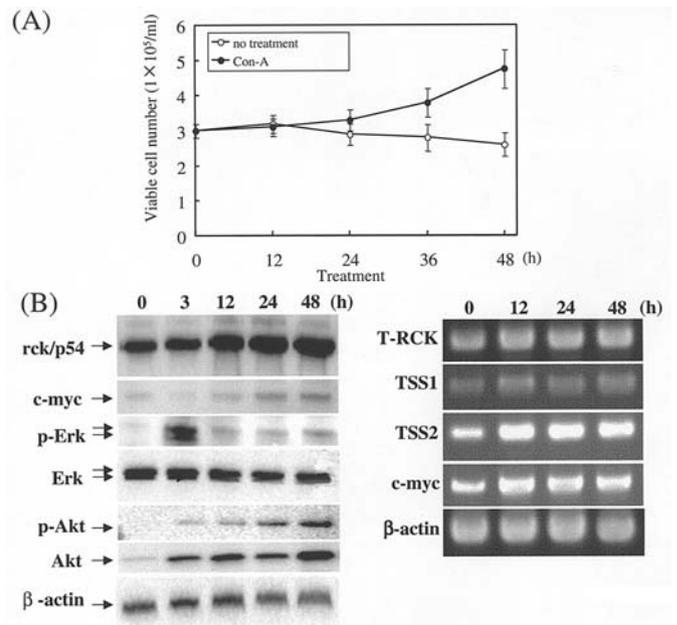


Figure 2. Changes in the expression levels of rck/p54 after treatment of human blood peripheral blood lymphocytes (PBL) with concanavalin-A (Con-A). (A), Cell growth of PBL after the treatment with Con-A (15 μ g/ml). Data represent the mean \pm SD of 3 different experiments each carried out in duplicate. The treatment was started after 8 h of incubation of the cells in culture medium. The starting cell number was 3×10^5 /ml. (B), Western blot analysis of rck/p54 and other proteins (left panel) and RT-PCR of T-RCK, TSS1 and TSS2 (RCKs, right panel). The anti-rck/p54N was used for Western blot analysis. The expression of c-myc, pErk and pAkt proteins was also monitored. TSS1 corresponds to an RCK transcript from exon 1a; and TSS2, to one from exon 1b. T-RCK indicates the total amount of RCK transcripts. The expression of c-myc transcripts was also examined. The samples were collected at the indicated times. β -actin was used as an internal control.

Next we examined the expression levels of rck/p54 during cell proliferation and differentiation to decipher the role of rck/p54 in cell growth. Examination of peripheral blood lymphocytes stimulated with Con-A indicated that the expression level of rck/p54 gradually increased up to 48 h after the addition of the mitogen, which almost paralleled the change in cell number of the growth curve (Fig. 2). The level of T-RCK, which represents the total mRNA levels of RCK, also paralleled those of the protein expression; however, the increase in the ratio of TSS1 (transcripts from exon 1a) to T-RCK was greater than that in the ratio of TSS2 (transcripts from exon 1b) to T-RCK. The levels of c-myc mRNA and protein, which we previously reported to be high when rck/p54 expression was high (19), were similar to those of rck/p54 and T-RCK. We also monitored the levels of p-Erk and p-Akt as markers for cell proliferation and survival. The levels of c-myc and pAkt gradually increased along with the increase in the rck/p54 level, whereas the level of pErk increased transiently at 3 h. These results indicate that rck/p54 functions positively for cell proliferation in response to a mitogenic factor.

To further examine the role of rck/p54 in cell growth, we monitored the expression level of rck/p54 during differentiation induced by the treatment with a protein kinase C (PKC) stimulator, TPA, which is an inducer of differentiation. As shown in Fig. 3A, the number of viable cells was decreased even at 6 h, and then the expression of differentiation surface marker CD38 was detected at 24 h (Fig. 3B). After 24 h, some

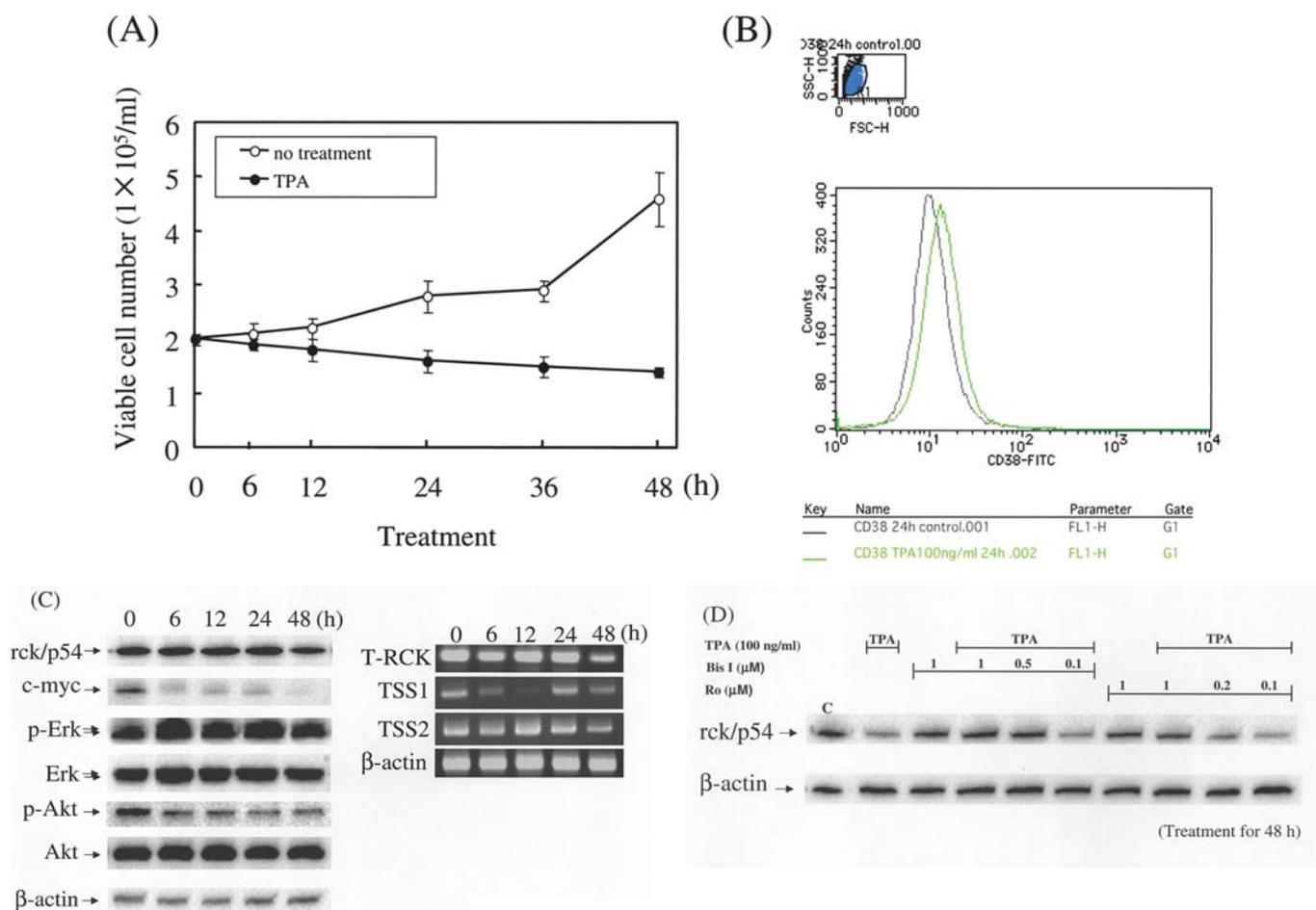


Figure 3. Changes in the expression levels of rck/p54 after treatment of U937 cells with TPA. (A) Cell growth after treatment with TPA (100 ng/ml). The starting cell number was $2 \times 10^5/\text{ml}$. Data represent the mean \pm SD of 3 different experiments, each carried out in duplicate. (B) Expression of the differentiation marker, CD38, at 24 h after the addition of TPA, as examined by FACS. (C) Western blot analysis of rck/p54 (left panel) and RT-PCR of RCKs (right panel). The anti-rck/p54N was used for Western blot analysis. The expression of c-myc, pErk and pAkt proteins was also monitored. The samples were collected at the indicated times. (D) The lowering of rck/p54 expression by TPA was canceled by PKC inhibitors. The inhibitors were co-incubated with TPA at the indicated concentrations. Anti-rck/p54N was used for Western blot analysis. β -actin was used as an internal control. Cells cultured in the presence of 0.05% DMSO were used as a control (C). Bis I, bisindolylmaleimide I; Ro, Ro-32-0432.

of the floating cells were attached to the plate; and apoptotic cells were seen (data not shown). The expression of c-myc and pAkt was also decreased in response to TPA treatment at 6 h. rck/p54 and T-RCK levels were eventually decreased at 48 h after treatment with fluctuation of the levels, as well as TSS1 and TSS2 during 6-24 h (Fig. 3C). Co-incubation with PKC inhibitors such as Bis I and Ro clearly canceled the effect of TPA on the rck/p54 expression at 48 h (Fig. 3D). The levels of expression were recovered in a concentration-dependent manner. At the same time, the PKC activity and number of viable cells in the presence of the PKC inhibitor recovered almost to those for the control (data not shown).

RCK gene silencing by RNAi. In order to confirm a role for rck/p54 in cell growth, we inactivated RCK expression in HeLa cells by using RNAi. The treatment with siRNA for RCK induced significant growth inhibition in HeLa cells, as shown in Fig. 4. The efficiency of transfection, as determined from the control siRNA-fluorescence, was $>90\%$ (data not shown). Apparently, in RCK siRNA-treated wells, the viable cell number was decreased to $\sim 50\%$, compared with that in the control (lipofection buffer-treated cells) at 36 h after the transfection (Fig. 4A). Although the growth of non-specific

(NS) siRNA-treated cells was slightly suppressed after the transfection, the efficacy of RCK RNAi was significant. Concurrently, the level of rck/p54 protein in siRNA-treated cells was reduced, compared with that in control cells or non-specific siRNA-treated cells, as found by Western blot analysis (Fig. 4B, upper panel). Moreover, the level of T-RCK also declined after the transfection in a manner almost parallel to that in the protein level (Fig. 4B, lower panel). Notably, cell-cycle analysis at 36 h after transfection revealed a typical S arrest pattern in siRNA-treated cells (Fig. 4C). Moreover, the level of c-myc which promotes cell cycle was considerably decreased in siRNA-treated cells. These results, like the above ones, indicate that rck/p54 contributed to the maintenance of cell growth and that the growth inhibition by RNAi was due to perturbed cell cycle.

rck/p54 interacts with eIF4E. The results from the RCK gene silencing of HeLa cells suggested that down-regulated rck/p54 may induce a deregulation of translation-initiation control, leading to cell growth inhibition, possibly by perturbation of expression of genes involved in the cell cycle. On the basis of a large amount of experimental data, one of the eukaryotic translation initiation factors, eIF4A, which forms part of the

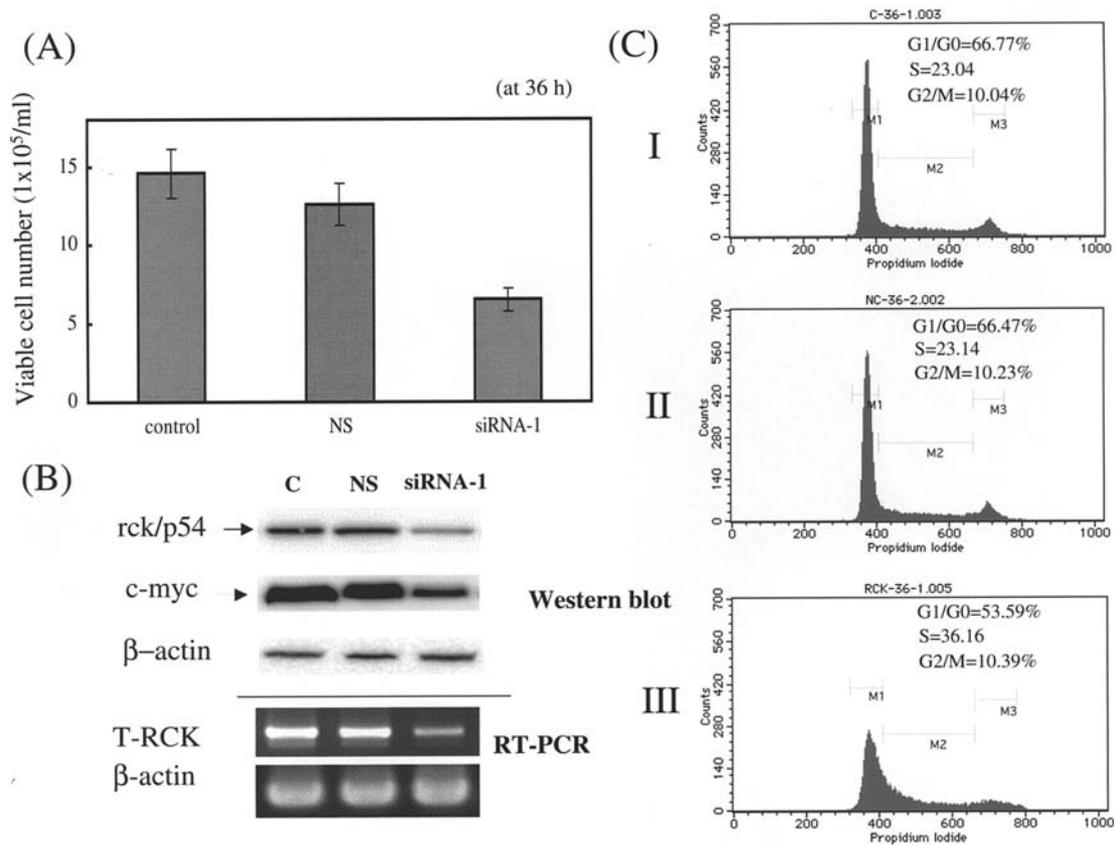


Figure 4. Growth inhibition of HeLa cells by RNAi of *RCK*. (A) Number of viable siRNA-treated or control cells at 36 h after the treatment. The treatment was performed at 24 h after inoculating cells into the culture dishes ($1 \times 10^5/\text{ml}$). Data represent the mean \pm SD of 3 different experiments, each carried out in duplicate. (B) Western blot and RT-PCR analyses of *RCK*. The anti-*rck/p54*N was used for Western blot analysis. RT-PCR of T-*RCK*. Lane contents are the same as in the upper panel. β -actin was used as an internal control. (C) Cell-cycle analysis of control or siRNA-treated cells by FACS. The percentage of cells in each phase is indicated: (I) cells incubated in the medium containing transfection buffer; (II) cells treated with non-specific control duplex; (III) cells treated with siRNA-1 of *RCK*. The experiment was performed at 36 h after transfection. Data representative of those from 3 independent experiments are shown.

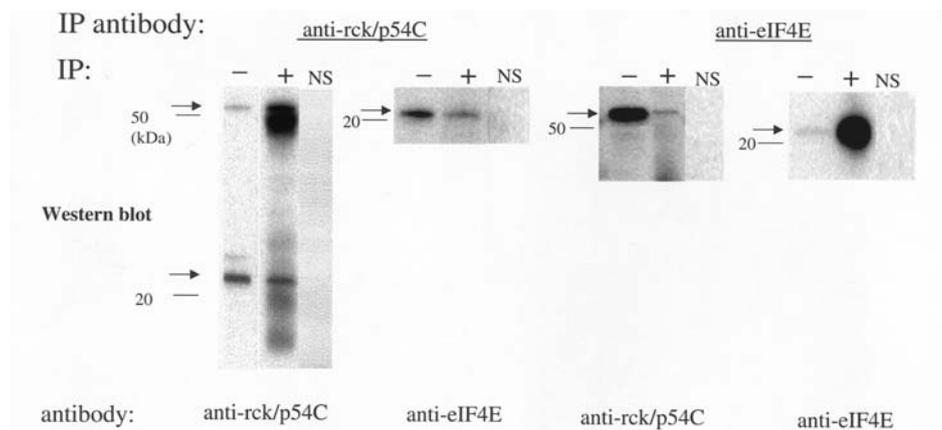


Figure 5. Immunoprecipitation of *rck/p54* or eIF4E from lysates of HeLa cells by using anti-*rck/p54C* or anti-eIF4E antibody. The cell lysates were firstly absorbed with rabbit or mouse anti-IgG coated protein A-Sepharose beads. *rck/p54*-specific immunoprecipitates (IP) (left panel) and eIF4E-specific IP (right panel) from cell lysates were analyzed by Western blotting using either antibody. For a negative control, we also performed immunoprecipitation with preimmune-serum ($\times 3,000$). The antibodies used for immunoprecipitation and Western blot analyses are indicated. In the first lane of each blot, cell lysate (-) was applied. IP sample, +; NC, negative control. In Western blot of *rck/p54* using the sample of *rck/p54*-specific IP, rabbit anti-*rck/p54C* antibody reacted with its IgG (~ 50 kb), and detected the form of proteolytic *rck/p54* (27 kb), which was also seen in *rck/p54*-specific IP.

cap-binding complex including eIF4E, is known to unwind or rearrange RNA-duplex structures at the 5'-end of eukaryote mRNA to prepare it for scanning by the small ribosome subunit (23). In addition, *rck/p54* is known to repress translation by

enhancing decapping (24). In order to confirm the association of *rck/p54* with the eIF4E, we performed immunoprecipitation using anti-*rck/p54C* antibody or anti-eIF4E antibody and non-treated HeLa cells. As shown in Fig. 5, *rck/p54* was clearly

demonstrated to be co-precipitated with eIF4E and, conversely, eIF4E with rck/p54.

Discussion

So far, we have consistently reported data showing that high expression of rck/p54, a human DEAD-box/RNA unwindase, is associated with malignant transformation, especially by contributing to enhancement of potentiality for carcinogenesis by the deregulation of translation initiation control (12,14,17-19,25). In the present study, we demonstrated that rck/p54 plays a role in the maintenance of cell growth probably by modulating the expression of genes involved in cell proliferation at the translation-initiation step, which is in line with the facts that rck/p54 has been verified as an RNA unwindase (14), its homologues have been reported to be associated with translational control (15,24,25) and, in the current study, rck/p54 interacted with eIF4E.

Our results on the *RCK* genomic structure and the characteristics of the transcription start sites and their possible promoter region showed that the TSS2 transcript, originating from exon 1b, was the major one. TSS1, the longer transcript from exon 1a, may function as a reservoir, because the change in the ratio of TSS1 to T-RCK was greater than that in the ratio of TSS2 to T-RCK in response to growth or differentiation stimuli. It is certain that variation in their usage in response to various external stimuli exists. In the tumor cell lines tested, the TSS1 transcript may be relatively more prevalent in the cell lines having a higher growth rate, such as U937, K562, SW480, and PC3 cells, than in those having a lower growth rate, such as HL60, NB4 and LNCaP (Fig. 1E). Based on the nucleotide sequence of the possible promoter region, there was only 1 SP1 binding site in the upstream of exon 1b, whereas there were several E2F and AP1 binding sites in the upstream of exon 1a. Therefore, TSS1 would be more responsive to external stimuli, whereas TSS2 may be constitutively generated. Further detailed functional studies are needed for better understanding of the significance of these differences.

The RNA metabolism processes include, among others, pre-mRNA splicing, ribosome biogenesis, mRNA export, translation initiation, organellar gene expression and RNA decay. DEAD-box proteins are known to be associated with all processes of RNA metabolism (26). They probably serve as links or control elements for such processes. rck/p54 belongs to the RNA helicase SF2/DDX6 subfamily, which is highly conserved from trypanosomes to humans (15). This subfamily includes *Xenopus* Xp54 (27), *Drosophilla* Me31B (28), *C. elegans* Chh-1 (29) and *Saccharomyces cerevisiae* Dhh-1 (24). Members of the DEAD-box superfamily are involved in a variety of cellular processes, including splicing, ribosome biosynthesis, RNA transport, translation initiation and RNA decay.

Xenopus Xp54, an integral component of stored mRNP in oocytes, is present at constant levels throughout oogenesis (27). This RNA helicase was shown to possess ATP-dependent duplex unwinding activity, to be a shuttling protein implicated in the nuclear assembly of stored mRNP particles and, further, to repress translation 3- to 5-fold; however, mutations in its DEAD and HRIGR helicase motifs activated translation to a

3- to 4-fold greater extent relative to the control (30). Furthermore, the *Drosophilla* homologue, Me31B, participates in the translational silencing of oscar and BicD mRNAs in oocytes (28), whereas yeast Dhh1 enhances decapping and interacts with both the decapping and deadenylase complexes in discrete cytoplasmic processing bodies (24). Collectively, these findings indicate that the conserved role of this RNA helicase family is down-regulating mRNA expression mainly by decapping at the level of eIF4E interaction, in which the cap-binding protein upon oligomerization is addressed to the 5' cap structure.

So far, in our examination of the expression of rck/p54 in malignant cell lines, all the cell lines tested expressed a large amount of rck/p54, even the malignant cell lines that originated from the tissues in which rck/p54 was shown to be poorly expressed (12). However, genomic aberrations such as point mutation, amplification and deletion specific to *RCK* were not observed in cancers. In typical induction systems of differentiation using TPA to treat hematopoietic tumor cell line U937, the level of rck/p54 expression was decreased during differentiation and almost paralleled the changes in the levels of c-myc and pAkt, which quickly respond to growth and survival signals. Such findings were also observed during differentiation induced by dibutyryl cyclic AMP in HL60 cells (data not shown). RNAi of *RCK* in HeLa cells induced significant growth inhibition with S arrest and down-regulation of c-myc (31). Thus, rck/p54 appears to be necessary for maintaining cell growth, even in quick response to external growth stimuli, but not for gene expression directed toward cell differentiation. Based on these findings, we could not conclude that rck/p54 functions only as a translational repressor in cancer cells. According to the effect of rck/p54 on the cell cycle, it was reported that *Saccharomyces cerevisiae* Dhh1 modulated mRNA metabolism in the recovery from G1/S cell-cycle arrest following DNA damage (32).

Minshall *et al* already reported the interaction of Xp54 with eIF4E in *Xenopus* oocytes (27). In the current study, we also demonstrated the interaction of rck/p54 with eIF4E. It is reasonable to think that high-expression of rck/p54 in cancer cells could positively function in cell-cycle progression, i.e. being a driving force for the expression of cell growth-related genes. Our study on crystallization of rck/p54 protein (33) and surface plasmon resonance assay indicated that rck/p54 has very high affinity for RNAs. Moreover, we previously reported that rck/p54 stabilized *c-myc* mRNAs and increased the translation-initiation efficiency in colon cancer SW480 cells (19). Collectively, the data suggest that rck/p54 probably promotes cell growth-related gene expression at translation-initiation machinery by cooperating with eIF4E or eIF4E-complex proteins in cancer cells.

It is physiologically significant that rck/p54 expression levels of peripheral blood lymphocytes were increased in response to Con-A stimulation, because rck/p54, a modifier of RNA structure, functions to promote cell growth, even in non-cancerous cells in response to external growth stimuli. The signal cascades for cell growth affected the expression levels of rck/p54. Of course, this is not necessarily linked to carcinogenesis directly; however, it is possible that persistent external growth stimuli enhance the levels of rck/p54, resulting in the elevated translation efficiency of oncogenes associated with cell-cycle progression, such as *c-myc* and *ras*, which would

cause a genomic instability leading to higher potentiality for carcinogenesis. It is important to disclose the signal cascade upstream of rck/p54 which has possible phosphorylation sites (34).

Attacking cancer cells at the molecular level is a new therapeutic approach in cancer treatment with a tremendous amount of promise. Many hurdles still limit the overall success of developing drugs which interfere in carcinogenesis. Different strategies are being pursued; some of the most promising are signal-transduction inhibitors, apoptosis-inducing substances, cell growth inhibitors, RNAi and cancer vaccines. Based on our results, it is necessary to disclose mechanisms that influence mRNA biogenesis, quality control and metabolic fate, involving rck/p54 and other RNA-binding proteins associated with RNA metabolism, in order to achieve a fully comprehensive view of post-transcriptional processes operating in carcinogenesis. A better understanding of such mechanisms will provide new strategies for cancer therapy and prevention.

Acknowledgements

We thank K. Nishizawa and H. Yi for their excellent assistance. This study was supported by a Grant-in-Aid for scientific research (to YA, No. 14657061) from the Ministry of Education, Science, Sports and Culture of Japan.

References

- Linder P, Lasko PF, Ashburner M, Leroy P, Nielsen PJ, Nishi K, Schmier J and Slonimski PP: Birth of the D-E-A-D box. *Nature* 337: 121-122, 1898.
- Wassarman DA and Steitz JA: Alive with DEAD proteins. *Nature* 349: 463-464, 1991.
- Pause A, Methot N, Svitkin Y, Merrick WC and Sonenberg N: Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. *EMBO J* 13: 1205-1215, 1994.
- Gorbalenya AE and Koonin EV: Helicases: amino acid sequence comparisons and structure-function relationship. *Curr Opin Struct Biol* 3: 419-429, 1993.
- Semenza GL: Transcriptional regulation of gene expression: mechanisms and pathophysiology. *Hum Mutat* 3: 180-199, 1994.
- Arrick BA, Grendell RL and Griffin LA: Enhanced translational efficiency of a novel transforming growth factor beta 3 mRNA in human breast cancer cells. *Mol Cell Biol* 14: 619-628, 1994.
- Harigai M, Miyashita T, Hanada M and Reed JC: A cis-acting element in the BCL-2 gene controls expression through translational mechanisms. *Oncogene* 12: 1369-1374, 1996.
- Rogers JT, Leiter LM, McPhee J, Cahill CM, Zhan SS, Potter H and Nilsson LN: Translation of the Alzheimer amyloid precursor protein mRNA is up-regulated by interleukin-1 through 5'-untranslated region sequences. *J Biol Chem* 274: 6421-6431, 1999.
- Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD: *Molecular Biology of the Cell*. Garland Publishing. New York, NY, 1989.
- Akao Y, Seto M, Takahashi T, Kubonishi I, Miyoshi I, Nakazawa S, Tsujimoto Y, Croce CM and Ueda R: Molecular cloning of the chromosomal breakpoint of a B-cell lymphoma with the t(11;14)(q23;q32) chromosomal translocation. *Cancer Res* 51: 1574-1576, 1991.
- Akao Y, Seto M, Yamamoto K, Iida S, Nakazawa S, Inazawa J, Abe T, Takahashi T and Ueda R: The RCK gene associated with t(11;14) translocation is distinct from the MLL/ALL-1 gene with t(4;11) and t(11;19) translocations. *Cancer Res* 52: 6083-6087, 1992.
- Akao Y, Marukawa O, Morikawa H, Nakao K, Kamei M, Hachiya T and Tsujimoto Y: The rck/p54 candidate proto-oncogene product is a 54-kilodalton D-E-A-D box protein differentially expressed in human and mouse tissues. *Cancer Res* 55: 3444-3449, 1995.
- Rabbitts TH: Chromosomal translocations in human cancer. *Nature* 372: 143-149, 1994.
- Akao Y, Yoshida H, Matsumoto K, Matsui T, Hogetsu K, Tanaka N and Usukura J: A tumour-associated DEAD-box protein, rck/p54 exhibits RNA unwinding activity toward c-myc RNAs *in vitro*. *Genes Cells* 8: 671-676, 2003.
- Minshall N, Thom G and Standart N: A conserved role of a DEAD box helicase in mRNA masking. *RNA* 7: 1728-1742, 2001.
- Cougot N, Babajko S and Seraphin B: Cytoplasmic foci are sites of mRNA decay in human cells. *J Cell Biol* 165: 31-40, 2004.
- Nakagawa Y, Morikawa H, Hirata I, Shiozaki M, Mastumoto A, Maemura K, Nishikawa T, Niki M, Tanigawa N, Ikegami M, Katsu K and Akao Y: Overexpression of rck/p54, a DEAD box protein, in human colorectal tumours. *Br J Cancer* 80: 914-917, 1999.
- Miyaji K, Nakagawa Y, Matsumoto K, Yoshida H, Morikawa H, Hongou Y, Arisaka Y, Kojima H, Inoue T, Hirata I, Katsu K and Akao Y: Overexpression of a DEAD box/RNA helicase protein, rck/p54, in human hepatocytes from patients with hepatitis C virus-related chronic hepatitis and its implication in hepatocellular carcinogenesis. *J Viral Hepat* 10: 241-248, 2003.
- Hashimoto K, Nakagawa Y, Morikawa H, Niki M, Egashira Y, Hirata I, Katsu K and Akao Y: Co-overexpression of DEAD box protein rck/p54 and c-myc protein in human colorectal adenomas and the relevance of their expression in cultured cell lines. *Carcinogenesis* 22: 1965-1970, 2001.
- Akao Y, Mizoguchi H, Ohishi N and Yagi K: Growth inhibition by overexpression of human DEAD box protein rck/p54 in cells of a guinea pig cell line. *FEBS Lett* 429: 279-283, 1998.
- Mishima S, Narita Y, Chikamatsu S, Itoh Y, Ohta S, Yoshida C, Araki Y, Akao Y, Suzuki K and Nozawa Y: Effects of propolis on cell growth and gene expression in HL-60 cells. *J Ethnopharmacol* 99: 5-11, 2005.
- Matsumoto K, Oh-Yong K, Hyungtae K and Akao Y: Expression of rck/p54, a DEAD-box RNA helicase, in gametogenesis and early embryogenesis of mice. *Dev Dyn* 233: 1149-1156, 2005.
- Rogers GW Jr, Komar AA and Merrick WC: eIF4A: the godfather of the DEAD box helicases. *Prog Nucleic Acid Res Mol Biol* 72: 307-331, 2002.
- Coller JM, Tucker M, Sheth U, Valencia-Sanchez MA and Parker R: The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA* 7: 1717-1727, 2001.
- Abdelhaleem M: Overexpression of RNA helicases in cancer. *Anticancer Res* 24: 3951-3953, 2004.
- Rocak S and Linder P: DEAD-box proteins: the driving forces behind RNA metabolism. *Nature Rev Mol Cell Biol* 5: 233-241, 2004.
- Ladomery M, Wade E and Sommerville J: Xp54, the *Xenopus* homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res* 25: 965-973, 1997.
- Nakamura A, Amikura R, Hanyu K and Kobayashi S: Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during *Drosophila* oogenesis. *Development* 128: 3233-3242, 2001.
- Navarro RE, Shim EY, Kohara Y, Singson A and Blackwell TK: cgh-1, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. *Development* 128: 3221-3232, 2001.
- Minshall N and Standart N: The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. *Nucleic Acids Res* 32: 1325-1334, 2004.
- Beer S, Zetterberg A, Ihrie RA, McTaggart RA, Yang O, Bradon N, Arvantis C, Attardi LD, Feng S, Reubner B, Cardiff RD and Felsher DW: Developmental context determines latency of MYC-induced tumorigenesis. *PloS Biol* 2: 332, 2004.
- Bergkessel M and Reese JC: An essential role for the Saccharomyces cerevisiae DEAD-box helicase DHH1 in G1/S DNA-damage checkpoint recovery. *Genetics* 167: 21-33, 2004.
- Matsui T, Hogetsu K, Akao Y, Tanaka M, Sato T, Kumasaka T and Tanaka N: Crystallization and X-ray analysis of the N-terminal core domain of a tumour-associated human DEAD-box RNA helicase, rck/p54. *Acta Crystallogr D Biol Crystallogr* 60: 156-159, 2004.
- Smillie DA and Sommerville J: RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles. *J Cell Sci* 115: 395-407, 2004.