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RNA-BINDING PATTERNS IN TOTAL HUMAN TISSUE PROTEINS: ANALYSIS BY NORTHWESTERN BLOTTING

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We have developed a reproducible Northwestern (NW) blotting method to identify general patterns of RNA-binding proteins in total human tissue homogenates and have identified some of the factors contributing to this reproducibility. Unfractionated homogenates of human brain tissue were separated by SDS-PAGE, electrotransferred wet to nitrocellulose, and probed with *in-vitro*-transcribed labeled RNAs. Approximately ten size classes of RNA-binding proteins were observed consistently and reproducibly. Although sequence-independent electrostatic RNA-protein interactions likely contributed to most of the binding, binding to some proteins was shown to be more dependent on protein conformation: binding was not blocked by preincubation with single- or double-stranded DNA, nor with poly(A) RNA, but preincubation with tRNA revealed a distinct subset of RNA-binding proteins. In addition, preincubation with RNA, but not DNA, revealed a previously undetected RNA-binding protein of approximately 90 kDa. The NW blotting method described here can be used to reveal tissue-specific differences in RNA-binding patterns.

RNA in living cells is often associated with, or bound to, proteins. A major advance in the analysis of protein-nucleic acid interactions occurred in 1980, when Bowen et al. (1) established a method to detect protein-DNA interactions by binding labeled DNA to proteins which had been separated by SDS-PAGE and transferred to nitrocellulose ("Southwestern blotting"); they extended this procedure to protein-RNA binding ("Northwestern blotting") by demonstrating protein binding to ^{125}I-labeled Rous sarcoma virus RNA (1).

Most applications of NW blotting have been performed on purified proteins and RNA (2-7). There are few examples of the application of NW blotting to detect interactions between specific proteins and mature mRNAs, even though these interactions are important in the translational

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Abbreviations: APP, amyloid β precursor protein; bp, base pair(s); Denhardt's solution, 0.04% each of polyvinylpyrrolidone, Ficoll, and bovine serum albumin; DTT, dithiothreitol; kDa, kilodaltons; NW, northwestern; nt, nucleotide(s); PBS, phosphate-buffered saline; PGAM-B, B isoform of phosphoglycerate mutase; PMSF, phenylmethylsulfonyl fluoride; RNAsin, pancreatic RNAse inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEVAG, 24:1 chloroform:isoamyl alcohol.

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control of gene expression (8-11), and even fewer examples of the use of the NW method to detect generalized protein-mRNA interactions. One reason for this is that the NW blotting procedure as currently described is difficult to apply in a more generalized manner, as the application of this method to search for RNA-binding proteins in the context of a population of hundreds or thousands of proteins is usually confounded by high backgrounds and lack of reproducibility.

It was an aim of this work to provide a more reproducible method to identify general patterns of RNA-binding proteins in unfractionated human tissue, and to explore and identify some of the factors contributing to this reproducibility.

MATERIALS AND METHODS

Tissue preparation

Fibroblast and lymphoblast cell lines were obtained from the tissue repository of the National Institute on Aging. Brain (temporal cortex) samples were a kind gift of Dr. P. Gambetti (CaseWestern Reserve University). Approximately 500 mg of tissue or cells were washed in PBS and then dispersed in 5 volumes of suspension buffer (100 mM NaCl, 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1μg/ml aprotinin [Sigma] and 100 μg/ml PMSF [Sigma]) with a homogenizer. An equal volume of 2 x SDS gel-loading buffer (100 mM Tris·HCl, pH6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added immediately and the sample was boiled for 10 minutes and stored in -70°C. The protein concentration was determined as described (12).

SDS-PAGE and electrotransfer

Electrophoresis of proteins (approximately 100 μg/lane) was carried out using SDS-polyacrylamide gels (2.6% crosslinking) as described (13). After the electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris·HCl, pH 8.3, 192 mM glycine, and 20% methanol) for 30 min and the proteins were then electrotransferred onto a nitrocellulose membrane (Bio-Rad) (14). After electrotransfer the proteins were allowed to renature by washing the membrane in binding buffer (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1x Denhardt's solution) at 4°C for at least 1 hour.

In vitro transcription of cloned plasmid DNAs

Clone pHB4.4 is a partial-length human amyloid β precursor protein (APP) cDNA clone encoding 2110 nt of APP mRNA sequences derived from the 5' end of the APP gene, extending from nt -61 to +1794 (numbering of Ref. 15), and including a 225-nt insertion of the "extra-exon" domain (16) between nt-865 and nt-866. Clones pAPP5.11 and pAPP5.1 are partial-length cDNA clones encoding APP mRNA sequences derived from the 3' end of the APP gene, extending from nt 1933-3208 and 866-3208, respectively. Clone pAPP6.3 contains a nearly-full-length APP cDNA 3510 bp in length, extending from nt -47 to +3208 and including the 225-nt extra exon domain. Clone pPGAM-B contains a 1.8-kb full-length human PGAM-B cDNA (clone pPGAMB.FL in Ref. 17).

Plasmid DNA was purified by alkaline lysis (18) and was linearized by restriction endonuclease digestion, treated with RNAseA, and then extracted with phenol/SEVAG for three times prior to ethanol precipitation. ^{32}P-labeled RNA was prepared by *in vitro* transcription (19) from these DNA templates. Briefly, 2 μg of template DNA was incubated with 50 mCi of [α-^{32}P]GTP (3000 Ci/mmol; DuPont-New England Nuclear), 0.5 mM each of CTP, ATP, and UTP, and 7.5 units of T3 or T7 RNA polymerase (Promega) in transcription buffer (20 mM Tris·HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM spermidine, 10 mM DTT, and 30 units RNAsin) at 37°C for 20 min, followed by a cold chase with 0.5 mM GTP for 40 min. After digestion with 10 ng RNAse-free DNAse for 30 min at 37°C, the RNA product was purified by phenol/SEVAG extraction and two ethanol precipitations, and checked for integrity by electrophoresis through a formaldehyde-agarose gel (20). Specific activities were typically 5 x 10^8 cpm/μg.

RNA-protein binding

Approximately $1x10^8$ cpm radiolabelled RNA probe was added to 10 ml binding buffer and incubated with the nitrocellulose filter containing the transferred proteins at the indicated temperature for 1 hour. The filter was then washed with the same buffer twice for 5 min and the dried nitrocellulose membrane was exposed to X-ray film for autoradiography.

RESULTS

General pattern of RNA-binding to human brain tissue proteins

Using the protocol described above, we probed human brain tissue (temporal cortex) homogenates separated by SDS-PAGE with *in-vitro*-transcribed sense-orientation APP mRNA derived from pHB4.4. We reproducibly observed a general pattern of RNA binding proteins which differed from the pattern of total proteins as revealed by silver staining (not shown), consisting of approximately 10 size classes of proteins that bound the probe strongly (denoted a-j in Fig. 1A), plus numerous more weakly-binding bands. On 10% polyacrylamide gels, we observed bands migrating at positions corresponding to approximately 103, 94, 76, 72, 50, 47, 40, 38, 35, and 21 kDa (Fig. 1A, lane 1). On 15% gels, we observed bands located at 35.5, 33.5, 32.5, 22, 20, 9, and 8.5 kDa (Fig. 1A, lane 2); the larger-sized proteins did not transfer well from these gels. The intense signal derived from region "j", located at 20-22 kDa, may be due to RNA binding to histone H1, which is in this size range.

Effect of monovalent and divalent cations

We incubated the filters in binding buffer containing 50 mM NaCl alone, or in buffer containing 50 mM NaCl plus 200 mM KCl, 10 mM $MgCl_2$ or 10 mM $CaCl_2$. Sodium ion alone gave the best result (Fig. 1B); both 200 mM KCl and 10 mM $MgCl_2$ reduced RNA-binding significantly, while 10 mM $CaCl_2$ increased the background signal severely, to the point of obscuring the signal when the autoradiogram was exposed for a length of time identical to that used with the other buffer conditions. The effect of NaCl concentration on the efficiency of RNA binding was concentration-dependent: as the NaCl concentration was increased from 50 to 100 to 200 mM, binding decreased successively (Fig 1C); KCl in the same concentration range showed a similar effect (data not shown). The RNA-binding was also inhibited in a concentration-dependent manner upon addition of $MgCl_2$ at 1, 5, and 10 mM (Fig 1D).

Effect of temperature

We compared binding at room temperature (22°C) and at physiological temperature (37°C); little difference in the binding pattern was observed (Fig. 1E). Based on these results, our standard NW binding protocol for all the subsequent experiments was performed in binding buffer containing 50 mM NaCl (see Methods) and with all incubations performed at 22°C. The addition of tRNA to the binding buffer is considered optional where appropriate (see below).

Effect of different RNA probes

In order to determine whether the general RNA binding pattern we observed was sequence-dependent, we compared the NW blots using sense and anti-sense APP RNA probes transcribed

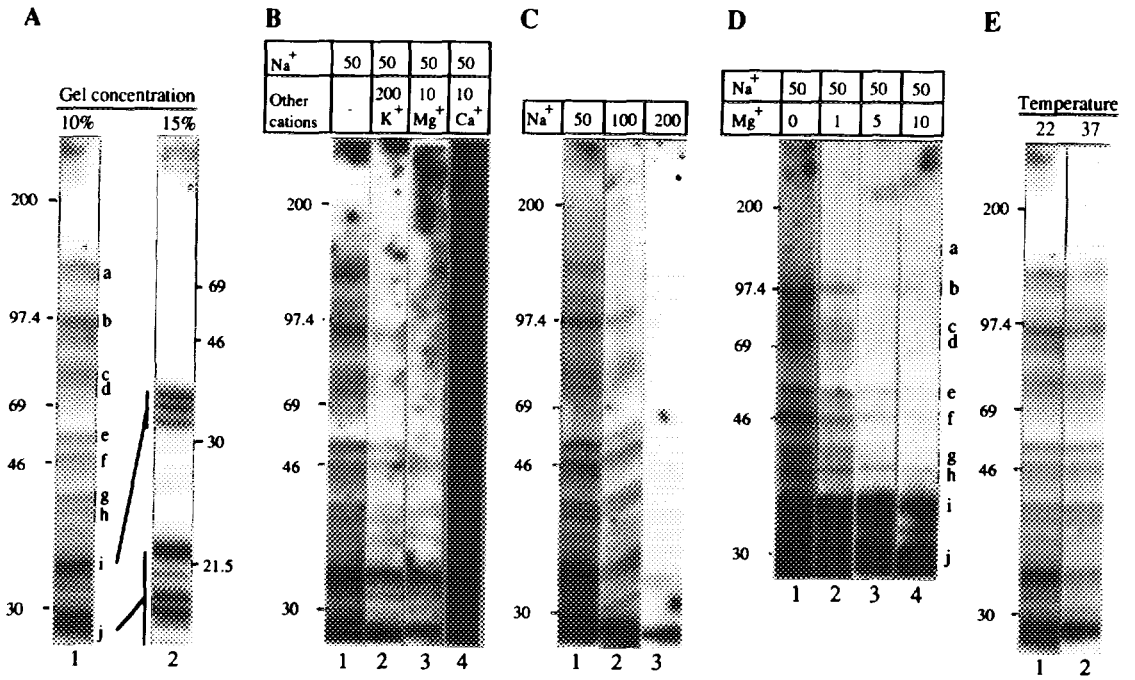

Fig. 1. NW blotting of brain proteins probed with labelled sense-strand APP RNA transcribed from clone pHB4.4 (see Fig. 3A). **A.** The pattern of RNA-binding proteins separated on 10% and 15% SDS-polyacrylamide gels. Approximately 10 size classes of RNA-binding proteins (denoted "a-j" here and in the subsequent figures) were observed. **B.** Effect of cations (KCl, $MgCl_2$, $CaCl_2$; concentrations in mM) in the standard binding buffer; the buffers with Mg^{++} and Ca^{++} had no EDTA. **C.** Effect of increasing NaCl concentration (mM) in the standard binding buffer. **D.** Effect of increasing $MgCl_2$ concentration (mM) in the standard binding buffer (no EDTA present). **E.** Effect of temperature of binding (standard conditions). Molecular weight standards (Amersham) in this and the following figures are indicated at the side, in kDa.

from clone pHB4.4 (Fig. 2A). Similarly, in order to determine whether RNA secondary structure played a role in the generalized binding pattern, we compared the pattern obtained with the sense-orientation APP RNA probe derived from pHB4.4 with that obtained using the sense-orientation of the PGAM-B gene (Fig. 2A). In both cases, we observed no significant differences in the binding patterns relative to the sense-orientation APP RNA probe used initially (Fig 2B). Fundamentally identical blotting patterns were obtained when we probed the filter with APP subregional RNAs transcribed from the pAPP5.11, pAPP5.1, and pAPP6.3 clones (Fig 2C).

Effect of nucleic acid blocking agents

We also compared the RNA binding in the buffers containing 100 μg/ml each of single-stranded DNA (sonicated salmon sperm DNA boiled for 10 min), double-stranded DNA (unboiled sonicated salmon sperm DNA), poly(A) RNA, and yeast tRNA. No significant effects were observed when binding was performed in the presence of single-or double-stranded DNA (Fig. 3). However, in the presence of yeast tRNA in the binding buffer (Fig. 3, lane 5) the signal derived from most of the proteins was strongly suppressed, with a concomitant increase in the

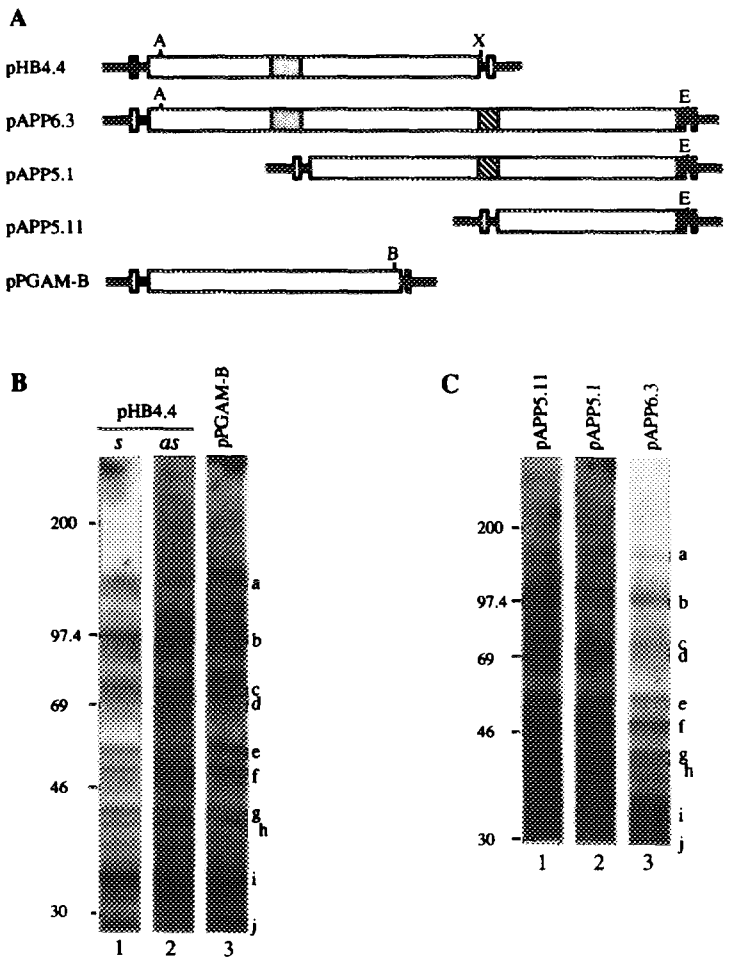

Fig. 2. Use of different probes in NW blotting (standard conditions). **A.** Maps of the cDNA clones (identified at the left of each map) used as sources of the RNA probes. The flanking solid lines denote the Bluescribe vector. The small vertical boxes denote T3 (open boxes) and T7 (solid boxes) promoters flanking the cDNA insert (large open boxes). The top 4 clones show segments of the APP cDNA, containing the A4 (hatched box) and extra exon (shaded box) domains of the human β-amyloid protein precursor gene; the polyadenylation signal is denoted by the horizontally hatched box. The bottom map denotes the human PGAM-B cDNA. Restriction enzymes used for linearizing the plasmids to prepare templates for *in vitro* transcription are shown (A, *ApaI*, B, *Bam*HI, E, *Eco*RV, X, *Xba*I). **B.** NW blotting with APP sense (s) and anti-sense (as) RNA transcribed from clone pHB4.4 and with sense RNA transcribed from pPGAM-B. **C.** NW blotting with sense-orientation APP subregion RNA probes.

signal from the few remaining "unsuppressed" bands (e.g. bands b, i, and j). In addition, one extra band located at approximately 90 kDa, which had displayed a weak signal under the standard conditions, was now revealed (arrow in Fig. 3). Preincubation with poly(A) RNA did not suppress any binding, but, like tRNA, also revealed the new binding protein.

RNA-binding pattern of proteins derived from different human tissues

Protein homogenates from human brain, heart, kidney, lung, and liver were compared for their RNA-binding properties. The general RNA-binding patterns were remarkably similar among

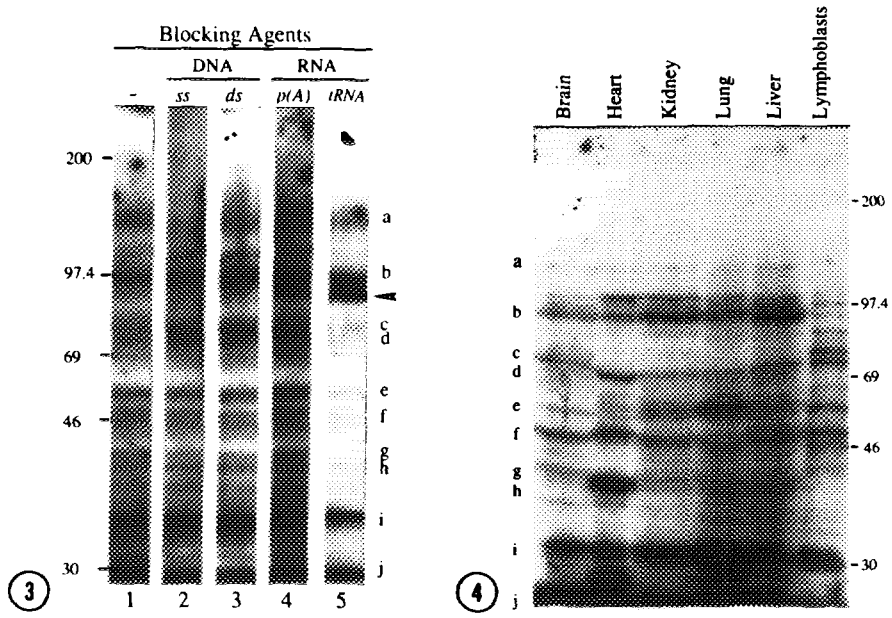

Fig. 3. NW blotting (standard conditions) in the presence of nucleic acids. Preincubation with 100 µg/ml each of heat-denatured sonicated salmon sperm DNA (ss), undenatured sonicated salmon sperm DNA (ds), poly (A) RNA, and yeast total tRNA (lanes 2-5, respectively) is compared to binding in standard conditions without preincubation in the presence of added nucleic acids (lane 1). Arrow denotes a 90-kDa band revealed upon preincubation with RNA.

Fig. 4. NW blotting (standard conditions) of protein homogenates from different human tissues, using the sense-orientation of APP RNA transcribed from pHB4.4 as probe.

these extensively differentiated tissues (Fig. 4). Only a few RNA-binding proteins were found to be specific to, or overexpressed in, any one tissue (see, for example, the patterns in heart or lung). Overexpression of 50- and 72-kDa RNA-binding proteins (bands "c" and "e") was also evident in the EBV-transformed lymphoblasts relative to the general pattern observed in the other tissues.

DISCUSSION

We have modified the NW blotting procedure to provide a reproducible method to detect generalized RNA-binding proteins in tissue homogenates. We have found that the tissue has to be as fresh as possible (i.e. either used directly or frozen at -70°C and thawed just prior to use; re-freezing of thawed samples should be avoided), and the homogenized sample has to be treated with SDS and denatured as soon as possible in order to prevent protein degradation. Electrotransfer must be performed "wet" (14). We found that semi-dry transfer not only produced poor transfer of large protein molecules, but also diminished the RNA-binding capability of those proteins that were transferred. We suspect that semi-dry transfer causes excessive heating of the samples, and also inhibits renaturation of the transferred proteins, even during the subsequent renaturing step. The proteins must be allowed to renature as much as possible by washing the filter

in binding buffer for at least 1 hour at 4°C. Moreover, the RNA probe must be free of unincorporated nucleotides and must be undegraded.

The RNA-binding affinity of these proteins was greatly diminished by increased concentrations of both monovalent (i.e. Na^+ and K^+) and divalent (i.e. Mg^{++} and Ca^{++}) cations, and there was also no apparent sequence-specificity to the binding. We conclude that most of the RNA-binding detected by the blotting method described here depended on electrostatic interactions. This is not inconsistent with the fact that the binding properties of many regulatory RNA-binding proteins are apparently highly sequence-specific. It is likely that a large group of RNA binding proteins exist which have little or no sequence specificity (i.e. electrostatic binding only), many of which presumably play a constitutive (housekeeping) role in the cell. Such proteins would likely be more stable and more abundant than sequence-specific regulatory RNA-binding proteins, and hence would be more easily detected in a generalized NW blotting protocol.

RNA binding could not be blocked by preincubation with poly(A) RNA, nor with single- or double-stranded DNA. However, preincubation with tRNA, which has significant secondary structure (as opposed to poly(A) RNA, which has both low complexity and little, if any, secondary structure), was able to block binding of the probe in a protein-specific manner. In other words, tRNA blocked the binding to most, but not all of the blotted proteins, and the strength of the signal derived from those proteins which were not blocked was enhanced. We suspect that the enhancement of signal was due to an increase in the effective concentration of free probe in the binding buffer. These results indicate first, that most of the binding in the NW protocol described here is RNA-specific, and second, that the binding to some (but certainly not all) of the target proteins is dependent to a significant degree on RNA secondary structure. The reduction in the number of RNA-binding protein bands in the presence of added tRNA was significant enough to warrant the incorporation of tRNA in the NW protocol when a more defined picture of the RNA-binding pattern in a tissue is desired.

In practice, because most RNA-binding proteins in a tissue can be blocked by increasing the amount of cation or by adding tRNA, it should be possible to "titrate out" the majority of nonspecific binding proteins and inhibit most background binding so as to highlight specific binding proteins of interest. Thus, although the NW method has been used mainly for the analysis of purified proteins, we believe that specific RNA-binding proteins may also be observable reproducibly in total tissue homogenates. For example, preincubation of the filter with either poly(A) RNA or tRNA revealed a specific band migrating at approximately 90 kDa (Fig. 3). This result may have been due to the fact that this 90-kDa protein bound the probe RNA only when a specific protein conformation was achieved upon incubation with the high concentration of blocking RNA. In other words, the blocking RNA caused a [cooperative?] change in conformation of the 90-kDa protein that increased its ability to bind the probe RNA. It is not known at present whether the "presentation" of the 90-kDa band depends on either the specific type of RNA used for preincubation or the specific RNA used as the probe. Nevertheless, the identification of a specific "RNA-dependent" RNA-binding protein exemplifies the type of analysis that can be done on total tissue homogenates using the NW protocol described here.

In NW blotting of proteins from different sources, several tissue-specific RNA-binding proteins were distinguished from the general binding pattern (Fig. 4). We note that the tissue-

specific differences in the RNA-binding patterns as observed by autoradiography did not correspond to differences in the abundance of proteins in any one tissue as observed by silver-staining of the gel (data not shown). Thus, our NW protocol is capable of identifying even low-abundance RNA-binding proteins, in spite of the presence of highly abundant tissue-specific proteins in the homogenate (e.g. myosin in heart or albumin in liver). The observation that some RNA-binding proteins were overexpressed in EBV-transformed lymphoblast cells suggests that the transition of a cell from the normal to the transformed phenotype may involve the altered expression of these proteins (see, for example Ref. 21).

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