

## Chromosome 6 encoded RNaseT2 protein is a cell growth regulator

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### Abstract

We have previously shown by chromosome transfer technique that chromosome 6 alters the phenotype of a variety of tumour cells and SV40 immortalized cells. We present here the phenotypic effects of the ectopic expression of RNaseT2, a highly conserved ribonuclease encoded by chromosome 6q27, in SV40 immortalized cell lines. We contrast our findings with those reported for ovarian carcinoma cell lines and an SV40 immortalized cell line transfected with RNaseT2. Although RNaseT2 expression is elevated in normal diploid fibroblasts approaching senescence (passage 64), forced expression of the gene in immortalized cells does not cause them to senesce. A significant reduction was observed in colony forming efficiency, anchorage independence and growth rate of cells transfected with RNaseT2. The levels of transcripts involved in Akt signalling pathway, cell cycle control and pathways related to cell proliferation decreased 2–10-folds in SV40 immortalized cells in response to RNaseT2 expression. Interestingly, some immortalized cells expressed alternatively spliced transcript variants instead of the full-length RNaseT2 transcript. Our results are consistent with the conclusion that RNaseT2 is a cell growth regulator and it does not induce senescence in SV40 immortalized cell lines.

**Keywords:** RNaseT2 • 6q27 • cell senescence • cell growth regulation • cell immortalization

### Introduction

Oncogenes and tumour suppressor genes regulate the growth and development of normal cells. Mutations and aberrant expression of these genes have been observed in a variety of human cancers [1, 2]. Cancer cells are characterized by their ability to escape senescence, which is a form of programmed growth arrest that limits the replicative potential of most somatic cells and also functions as a mechanism of tumour suppression [3–5]. The senescent cells typically have flattened shape and large cytoplasmic vacuoles [6], and can be distinguished histologically by staining with a chromogenic substrate for  $\beta$ -galactosidase [7].

A number of genes that induce replicative senescence in human cancer cell lines have been successfully mapped by chromosome transfer experiments [8–10]. Chromosome 6 from normal human diploid cells, when transferred into tumour cells and SV40 immortalized cells, was shown to confer senescence on the recipient cells [11, 12]. Moreover, immortal revertants of the

senescent clones were found to have consensus deletions in four specific regions of the donor chromosome 6 (R.S. Athwal, unpublished observations). The characterization of one of these regions indicated the deletion of RNaseT2 (formerly known as RNase 6PL), an evolutionarily conserved ribonuclease that has also been identified as a candidate tumour suppressor gene by two other groups using different approaches [13, 14]. RNaseT2 homologues were recognized as regulatory proteins in plants [15] and their presence was subsequently confirmed in bacteria [16], viruses [17], *Drosophila* [18] and human beings [19].

The full-length human RNaseT2 has 256 amino acids and a predicted size of 29 kD. The gene has nine exons that show differential splicing variants in some cDNA isolates [14]. The levels of RNaseT2 were found to be significantly reduced in ovarian carcinoma cell lines, *but* no mutations in this gene were found [13]. While the pRPC and SG10G cells transfected with RNaseT2 retained the parental phenotype, HEY4 and XP12RSOV transfectants acquired a senescent phenotype [13]. Subsequent studies confirmed that the human protein is a catalytically active RNase [20], although it was reported that abolition of its catalytic activity did not alter its effect on tumorigenesis or metastasis [21]. Campomenosi *et al.* [20] showed that the full-length RNaseT2 is a secreted protein, although its proteolytic fragments remain in lysosomes. The mechanism by which a secretory RNase might

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affect tumorigenesis of the producing cell remains a subject of speculation. We describe here previously unreported RNaseT2 splice variants and the effect of ectopic expression of RNaseT2 on the growth properties of two SV40-immortalized cell lines.

## Materials and methods

### Cell lines and culture conditions

The cells used in this study included normal diploid fibroblasts: GM03468A and FS-2; SV40 immortalized human cell lines: CRL-9609, AR5, XPD, XPV2, CSG2 and GM0847; human ovarian tumour cell lines: OVCAR3 and SKOV-3; melanoma cell line WM239A; lung cancer cell line A549 and glioblastoma cell line U87MG3. Some of these cell lines were purchased from commercial sources while others were either produced in the laboratory or received as gift from other investigators. RA6A-1-1 and RA6A-1-2 are immortal revertant clones which arose from senescent microcell hybrids of c139 carrying a normal human donor chromosome 6 [11]. The c139 is an SV40 immortalized clone of human diploid fibroblast. All cell lines were cultured in DF-12 medium containing 10% foetal bovine serum, 1% L-glutamine and 1% antibiotic and antimycotic solution. The medium was supplemented with 400 µg/ml G418 (Sigma, St. Louis, MO, USA) for selection of gene transfer clones.

### Cloning of full length RNaseT2 cDNA and transfection into mammalian cells

Full-length RNaseT2 cDNA was obtained by RT-PCR of RNA from FS2 cells. The complete RNaseT2 coding sequence was cloned in a mammalian expression vector pCDNA3.1<sup>+</sup> in frame with a FLAG epitope tag. Forward primer F-KpnI-Kozak-Flag-365 (5'-CGG GGT ACC GCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG ATG CGC CCT GCA GCC CTG-3') has an in frame KpnI site, Kozak sequence and a 27 base FLAG sequence followed by the start codon and 15 bases specific to the full length RNaseT2 coding sequence. Reverse primer R-XbaI-Stop-RNaseT2 (5'-GC TCT AGA TCA ATG CTT GGT CTT TTT AGG TGG-3') has an XbaI site followed by the reverse complement of the stop codon and 21 bases of RNaseT2 specific sequence immediately upstream of the stop codon. The first strand cDNA generated by RT was PCR amplified using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR product was purified using GeneClean kit (Qbiogene, Inc., Carlsbad, CA, USA) and digested with KpnI and XbaI. The purified fragment was subsequently ligated to pCDNA3.1<sup>+</sup> vector (Invitrogen) that had been digested with KpnI and XbaI restriction enzymes. The ligated mixture was used to transform DH5 $\alpha$  competent cells. The specific clones were verified by sequencing and preserved for further experiments.

The full-length cDNA, cloned into pCDNA3.1<sup>+</sup>, was transfected into cultured cells using the calcium phosphate precipitation method. Approximately 10 µg of DNA was dissolved in 250 µl of 2 $\times$  HBS medium and mixed with 250 µl of CaCl<sub>2</sub> (250 mM) at room temperature to prepare a CaCl<sub>2</sub>/HBS/DNA precipitate in a sterile 1.5 ml tube. The DNA precipitate was kept at room temperature for 20 min. before adding to a culture dish containing a monolayer of cells (2  $\times$  10<sup>5</sup>), and the dish was incubated at 37°C. The medium was replaced with fresh complete medium after 24 hrs.

Following 48 hrs of incubation, the medium was replaced with selection medium containing G418 (400 µg/ml). The colonies that formed in the selection medium were either isolated individually or their phenotypes were observed in parent plates.

### Analysis of gene transfer clones

Gene transfer colonies were maintained in selection medium and examined for cell and colony morphology using a phase contrast microscope. The expression of RNaseT2 protein in cells from each colony was analysed by Western blotting.

### Western blot hybridization

The cells were lysed with RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM ethylenediaminetetraacetic acid and 50 mM Tris-HCl, pH 7.5) and total protein was quantified using Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA). The lysate (~25 µg proteins) was fractionated on 8% polyacrylamide-SDS gel and the proteins were electrotransferred onto a nitrocellulose membrane. The membrane was incubated in a PBS solution containing 10% non-fat dried milk (Bio-Rad Laboratories, Hercules, CA, USA) and 0.1% Tween (Sigma) for 1 hr. The membrane was subsequently incubated with a monoclonal mouse anti-Flag antibody M5 (1:70 dilution) in PBS solution containing 4% non-fat dried milk and 0.1% Tween. Following an incubation of 12 hrs, the membrane was washed six times with 0.1% Tween in PBS and incubated with secondary antibody at a dilution of 1: 2500 in 4% non-fat dried milk solution at room temperature for 1 hr. The membrane was washed and signal was detected using a chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA, USA) according to the manufacturer's protocol. Subsequently, membranes were stripped and incubated with monoclonal mouse anti-human  $\beta$ -actin antibody (Sigma) to assess variations in the protein loading.

### Cell growth, colony formation and anchorage independency assays

Cell proliferation was measured using TACS – MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation assay kit (Trevigen, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, cells (2.5  $\times$  10<sup>3</sup>) were incubated in 100 µl of culture medium in a flat-bottomed 96-well plate (Corning Inc., Corning, NY, USA), and cell density was measured every 24 hrs for a period of 7 days. At each time-point MTT reagent (10 µl) was added to each well and plates were incubated for 4 hrs at 37°C in the dark to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. The formazan dye was solubilized with a detergent and the absorbance was measured at 570 nm in a microplate reader (Beckman Coulter Inc., Fullerton, CA, USA). The average value of six independent absorbance readings was calculated and plotted against each time-point.

For colony formation assay, 250 cells were plated in a 10 cm tissue culture dish either in complete DF-12 medium or selection medium containing G418. After 10 days, plates were fixed with methanol for 10 min. and stained with crystal violet to count colonies.

To measure anchorage independence, 5000 viable cells were suspended in 0.35% agar and overlaid on 0.5% agar in 60 mm tissue culture dishes. The dishes were incubated at 37°C in 7.5% CO<sub>2</sub> incubator for 14 days and colonies were counted and photographed following staining with 0.05% NTB (Sigma).

## Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed to compare the relative levels of RNaseT2 expression in different cell lines. An aliquot (2  $\mu$ l) of the first strand cDNA, prepared from total cellular RNA, was amplified in a 25  $\mu$ l reaction mixture containing 2 mM MgCl<sub>2</sub>, 1 $\times$  PCR buffer, 200  $\mu$ M of dNTPs, 1.0  $\mu$ M of primers specific for RNaseT2, 1  $\mu$ M of primers specific for GAPDH and 1 unit Taq polymerase (Promega, Madison, WI, USA). Amplifications were performed with initial denaturation at 96°C for 5 min., followed by varying number of cycles of incubations at 94°C (1 min.)/58°C (1 min.)/72°C (2 min.). PCR products at cycles 18, 22, 26, 30 and 34 were compared among different cell types. The GAPDH product, which was amplified in the same tube was used as an internal control. PCR products were fractionated by electrophoresis in 2% agarose gel and the density of each band was analysed using Alphamager™ 2200 (Alpha Innotech, Alexandria, VA, USA). The amplification of GAPDH and RNaseT2 transcripts in the same tube was compared with amplification of individual transcripts performed separately.

## Sequences of primers

The sequences of some primers have been described in the text in specific sections of the methods or results. The sequences of other primers used for PCR are presented here:

F180: 5'-CGC GAG ATA CTG GTT TAG GC-3'

R588: 5'-CAT CCT TCA CTT TTA TCG GGC-3'

RNaseT2 F583: 5'-AGG ATG TAA TAG ATC GTG GC-3'

RNaseT2 R1158: 5'-AGA ATA TTT CCA AAA CTT GGG-3'

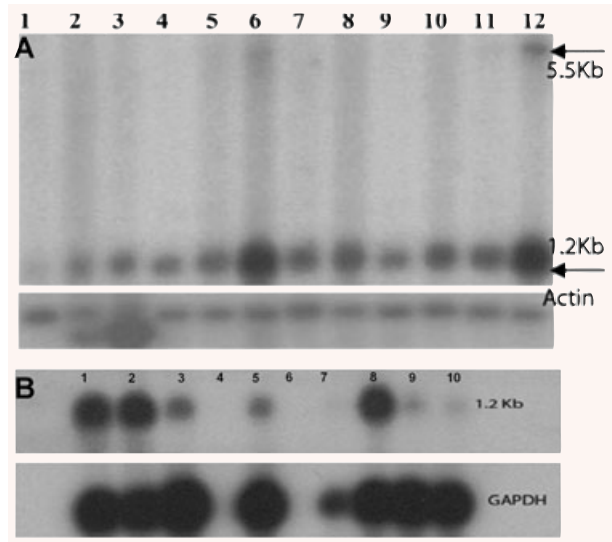
## Northern blot hybridization

Multiple-tissue Northern blots were purchased from CLONTECH (GIBCO BRL Life Technologies, Carlsbad, CA, USA), and the polyA RNA blots for the tumour cell lines were prepared in the lab. Poly(A)<sup>+</sup> RNA, purified from cultured cell lines using the oligotex mRNA midi kit (Qiagen Inc., Valencia, CA, USA), was fractionated in 0.8% denaturing agarose gels and transferred to a nylon membrane using standard protocols [22]. Blots were hybridized with a [<sup>32</sup>P] labelled 1.2 kb RNaseT2 cDNA probe. The probe DNA bound to membranes was removed by incubation in 0.5% SDS at 100°C and the membranes were subsequently hybridized with a GAPDH probe to assess for the loading variations.

## Results

### RNaseT2 expression in human tissues and cell lines

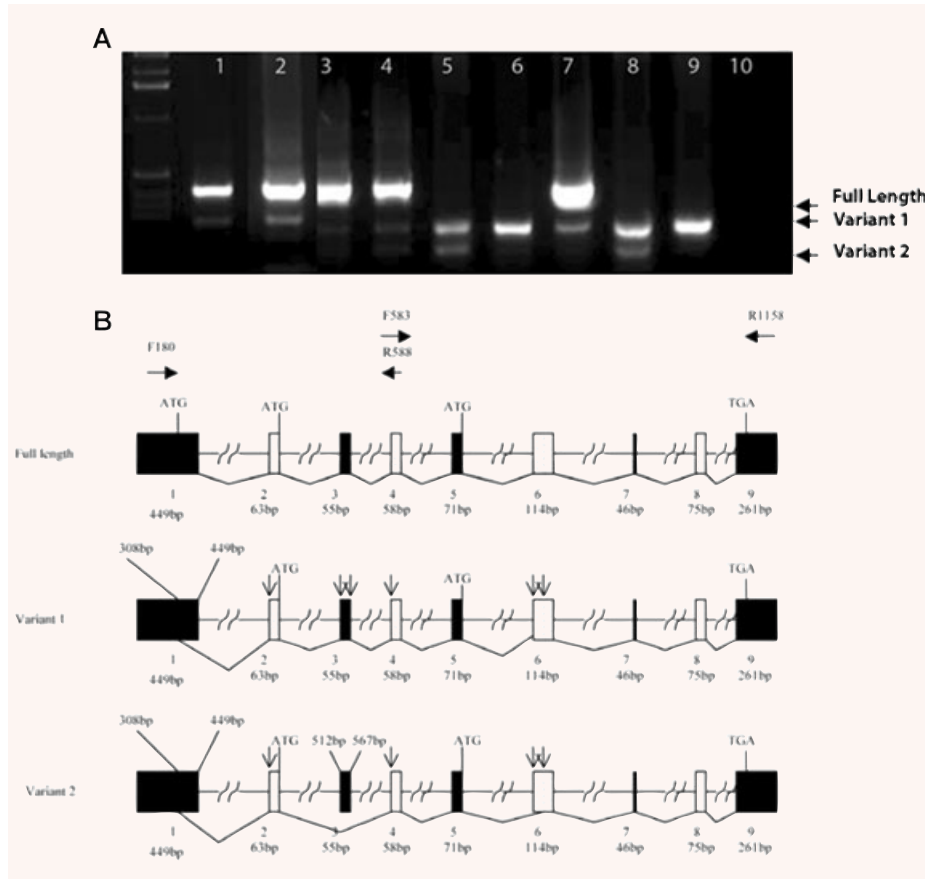
Multitissue Northern blot revealed a 1.2 kb RNaseT2 transcript in all 12 tissues tested (Fig. 1A). However, the levels of transcript



**Fig. 1** RNaseT2 transcript expression in human tissues, tumour cell lines and SV40 immortalized human cell lines. **(A)** RNaseT2 full-length cDNA probe was hybridized to total RNA from brain (lane 1), heart (lane 2), muscle (lane 3), colon (lane 4), thymus (lane 5), spleen (lane 6), kidney (lane 7), liver (lane 8), small intestine (lane 9), placenta (lane 10), lung (lane 11) and peripheral blood leucocytes (lane 12). The arrows indicate the positions of RNaseT2 transcripts. **(B)** The probe RNaseT2 cDNA was hybridized to total RNA isolated from various cell lines such as FS-2 (lane 1), GMO3468A (lane 2), WM239A (lane 3), XPV2 (lane 5), XPD (lane 7), OVCAR3 (lane 8), CSG2 (lane 9) and CRL-9609 (lane 10). Lanes 4 and 6 have no RNA. The amount of total RNA was normalized by hybridization of the same blot to a radioactive  $\beta$ -actin or GAPDH probe.

were variable among different tissues. While its expression was relatively low in brain, the transcript was most abundant in spleen and peripheral blood leucocytes. Moderate amounts of RNaseT2 transcript were present in heart, colon and small intestine as compared to muscle, thymus, kidney, placenta and lung, where the expression was slightly more. A prominent larger transcript of 5.5 kb was also observed in spleen and peripheral blood lymphocytes. The levels of 1.2 kb transcript were approximately 8-fold higher than the 5.5 kb transcript in these tissues. The existence of 5.5 kb transcript has been reported by other investigators [14], but remains uncharacterized.

The analysis for RNaseT2 expression in a variety of human immortal cell lines revealed that the transcript levels in carcinoma cells and SV40 immortalized cell lines were much less than the normal diploid fibroblast cells (Fig. 1B). RNaseT2 expression appeared to be approximately 5-fold lower in WM239A and XPV2 cells, whereas it was approximately 10-folds less in XPD, CSG2 and CRL9609 cells as compared to normal fibroblasts. Interestingly, the levels of RNaseT2 transcript in ovarian carcinoma cell line OVCAR3 were comparable to normal diploid fibroblast cells (Fig. 1B).



**Fig. 2** Splice variants and gene structure of RNaseT2. **(A)** Aliquots of total RNA from human cell lines FS-2 (lane 1), GM03468A (lane 2), AR5 (lane 3), GM0847 (lane 4), 6A-1-1 (lane 5), 6A-2-2 (lane 6), SKOV-3 (lane 7), A549 (lane 8) and U87MG3 (lane 9) were amplified by RT-PCR using primer pair F180/R588 that corresponds to Exon 1 and Exon 4. Lane 10 is a no-template control. The arrows indicate the amplified products of spliced variants 1 and 2. The figure is a composite of two gel pictures. **(B)** The boxes represent the relative positions of exons. The position of primer sequences that correspond to 5' and 3' ends of the gene are indicated by horizontal arrows. In-frame start codons are marked as ATG sequences and the vertical arrows indicate out-of-frame start codons.

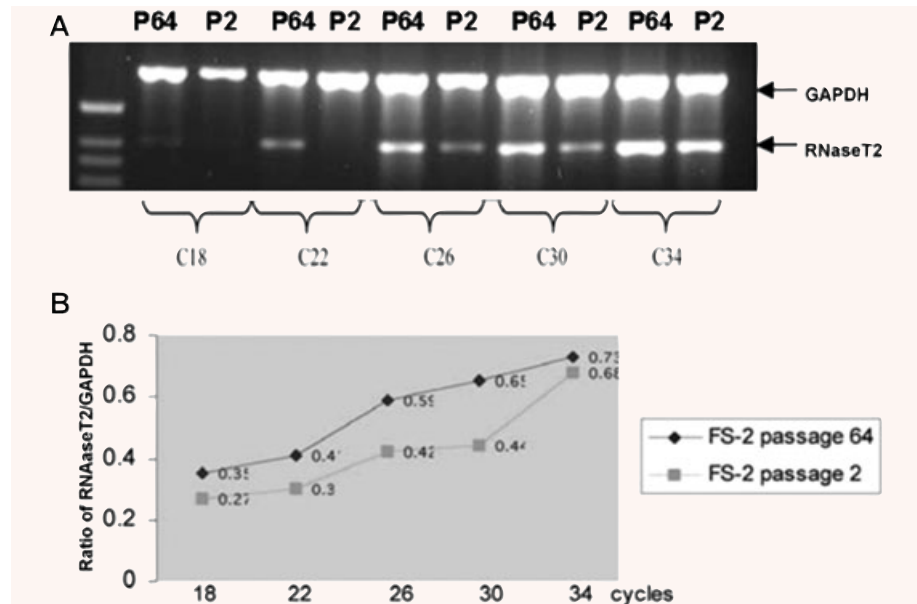
### Alternative splicing of RNaseT2 transcript

The exon composition of transcripts observed in Northern blots was further investigated by exon-specific RT-PCR. Total RNA samples, isolated from normal diploid fibroblasts and variety of immortal cell lines, were amplified by using the primer pairs F180/R588 and F583/R1158. Variations in sizes of amplified products were detected at the 5' end of the transcript (Fig. 2A) but not at the 3' end (data not shown). The RT-PCR amplification of mRNA from normal diploid fibroblast cells with F180/R588 primers yielded a major 409 bp product (expected from the previously characterized 1.2 kb RNaseT2 mRNA) and a minor 268 bp product. While the 268 bp product was amplifiable from all cell lines tested, the 409 bp product was undetectable in A549 lung carcinoma, U87MG3 glioblastoma and in clones 6A-1-1 and 6A-2-2, the two immortal revertants of senescent chromosome 6 transfer clones. A 213 bp product was also detected in the RT-PCR products of mRNA isolated from clone 6A-1-1 and A549 lung carcinoma cell line. The sequencing of the 268 bp products of several cell lines revealed identical nucleotide sequences. Similarly, the sequences of the 213 bp product from RT-PCR of 6A-1-1 and

A549 mRNAs also yielded identical sequences. The analyses of these sequences confirmed that the 268bp and 213 bp products are splice variants of RNaseT2.

The positions of PCR primers, exon/intron boundaries and alternative splicing variants are schematically shown in Fig. 2B. The comparison of sequences of 268 bp (variant 1) and 213 bp (variant 2) products revealed that these fragments are missing 141 bases from the 3' end of exon 1, thereby losing the putative start codon for RNaseT2. Furthermore, the 213 bp (variant 2) has also excluded the 55 bp exon 3. These results demonstrate that several cell lines such as A549, U87MG3, SV40-immortalized cell line GM0847 and subclone 6A-1-1 efficiently transcribe the RNaseT2 gene but fail to produce mRNA that encodes full-length RNaseT2. Amplification of the 3' end of the transcript with F583/R1158 failed to detect previously reported variants [14] or any other splice variants in nine cell lines that we tested (data not shown). It warrants mention that the splicing pattern observed for CRL-9609 and CSG2 cells were identical to the other SV40 immortalized cell lines AR5 and GM0487 (data not shown). We note that these primer pairs cannot detect splice variants that lack exon 4 or are much longer or much less abundant than the predominant isoform.

**Fig. 3** Comparison of RNaseT2 expression in young and aged FS-2 cells by semi-quantitative RT-PCR. **(A)** RT-PCR products of RNA samples from FS-2 cells at passage 64 (P64) and passage 2 (P2) with primers for RNaseT2 and GAPDH. Lanes show amplified products after 18, 22, 26, 30 and 34 cycles of PCR. **(B)** A plot of density ratios of RNaseT2 and GAPDH in each lane of the gel shown in part A.



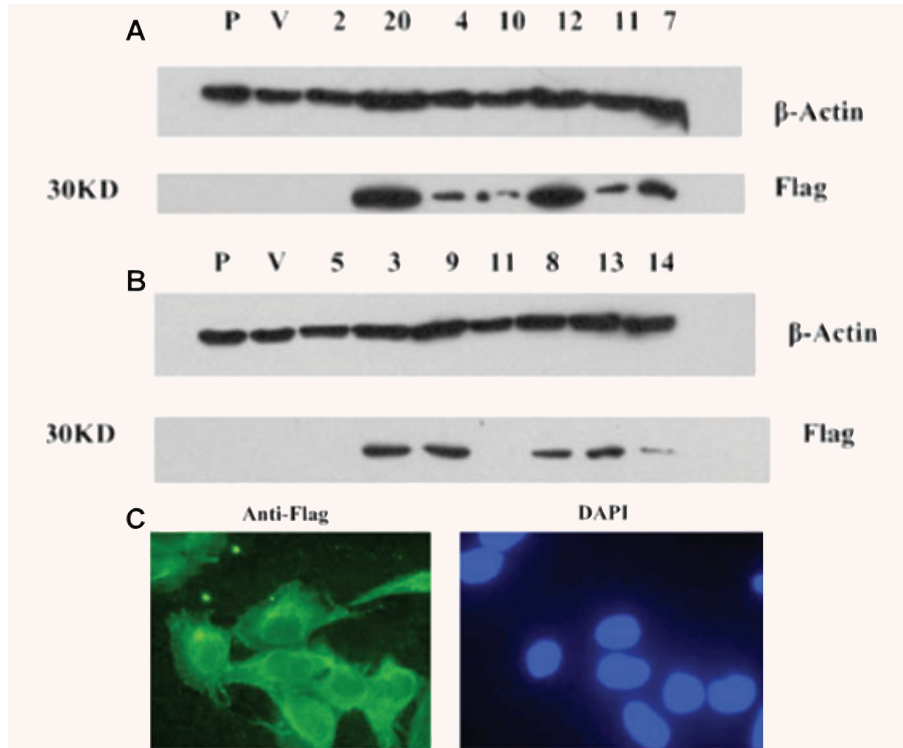
### RNaseT2 expression in early and late passage cells

A semi-quantitative RT-PCR comparison of RNaseT2 expression in young (passage 2) and aged (passage 64) FS-2 cells, using primer pair F180/R588, revealed a significantly higher level in late passage cells relative to early passage cells (Fig. 3A). The cDNA product of mRNA from aged cells was visible after 18 cycles of amplification, but it was detectable only after 26 cycles in young cells. A densitometric analysis of the amplified products of GAPDH and RNaseT2 transcripts confirmed the quantitative changes in early and late passage cells at different cycles of amplification (Fig. 3B).

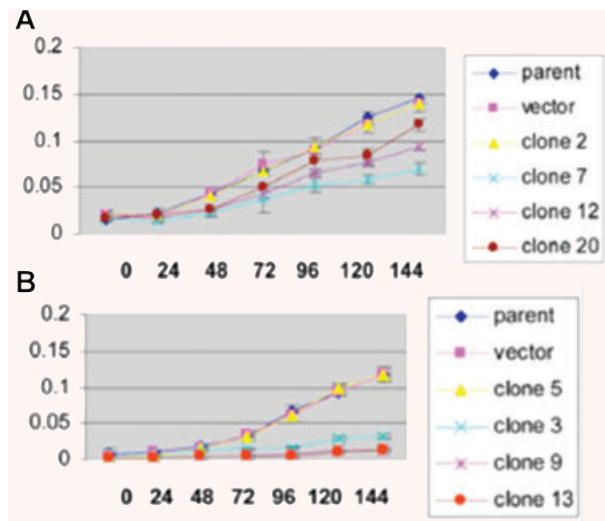
### Effect of ectopic expression of RNaseT2 in SV40 immortalized cell lines

Because SV40 immortalized cell lines, CRL9609 and CSG2, displayed reduced endogenous expression of RNaseT2, these cell lines were selected to investigate the effect of ectopic expression of RNaseT2 gene. The 1.2 kb RNaseT2 cDNA was cloned in frame with a FLAG epitope in pCDNA3.1<sup>+</sup> and transfected into CRL9609 and CSG2 cells. Some transfected cells in G418 selection medium exhibited slow-growing phenotype, while other transfected cells showed a fast-growing phenotype. Representative slow-growing and fast-growing colonies were analysed for the expression of the FLAG-tagged RNaseT2 protein, growth properties, colony forming efficiency, anchorage independence and survival in culture medium through multiple passages for a 6-month period in comparison with parent cell lines and vector controls.

Western blot analysis revealed the expression of the fusion protein in slow-growing clones, but not in fast-growing clones (Fig. 4A and B). In situ visualization of the tagged RNaseT2, by immunofluorescence, showed uniform distribution throughout the cytoplasm with a lesser signal associated with the nucleus (Fig. 4C). Even after culturing for 6 months, five clones of CSG2/RNaseT2 and six clones of CRL-9609/RNaseT2 showed expression of RNaseT2 protein. The cells from these clones continued to multiply at an appreciably slower, but measurable rate, relative to the parental cells and vector control. While the growth rate of CRL-9609/RNaseT2 clones was slightly slower than the parent cell line (Fig. 5A), the growth of CSG2/RNaseT2 clones was reduced dramatically (Fig. 5B). Similar effects were also seen on cloning efficiency and anchorage-independent growth of cells expressing RNaseT2 (Figs 6 and 7). The cloning efficiency of parent CRL-9609 cells, vector transfected clone and a non-expressing transfected clone varied between 52% and 56% as compared to 17–18% observed for RNaseT2 expressing clones of CRL-9609 (Fig. 6, parts A and B). Similarly the colony forming efficiencies for CSG2 parental cells, a vector transfected clone and a non-expressing clone varied between 53% and 65%, but RNaseT2 expressing clones of CSG2 had a significantly reduced cloning efficiency that varied between 4% and 6% (Fig. 6, parts C and D). Moreover, the CSG2/RNaseT2 clones formed much smaller colonies relative to the CRL9609/RNaseT2 clones. In soft agar, CRL-9609 and CSG2 gave an average of approximately 2000 and 2500 colonies, respectively. However, RNaseT2 expressing clones of CRL-9609 formed an average of 18, 144 and 1059 colonies for three different clones, respectively. Three RNaseT2 expressing CSG2 clones, on the other hand, formed an average of 17, 27 and 316 colonies, respectively (Fig. 7). Thus the colony forming efficiency and the ability to grow in soft agar of RNaseT2 expressing clones was significantly reduced relative to the controls.



**Fig. 4** Analysis for the expression of Flag tagged RNaseT2. **(A)** Total cell lysates of SV40 immortalized cell line CRL-9609 and gene transfer clones were hybridized with antibodies against FLAG protein and  $\beta$ -actin, respectively. **(B)** Total cell lysates of CSG2 cells and gene transfer clones were probed with antibodies as above. Lanes represent lysates from parental cells (P), cells transfected with the empty vector (V) and various gene transfer clones designated by clone numbers. The 30 kD FLAG-tagged RNaseT2 and actin protein are indicated. Note that clone 2 in part A and clones 5 and 11 in part B did not express the transferred gene. **(C)** CSG2 cells were transfected with FLAG-tagged RNaseT2 construct and processed to detect the presence of FLAG epitope with a specific antibody. The cell nuclei were subsequently stained with DAPI as indicated.



**Fig. 5** Effect of RNaseT2 overexpression on the growth of SV40 immortalized human epithelial CRL-9609 and fibroblast CSG2 cells. **(A)** The multiplication of CRL-9609 cells, stably expressing RNaseT2, measured using MTT assay at different time-points. Each point in the curve represents the average of six replicates. Clones 7, 12 and 20 express FLAG-tagged RNaseT2 recombinant protein, and clone 2 is a negative transfer clone. **(B)** The growth of CSG2 cells, expressing RNaseT2 was measured as above. Clones 3, 9 and 13 express FLAG-tagged RNaseT2 recombinant protein, and clone 5 is a negative gene transfer clone.

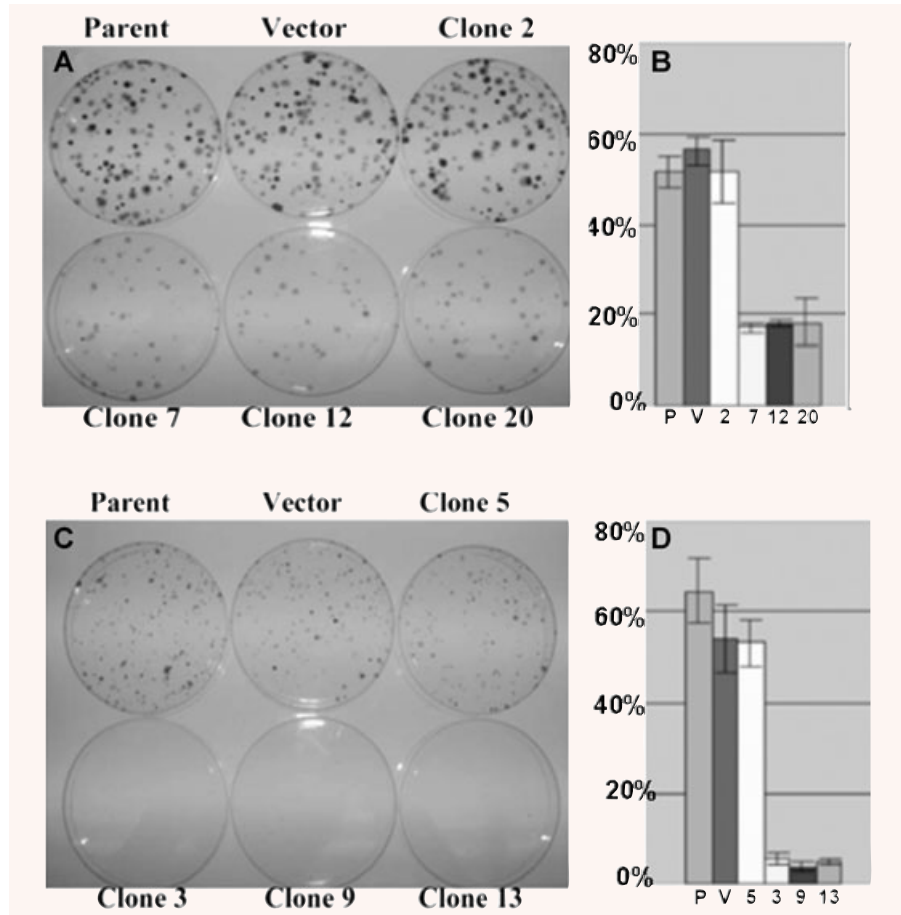
### Superarray analysis of RNaseT2 transfected CSG2 cells

To characterize possible pathways mediating the phenotypic effects of RNaseT2, RNA isolated from parent CSG2 cells and an RNaseT2 expressing clone of CSG2 cells, clone #13, was hybridized to an array of 23 genes representing the Akt pathway. A comparison of the signal intensities between parent cells and gene transfer clone revealed that the transcript levels for most of the genes involved in the Akt pathway, cell proliferation and cell cycle control were reduced in response to the expression of RNaseT2 (Table 1). A significant decrease varying between 2.6- and 10.7-fold was observed for transcripts corresponding to c-jun (10.7-fold), cyclin D1 (4.3-fold), PDK-1 (3.25-fold), matrix metalloproteinase (MMP)-7 (2.8-fold), bcl-2 (2.8-fold) and c-myc (2.6-fold) in CSG2 cells transfected with RNaseT2.

### Discussion

RNaseT2 is widely expressed in human tissues. Most immortalized cell lines under express RNaseT2, but at least one ovarian tumour cell line (OVCAR3) showed high levels of its transcript. It is possible that RNaseT2 transcript, in OVCAR3 cells, is either

**Fig. 6** Effect of RNaseT2 overexpression on colony forming efficiency. **(A)** Photograph of plates containing parent CRL-9609 cells, cells transfected with empty vector, negative transfer clone 2 and RNaseT2 expressing clones 7, 12 and 20. **(B)** A bar diagram showing average numbers of colonies for three independent experiments corresponding to cells and clones shown in part A. **(C)** Photograph of plates containing parent CSG2 cells, cells transfected with empty vector, negative transfer clone 5 and RNaseT2 expressing clones 3, 9 and 13. **(D)** A bar diagram showing an average of colony counts of three independent experiments each for cells and clones shown in part C.

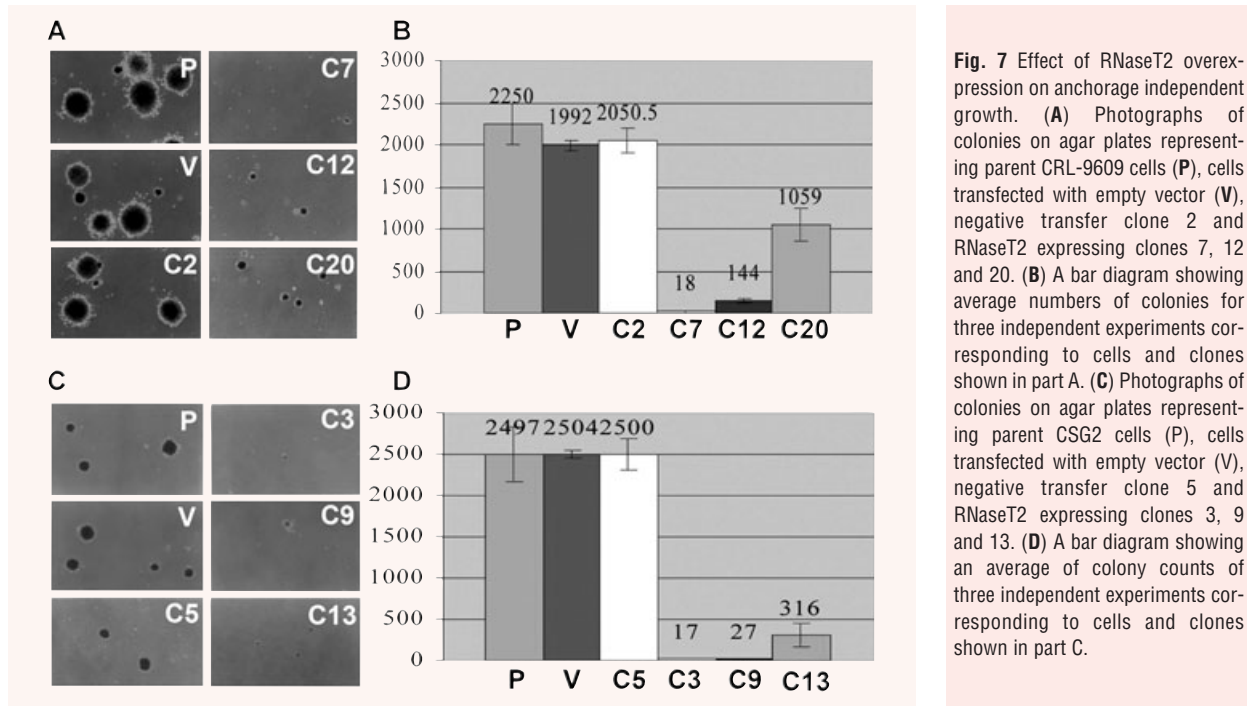


truncated or mutated and thus failed to translate into a functional protein. A systematic search for splice variants that could be detected by RT-PCR revealed a splice variant lacking 141 nt of exon-1 in every cell line examined. Although the spliced isoform appeared to be a significant fraction of the total transcript, it was not detected in Northern blots. We attribute this to the minor differences in transcript size that could not be resolved on agarose gels used to fractionate total RNA, prior to their transfer onto membranes. However, we believe the resolution of these spliced variants by RT-PCR is conclusive. The identities of the spliced variants were further supported by the sequencing of the RT-PCR products. The existence of splice variants of RNaseT2 has previously been reported in cDNA libraries [14]. This observation suggests that alternative splicing may be a significant mode of regulation of RNaseT2, at least in immortalized cell lines. The full-length mRNA was not detected at all in four of the immortalized cell lines. Interestingly, the excluded part of exon-1 encodes the first 27 codons of the predicted full-length protein. These codons include the normal start codon and the signal sequence, which is believed to direct the protein into the endoplasmic reticulum and then out of

the cell *via* endosomes. If the splice variant is translated from an internal start codon, it would lack the secretory signal peptide and will remain in the cytoplasm.

To test the effect of RNaseT2 on cell phenotypes, we made an RNaseT2 construct tagged with a FLAG epitope at the 5' end in a cDNA expression vector and transfected into cells. The 30 kD fusion protein showed cytoplasmic expression and induced the inhibition of growth rate of the cells. Furthermore, RNaseT2 appeared to affect growth rate in a cell line specific manner as revealed by its effect on the growth of CSG2 and CRL-9609 cells, respectively. Although RNaseT2 expression is elevated in aged cells, RNaseT2 expressing clones of CSG2 and CRL-9609 cells lacked characteristic senescent cell morphology as well as acid  $\beta$ -galactosidase activity (data not shown). We believe the RNaseT2 induced senescence, observed by Acquati *et al.* [13], perhaps was due to the cell lines used in their studies. Future studies will be continued to test the effect of RNaseT2 expression in different cell lines, using an inducible expression vector system.

The growth regulatory effect of cytoplasmic RNaseT2 is consistent with its ability to alter the levels of Akt pathway transcripts.



**Fig. 7** Effect of RNaseT2 overexpression on anchorage independent growth. **(A)** Photographs of colonies on agar plates representing parent CRL-9609 cells (P), cells transfected with empty vector (V), negative transfer clone 2 and RNaseT2 expressing clones 7, 12 and 20. **(B)** A bar diagram showing average numbers of colonies for three independent experiments corresponding to cells and clones shown in part A. **(C)** Photographs of colonies on agar plates representing parent CSG2 cells (P), cells transfected with empty vector (V), negative transfer clone 5 and RNaseT2 expressing clones 3, 9 and 13. **(D)** A bar diagram showing an average of colony counts of three independent experiments corresponding to cells and clones shown in part C.

**Table 1** Alterations in Akt pathway transcripts in RNaseT2 transfected CSG2 cells, relative to parent CSG2 cells. Total RNA from parent CSG2 cells and RNaseT2 transfected cells from clone #13 were hybridized to Akt pathway superarray blots separately. The intensity of spots on the two blots was determined and ratio calculated. The numbers greater than one indicate the fold decrease in specific transcripts in the RNaseT2 transfected cells

Parent cell expression/clone expression (ratio)	Transcripts (Akt pathway)
1.1	Akt-1
1.4	Akt-2
0.9	bad
2.8	Bcl-2
1.8	Caspase9
0.6	Caspase3
10.7	c-jun
2.6	c-myc
1.3	β-actin
1.9	CREB
4.3	Cyclin D1
1.7	Fibronectin
1.5	FKHRL1
1.6	GSK3
1.0	GAPDH
2.8	MMP-7
1.1	p70S6 kinase
3.2	PDK-1
1.8	PDK-2
1.4	PI3K p110b
1.5	PTEN
1.3	Raf
1.6	S6



Akt, a serine-threonine kinase with a Pleckstrin homology (PH) domain, can phosphorylate a variety of proteins that in turn can influence processes such as cell survival, cell proliferation and cell growth [23, 24]. The phosphorylated form of Akt inhibits specific proteins that facilitate apoptosis, alters the balance of some proteins that are involved in cell cycle control, and influences important factors that control the rate of protein synthesis in cell [25]. The majority of Akt pathway transcripts that are altered in RNaseT2 transfected cells code for regulators of cell cycle and cell growth. While the levels of most Akt pathway transcripts go down as a result of RNaseT2 over expression in the cytoplasm, some are unaffected or increase slightly. These data indicate that RNaseT2 affects mRNA levels of specific genes. Among these transcripts PDK-1, c-jun and cyclin D1 are particularly noteworthy. PDK-1 (3-phosphoinositide-dependent protein kinase 1) is an enzyme that phosphorylates human Akt (also known as protein kinase B) in the presence of the phospholipid products of phosphoinositide (PI)2 3-kinase [26, 27]. The proto-oncogene c-Jun is a major component of activating protein-1 (AP-1) transcription factor involved in many cellular processes including cell proliferation, apoptosis, differentiation and cell transformation [28]. Cyclin D1, on the other hand, controls cell cycle progression through the G1 phase and the transition from the G1 to S phase

[29, 30]. We note that ribonuclease overexpression studies must be interpreted with special caution, because large quantitative differences in the amount of the protein could conceivably result in a qualitative change in substrate targeting.

In conclusion, the decrease in colony formation efficiency, reduction in anchorage independent growth and low rate of proliferation of RNaseT2 transfected SV40 immortalized cells clearly indicate RNaseT2 to be a growth regulator. The lack of senescence-associated morphological and histological hallmarks, following RNaseT2 expression in SV40 immortalized cells, suggest that RNaseT2 does not function as a senescence-inducing gene when overexpressed by itself in these cells. Based on its growth regulatory effects, we believe that the pathways governed by RNaseT2 may serve as potential therapeutic targets.

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## References

- Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004; 10: 789-99.
- Futreal PA, Coin L, Marshall M, et al. A census of human cancer genes. *Nat Rev Cancer*. 2004; 4: 177-83.
- Hayflick L. The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res*. 1965; 37: 614-36.
- Jazwinski SM. Longevity, genes, and aging. *Science*. 1996; 273: 54-9.
- Smith JR, Pereira-Smith OM. Replicative senescence: implications for *in vivo* aging and tumor suppression. *Science*. 1996; 273: 63-7.
- Angello JC, Pendergrass WR, Norwood TH, et al. Cell enlargement: one possible mechanism underlying cellular senescence. *J Cell Physiol*. 1989; 140: 288-94.
- Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA*. 1995; 92: 9363-7.
- Sugawara O, Oshimura M, Koi M, et al. Induction of cellular senescence in immortalized cells by human chromosome 1. *Science*. 1990; 247: 707-10.
- Uejima H, Shinohara T, Nakayama Y, et al. Mapping a novel cellular-senescence gene to human chromosome 2q37 by irradiation microcell-mediated chromosome transfer. *Mol Carcinog*. 1998; 22: 34-45.
- Reddy DE, Sandhu AK, DeRiel JK, et al. Identification of a gene at 16q24.3 that restores cellular senescence in immortal mammary tumor cells. *Oncogene*. 1999; 18: 5100-17.
- Sandhu AK, Hubbard K, Kaur GP, et al. Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6. *Proc Natl Acad Sci USA*. 1994; 91: 5498-502.
- Sandhu AK, Kaur GP, Reddy DE, et al. A gene on 6q 14-21 restores senescence to immortal ovarian tumor cells. *Oncogene*. 1996; 12: 247-52.
- Acquati F, Morelli C, Cinquetti R, et al. Cloning and characterization of a senescence inducing and class II tumor suppressor gene in ovarian carcinoma at chromosome region 6q27. *Oncogene*. 2001; 20: 980-8.
- Liu Y, Emilion G, Mungall AJ, et al. Physical and transcript map of the region between D6S264 and D6S149 on chromosome 6q27, the minimal region of allele loss in sporadic epithelial ovarian cancer. *Oncogene*. 2002; 21: 387-99.
- Clark KR, Okuley JJ, Collins PD, et al. Sequence variability and developmental expression of S-alleles in self-incompatible and pseudo-self-compatible petunia. *Plant Cell*. 1990; 2: 815-26.
- Meador J 3rd, Kennell D. Cloning and sequencing the gene encoding Escherichia coli ribonuclease I: exact physical mapping using the genome library. *Gene*. 1990; 95: 1-7.
- Schneider R, Unger G, Stark R, et al. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. *Science*. 1993; 261: 1169-71.
- Hime G, Prior L, Saint R. The Drosophila melanogaster genome contains a member of the Rh/T2/S-glycoprotein family of ribonuclease-encoding genes. *Gene*. 1995; 158: 203-7.
- Trubia M, Sessa L, Taramelli R. Mammalian Rh/T2/S-glycoprotein ribonuclease family genes: cloning of a human member located in a region of chromosome 6 (6q27) frequently deleted in human malignancies. *Genomics*. 1997; 42: 342-4.
- Campomenosi P, Salis S, Lindqvist C, et al. Characterization of RNASET2, the first human member of the Rh/T2/S

- family of glycoproteins. *Arch Biochem Biophys.* 2006; 449: 17–26.
21. **Acquati F, Possati L, Ferrante L, et al.** Tumor and metastasis suppression by the human RNASET2 gene. *Int J Oncol.* 2005; 26: 1159–68.
  22. **Sambrook J, Russel D.** Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor: CSHL Press; 2001.
  23. **Altomare DA, Testa JR.** Perturbations of the AKT signaling pathway in human cancer. *Oncogene.* 2005; 24: 7455–64.
  24. **Du K, Tsiichlis PN.** Regulation of the Akt kinase by interacting proteins. *Oncogene.* 2005; 24: 7401–9.
  25. **Ruggero D, Sonenberg N.** The Akt of translational control. *Oncogene.* 2005; 24: 7426–34.
  26. **Alessi DR, James SR, Downes CP, et al.** Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B-alpha. *Curr. Biol.* 1997; 7: 261–9.
  27. **Stokoe D, Stephens LR, Copeland T, et al.** Dual role of phosphatidylinositol 3,4,5-trisphosphate in the activation of protein kinase B. *Science.* 1997; 277: 567–70.
  28. **Karin M, Liu Z, Zandi E.** AP-1 function and regulation. *Curr Opin Cell Biol.* 1997; 9: 240–6.
  29. **Weinberg RA.** The retinoblastoma protein and cell cycle control. *Cell.* 1995; 81: 323–30.
  30. **Sherr CJ.** Cancer cell cycles. *Science.* 1996; 274: 1672–7.