# Growth inhibition by overexpression of human DEAD box protein rck/p54 in cells of a guinea pig cell line

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**Abstract We transfected cells of a guinea pig cell line with RCK cDNA inserted in a pIRES1neo expression vector. The overexpression of rck/p54 was confirmed by Western blot and RT-PCR analysis. In two clones expressing rck/p54, the cell growth was highly inhibited; and their anchorage-independent growth, which is an important character of malignant transformation, was not found. These findings are the first evidence that the overexpression of a DEAD box protein/RNA helicase could inhibit substantially cell growth at the translational level.**



*Key words:* RCK gene; rck/p54; DEAD box/RNA helicase; Gene transfection; Growth inhibition; Guinea pig cell

## 1. Introduction

The RCK gene was originally found and characterized by a study on the t(11;14)(q23;q32) chromosomal translocation in a B-cell lymphoma cell line, RC-K8 [1,2]. This gene is fused to an immunoglobulin heavy chain (IgH) gene upon t(11;14) chromosomal translocations, which are observed in about 5% of B-cell lymphomas carrying 14q32 translocations. Although the RCK gene was decapitated at its first intron, the nature of the RCK gene product (rck/p54) is not changed by the t(11;14) chromosomal translocation [2].

It is well known that genes fused to IgH, such as *c-myc* of t(8;14) in Burkitt's lymphomas, and *bcl-2* of t(14;18) in follicular B-cell lymphomas, overexpress their products and show alteration of their regulation, which dysfunction can contribute to tumorigenesis [3]. In the case of t(11;14) in RC-K8 cells, the molecular mechanism of RCK gene in tumorigenesis is also considered to be overexpression of its protein due to deregulation by the fusion to the IgH gene, although the level of mRNA of 7.5 kb is not elevated so much as in the case of *c-myc* or *bcl-2*. We identified the product of the RCK gene as a 54-kDa cytoplasmic protein [4], and rck/p54, of 472 amino acids, was found to be a putative RNA helicase belonging to the DEAD box (D-E-A-D is the single letter code of Asp-Glu-Ala-Asp) protein family, some of which act as modulators of RNA structure. DEAD box proteins have been isolated from organisms ranging from bacteria to humans. rck/p54 possesses eight highly conserved amino-acid motifs shared by the DEAD box family. DEAD box proteins, such as mouse eIF-4A [5], human p68 [6], and *Drosophila* Vasa [7], were verified to have RNA helicase activity. The *Xenopus* rck/p54 homolog, Xp54, which is homologous to rck/p54 by 94%, was also shown to have RNA helicase activity and to facilitate the translation of the genes for oogenesis [8]. The best characterized DEAD box protein, eIF-4A, plays a central role in translation initiation together with two other factors, eIF-4B and the cap-binding protein complex eIF-4F. In cells, eIF-4A exists in a free form or in the eIF-4F complex, which interacts with mRNA to facilitate the translation of the mRNA [9]. Recently, it was reported that a yeast DEAD box protein, Ded1p, is required for the initiation step of translation [10].

The mRNA of RCK is ubiquitously present in human tissues [4]. However, differences in rck/p54 expression between normal tissues and cancer cells originated from these tissues exist to some extent in the brain, skeletal muscle, and lung tissues. Transformed cell lines of brain tumors, rhabdomyosarcomas, and lung cancers were shown to have good expression in spite of the poor expression in the corresponding normal tissues [4]. These findings suggest that the RCK gene is involved in cell growth and carcinogenesis. Therefore, it is of interest to determine how RCK contributes to tumorigenesis in malignant lymphomas and in some solid tumors.

Based on these findings, we wanted to test the hypothesis that overexpression of rck/p54 results in alteration of cell growth, and so we transfected various cell lines with the RCK gene and obtained a guinea pig cell line that showed good expression of human rck/p54. Differing from our supposition, we found that rck/p54 functions to inhibit cell growth and to reduce the tumorigenicity of these cells.

## 2. Materials and methods

### 2.1. Cells

The guinea pig cell line used, 104C1, was established by Evans and DiPaolo [11]. This cell line was originated from a 43-day fetus skin, and established by the treatment with a chemical carcinogen, benzopyrene. The 104C1 cell line possesses tumorigenicity as evidenced by colony formation in soft agar and tumor formation in nude mice and shows the characteristics of anaplastic fibrosarcoma with a loss of orientation including piling up and crisscrossing of cells. The karyotype analysis of 104C1 showed a diploid pattern without any marker chromosomes.

### 2.2. Construction of expression vector for human rck/p54

A human RCK cDNA clone, SA30, was digested with *Bam*HI; and the resultant 1.8-kb fragment including the entire coding region was inserted into a pIRES1neo vector of 5.3 kb, as shown in Fig. 1. The pIRES1neo vector, derived from pCIN4, contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus, and permits the translation of two open reading frames, RCK and neomycin phosphotransferase II (NPT II) cDNA, from one messenger RNA [12]. Cells expressing a high level of rck/p54 can be selected by neomycin

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*Abbreviations:* FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction


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resistance. The final construct, designated pIRES-RCK, was used for gene transfection (Fig. 1). The pIRES1neo and pIRES-fRCK, which contains the 3′ flanking *Hin*dIII 0.8-kb fragment of RCK cDNA [2] in pIRES1neo vector, were used for transfection as controls.

*2.3. Transfection of 104C1 cells*

Cells of the 104C1 guinea pig cell line were cultured in RPMI 1640 medium supplemented with 10% FBS. Exponentially growing cells were transfected with the plasmid by the method using cationic liposomes [13]. Briefly, the cells ($5\times10^5$ cells/60-mm dish) were cultured overnight and then incubated with liposome-entrapped pIRES-RCK, pIRES1neo, or pIRES-fRCK (1 μg DNA/100 nmol of lipids in 1 ml of the medium). After incubation for 16 h, the cells were cultured in fresh medium for one day and then selected with neomycin (G418) at a concentration of 1000 μg/ml, exchanging the medium every three days. After selection for seven days, the living cells were segregated by limiting dilution. Two clones, which were shown by the polymerase chain reaction (PCR) to carry a part of the pIRES-RCK DNA fragment, were obtained at day 20 (the day of transfection is designated as day 0).

*2.4. PCR for detection of pIRES-RCK DNA fragment in RCK gene transfectants*

Cellular DNA from 104C1 and transfected cells was extracted as described previously [14]. Each DNA was amplified by use of two pairs of primers specific for pIRES-RCK: $P_1$ (sense), 5′-TCGCGATGTACGGGCCAGAT; and $P_2$ (antisense), 5′-AATGCAGGGAAGCACCTGTA (Fig. 1). The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). The PCR products were analyzed by electrophoresis on 2% agarose gels.

*2.5. Detection of RCK mRNA by RT-PCR*

Total cellular RNA was isolated by the phenol/guanidium thiocyanate method with DNase I treatment [15]. After reverse transcription of 2 μg of total RNA, cDNA was generated. PCR primers that were used to amplify the cDNA sequences were as follows: for RCK, $P_3$ (sense) 5′-ACTTCGGCGGCGCCACGAGA and $P_4$ (antisense) 5′-AGATCCAGGATTCTCCCAGG; for NPT II, $P_5$ (sense) 5′-ATGGGATCGGCCATTGAACA and $P_6$ (antisense) 5′-TGATCCCCTCAGAAGAACTC (Fig. 1). The 661-bp guinea pig β-actin cDNA product was used for an internal standard. The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). The PCR products were analyzed by electrophoresis on 2% agarose gels.

*2.6. Western blot analysis*

Parent 104C1 cells and transfectants were washed twice with PBS, treated with lysis buffer (2×PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1 mM phenylmethanesulfonyl fluoride), and then homogenized with an ultrasonic homogenizer (Heat Systems-Ultrasonics). The homogenized samples were used without centrifugation. Ten micrograms of lysate protein were separated by SDS-PAGE using a 12% polyacrylamide gel and electroblotted onto a PVDF membrane (Du Pont). After blockage of non-specific binding sites for 1 h with 5% non-fat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4°C with anti-human rck/p54 antibody [4] at a dilution of 1:300. The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega) at room temperature, and then washed three times with TPBS. The immunoblot was visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs).

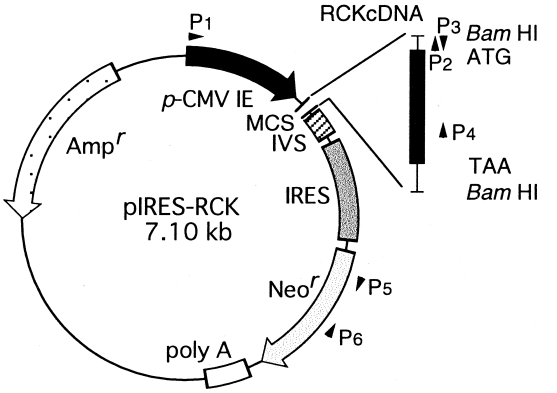


Fig. 1. Schematic diagram of the expression vector for human RCK. The relative locations of functional elements in the expression vector pIRES-RCK are indicated. The locations of primers used in this study are indicated by arrowheads.

*2.7. Evaluation of cell growth characteristics*

Cells in mass culture or in colonies developing from sparsely seeded cells were inspected for cell number and morphological changes by phase-contrast microscopy. Cell viability was assessed by a dye exclusion test. Plating efficiencies (the average number of colonies per 60-mm dish divided by the number of cells seeded per dish×100) were determined 14 days after inoculation of 500 cells. The ability of cells to grow as colonies in or on the soft agar was assessed. Briefly, $10^3$ individual trypsinized cells were suspended in 5 ml RPMI 1640 medium/10% FBS/0.35% liquid Bacto-agar (Difco Laboratories, Inc.) at 40°C, immediately poured onto a 4-ml basal layer of RPMI 1640 medium/10% FBS/0.5% Bacto-agar in a 60-mm dish, and incubated at 37.5°C. Colony formation was examined for the presence of colonies growing in the 0.35% agar layer or on the soft agar by phase-contrast light microscopy at 7, 14, 21, and 28 days after seeding. In all procedures, neomycin selection was continued.

## 3. Results

*3.1. Gene transfection and isolation of rck/p54-expressing cells*

The expression vector construct including RCK cDNA encoding a 472 amino acid sequence is shown in Fig. 1. This construct was used for transfection of 104C1 cells by means of cationic liposomes, and the pIRES1neo and pIRES-fRCK vectors were employed to transfect 104C1 cells for controls. After the transfection we selected the transfected cells with neomycin at 1000 μg/ml. We started to isolate the transfected cells by limiting dilution on day 7 in each transfection. As a result, we got transfectants of pIRES1neo and pIRES-fRCK

Table 1
Growth properties of clones expressing rck/p54 and those of controls

| Cell line | Growth in monolayer cells | | | Colony formation in or on agar |
|---|---|---|---|---|
| | Doubling time (h)[a] | Total RNA[b] (μg) | Plating efficiency[c] (%) | |
| 104C1 | 24 ± 1.1 | 58.2 | 82.2 | +[d] |
| 104C1-IRES | 44 ± 2.8 | 43.3 | 21.8 | +[d] |
| 104C1-RCK-1 | 96 ± 2.1 | 30.9 | 2.1 | 0 |
| 104C1-RCK-2 | 86 ± 2.0 | 30.2 | 2.6 | 0 |

[a]Mean ± S.D. is given ($n=12$).
[b]The mean value of total RNA obtained from $5\times10^5$ cells.
[c]Average number of colonies per dish obtained 10 days after seeding of 500 cells/dish.
[d]Formation of 1 or more colonies in or on agar per $10^3$ seeded cells.

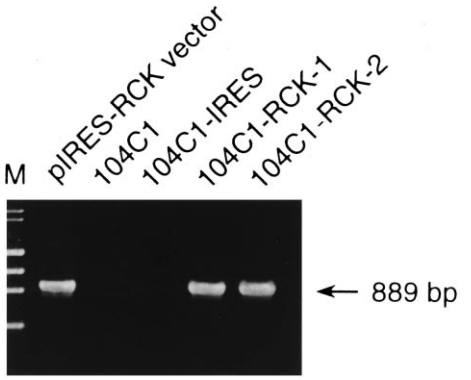


Fig. 2. PCR detection of the DNA fragment containing the promotor of pIRES1neo expression vector and a part of RCKcDNA with $P_1$ and $P_2$. DNA samples were from the following: pIRES-RCK vector, 104C1 cells, 104C1-IRES cells, 104C1-RCK-1 cells, 104C1-RCK-2 cells. The products were applied to a 2% agarose gel and stained with ethidium bromide. The arrow shows the expected size of the PCR product.

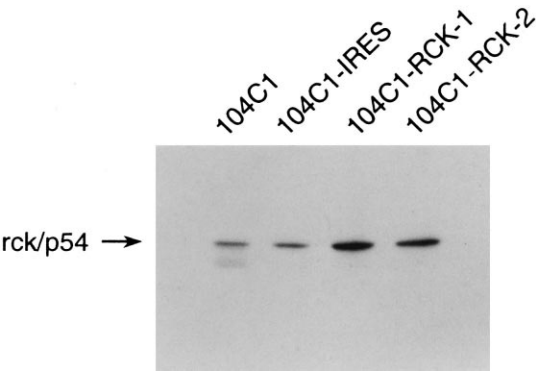


Fig. 3. Western blot analysis of rck/p54. Cell protein lysates containing 10 μg/protein from 104C1, 104C1-IRES, 104C1-RCK-1, and 104C1-RCK-2 cells were subjected to SDS-PAGE in a 12% gel. Lanes are as indicated in the figure.

vector and each transfectant was named 104C1-IRES and 104C1-fRCK cells, respectively. Among them, 104C1-IRES cells were used for a representative control except for the experiment of cell growth curve.

PCR study using pIRES-RCK specific $P_1$ and $P_2$ primers (Fig. 1) showed that among pIRES-RCK transfectants, two clones contained the DNA fragments from the pIRES-RCK vector. These two clones were named 104C1-RCK-1 and -2. The result of PCR analysis of 104C1-RCK transfectants is shown in Fig. 2. The 889-bp DNA fragment was detected only in pIRES-RCK vector, 104C1-RCK-1, and -2 cells.

The amount of rck/p54 protein, of approximately 54 kDa, in these cell lines was determined by Western blot analysis (Fig. 3). Both pIRES-RCK-transfected cell lines, 104C1-RCK-1 and -2, produced a much larger amount of human rck/p54 than parent or 104C1-IRES cells. The amount of rck/p54 in 104C1-RCK-1 cells was approximately two times larger than that in 104C1-RCK-2 (Fig. 3). Earlier, we cloned the mouse RCK homologue and found its deduced amino acid identity to human rck/p54 to be 92.5% [16]. Since it is reasonable that anti-human rck/p54 antibody crossreacts with the endogenous guinea pig rck/p54 and its molecular weight seems to be similar to that of human rck/p54, we performed RT/PCR to confirm human RCK mRNA expression by using human-specific primers $P_3$ and $P_4$ for RCK cDNA (Figs. 1 and 4). Although in 104C1 and 104C1-IRES cells the RT-PCR product was almost undetectable, both 104C1-RCK-1 and -2 cells were shown to produce large amounts of the DNA fragments. Therefore, the difference between the amount of rck/p54 protein in 104C1-RCK-1 or -2 cells and that in parent 104C1 or 104C1-IRES cells shown by Western blot analysis is due to the introduced pIRES-RCK vector. Thus, we confirmed that 104C1-RCK-1 and -2 cells expressed human rck/p54.

### 3.2. *Evaluation of cell growth characteristics in rck/p54-expressing cells*

As was shown in Fig. 3, 104C1-RCK-1 cells expressed rck/p54 more than did 104C1-RCK-2 cells. For each clone, the cell growth curve is shown in Fig. 5. The growth properties of cells expressing rck/p54 were markedly different from those of parent and pIRES-transfected cells (Table 1). The growth of both 104C1-RCK-1 and -2 cells was extremely suppressed compared with that of parent, 104C1-IRES, or 104C1-fRCK cells. There was no significant variation in control transfectants. A good correlation between the rck/p54 expression and suppression of growth rate was shown in these three cell lines, i.e. 104C1-IRES, 104C1-RCK-1, and -2 cells (Figs. 3 and 5, Table 1). We consider that the difference in growth rate between parent and 104C1-IRES or 104C1-fRCK cells is derived from the effect of these vectors themselves. The ratio of percent planting efficiency of 104C1-RCK-1 and 104C1-IRES cells was approximately 1:10. Also, the doubling time of 104C1-RCK-1 and -2 cells was much longer than that of 104C1-IRES cells or parent cells (Table 1). Thus, the cell growth of 104C1-RCK-1 and -2 cells was extremely inhibited in comparison with that of parent, 104C1-IRES, and 104C1-fRCK cells.

We then examined the effect of expression of rck/p54 on the

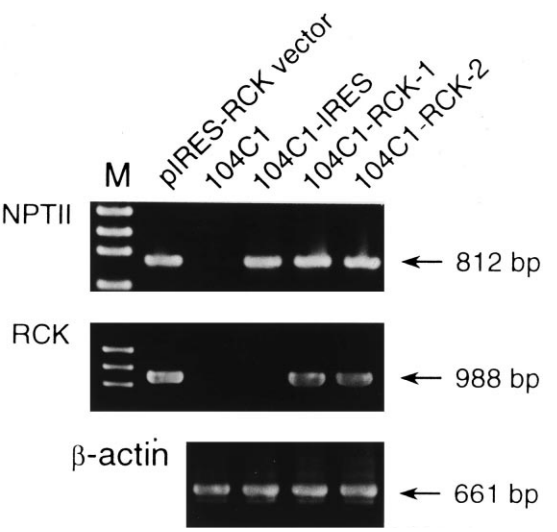


Fig. 4. RT-PCR for detection of mRNA expression of NPT II and RCK cDNA. cDNA samples were prepared from the following: pIRES-RCK vector and 104C1 cells, 104C1-IRES, 104C1-RCK-1, and 104C1-RCK-2 cells. The product of β-actin was used as an internal standard. The products were applied to 2% agarose gels and stained with ethidium bromide. Arrows show the expected sizes of PCR products.

transforming activity of the cells in terms of the formation of colonies in or on agar. Although 104C1-IRES cells had formed several colonies by 14 days after seeding on the soft agar, 104C1-RCK-1 and -2 cells formed no colonies either in or on the soft agar even by 28 days after seeding. The saturation density for piling up growth was also much lower in 104C1-RCK-1 and -2 cells than in 104C1-IRES cells and parent cells (data not shown). Clearly, 104C1 cells expressing rck/p54 lost their anchorage independence, which correlates with tumorigenicity in rodent cells [17], and which is an important trait of malignant transformed cells.

### 3.3. Morphological changes in cells expressing rck/p54

104C1-RCK-1 and 104C1-IRES cells were seeded at a density of $5\times10^2$ in 60-mm diameter dishes. The cells and colonies were examined morphologically by phase-contrast light microscopy at 7 and 14 days after seeding. In contrast to those of 104C1-IRES colonies, the diameters of 104C1-RCK-1 colonies at 7 days after seeding were smaller (20–40 cells); and the peripheral cells of the 104C1-RCK-1 colonies tended to be oval-shaped, not spindle, and showed degenerative changes such as marked vacuolation and increased cell volume. Moreover, 104C1-IRES cells lined up directionally, whereas cells of 104C1-RCK-1 were scattered randomly and seemed unable to make contact with each other regularly (Fig. 6). Nuclear changes such as chromatin condensation and segmentation that are characteristic of apoptosis were not found.

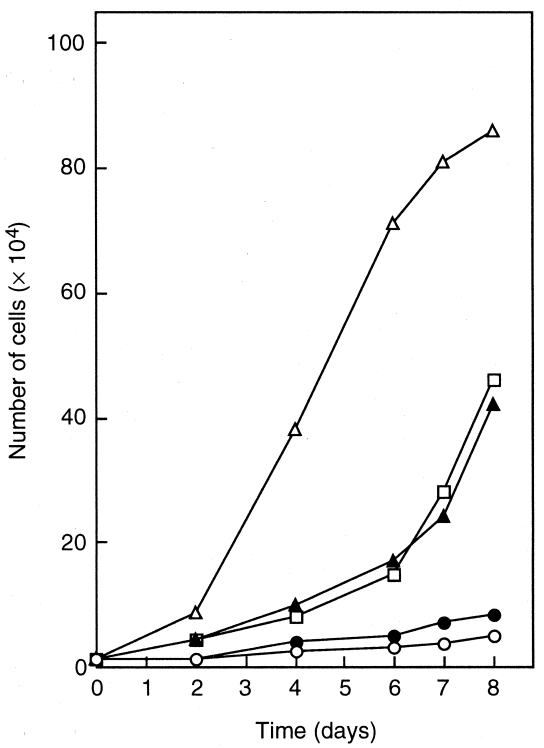


Fig. 5. Growth curves of cells of transfectants expressing rck/p54 and control cell lines. Cells were seeded at $1\times10^4$ cells per 60-mm dish and grown in RPMI 1640 medium supplemented with 10% FBS. Cells were counted on the indicated days. All results are the mean of three experiments carried out in duplicate. ○, 104C1-RCK-1 cells; ●, 104C1-RCK-2 cells; △, parent 104C1 cells; ▲, 104C1-IRES cells; □, 104C1-fRCK cells.

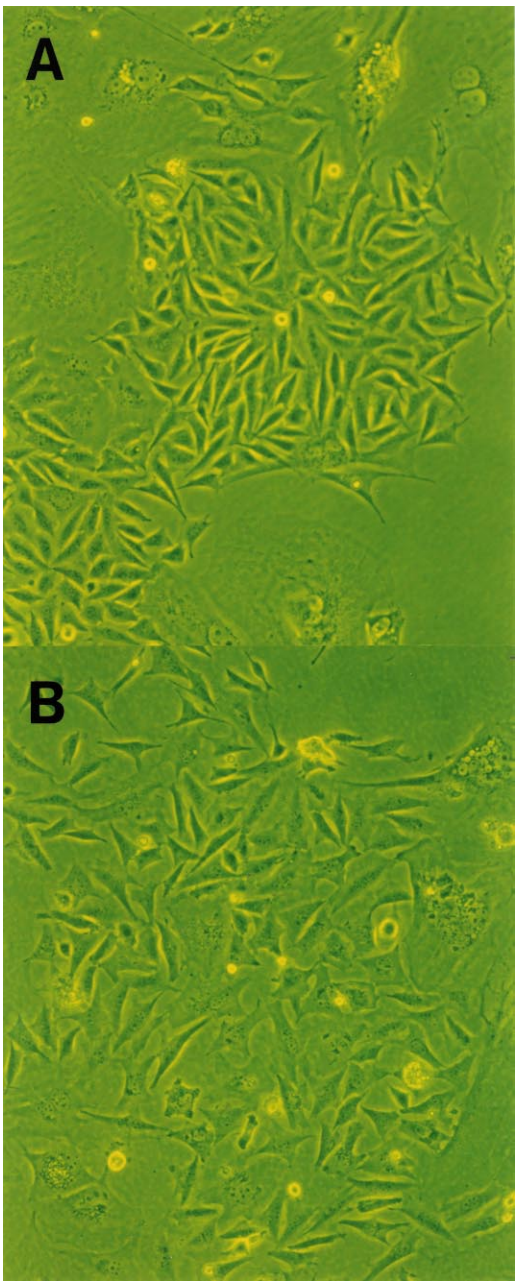


Fig. 6. Morphological aspects of 104C1-IRES and 104C1-RCK-1 cells observed by phase-contrast light microscopy. A: 104C1-IRES cells at 7 days after seeding; B: 104C1-RCK-1 cells at 14 days after seeding.

## 4. Discussion

In the present study, we have provided data that human rck/p54 overexpressed in cells of a guinea pig cell line chemically induced from a fetal carcass caused suppression of cell growth and loss of tumorigenicity. These findings suggest that the regulation at the translational level by the DEAD box protein rck/p54 could be important for cell growth and carcinogenesis.

In our previous study, the amount of rck/p54 in RC-K8 cell line studied by Western blot analysis was larger than that in peripheral blood lymphocytes or in some leukemia cell lines. In the brain, skeletal muscle, and lung tissues, the expression

of rck/p54 is very poor, but tumors that originated from these tissues expressed a significant amount of rck/p54 [4]. Thus, the deregulation, i.e. overexpression, of rck/p54 expression may be linked to tumorigenesis in some tumors.

Recently, a DEAD box protein, MrDb, was reported to be one of the target proteins of the *c-myc* gene that positively functions in cell proliferation [18]. From the report of Ladomery et al. [8] that Xp54, *Xenopus* homologue of rck/p54, possesses RNA helicase activity, rck/p54 could also have an RNA helicase activity. Accordingly, it was expected that rck/p54 could be associated with the translation of oncogenes or growth-related genes.

The reason why our experiments gave results contradictory to those found for the B-cell line RC-K8 is not clear at present, but we can suggest two hypotheses. First, if the expression of certain oncogenes or growth factors is tightly controlled at the translational level by the availability of rck/p54 homologues, and human rck/p54 overexpressed combines with the guinea pig translation initiation factors, its complex may work against the guinea pig rck/p54 homologue in a dominant negative manner, resulting in suppressed translation of genes that are necessary for cell proliferation. In this regard, we cloned the partial cDNA of guinea pig RCK gene and its deduced 472-amino acid sequence showed a good homology to human rck/p54 by 99.1% with complete agreement in eight consensus motifs of DEAD box protein (unpublished data). Among four differences of amino acids, the two localed relatively close to each other between consensus motifs VI and VII, are transversions from serine to proline and isoleucine to threonine. These transversions may affect the higher structure of protein. Second, human rck/p54 may promote the translation of genes for cell differentiation in guinea pig cell line 104C1. This idea stems from the finding that DEAD box/RNA helicase genes such as Ste13 and ME31B, which are homologous to rck/p54 by more than 70%, function in sex differentiation. For example, ME31B is expressed only in oocytes to yield a protein that functions during early embryogenesis [19]. Also, Ste13 is essential for nitrogen starvation-induced $G_1$ arrest and promotes cells to enter the $G_0$ phase for initiation of sexual development in the fission yeast *Schizosaccharomyces pombe* [20]. Thus, other DEAD box proteins in the subfamily, in which rck/p54 is included [4], are associated with embryogenesis and sexual differentiation.

In any case, our data indicate that the deregulation of the RCK gene encoding a DEAD box protein could lead to inhibition of cell proliferation and of malignant transformation of 104C1 cells.

As for the substrate specificity of RNA helicase, it is known that eIF-4A and the proteins involved in pre-mRNA splicing have specific target sequences or structures in the RNA [5]. However, until recently there has been no report on specific RNA sequence requirements of DEAD box proteins for their in vitro activities of ATPase and RNA helicase. DEAD box protein such as rck/p54 may play different roles in various cells and stages of cell differentiation depending on its substrate and regulation.

These contrary results are true in the case of the *c-myc* oncogene, which is required substantially to lead to physiological cell death or apoptosis [21]. Further, the Wilms' tumor gene (WT1), a suppressor oncogene, when overexpressed in leukemic cells, was shown to be associated with leukemogenesis [22].

For understanding the mechanism of growth suppression by rck/p54, it will be necessary to transfer the RCK gene to additional cell lines to examine whether the phenomenon is specific to 104C1 cells or not, and to use deletion mutants of this gene to analyze which consensus motifs are necessary for this result in the 104C1 cell line. The use of RCK gene transgenic mice may give us a clue to unveil the function of the RCK gene.

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