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(57) Abstract: The invention relates to peptides derived from neural-restrictive silencer factor which modulate expression of CD59 and thus affect antibody-mediated complement cytotoxicity in tumours.



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PEPTIDES

The present invention relates to peptides which are of use in the treatment of cancer, to nucleic acids encoding the peptides, constructs
5 containing the nucleic acids and cells transformed or transfected with the constructs. In particular, the invention relates to peptides derived from the neural-restrictive silencer factor (REST) which are of use in modulating expression of the complement regulator CD59 on tumour cells and so sensitising the tumour cells to immunotherapy.

10 Most tumours initially respond to chemotherapy and monoclonal antibody (mAb) therapy, but often relapse into a multidrug resistant state. Outcome in neuroblastoma patients is particularly poor. Sensitising tumours to immune attack, for example by blocking complement regulators, might aid therapy, but current approaches, while effective *in vitro* (siRNA) or in xenograft animal
15 models (blocking mAb) of human cancer, cannot easily be applied *in vivo*. To address these limitations we set out to develop a new generation of agents that target tumours and inhibit expression of complement regulator genes. We identified molecular mechanisms involved in overexpression of CD59 in neuroblastoma and designed peptides that inhibit CD59 expression and
20 sensitise cells to immunotherapy.

Complement is a major component of innate immunity (Walport, 2001). Complement can be activated on tumour cells by antibodies (Magyarlaki et al., 1996), immune complexes (Lucas et al., 1996), as a consequence of apoptosis (Matsumoto et al., 1997) or through proteolytic processes (Björge et al., 1997).
25 Normal and malignant cells are protected by membrane-bound complement regulators (mCReg) that act as physiologic brakes to complement amplification either by limiting formation of the C3/C5 convertase enzymes (CD35, CD46, CD55), or of the assembly of the cytolytic membrane attack complex (CD59) (Walport, 2001). In many tumours, mCReg expression is greater than in normal
30 surrounding tissue (Björge et al., 1997; Rushmere et al., 2004). Consequently, the increased complement resistance conferred by these mCReg has been

proposed as a mechanism that facilitates survival of the tumour or the metastasising tumour cell when it enters the circulation (Gorter and Meri, 1999).

Most monoclonal antibodies (mAbs) used in anticancer immunotherapies activate complement; however, strong evidence for a role of complement in cancer regression exists only for Rituximab (anti-CD20) (Maloney et al., 2002). For the majority of therapeutic mAb, complement likely plays little or no role in tumour clearance because the tumour abundantly expresses mCReg (Imai et al., 2005). Indeed, repeated sub-optimal Rituximab treatment caused resistance to complement killing in the B-cell line RAMOS by inducing increased expression of CD55 and CD59 (Takei et al., 2006). Blocking of CD55 and CD59 increased the effectiveness of therapeutic mAb killing in B-cell lines by 5-6 fold, confirming the protective role of mCReg (Golay et al., 2001). Although blocking of the mCReg with mAbs enhances complement-mediated immunoclearance of tumours, their high molecular mass and the ubiquitous expression of their targets are serious limitations for their application in humans. An alternative approach, downmodulation of mCReg, has been successfully achieved in vitro by RNA interference; however, there are numerous problems (e.g. *in vivo* stability, tissue specific targeting, and unwanted immune system activation) currently preventing use *in vivo* (Kurreck, 2005).

These facts justify development of new strategies to overcome the stated drawbacks. A novel approach that could considerably enhance the therapeutic potential of currently used anticancer immunotherapies would be to inhibit expression of mCReg genes by targeting their transcriptional regulators. Little is currently known about the mechanisms that control expression of the mCReg. We have recently demonstrated a modulation of CD59 expression by p53 during treatment of neuroblastoma cells with chemotherapeutics (Donev et al., 2006).

The present invention results from an extension of this work and in particular from the identification of additional and novel molecular mechanisms leading to overexpression of CD59 in neuroblastoma. We implicated the neural-restrictive silencer factor (REST) as an important regulatory component of the transcriptional machinery of the CD59 gene. REST was originally described as a transcriptional repressor of neuronal gene expression (Chong et al., 1995;

Schoenherr and Anderson, 1995); however, recently it has emerged as a tumour suppressor capable of transforming epithelial cells when mutated (Westbrook et al., 2005). So far, REST has been found to be a target for several different types of mutations in neuroblastoma (Palm et al., 1999), small cell lung carcinoma
5 (Coulson et al., 2000) and colorectal cancer (Westbrook et al., 2005).

The present invention has its basis in our finding that REST is involved in modulation of CD59 expression in neuroblastoma and in particular relates to novel REST peptides that target identified transcriptional regulator sites of CD59, reduce CD59 expression and sensitise tumour cells to complement-
10 mediated killing triggered by a mAb used in neuroblastoma immunotherapy.

Therefore, in a first aspect of the present invention there is provided a peptide comprising SEQ ID NO: 2 or a homologue or derivative thereof; provided that the peptide is not full-length REST.

The skilled person will appreciate that homologues or derivatives of the
15 peptide of the invention will also find use in the context of the present invention, ie as suppressors of CD59 gene expression. Thus, for instance proteins or polypeptides which include one or more additions, deletions, substitutions or the like are encompassed by the present invention. In addition, it may be possible to replace one amino acid with another of similar "type". For instance replacing one
20 hydrophobic amino acid with another. One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment.
25 A program like BLASTx will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of analysis are contemplated in the present invention.

Suitably, a homologue of a peptide such as those of SEQ ID NO: 2, SEQ
30 ID NO: 4 or SEQ ID NO: 6 will be at least 70% homologous to that peptide, but more suitably it will have at least 80% and, increasing order of suitability, 85%, 90%, 95% or 99% homology to the given peptide.

SEQ ID NO: 2 consists of zinc-finger domains 6, 7 and 8 of full-length REST plus their interdomain sequences.

The peptide may consist solely of the sequence of SEQ ID NO: 2 or a homologue or derivative thereof.

- 5 Alternatively, however, the peptide may comprise the sequence of SEQ ID NO: 2 or a homologue or derivative thereof linked at the 5' and/or 3' end to a nuclear localisation signal, which is optionally separated from SEQ ID NO: 2 by a spacer.

10 The nuclear localisation signal may be derived from the full-length REST protein, in which case it is suitably zinc-finger domain 5 of full-length REST. An example of such a peptide is REST58 (SEQ ID NO: 4) or a homologue or derivative thereof, which comprises domain 5 of full length REST, which is responsible for nuclear localisation, and domains 6, 7 and 8 (Figure 4A) that are essential for DNA binding. REST58 is delivered to the nucleus via a REST-specific receptor (Shimojo and Hersh, 2003) recognised by the zinc-finger
15 domain 5 of the protein. The REST-specific receptor has been identified only recently and its efficacy in delivering REST to the nucleus has not been investigated.

20 The peptide may additionally comprise an N' terminal methionine residue and an example of such a peptide is shown as SEQ ID NO: 6.

25 Alternative nuclear localisation signals may also be used, for example in the peptide REST68 (SEQ ID NO: 8) or a homologue or derivative thereof. This peptide is similar to REST58 except that domain 5 has been replaced by the classical nuclear localisation signal (NLS) which has the sequence PKKKRKV and which delivers proteins to the nucleus via importin α 1 (Nadler et al., 1997).

 In a second aspect of the invention, there is provided nucleic acid molecule encoding a peptide of the first aspect of the invention or which hybridises under stringent conditions to nucleic acid encoding a peptide of the first aspect of the invention.

30 The nucleic acid may encode a peptide having SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8. In particular, there is provided SEQ ID

NO: 1, which encodes SEQ ID NO: 2, SEQ ID NO: 3, which encodes SEQ ID NO: 4, SEQ ID NO: 5, which encodes SEQ ID NO: 6 or SEQ ID NO: 7, which encodes SEQ ID NO: 8 or homologues thereof or nucleic acid which hybridises to SEQ ID NO: 1, 3, 5 or 7 under stringent conditions.

5 When comparing nucleic acid sequences for the purposes of determining the degree of homology or identity one can use programs such as BESTFIT and GAP (both from the Wisconsin Genetics Computer Group (GCG) software package) BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be
10 aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention compare when discussing identity of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

 Suitably, a homologue of a nucleic acid sequence of the invention, for
15 example, SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5 will be at least 70% homologous to that nucleic acid sequence, but more suitably it will have at least 80% and, increasing order of suitability, 85%, 90%, 95% or 99% homology.

 Reference herein to stringent conditions includes reference to either increasing the temperature of incubation to at least 45°C but more suitably to
20 65°C and/or washing the annealed molecules using a salt solution having an ionic strength of from 1.0M sodium chloride to 0.02M sodium chloride.

 The invention also provides a construct comprising the nucleic acid molecule of the second aspect of the invention and, further, the invention also provides a cell transformed or transfected with the construct.

25 Furthermore, there is provided a process for the preparation of a peptide according to the first aspect of the invention, the process comprising expressing the nucleic acid molecule of the second aspect of the invention.

 We have found that the peptides of the present invention inhibit expression of CD59 both at mRNA level and at protein level. REST68 is
30 particularly effective and has a 4-fold effect at mRNA level and a 2.5-fold effect at protein level (Figure 4). Taking into account the fact that cell-bound CD59 turns over relatively rapidly (Davies et al., 2005), and that transfected

neuroblastoma cells were cultured for up to two weeks before studying the effect of REST68, the observed discrepancy between mRNA and protein change might be a result of compensatory mechanism(s) that affects either the stability or the expression of CD59 at protein level.

5 A key finding is that the peptides of the invention do not affect significantly expression of CD59 in cells expressing predominantly the full-length REST (Figure 4). Endogenously expressed REST has already downmodulated the expression of CD59 and a further introduction of a peptide of the invention does not show a significant effect. These findings lead us to suggest that the peptides
10 of the invention would not change significantly the expression of other REST-controlled genes in normal cells. The selective effect of the peptides of the invention on neuroblastoma cells is a critical feature that may remove the need to target this therapeutic peptide to tumours. Expression of the truncated isoform of REST, while not reported in normal cells, is also described in small cell lung
15 carcinoma and colorectal cancer (Coulson et al., , 2000; Westbrook et al., 2005). This new approach to sensitising tumour cells to C attack modelled here in neuroblastoma may therefore be of broader relevance to tumour immunotherapy.

 Therefore, in a further aspect of the invention there is provided a peptide
20 of the first aspect of the invention, nucleic acid of the second aspect of the invention or a construct comprising said nucleic acid for use in medicine, particularly in the treatment of cancer.

 Particular forms of cancer which may be treated include especially neuroblastoma but also other types of cancer such as small cell lung carcinoma
25 and colorectal cancer.

 The peptide, nucleic acid molecule or construct will generally be used in combination with an immunotherapeutic agent targeted to the type of tumour being treated.

 The invention also provides the use of a peptide of the first aspect of the
30 invention, nucleic acid molecule of the second aspect of the invention or a construct comprising said nucleic acid molecule in the preparation of an agent

for the treatment of cancer, in particular neuroblastoma but also other types of cancer such as small cell lung carcinoma and colorectal cancer.

There is also provided a product comprising a peptide of the first aspect of the invention, nucleic acid molecule of the second aspect of the invention or a
5 construct comprising said nucleic acid molecule and an immunotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer.

In a further aspect of the invention, there is provided a pharmaceutical composition comprising a peptide of the first aspect of the invention, nucleic acid
10 molecule of the second aspect of the invention or a construct comprising said nucleic acid molecule together with a pharmaceutically acceptable excipient.

Suitably, the composition may contain an immunotherapeutic suitable for the treatment of cancer, particularly a cancer of one of the types specified above.

15 In yet another aspect of the invention there is provided a method for the treatment of cancer, the method comprising administering to a patient in need of such treatment effective amounts of a first agent selected from the group consisting of a peptide of the first aspect of the invention, nucleic acid molecule of the second aspect of the invention and a construct comprising said nucleic
20 acid molecule; and a second agent which is an immunotherapeutic agent.

As with other aspects of the invention, the cancer to be treated is suitably neuroblastoma but the method may also be used for the treatment of other types of cancer such as small cell lung carcinoma and colorectal cancer. Peptides and related agents will be administered in different ways, such as intravenously,
25 intraperitoneally, intramuscularly, subcutaneously, topically or orally as a powder formulation or more suitably as a liquid formulation, as appropriate for the tumour type. For some tumour types, directed delivery into the supplying artery or even direct intratumoural administration may be possible.

The immunotherapeutic agent is suitably chosen such that it is
30 appropriate for use in the treatment of the particular type of cancer being targeted. In particular, it may be an antibody, especially a monoclonal antibody and will bind specifically to cells of the targeted tumour.

The invention will now be described in greater detail with reference to the non-limiting examples and to the drawings in which:

Figure 1. A 35bp positive responsive element from the CD59 promoter is essential for overexpression of the gene in neuroblastoma. (A) Schematic presentation of the CD59 promoter fragments used in the EGFP-reporter assays. Four different fragments containing parts of exon 1 (solid box) along with variable portions of the 5' flanking region (represented by lines) were prepared and ligated into a promoterless pEGFP-1 vector upstream of the EGFP gene. The arrow points in the 3' direction. (B) Kelly cells were transfected with the constructs shown in (A) and the expression of EGFP was assessed by flow cytometry. Values are mean $\pm$ SD for three independent experiments. (C) The 35bp sequence that differs between the -35 and -70 constructs and upregulates expression of the reporter EGFP gene.

Figure 2. Expression of truncated REST in neuroblastoma cells correlates with high expression of CD59. (A) RT-PCR analysis of expression of REST isoforms in different neuroblastoma cell lines and in primary neurons. Specific primers either annealed on each side of the inserted sequence or a nested reverse primer, were designed within the insert amplifying only the cDNA encoding the truncated REST isoform. (B) Expression of CD59 on the cell surface of neuroblastoma cells was assessed by flow cytometry. The data are mean $\pm$ SD of triplicates and representative of two experiments. (C) RT-PCR analysis of expression of REST isoforms in clinical neuroblastoma samples (NT1 – NT10). GAPDH was monitored as an indicator for the quality of RNA prepared from clinical tissue samples.

Figure 3. Full-length REST is a suppressor of CD59 expression. (A) EMSA with oligonucleotides labelled with biotin, incubated with 5 μ g of nuclear protein extracts from normal brain and neuroblastoma cell lines Kelly and IMR32, in the presence of 0.1 μ g/ μ l poly-d(IC) and 1 μ g/ μ l salmon sperm DNA.

Ab against REST was added in some reactions (+) to test whether the oligonucleotide retardation is a result of REST binding. Oligonucleotides were separated in a 6% polyacrylamide gel for 1h at 100V, transferred onto a nitrocellulose membrane, and detected by ExtrAvidin-HRP. (B) Autoradiograph
5 obtained under the same conditions as described above, however the gel was run for 3h at 100V for better visualisation of the retardation, due to the high molecular mass of the complexes. (C) Knockdown of expression of REST, both full-length and truncated, in IMR32 and Kelly cells and increase of expression of CD59 in IMR32 cells by RNA interference (restRNAi). Scrambled siRNA
10 sequence (cntrRNAi) was used as a control and expression of the GAPDH housekeeping gene was monitored in addition.

Figure 4. Peptides derived from the DNA-binding domain of REST suppress expression of CD59. (A) The REST repressor protein is shown
15 schematically (left hand-side), illustrating the eight zinc finger DNA binding domains and two repressor domains. Domains included in REST58 (110 amino acid residues, AA) and REST68 (includes 82AA from the REST plus the 7AA-long NLS) are shown. RT-PCR analysis of expression of CD59 mRNA in IMR32 and Kelly cells transfected either with REST58, REST68, or an empty
20 expression vector. (B) QPCR for expression of CD59 mRNA in IMR32 and Kelly cells transfected as above. Expression in cells transfected with empty vector was set as 100%. Results from two independent measurements ($\pm$ SD; *, $p > 0.001$). (C) Flow cytometry analysis of expression of CD59 on the surface of IMR32 and Kelly cells transfected as above. (D) Flow cytometry analysis of
25 expression of CD59 on the surface of NMB7, La-N-1, La1-5S, SH5Y, SK-N-ER, SK-N-SH, and La1-55N 96h after cells were transfected either with REST68 or an empty expression vector. Bars represent mean ($\pm$ SD) from two independent measurements (*, $p < 0.001$).

30 Figure 5. REST68 peptide suppresses expression of CD59 gene by blocking binding of transcriptional activators to the 35bp responsive element. ChIP was performed on Kelly cells transfected with either REST68 expression

construct or empty vector with antibodies against Sp1, AP2, CPBP, and REST. Immunoprecipitation with non-immune rabbit IgG was carried out as a control for the assay background. The pulled-down DNA was characterised for presence of the 35bp element by QPCR. Highest levels of binding for each transcription factor, regardless of the plasmid with which cells were transfected, were set as 100%. Values are mean $\pm$ SD for two independent ChIP experiments each analysed in duplicate (*, $p < 0.001$).

Figure 6. REST68 peptide sensitises neuroblastoma cells to C-mediated lysis. Kelly, La1-5S, SK-N-ER, and NMB7 cells transfected either with REST68 expression construct (-■-) or empty vector (-▲-) were tested for their sensitivity to C-dependent cytotoxicity triggered by anti-GD2 mAb. Lysis assay with preincubation of both cell lines (REST68, -□-; empty vector, -△-) with CD59-blocking Fab fragment was carried out as a control. Values are mean $\pm$ SD for three independent experiments.

EXAMPLES

Example 1 – Identification of a 35bp Positive Regulatory Sequence within the CD59 Promoter in Neuroblastoma

In our investigation of the link between REST and CD59 expression, we first searched for the sequence within the CD59 promoter that is responsible for elevated expression of CD59 in neuroblastoma. Similar investigations have been carried out previously for human Burkitt lymphoma and chronic myelogenous leukemia using luciferase-reporter constructs (Holguin et al., 1996). Here we used a pEGFP-1 promoterless vector and generated EGFP-reporter constructs containing either the entire promoter of the CD59 gene (-2140) or parts of it (-35, -70, -151) located upstream of the EGFP coding sequence (Figure 1A). We introduced these reporter constructs into the Kelly neuroblastoma line and selected transfectants by adding G418 (neomycin). Expression of EGFP by the different constructs was then analysed by flow cytometry (Figure 1B). The -2140, -151 and -70 constructs all produced similar amounts of EGFP

expression; however, expression from the -35 construct was reduced by approximately 3-fold. These data demonstrate the importance of the additional 35bp sequence between the -35 and -70 promoter constructs (Figure 1C) for elevated expression of the CD59 gene in neuroblastoma.

- 5 We next analysed the 35bp positive regulatory sequence in the CD59 promoter for potential binding to transcription factors. Using MatInspector™ (Genomatix Software GmbH, München, Germany), we identified several transcriptional activators (e.g. Sp1, AP2, CPBP, PLAG1) that may bind to the positive regulatory sequence (Table 1). However, we also found putative binding
10 sites for the transcriptional suppressors REST and ZBP-89. It is likely that interplay between at least some of these potential negative and positive regulators will determine the expression status of the CD59 gene.

Table 1 Transcription factors predicted to bind within the 35bp responsive element in the CD59 promoter. Positions refer to the fluorescent constructs
15 shown in Figure 1.

Transcription factor	Positions, from-to
Core promoter-binding protein (CPBP)	-70 to -48
Stimulating protein 1 (SP1)	-64 to -50
Pleomorphic adenoma gene (PLAG) 1	-63 to -43
MYC-associated zinc finger protein related transcription factor	-62 to -50 -61 to -41
Pleomorphic adenoma gene (PLAG) 1	-60 to -40
Neural-restrictive-silencer-element	-60 to -38
Kruppel-like zinc finger protein 219	-59 to -45
Stimulating protein 1 (SP1)	-57 to -35
Zinc finger transcription factor ZBP-89	-56 to -42
Activator protein 2 (AP2)	-55 to -39
EGR1, early growth response 1	-51 to -37
Stimulating protein 1 (SP1)	

Cell lines, patient samples and preparation of nuclear lysates

Human neuroblastoma cell lines IMR32, SH5Y, Kelly, La-N-1, La1-55N, SK-N-SH, La1-5S (European Collection of Animal Cell Cultures, Salisbury, UK),
5 NMB7, and SK-N-ER (kind gift from Dr. P. Gasque, University of la Reunion, Saint Denis, Ile de la Reunion) were maintained in RPMI1640 with 10% heat-inactivated FCS, supplemented with glutamine, penicillin, and streptomycin (Invitrogen, Paisley, UK). Neuroblastoma clinical samples (NT1 - NT10) were obtained via the CCLG Biological Studies Tumor Bank, UK (Study number: 2007
10 BS 08). Nuclear protein extracts were prepared from all neuroblastoma cell lines as described previously (Donev et al., 2003).

Design of promoter constructs

Expression constructs were prepared by ligating the CD59 promoter
15 fragments into the pEGFP-1 vector (Clontech, United Kingdom). This promoter-less vector contains a cloning site immediately upstream of the EGFP reporter gene. The promoter fragments were amplified from human genomic DNA using a common reverse primer containing restriction site (underlined) for Age I enzyme (GCACCGGTAAGATCCTCTTCCAGCCTCGA; SEQ ID NO: 9) and a
20 series of forward primers with Kpn I restriction site (underlined):

CGCCGGTACCTGAATTCAGATTTGTGCACA (SEQ ID NO: 10) for the
-2140 construct;

CGCCGGTACCTCCGCGCGGGGGTGGAGGGAGA (SEQ ID NO: 11) for
the -151 construct;

25 ATTAGGTACCAAGGGCATCCTGAGGGGC (SEQ ID NO: 12) for the -70
construct; and

ATTAGGTACCCCTTGCGGGCTGGAGCGAA (SEQ ID NO: 13) for the -
35 construct.

The amplified fragments and the plasmid were digested with Age I and
30 Kpn I. After ligation into pEGFP-1, the nucleotide sequence of the inserts was determined by sequencing to ensure that PCR artefacts had not been introduced.

The reporter constructs were transfected into Kelly neuroblastoma cells using the Effectene reagent (Qiagen, Crawley, West Sussex, United Kingdom). Transfected cells were selected by inclusion of 400 μ g/ml G418 (Clontech) in the culture medium. Cells were then analysed for expression of EGFP by flow
5 cytometry.

Example 2 – REST Modulates Expression of the CD59 Gene

It has been previously shown that REST is expressed as a truncated form in neuroblastoma tumours due to an insertion within the gene that introduces a
10 stop codon (Palm et al., 1999). Recently it was suggested that expression of this truncated isoform plays a role in tumour progression (Coulson, 2005; Majumder, 2006). Taking into account that full-length REST may bind within the 35bp responsive element in the CD59 promoter, we addressed here the role of the expression of truncated REST in CD59 overexpression in neuroblastoma.

15 RT-PCR analysis of expression of REST using a primer pair that anneals on both sides of the inserted sequence (Figure 2A) showed that eight of nine studied neuroblastoma cell lines express predominantly the truncated isoform. We did not detect the insertion in primary neurons and IMR32 cells indicating that they expressed exclusively or predominantly full-length REST. However,
20 when we carried out a nested RT-PCR with reverse primer within the REST insertion, the insertion was detected at low level in the IMR32 cells but not in primary neurons. Importantly, we found that high expression level of CD59 coincided with expression of the truncated REST isoform in the various cell types (Fig. 2B). To further explore clinical importance, RT-PCR analysis of
25 expression of REST was tested in ten clinical neuroblastoma samples (Fig. 2C). Truncated REST was present in nine of ten specimens. The sample without truncated REST (N1) contained only trace amounts of full-length REST. These findings demonstrate the clinical relevance of the switch to expression of truncated REST in neuroblastoma. Two of the tumors (NT6 and NT7) showed
30 presence of an additional alternative REST transcript that is not further defined.

In order to confirm that REST binds in the identified 35bp responsive element, we performed an electrophoretic mobility shift assay (EMSA) with nuclear protein extracts from normal human brain and neuroblastoma cells (Figure 3A, B). We detected protein binding to the 35bp regulatory element in all the lanes. However, a supershift with antibody against REST was observed only in nuclear extracts from cells expressing the full-length REST. In Kelly cells that expressed only the truncated isoform, no binding between the 35bp element and REST was detected. This finding, together with the observation that higher expression of CD59 coincides with the expression of truncated REST (Figure 2B), suggest that the full-length protein suppresses expression of CD59 by binding within the 35bp responsive element. We tested this by transfecting IMR32 and Kelly cells with plasmid expressing siRNA against both isoforms of REST. The knockdown efficiency for both REST isoforms was approximately 90% (Figure 3C). Knockdown of REST resulted in a 7-fold increase in expression of CD59 mRNA in IMR32 cells that predominantly express full-length REST (Figure 3C). In contrast, knocking-down truncated REST in Kelly cells did not affect CD59 mRNA expression.

Electrophoretic Mobility Shift Assay

Biotinylated sense and antisense strands of the 35bp regulatory sequence (Figure 1C) were purchased from Biomers.net GmbH (Ulm, Germany). Oligonucleotides (200pmol each) were mixed in equimolar amounts in 50µl of annealing buffer (50mM KCl, 1.5mM MgCl₂, Tris-HCl, pH 8.3), placed in a boiling water bath for 2 minutes, and allowed to cool slowly to room temperature. The annealed DNA probe (10pmol per reaction) was incubated with 5µg of nuclear protein extracts from IMR32, Kelly, or normal human brain (Active Motif, Rixensart, Belgium) as previously described (Enukashvily et al., 2005). Each reaction contained 0.1 mg/ml poly(deoxyinosinicdeoxycytidylic acid) to block non-specific electrostatic interactions and 1mg/ml salmon sperm DNA as a competitor (both purchased from Sigma-Aldrich). In some of the reactions, a rabbit polyclonal anti-REST (H-290) antibody raised against amino acids 1-290

of the protein (Santa Cruz Biotechnology, Santa Cruz, California, USA) was added to identify complexes containing the protein. This antibody recognises both the full-length and the truncated REST isoforms. Each reaction was separated in 6% polyacrylamide gels and transferred onto nitrocellulose membranes. DNA was detected using ExtrAvidin-HRP (Sigma-Aldrich) followed by chemiluminescence development (Bio-Rad, Herts, United Kingdom).

RNA interference

A siRNA sequence efficiently knocking-down expression of REST (Kim et al., 2004) was designed into a short hairpin RNA expressing plasmid pLKO.1-TRC (Addgene Inc, Cambridge, MA, USA). Sense and antisense oligonucleotides containing either the active sequence (GATGCACAACTGTTCTTC; SEQ ID NO: 14) or the scrambled one (CATTGCGGTTTACGACTAA; SEQ ID NO: 15) were purchased from Biomers.net GmbH. Single stranded oligonucleotides were annealed as described above for the EMSA. They were designed in such a manner that the resultant double stranded constructs contained sticky Age I and Eco RI 5'- and 3'-ends, respectively. After digestion of the pLKO.1-TRC with these two enzymes, the annealed constructs were ligated into the plasmid by T4 DNA ligase (Invitrogen). Ligated plasmids were transformed into E.coli, amplified, and purified with GenElute plasmid miniprep kit (Sigma-Aldrich). These two constructs were transfected into IMR32 and Kelly cells using Effectene reagent. Transfected cells were then selected by adding of 1µg/ml or 10µg/ml of puromycin (Invitrogen) to the culture medium of IMR32 and Kelly cells, respectively, and cells were analysed for expression of REST, CD59 and GAPDH.

Example 3 – Design of Peptides for Suppression of Expression of CD59

Our next step was to design peptides that can suppress expression of CD59 in neuroblastoma. Based on our observation that only the full-length REST binds within the 35bp responsive element to suppress expression of CD59, and on previously characterised roles of REST domains (Shimojo et al.,

2001), we designed two constructs expressing REST-derived peptides predicted to bind to the CD59 promoter and suppress expression of the gene. One of the peptides, named REST58, comprises domain 5, which is responsible for nuclear localisation, and domains 6, 7 and 8 (Figure 4A) that are essential for DNA binding. In the second peptide, named REST68, domain 5 has been replaced by the classical nuclear localisation signal (NLS) (Nadler et al., 1997). We transfected these two expression constructs into IMR32 and Kelly cells and investigated their effects on the expression of CD59. A conventional RT-PCR (Figure 4A) clearly showed that both peptides had considerable suppressive effect on CD59 expression in Kelly cells lacking the full-length REST; however, the peptides had no significant effect in IMR32 cells expressing mainly the full-length protein. We further clarified this issue by quantifying the effect of REST-derived peptides using real time quantitative PCR (QPCR). In Kelly cells, REST58 lowered expression of CD59 mRNA by 2.5-fold, while transfection with REST68 constructs yielded a 4-fold suppression (Figure 4B). In IMR32 cells there was a non-significant trend towards reduction of CD59 mRNA expression by the two constructs. The effect of REST58 and REST68 on expression of CD59 at protein level (Figure 4C) was similar to that observed at mRNA level, with a reduction in expression in Kelly cells of approximately 2-fold and 2.5-fold, respectively. We next studied the effect of REST68 on CD59 expression in several other neuroblastoma cell lines (Fig. 4D). In all of them CD59 expression on the cell surface was reduced within 96h of the transfection by at least 2-fold. In NMB7 cells, this effect was significantly greater and we observed almost a complete suppression of CD59 by the REST peptide (10% of that in cells transfected with an empty vector).

Next we elucidated the mechanism by which REST-derived peptides suppress expression of CD59 in neuroblastoma cells. However, the utility of the peptides of the invention is not dependent on the correctness or otherwise of our deductions concerning this mechanism.

We carried out chromatin immunoprecipitation (ChIP) assays in Kelly cells transfected with the REST68 expressing construct using antibodies against transcription factors predicted to bind within the 35bp responsive element from

the CD59 promoter (Table 1 above). In cells expressing the truncated REST (Kelly/pDR2) we detected strong binding of transcriptional activators Sp1, AP2 and CPBP within the 35bp regulatory element, and no binding of truncated REST was observed (comparable with that for the background control with non-immune IgG). However, when these cells were transfected with the REST68 expression plasmid, the expressed peptide bound within the regulatory sequence sequestering binding of all three transcriptional activators by approximately 5-fold (Figure 5).

Design of peptides for suppression of expression of CD59

To test the effect of REST- and p53-derived peptides on expression of CD59, several pDR2 Δ EF1 α -based constructs for expression in mammalian cells were designed. Sequence encoding REST58 was amplified from IMR32 cDNA using the following pair of primers:

CGATCTAGAGCCACCATGTATAAATGTGAACTT (SEQ ID NO: 16) forward,
and:
AGAGGATCCTCAATGCTTAGATTGAAGT (SEQ ID NO: 17) reverse.

Forward and reverse primers contained restriction sites (underlined) for XbaI and Bam HI, respectively, which were used for cloning into the pDR2 Δ EF1 α vector after digestion with the same enzymes. The forward primer also contains a Kozak sequence to improve RNA translation. For the REST68 expression construct domain 5 from REST58 was replaced with a NLS. For this purpose the same reverse primer was used; however, to accommodate the long extension at the 5'-end, a series of five forward primers was designed, F1 to F5, that partially overlap, and five sequential amplifications, starting with F1 and finishing with F5, were performed. The sequences of these primers were:

F1 – GGGTGGTGGTTTTAAATGTGATCAGT (SEQ ID NO: 18);

F2 – AACGTAAAGTGGGTGGTGGTTTTAAA (SEQ ID NO: 19);

F3 – CCAAAAAAAAAACGTAAAGTGGGTGG (SEQ ID NO: 20);

F4 – AGCCACCATGCCAAAAAAAAAACGTA (SEQ ID NO: 21);

5 F5 – CGATCTAGAGCCACCATGCCAAAA (SEQ ID NO: 22).

As with the forward primer for the REST58 construct, F5 primer contained XbaI restriction site (underlined) and Kozak sequence. All constructs and products were sequenced to ensure their fidelity.

10 All constructs were introduced into neuroblastoma cells using Effectene reagent and transfected cells were selected in medium containing either 100µg/ml (IMR32) or 400µg/ml (rest of the cell lines) hygromycin B (Invitrogen).

Chromatin Immunoprecipitation

15 Kelly cells transfected either with the REST68 expression construct or the vector alone as a control were fixed for 10 minutes at room temperature in tissue culture medium containing 1% formaldehyde. All further steps of this assay have been described previously (Orlando and Paro, 1993). Chromatin was sonicated to produce DNA fragments in the range of 200 to 800 bp (electrophoretically determined in 1.5% agarose). The immunoprecipitation was carried out either
20 with rabbit polyclonal anti-Sp1 (Merck, Nottingham, United Kingdom), rabbit polyclonal anti-AP2 (Merck), sheep polyclonal anti-CPBP (R&D Systems Europe Ltd, Abingdon, United Kingdom), or a mixture of rabbit polyclonal anti-REST (H-290) and goat polyclonal anti-REST (P-18) (Santa Cruz Biotechnology) raised against peptides mapping within the N-terminus and the internal region of the
25 protein, respectively. Mixing both anti-REST antibodies was necessary to ensure recognition of both the truncated isoform of REST and the REST68 peptide. Non-immune rabbit IgG was used as a control for the background of these experiments. The naked co-immunoprecipitated DNAs were then used as templates in QPCR assays as described below. Statistical significance of the
30 data was assessed by the Student's t test.

Reverse transcription-PCR and quantitative PCR

Total RNA from frozen patient samples and neuroblastoma cell lines was purified using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). Total RNA from normal primary neurons was obtained from TCS Cellworks (Buckingham, United Kingdom). To detect the REST isoforms expressed by neuroblastoma cells, a conventional RT-PCR was performed using either a pair of primers on each side of the mutated sequence or with a nested reverse primer within the insert (Coulson et al., 2000). For semiquantification of CD59 expression, CD59-specific primers:

- 10 (TGCAATTTCAACGACGTCACA; SEQ ID NO: 25 – forward, and:
GAAATGGAGTCACCAGCAGAAGA; SEQ ID NO: 26 – reverse)
and a GAPDH specific primer pair (Coulson et al, 1999) as a control, were used. cDNA were synthesised using TaqMan Reverse Transcription reagents (Applied Biosystems, Warrington, United Kingdom) and the amplification was carried out
15 with Platinum Blue PCR SuperMix (Invitrogen).

- To quantify the CD59 copies in purified RNAs and to analyse the data, we followed the procedure described previously (Donev et al., 2006). At least two independent experiments were done for each mRNA, and Student's t test was applied to calculate significance in changes of expression pattern. In a similar
20 manner we quantified the binding of different transcription factors to the CD59 promoter. Immunoprecipitated DNAs were used as templates (10ng per reaction) in a quantitative assay with primer pairs for detection of either the 35bp positive regulatory sequence (AAGGGCATCCTGAGGGGC; SEQ ID NO: 27 – forward; TTTCGCTCCAGCCCGCAAG; SEQ ID NO: 28 – reverse). Two
25 independent analyses of the immunoprecipitated DNAs were carried out for each antibody.

Flow cytometry

- The effect of different expression constructs on expression of CD59 at
30 protein level was assessed by staining the neuroblastoma cells (3×10^5) with mouse monoclonal anti-CD59 antibody (BRIC229) for 30 minutes on ice. The unbound antibody was removed by three washes with flow cytometry buffer

(FCB) (PBS containing 10 mM EDTA, 1% bovine serum albumin, pH 7.4). The cells were then incubated for another 30 minutes with 1:100 dilution of FITC-conjugated anti-mouse immunoglobulins (The Binding Site, Birmingham, United Kingdom), washed three times with FCB and analysed on a BD FACSCalibur (BD, Oxford, United Kingdom). All measurements were made in duplicate and each experiment was replicated twice. Results were combined and statistically analysed by Student's t test. $P < 0.05$ was considered to show statistically significant differences.

10 **Example 4 – REST-derived peptides sensitise neuroblastoma cells to killing by therapeutic mAb and C**

Finally, we tested the effect of the constructs modulating expression of CD59 on C-mediated cytotoxicity triggered by anti-GD2 mAb used in neuroblastoma immunotherapy (Figure 6). We carried out the lysis assays using different concentrations of human serum as a source of C. We found that maximum lysis achieved for Kelly cells with no modulation of expression of CD59 was around 55%. However, cells transfected with the REST68 expression plasmid were more susceptible to C-dependent killing at all serum doses and approximately 80% were lysed at maximum. To confirm that reduced expression of CD59 was responsible for the observed sensitisation to C-dependent cytotoxicity, we measured the lysis of Kelly cells transfected either with REST68 expressing construct or empty vector, in which CD59 was first blocked by Fab fragment prepared from MEM43 antibody that suppresses the protective role of CD59 in C-mediated lysis (Bodian et al., 1997). After blocking CD59, lytic susceptibility was increased and was similar for cells transfected with REST68 or empty vector, confirming that increased susceptibility of REST68 treated cells to C lysis was a result of decreased CD59 expression. Heat-inactivated normal human serum did not cause specific lysis of Kelly (data not shown).

Similar lysis assays were carried out for La1-5S, SK-N-ER, and NMB7 lines (Fig. 6) for which we established populations of transfected cells by selecting with Hygromycin B. La1-5S and SK-N-ER cells transfected with REST68 showed approximately 30% higher maximum lysis compared to these

transfected with an empty vector. In NMB7, the maximum lysis achieved in REST68 transfected populations was around 95% (35% higher than in empty vector transfected control). This almost complete C killing was most likely a result of the very efficient suppression of CD59 in this cell line and of the fact
5 that NMB7 do not express CD55 and CD46 at levels detectable by western blotting (van Beek et al., 2005).

Complement lysis assay

Normal human serum (NHS), obtained by cubital vein puncture from
10 healthy volunteers, separated promptly, and stored at -80°C until use, was the source of complement in all experiments.

Neuroblastoma cells transfected with either empty pDR2ΔEF1α vector or constructs expressing REST68 were suspended in RPMI 1640 culture medium with no FCS and transferred into 96-well plates (10⁴ cells/well) with anti-GD2
15 monoclonal antibody, clone 14.2Ga (Chemicon, Chandlers Ford, Hampshire, United Kingdom) at a concentration of 10μg/ml, which was previously shown to yield a maximum lysis effect at this conditions (Mujoo et al., 1987). In experiments with CD59 blocking, excess of Fab fragment (10μg/ml) generated from MEM43 mAb against CD59 (ImmunoPure Fab preparation kit, Perbio
20 Science UK Ltd, Cramlington, UK) was preincubated with the cells for 30 minutes at 37°C. NHS was diluted as appropriate in RPMI 1640 medium and added to cells. The lysis assay was carried out using Colorimetric Cytotoxicity assay kit (Oxford Biomedical Research, Oxford, MI, USA) that measures the release of lactate dehydrogenase (LDH) by the cells. To determine the
25 maximum LDH release the cells were lysed with the lysing reagent provided in the kit. Spontaneous release was assessed by incubation without mAb and with heat-inactivated NHS (15 minutes at 56°C). All experiments were performed in triplicate for each condition. The percentage of lysed cells was calculated using the following formula:

30 % Lysis = [(measured LDH release – spontaneous release) / (maximum release – spontaneous release)] x 100

The experiment was replicated twice and data were analysed by Student's test.

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25

CLAIMS

1. A peptide comprising SEQ ID NO: 2 or a homologue or derivative thereof; provided that the peptide is not full-length REST.

5

2. A peptide as claimed in claim 1 consisting of SEQ ID NO: 2 or a homologue or derivative thereof.

3. A peptide as claimed in claim 1 comprising SEQ ID NO: 2 or a homologue or derivative thereof linked at the 5' and/or 3' end to a nuclear localisation signal, which is optionally separated from SEQ ID NO: 2 by a spacer.

4. A peptide as claimed in claim 3, wherein the nuclear localisation signal is derived from the full-length REST protein.

5. A peptide as claimed in claim 4, wherein the nuclear localisation signal is zinc-finger domain 5 of full-length REST.

6. A peptide as claimed in claim 5 which is REST58 (SEQ ID NO: 4) or a homologue or derivative thereof.

7. A peptide as claimed in any one of claims 1 to 6 further including an N' terminal methionine residue.

25

8. A peptide as claimed in claim 7 which is SEQ ID NO: 6.

9. A peptide as claimed in claim 3, wherein the nuclear localisation signal is the classical nuclear localisation signal (NLS) having the sequence PKKKRKV.

30

10. A peptide as claimed in claim 9 which is REST68 (SEQ ID NO: 8) or a homologue or derivative thereof.
11. Nucleic acid molecule encoding a peptide as claimed in any one of
5 claims 1 to 10.
12. Nucleic acid molecule which hybridises under stringent conditions to nucleic acid as claimed in claim 11.
13. Nucleic acid molecule as claimed in claim 11 having the sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 7 or a
10 homologue of any of these.
14. Nucleic acid molecule as claimed in claim 12 which hybridises to SEQ ID NO: 1, 3, 5 or 7 under stringent conditions.
15. A construct comprising nucleic acid molecule as claimed in any
15 one of claims 11 to 14.
16. A cell transformed or transfected with a construct as claimed in claim 15.
17. A process for the preparation of a peptide as claimed in any one of
20 claims 1 to 10, the process comprising expressing the nucleic acid as claimed in claim 11 or claim 13.
18. A peptide as claimed in any one of claims 1 to 10, nucleic acid
25 molecule as claimed in any one of claims 11 to 14 or a construct as claimed in claim 15 for use in medicine, particularly in the treatment of cancer.
19. The use of a peptide as claimed in any one of claims 1 to 10, nucleic acid molecule as claimed in any one of claims 11 to 14 or a construct as
30 claimed in claim 15 in the preparation of an agent for the treatment of cancer.

20. A product comprising a peptide as claimed in any one of claims 1 to 10, nucleic acid molecule as claimed in any one of claims 11 to 14 or a construct as claimed in claim 15 and an immunotherapeutic agent as a combined preparation for simultaneous, separate or sequential use in the treatment of cancer.

21. A pharmaceutical composition comprising a peptide as claimed in any one of claims 1 to 10, nucleic acid molecule as claimed in any one of claims 11 to 14 or a construct as claimed in claim 15 together with a pharmaceutically acceptable excipient.

22. A pharmaceutical composition as claimed in claim 21, further comprising an immunotherapeutic agent suitable for the treatment of cancer.

23. A method for the treatment of cancer, the method comprising administering to a patient in need of such treatment effective amounts of a first agent selected from the group consisting of a peptide as claimed in any one of claims 1 to 10, nucleic acid molecule as claimed in any one of claims 11 to 14 or a construct as claimed in claim 15 and a second agent which is an immunotherapeutic agent.

24. A peptide, nucleic acid molecule or construct as claimed in claim 18, the use as claimed in claim 19, a product as claimed in claim 20, a pharmaceutical composition as claimed in claim 21 or claim 22 or a method as claimed in claim 23, wherein the cancer is neuroblastoma.

25. A peptide, nucleic acid molecule or construct as claimed in claim 18, the use as claimed in claim 19, a product as claimed in claim 20, a pharmaceutical composition as claimed in claim 21 or claim 22 or a method as claimed in claim 23, wherein the cancer is small cell lung carcinoma or colorectal cancer.

26. A product as claimed in claim 20, a pharmaceutical composition as claimed in claim 21 or claim 22 or a method as claimed in claim 23, wherein the immunotherapeutic agent is an antibody which binds specifically to cells of the targeted tumour.

5

27. A product as claimed in claim 20, a pharmaceutical composition as claimed in claim 21 or claim 22 or a method as claimed in claim 23, wherein the immunotherapeutic agent is a monoclonal antibody which binds specifically to cells of the targeted tumour.

10

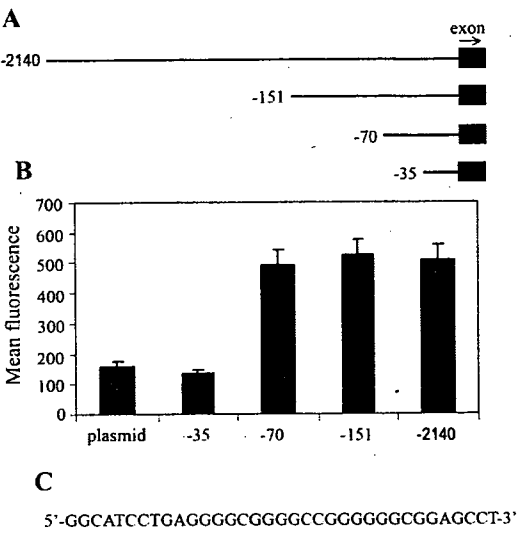


figure 1

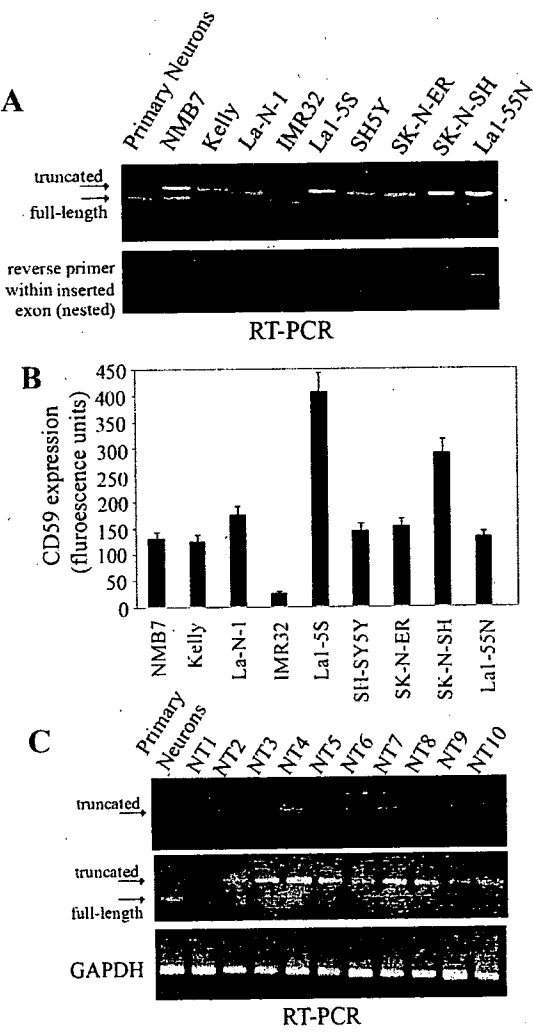


figure 2

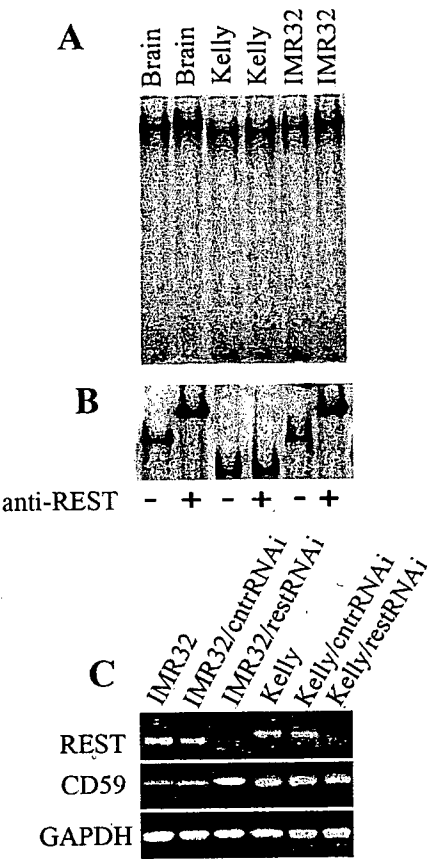


figure 3

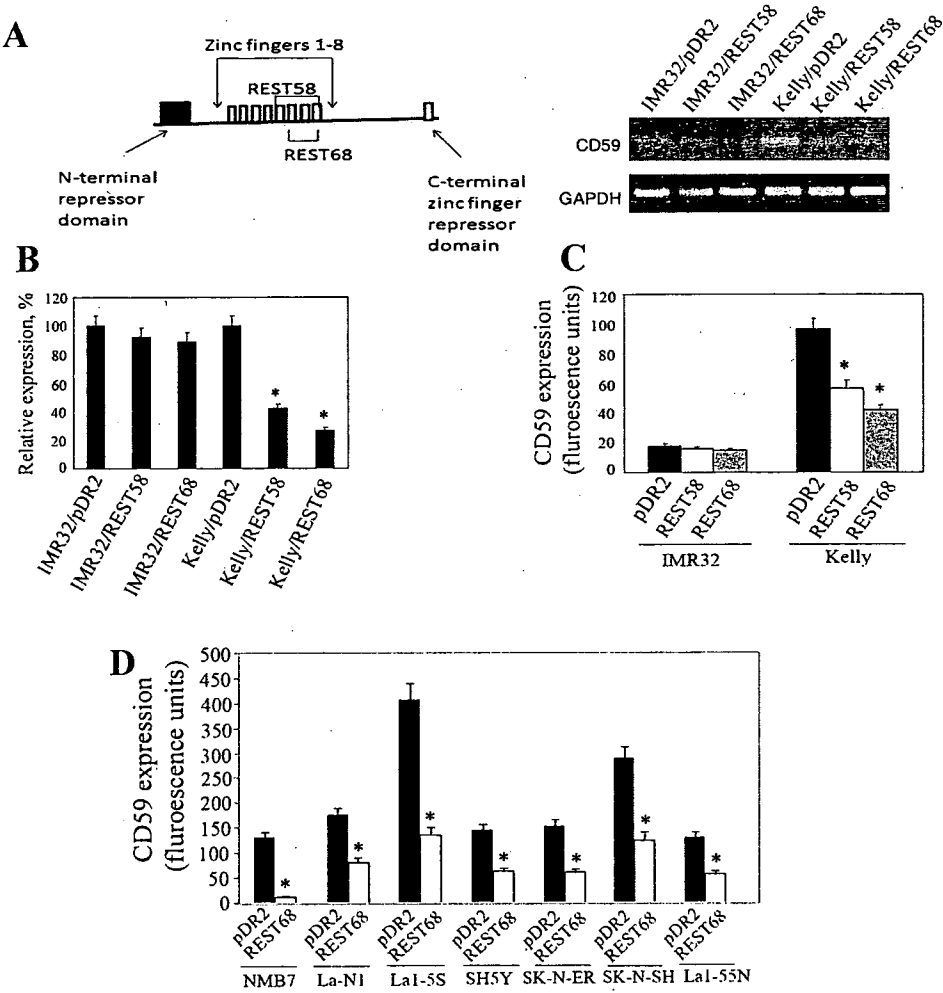


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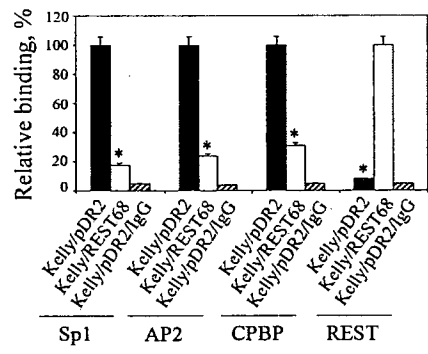


figure 5

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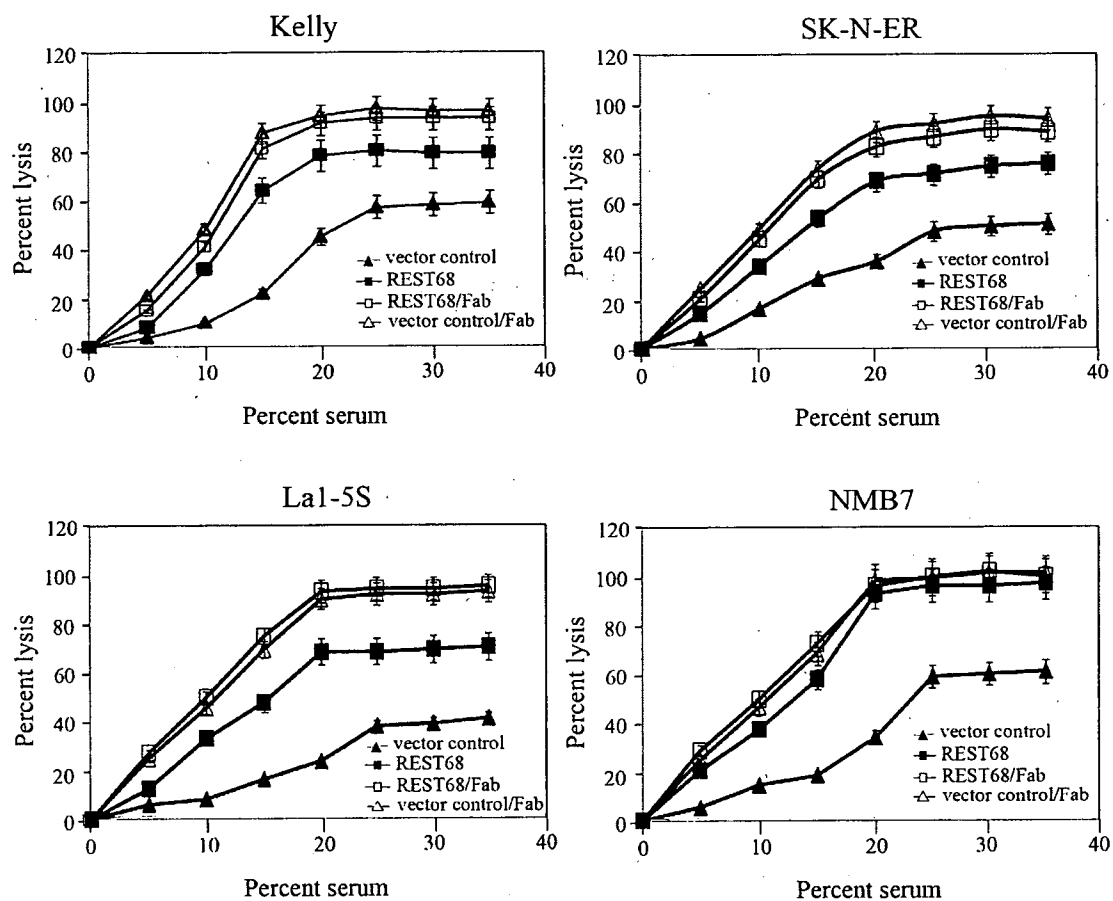


figure 6