Molecular Cancer

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Gene expression profiling by DNA microarray analysis in mouse embryonic fibroblasts transformed by ras^{V12} mutated protein and the E1A oncogene

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Published: 19 March 2003

Molecular Cancer 2003, **2**:19

Received: 24 January 2003
Accepted: 19 March 2003

This article is available from: http://www.molecular-cancer.com/content/2/1/19

Abstract

Background: Ras is an area of intensive biochemical and genetic studies and characterizing downstream components that relay ras-induced signals is clearly important. We used a systematic approach, based on DNA microarray technology to establish a first catalog of genes whose expression is altered by ras and, as such, potentially involved in the regulation of cell growth and transformation.

Results: We used DNA microarrays to analyze gene expression profiles of ras^{V12}/E1A-transformed mouse embryonic fibroblasts. Among the ~12,000 genes and ESTs analyzed, 815 showed altered expression in ras^{V12}/E1A-transformed fibroblasts, compared to control fibroblasts, of which 203 corresponded to ESTs. Among known genes, 202 were up-regulated and 410 were down-regulated. About one half of genes encoding transcription factors, signaling proteins, membrane proteins, channels or apoptosis-related proteins was up-regulated whereas the other half was down-regulated. Interestingly, most of the genes encoding structural proteins, secretory proteins, receptors, extracellular matrix components, and cytosolic proteins were down-regulated whereas genes encoding DNA-associated proteins (involved in DNA replication and reparation) and cell growth-related proteins were up-regulated. These data may explain, at least in part, the behavior of transformed cells in that down-regulation of structural proteins, extracellular matrix components, secretory proteins and receptors is consistent with reversion of the phenotype of transformed cells towards a less differentiated phenotype, and up-regulation of cell growth-related proteins and DNA-associated proteins is consistent with their accelerated growth. Yet, we also found very unexpected results. For example, proteases and inhibitors of proteases as well as all 8 angiogenic factors present on the array were down-regulated in transformed fibroblasts although they are generally up-regulated in cancers. This observation suggests that, in human cancers, proteases, protease inhibitors and angiogenic factors could be regulated through a mechanism disconnected from ras activation.

Conclusions: This study established a first catalog of genes whose expression is altered upon fibroblast transformation by ras^{V12}/E1A. This catalog is representative of the genome but not exhaustive, because only one third of expressed genes was examined. In addition, contribution to ras signaling of post-transcriptional and post-translational modifications was not addressed. Yet, the information gathered should be quite useful to future investigations on the molecular mechanisms of oncogenic transformation.

Background

Cancer is a disease caused by multiple genetic alterations that lead to uncontrolled cell proliferation. This process often involves activation of cellular proto-oncogenes and inactivation of tumour-suppressor genes. One of the earliest and most potent oncogenes identified in human cancer is the mutant *ras* [1,2]. *Ras* family of proto-oncogenes encodes small GTP-binding proteins that transduce mitogenic signals from tyrosine-kinase receptors [reviewed in [3]]. *In vitro*, oncogenic *ras* efficiently transforms most immortalized rodent cell lines but fails to transform mouse primary cells [4]. However, *ras* can transform primary mouse cells by cooperating with other oncogenic alterations such as overexpression of c-Myc, dominant negative p53, D-type cyclins, Cdc25A and Cdc25B, or loss of *p53*, *p16* or *IRF-1* [5–7]. Several viral onco-proteins can also cooperate with *ras*, for example SV40 T-antigen, adenovirus E1A, human papillomavirus E7 and HTLV-1 Tax [reviewed in [6,7]]. When expressed alone in primary cells, most of these alterations facilitate their immortalization [7]. Oncogenic transformation of primary cells by co-expression of *ras* and immortalizing mutations constitutes a model of multistep tumorigenesis that has been reproduced in animal systems [reviewed in [8,9]].

Ras has been an area of intensive biochemical and genetic studies [10]. These studies helped to characterize downstream signaling events and components that relay *ras*-induced mitogenic signals to the ultimate transcription factors which regulate expression of genes involved in cell growth and transformation. Downstream signaling elicited by the oncogenic form of Ras protein impairs regulation of gene expression with eventual disruption of normal cellular functions. Downstream transcription factors were found essential for *ras*-mediated cell transformation [11–13]. However, compared with our knowledge on *ras* signaling events, little is known on target genes involved in the phenotypic changes resulting from *ras* activation, such as cell transformation. Thus, identification of genes whose expression is altered during *ras*-mediated cell transformation would provide important information on the underlying molecular mechanism. In the present work, we used DNA microarray technology to analyze gene expression profiles of ras^{V12}/E1A-transformed primary mouse embryonic fibroblasts (MEFs), in order to identify genes whose expression is transformation-dependent.

Results

Analysis of gene expression changes after ras^{V12}/E1A-transformation

We used microarray analysis to compare expression profiles of ~12,000 genes in normal vs. ras^{V12}/E1A-transformed fibroblasts. Figure 1 shows the phenotypic changes of the ras^{V12}/E1A-transformed MEFs. With Affymetrix microarray technology, differential expression values greater than 1.7 are likely to be significant, based on internal quality control data. We present data which use a more stringent ratio, restricting our analysis to genes that are overexpressed or under-expressed at least 2.0 fold in ras^{V12}/E1A-transformed fibroblasts relative to the empty retrovirus-transduced MEFs. We summarize the highlights below and present the full profile in Figure 2.

Among the ~12,000 genes and ESTs analyzed, expression of 815 showed to be altered by at least 2.0 fold in the ras^{V12}/E1A-transformed fibroblasts, of which 203 corresponded to ESTs. Among known genes, 202 were up-regulated (Table 1)(see Additional file 1) whereas 410 were down-regulated (Table 2)(see Additional file 2) by ras^{V12}/E1A-transformation. It is interesting to note that about one half of genes encoding transcription factors, signaling proteins, membrane proteins, channels, or apoptosis-related proteins was up-regulated whereas the other half was down-regulated (Figure 2). However, after ras^{V12}/E1A-transformation most of genes encoding structural proteins, secretory proteins, receptors, proteases, protease inhibitors, extracellular matrix components, proteins involved in angiogenesis and cytosolic proteins, were down-regulated whereas genes encoding DNA-associated proteins (involved in DNA replication and reparation) and cell growth-related proteins were up-regulated (Figure 2). These data may explain, at least in part, the behavior of transformed cells. For example, down-regulation of structural proteins, extracellular matrix components, secretory proteins and receptors is consistent with reversion of the phenotype of transformed cells towards a less differentiated phenotype and up-regulation of cell growth-related proteins and DNA-associated proteins is consistent with their accelerated growth.

Transcription factors

57 genes encoding transcription factors were up-regulated and 45 down-regulated by ras^{V12}/E1A-transformation. The most strongly activated genes corresponded to the homeobox protein SPX1 (39 fold), myb proto-oncogene (25 fold) and the paired-like homeodomain transcription factor (19 fold), whereas the most repressed were the osteoblast specific factor 2 (123 fold), the p8 protein (51 fold), the H19 mRNA (21 fold) and the early B-cell factor (20 fold).

Structural proteins

Expressions of 10 genes encoding structural proteins were up-regulated in MEFs-transformed cells, 44 being down-regulated. The most important up-regulation was observed for cytokeratin (26 fold) and desmoplakin I (17 fold), the strongest down-regulations for smooth muscle calponin (115 fold), transgelin (49 fold), debrin (41

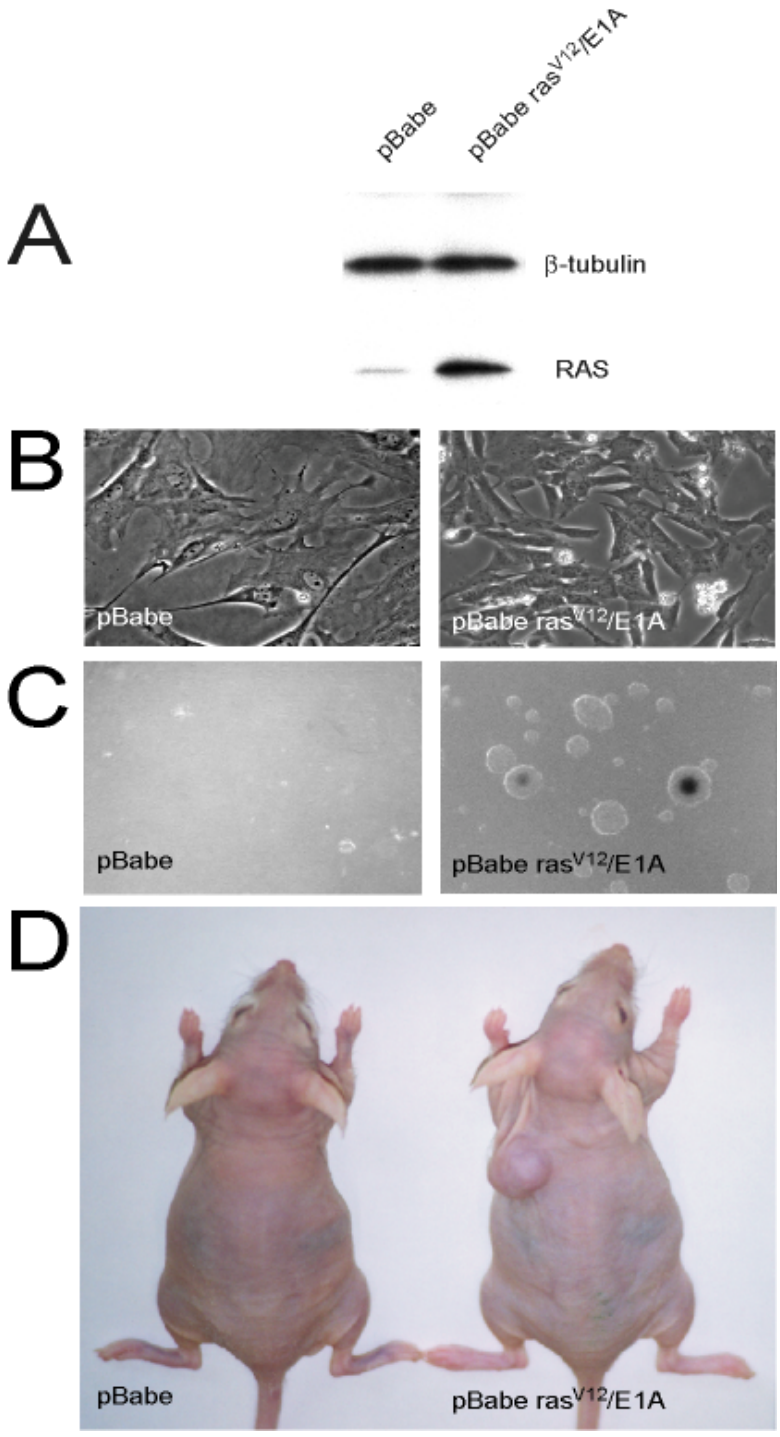

Figure 1
A. Expression of RAS was verified by immunoblot analysis in MEFs transduced with pBabe (control) or pBabe-ras^{V12}/E1A (transformed) retroviruses. B. Morphological aspect of the pBabe and pBabe-ras^{V12}/E1A transduced mouse embryonic fibroblats. C. Anchorage-independent growth of the ras^{V12}/E1A transformed MEF. Fifty thousand cells were plated on 0.6% agar in DMEM-10% FCS and overlaid on 0.6% agar in the same medium. Photomicrographs were taken 10 days after plating. D. ras^{V12}/E1A transformed MEF induce tumor formation. One million of pBabe and pBabe-ras^{V12}/E1A transduced mouse embryonic fibroblast were injected in 200 μl PBS as xenografts in nude mice. Representative mice at day 18.

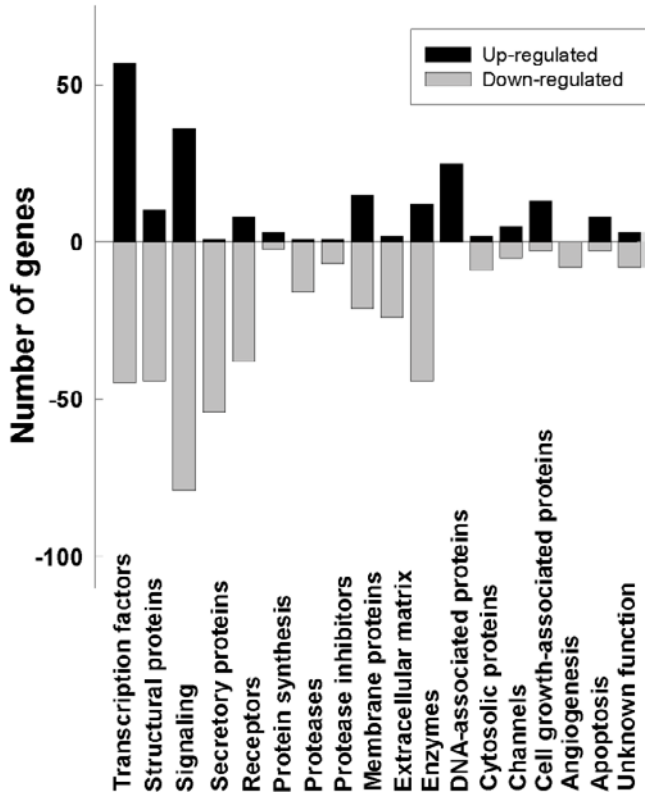

Figure 2
Gene expression changes after ras^{V12}/E1A-transformation. Number of genes up-regulated or down-regulated were grouped by function (Transcription factors, structural proteins, signaling, secretory proteins, receptors, protein synthesis, proteases, protease inhibitors, membrane proteins, extracellular matrix, enzymes, DNA-associated proteins, cytosolic proteins, channels, cell growth-associated proteins, angiogenesis, apoptosis and unknown function). Bars represent the number of genes in each group.

fold), p50b (35 fold) and vascular smooth muscle alpha-actin (34 fold).

Signaling factors
36 genes encoding proteins involved in numerous signaling pathways were up-regulated and 79 down-regulated in ras^{V12}/E1A-transformed MEFs. The EGP314 precursor (also known as the calcium signal transducer 1) was found 25 fold up-regulated, whereas the cysteine rich intestinal protein (41 fold) and ASM-like phosphodiesterase 3a (31 fold) were the most strongly down-regulated genes.

Secretory proteins
Only one gene, encoding the transforming growth factor alpha, was detected as up-regulated (3 fold) in transformed cells. By contrast, expressions of 54 secretory proteins were repressed after ras^{V12}/E1A-transformation. The most affected genes were those encoding cholecystokinin (112 fold), serum amyloid A3 (85 fold), PRDC (58 fold), insulin-like growth factor binding protein 5 (41 fold), gremlin (36 fold), follistatin (33 fold), the small inducible cytokine subfamily B (27 fold), cytokine SDF-1-beta (23 fold) and the small inducible cytokine A7 (22 fold).

Receptors
8 receptors were up-regulated and 38 down-regulated in transformed fibroblasts. Overexpression was observed for acetylcholine receptor beta (8 fold), tyrosine kinase receptor (3 fold), growth hormone releasing hormone receptor (3 fold), semaphorin M-sema G (3 fold) and amphiregulin (2 fold). Strongest down-regulations were found for integrin alpha 5 (43 fold), transient receptor protein 2 (19 fold), retinoic acid receptor alpha (14 fold), retinoic orphan receptor 1 (11 fold) and platelet derived growth factor receptor (12 fold).

Protein synthesis
3 genes involved in protein synthesis (BRIX, nucleolin, ribosomal protein L44 and SIK similar protein) were overexpressed and 2 (ribosomal protein S4X and ribosomal protein L39) were down-regulated, suggesting that protein synthesis is not strongly affected by transformation.

Proteases and protease inhibitors
Only the kallikrein B protease and the elafin-like protein II protease inhibitor were up-regulated (3 and 2 fold respectively) after ras^{V12}/E1A-transformation. By contrast, 16 proteases and 7 protease inhibitors were found repressed in transformed MEFs. The tolloid-like (41 fold) and meltrin beta (33 fold) were proteases most down-regulated and the tissue factor pathway inhibitor 2 (44 fold) and the plasminogen activator inhibitor (31 fold) were the most affected protease inhibitors.

Membrane proteins
15 genes encoding membrane proteins were up-regulated and 21 were down-regulated. Histocompability 2, D region locus, (16 fold) and melanoma differentiation associated protein (9 fold) were the most overexpressed genes, whereas Thy-1.2 glycoprotein (36 fold), cadherin 11 (14 fold) and vascular cell adhesion molecule 1 (13 fold) were the most repressed.

Extracellular matrix
Laminin gamma 1 (20 folds) and entactin-2 (6 folds) were the two extracellular matrix encoding genes found up-regulated during transformation, whereas 24 genes were down-regulated. Among them, procollagen type VI, alpha 1 (121 folds), procollagen type III, alpha 1 (56 folds), procollagen type I, alpha 1 (44 folds), procollagen type I, alpha 2 (37 folds), collagen type VI, alpha 3

subunit (21 folds) and decorin (19 folds) were the most affected.

Enzymes

Twelve enzymes involved in cellular metabolism were found overexpressed after ras^{V12}/E1A-transformation and 44 were found down-regulated. The most activated genes were serine hydroxymethyl transferase 1 (6 fold), acetyl coenzyme A dehydrogenase (5 fold) and the acetyltransferase (GNAT) family containing protein (4 fold), whereas the most repressed genes were lysozyme P (88 fold), lysyl oxydase (61 fold) and lysozyme M (55 fold). Interestingly, maximal overexpressions were 6, 5 and 4 fold, whereas down-regulations were 88, 61 and 55 fold indicating that in addition to the fact that more genes were down-regulated (44 vs. 12), change in expression was also more important for down-regulated genes.

DNA-associated proteins

25 genes encoding DNA-associated proteins were up-regulated, whereas no gene of this family was found down-regulated. The most strongly activated genes were nucleoside diphosphate kinase (9 fold), the topoisomerase-inhibitor suppressed (7 fold), the helicase lymphoid specific (6 fold) and the DNA2-like homolog (6 fold).

Cytosolic proteins

Expression of 2 genes encoding cytosolic proteins was activated after ras^{V12}/E1A-transformation, whereas expression of 6 genes was repressed. Genes coding for acyl-CoA-binding protein (3 fold) and tubulin-specific chaperone (2 fold) were overexpressed, whereas the most strongly repressed gene was that coding cytochrome P450 (61 fold).

Channels

5 genes encoding channels were up-regulated and also 5 were down-regulated. Chloride channel protein 3 was the most up-regulated gene (11 fold) and the channel beta-1 subunit (15 fold) was the most down-regulated gene.

Cell growth-associated proteins

As expected for transformed cells which grow more rapidly, 13 genes encoding proteins involved in cell growth were found overexpressed, whereas only 3 were found down-regulated in ras^{V12}/E1A-transformed MEFs. The most activated genes were those coding for cyclin-dependent kinase-like 2 (6 fold) and cell division cycle 7-like 1 (5 fold) whereas the most repressed gene was cyclin D2 (4 fold).

Angiogenesis

Angiogenesis is a key process in carcinogenesis. Contrary to the expected for a tumoral cell, we were unable to found angiogenesis-associated genes up-regulated by ras^{V12}/E1A-transformation. To our surprise, all 8 genes associated with angiogenesis showing differential expression were down-regulated. These included genes coding for thrombospondins 1 (15 fold), 2 (32 fold) and 3 (6 fold), pigment epithelium-derived factor (26 fold), pleiotrophin (24 fold), GRO1 oncogene (16 fold), angiogenin-related protein (4 fold) and tumor necrosis factor induced protein 2 (3 fold).

Apoptosis

8 apoptosis-related genes were up-regulated in transformed MEFs and 3 down-regulated. The p53 apoptosis effector related to Pmp22 was the most activated gene (19 fold) and death-associated protein 1 gene was the most under-expressed (4 fold) after transformation.

Unknown function

3 genes encoding proteins without well defined function were found up-regulated in mutated *ras*-E1A expressing fibroblasts, whereas 8 were found to be down-regulated.

As a proof-of-principle, we verified the relative expression levels of 11 of these 815 genes by Northern blot analysis. The following 11 genes were tested : p8, transgelin, serum amyloid A3, lysyl oxidase, thrombospondin 2, extracellular superoxide dismutase, biglycan, myb, cytokeratin, HMG2 and ezrin. In all of them Northern blot data confirmed microarray data. The first 7 were down-regulated in transformed MEFs, the 4 others being overexpressed (Figure 3).

Discussion

A number of *ras*-regulated genes have been identified by studies on immortalized cells or cancer cells expressing the oncogenic *ras* [14–21]. However, although these results are quite interesting, it is important to note that established cell lines are frequently subject to genetic and epigenetic changes that are selected during passaging or immortalization and may affect *ras* target-gene expression. Primary cultures, such as mouse embryonic fibroblasts, do not have that drawback. This is why, to identify *ras* target genes, we decided to analyze global gene expression shortly after retroviral transfer of an ectopic mutated *ras* in MEFs. Yet, because activated *ras* alone induces MEF senescence instead of transformation, we associated to it the adenovirus-derived oncogene E1A. The ras^{V12}/E1A transformation of MEFs (and of other non-immmortalized cells as well) is specific and controlled. Using the Affymetrix technology on ~12,000 genes, we found that expression of 6.8% of them was significantly modified in MEFs by ras^{V12}/E1A-transformation. Because oncogenic transformation of fibroblasts allows tumor development when cells are injected in the immunocompromised mouse (see Figure 1), studying target genes of activated *ras* should improve our understanding of the molecular mechanisms by which *ras* transforms cells and eventually

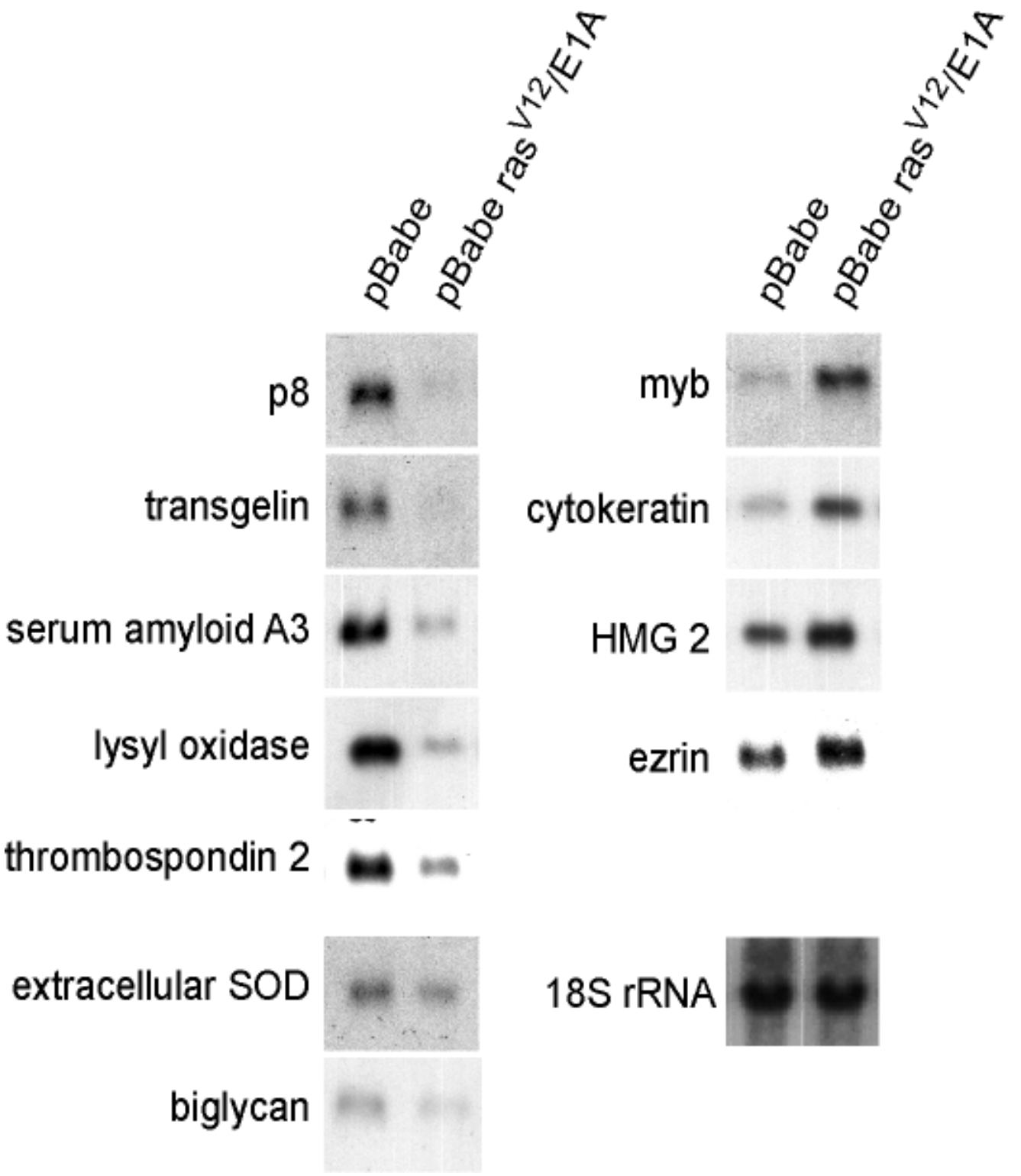

Figure 3
Confirmation of microarray results by Northern blot analysis. 18S rRNA was used as a loading control. Total RNA isolated from pBabe and pBabe-ras^{V12}/E1A transduced MEFs were blotted onto Hybond-N membranes and hybridized with ^{32}P-labeled probes as described in Material and Methods section.

allows tumor formation. It is interesting to note that only 24% of down-regulated and 40% of up-regulated genes showed strong modification (i.e.: >5 fold change) of its expression after transformation.

Several examples of genes up- or down-regulated upon *ras* transformation have already been reported [22–25]. Present data on systematic analysis of about one third of the expressed genome confirm those reports while extending considerably our knowledge of genes activated or repressed by oncogenic *ras* in association with the E1A adenoviral protein. Our results may explain the behavior of transformed cells. For example and as expected, virtually all of the genes coding for secreted factors or extracellular matrix component, which are associated with a differentiated phenotype, were down-regulated. Also, morphological changes observed after transformation (see Figure 1), may be explained by the fact that 44 genes encoding structural proteins were under-expressed. Another expected result was that cell growth-related proteins (involved in the regulation of the cell cycle or inducing cell proliferation) and DNA-associated proteins (involved in DNA replication and reparation) were up-regulated in transformed MEFs, in agreement with their accelerated growth. Also, it is not a surprise to find an altered expression for 56 enzymes involved in cell metabolism because, compared to normal fibroblasts, transformed cells show accelerated growth, increased migration capacity and strong morphological changes. These enzymes could be involved in some of these changes.

Several genes coding for transcription factors (n = 102) and proteins involved in signaling pathways (n = 115) were up- or down-regulated suggesting that modification of the amounts of these factors could be responsible for the dramatic changes in gene expression observed in transformed cells. It is interesting to note that approximately as many transcription factors were up-regulated (n = 57) as down-regulated (n = 45).

Besides data coherent with previous knowledge, we also found very unexpected results. For example, we found that genes coding for proteases and inhibitors of proteases were strongly down-regulated by ras^{V12}/E1A transformation. This was surprising since these factors are up-regulated and strongly involved in tumor progression involving mutated *ras*. This observation could suggest that in human cancers, proteases and protease inhibitors are activated through a mechanism disconnected from *ras* activation. We were similarly surprised by the fact that all 8 angiogenic factors present on the array were found down-regulated by ras^{V12}/E1A transformation. Like proteases and inhibitors of proteases, angiogenic factors are involved in tumour progression and still repressed during ras^{V12}/E1A-mediated transformation. It is therefore highly unlikely that their overexpression reported in several cancers is controlled by a *ras*-dependent pathway. Finally, it was also unexpected that only 5 genes involved in protein synthesis were up- or under-expressed, suggesting that protein synthesis is not strongly altered after ras^{V12}/E1A transformation.

Conclusions

In conclusion, this study of a large number of genes has identified those whose expression is altered upon fibroblast transformation by ras^{V12}/E1A. It is however not exhaustive because the analyzed genes are only representative of the genome (one third of the expressed genes was examined), and post-transcriptional and post-translational modifications were not addressed. Yet, information gathered should be quite useful to future investigations on the molecular mechanisms of oncogenic transformation.

Methods

Primary mouse embryo fibroblasts (MEFs)

Primary embryo fibroblasts were isolated from 14.5 day-old SV129J mouse embryos following standard protocols [26]. Cell were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicilin G and 100 μg/ml streptomycin.

Retroviral infection

Oncogenic *ras* transforms most immortal rodent cells to a tumorigenic state, whereas transformation of mouse primary cells requires either a cooperating oncogene or the inactivation of a tumour suppressor gene. The adenovirus E1A oncogene cooperates with *ras* to transform primary mouse fibroblasts [7] and abrogates *ras*-induced senescence [27]. Therefore, we transduced MEFs with the pBabe-ras^{V12}/E1A retroviral vector which expresses both the ras^{V12} mutated protein and the E1A oncogene to obtain transformed fibroblasts. pBabe-ras^{V12}/E1A [described in ref. [27]] and pBabe (as control) plasmids were obtained from S. Lowe. Bosc 23 ecotropic packaging (10^6) cells were plated in a 6-well plate, incubated for 24 hr, and then transfected with PEI with 5 μg of retroviral plasmid. After 48 hr, the medium containing the virus was filtered (0.45 μm filter, Millipore) to obtain the first supernatant. MEFs were plated at 2×10^5 cells per 35 mm dish and incubated overnight. For infections, the culture medium was replaced by an appropriate mix of the first supernatant and culture medium (V/V), supplemented with 4 μg/ml polybrene (Sigma), and cells were incubated at 37°C. As a control, we evaluated the ability of the retroviral vector to transduce MEFs by using a retroviral vector expressing the EGFP under control of the retroviral promoter located in the long terminal repeat. About 30% of MEFs expressed high levels of EGFP fluorescence 48 h after

transduction (data not shown), indicating that retroviral vectors are well adapted to our experimental set-up. Retrovirus-infected cells were selected with puromycin (0.7 μg/ml). Transformation of MEFs by the pBabe-ras^{V12}/E1A retroviral vector was evaluated by examining changes in their morphological aspect, by quantifying expression of the RAS protein by western blot, by monitoring cell proliferation, colony formation in soft-agar and tumors in nude mice. In soft-agar assays, pBabe-ras^{V12}/E1A transformed cells formed colonies at high frequency (Figure 1). Similarly, transformed cells produced tumors in all (3/3) athymic nude mice when injected subcutaneously, whereas control MEFs did not (0/3) (Figure 1).

Western blot analysis

One hundred μg of total protein extracted from cells was separated with standard procedures on 12.5% SDS-PAGE using the Mini Protean System (Bio-Rad) and transferred to a nitrocellulose membrane (Sigma). The intracellular level of RAS was estimated by Western blot using the H-ras (C-20) polyclonal antibody (1:200) purchased from Santa Cruz Biotechnology, Inc.

Microarray

Total RNA was isolated by Trizol (Gibco-BRL by Invitrogen). Twenty μg of total RNA was converted to cDNA with SuperScript reverse transcriptase (Gibco-BRL by Invitrogen), using T7-oligo-d$(T)_{24}$ as a primer. Second-strand synthesis was performed using T4 DNA polymerase and E. Coli DNA ligase followed by blunt ending by T4 polynucleotide kinase. cDNA was isolated by phenol-chloroform extraction using phase lock gels (Brinkmann). cDNA was *in vitro* transcribed using the T7 BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, N.Y.) to produce biotinylated cRNA. Labelled cRNA was isolated using an RNeasy Mini Kit column (Qiagen). Purified cRNA was fragmented to 200–300 mer cRNA using a fragmentation buffer (100 mM potassium acetate-30 mM magnesium acetate-40 mM Tris-acetate, pH 8.1), for 35 min at 94°C. The quality of total RNA, cDNA synthesis, cRNA amplification and cRNA fragmentation was monitored by micro-capillary electrophoresis (Bioanalizer 2100 by Bioanalyser 2100, Agilent Technologies). The cRNA probes were hybridized to an MGu74Av2 Genechip (Affymetrix, Santa Clara, CA). The MGu74Av2 Genechip represents ~6,000 sequences of mouse Unigene that have been functionally characterized and ~6,000 sequences ESTs clusters. Each sequence in the chip is represented by 32 probes : 16 "perfect match" (PM) probes that are complementary to the mRNA sequence and 16 "mismatch" (MM) probes that only differ by a single nucleotide at the central base (more detailed information about the MGu74Av2 Genechip can be obtained in the web site http://www.affymetrix.com. Fifteen micrograms of fragmented cRNA was hybridized for 16 h at 45°C with constant rotation (60 rpm). Microarrays were processed in an Affymetrix GeneChip Fluidic Station 400. Staining was made with streptavidin-conjugated phycoerythrin (SAPE) followed by amplification with a biotinylated anti-streptavidin antibody and a second round of SAPE, and then scanned using an Agilent GeneArray Scanner (Agilent Technologies). Expression value (signal) is calculated using Affymetrix Genechip software MAS 5.0 (for fully description of the statistical algorithms see http://affymetrix.com/support/technical/whitepapers/sadd_whitepaper.pdf. Briefly, signal is calculated as follow : First, probe cell intensities are processed for global background. Then, MM value is calculated and subtracted to adjust the PM intensity in order to incorporate some measure of non-specific cross-hybridization to mismatch probes. Then, this value is log-transformed to stabilize the variance. Signal is output as the antilog of the resulting value. The 20 probe pairs representing each gene are consolidated into a single expression level. Finally, software scales the average intensity of all genes on each array within a data set. Final value of signal is considered representative of the amount of transcript in solution.

Housekeeping controls β-actin and GAPDH genes serve as endogenous controls and are useful for monitoring the quality of the target. Their respective probe sets are designed to be specific to the 5', middle, or 3' portion of the transcript. The 3'/5' signal ratio from these probe sets is informative about the reverse transcription and *in vitro* transcription steps in the sample preparation. Then, an ideal target in which all transcripts was full-length transcribed would have an identical amount of signal 3' and 5' and the ratio would be equal to 1. Differences greater than three fold between signal at 3' and 5' for these housekeeping genes indicate that RNA was incompletely transcribed or target may be degraded. Ratio of fluorescent intensities for the 5' and 3' ends of these housekeeping genes was <2.

Hybridization experiments were repeated twice using independent cRNA probes synthezised with RNA from two independent sets of MEF-infected cells. Genes were considered as differentially expressed when both hybridizations showed >2 folds change. Data presented in this work represent the average of both hybridizations. The list of unchanged genes should be obtained from authors upon request.

Validation of gene expression profiles by Northern blot hybridization

Synthesis of probes: One microgram of total RNA from MEF cells was subjected to PCR with reverse transcription using the One Step RT-PCR kit (Gibco-BRL) according to the manufacturer's protocol to synthezise specific cDNA probes. PCR were carried out for 32 cycles, each cycle consisting in a denaturing step for 1 min at 94°C, an

annealing step for 2 min at 56°C, and a polymerization step for 2 min at 72°C. Selected RNA species were amplified using the following primers: p8, sense, 5'-ggagagagcagactaggcata-3' and antisense, 5'-gttgctgccacccaagggcat-3'; transgelin, sense, 5'-ccagccagctctgcagatggg-3'and antisense, 5'-gcaggcagatttctgagttc-3'; serum amyloid A3, sense, 5'-ggatgaagccttccattgcc-3' and antisense, 5'-gaagagctacaccgccactc-3'; lysyl oxidase, sense, 5'-taaaacgactgtccccaacc-3' and antisense, 5-tcacggccgttgttagtgta-3'; thrombospondin 2, sense, 5'-aagcccagtcgggcttacgg-3' and antisense, 5-tgctggagctggagccctgc-3'; extracellular superoxide dismutase, sense, 5'-ccttagttaacccagaaatct-3' and antisense 5'-gtacctcaaaggtgctcactgg-3'; biglycan, sense, 5'-ggctgctttctgctcacagg-3' and antisense 5'-gcaactgaccatcacctccta-3'; myb proto-oncogene, sense, 5'-ctaaaccatttcatgaggag-3' and antisense, 5-aacaaatgcaaaattcaccc-3'; cytokeratin, sense, 5'-ctggtctcagcagattgagg-3' and antisense, 5-ggtaggtggcaatctctgcc-3' ; high mobility group protein 2, sense, 5'-cgtctgccttctgcctgttttg-3' and antisense 5'-gcccttgacacggtatgcagc-3' and ezrin, sense, 5'-caacgaggagaagcggatca-3' and antisense 5'-gtgtgacacctgcctgcagtg-3'. Specificity of the PCR products was confirmed by direct DNA sequencing.

Northern blot hybridization: RNA samples (10 μg) were submitted to electrophoresis on a 1% agarose gel and vacuum blotted onto Hybond-N membranes (Amersham). The filters were hybridized with the ^{32}P-labeled probes for 16 h at 65°C in 5X SSPE (1X SSPE is 180 mM NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.5), 5X Denhardt solution, 0.5% SDS and 100 μg/ml single stranded herring sperm DNA. Filters were then washed four times for 5 min at room temperature in 2X SSC, 0.2% SDS, twice for 15 min at 50°C in 0.2X SSC, 0.2% SDS, and once for 30 min in 0.1X SSC at 50°C before autoradiography exposure on Kodak X-Omat films at -80°C from 8 hr to 4 days.

Authors' contributions

SV prepared cells and retroviruses, CM carried out RNA purification and Northern blot analysis, ELC and CL were in charge of microarray hybridization, PB participated in the analysis of gene expression data, JCD participated in the design of the study, JLI participated in the analysis of data and wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

Mouse embryo fibroblasts genes over-expressed upon ras^{V12}/E1A transformation *(*Microsoft Word document*). Genes found over-expressed by microarray analysis are listed, with their GenBank accession number, the over-expression factors (relative to control) observed in two separate experiments and the average over-expression factor.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1476-4598-2-19-S1.doc]

Additional File 2

Mouse embryo fibroblasts genes under-expressed upon ras^{V12}/E1A transformation *(*Microsoft Word document*). Genes found under-expressed by microarray analysis are listed, with their GenBank accession number, the under-expression factors (relative to control) observed in two separate experiments and the average under-expression factor.*
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Acknowledgements

We thank Dr. S. Lowe for the kind gift of pBabe-ras^{V12}/E1A and pBabe plasmids and R. Grimaud and F. Roche for technical assistance. We also thanks to Dr M.J. Pébusque for critical reading of the manuscript. This work was supported by a grant from the Ligue Nationale Contre le Cancer (LNCC) and Association pour la Recherche sur le Cancer (ARC).

References

1. Capon DJ, Chen EY, Levinson AD, Seeburg PH and Goeddel DV **Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue.** *Nature* 1983, **302:**33-37
2. Shih C and Weinberg RA **Isolation of a transforming sequence from a human bladder carcinoma cell line.** *Cell* 1982, **29:**161-169
3. Barbacid M **ras genes.** *Annu Rev Biochem* 1987, **56:**779-827
4. Newbold RF and Overell RW **Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene.** *Nature* 1983, **304:**648-651
5. H Land, LF Parada and RA Weinberg **Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes.** *Nature* 1983, **304:**596-602
6. Weinberg RA **Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis.** *Cancer Res* 1989, **49:**3713-3721
7. Ruley HE **Transforming collaborations between ras and nuclear oncogenes.** *Cancer Cells* 1990, **2:**258-268
8. Hunter T **Cooperation between oncogenes.** *Cell* 1991, **64:**249-270
9. Vogelstein B and Kinzler KW **The multistep nature of cancer.** *Trends Genet* 1993, **9:**138-141
10. Gibbs JB, Oliff A and Kohl NE **Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic.** *Cell* 1994, **77:**175-178
11. Langer SJ, Bortner DM, Roussel MF, Sherr CJ and Ostrowski MC **Mitogenic signaling by colony-stimulating factor 1 and ras is suppressed by the ets-2 DNA-binding domain and restored by myc overexpression.** *Mol Cell Biol* 1992, **12:**5355-5362
12. Johnson R, Spiegelman B, Hanahan D and Wisdom R **Cellular transformation and malignancy induced by ras require c-jun.** *Mol Cell Biol* 1996, **16:**4504-4511
13. Finco TS, Westwick JK, Norris JL, Beg AA, Der CJ and Baldwin AS **Oncogenic Ha-Ras-induced signaling activates NF-kappaB**

transcriptional activity, which is required for cellular transformation. *J Biol Chem* 1997, **272:**24113-24116
14. Krzyzosiak WJ, Shindo-Okada N, Teshima H, Nakajima K and Nishimura S **Isolation of genes specifically expressed in flat revertant cells derived from activated ras-transformed NIH 3T3 cells by treatment with azatyrosine.** *Proc Natl Acad Sci USA* 1992, **89:**4879-4883
15. Liang P, Averboukh L, Zhu W and Pardee AB **Ras activation of genes: Mob-1 as a model.** *Proc Natl Acad Sci USA* 1994, **91:**12515-12519
16. Jo H, Zhang H, Zhang R and Liang P **Cloning oncogenic ras-regulated genes by differential display.** *Methods* 1998, **16:**365-372
17. Jo H, Cho YJ, Zhang H and Liang P **Differential display analysis of gene expression altered by ras oncogene.** *Methods Enzymol* 2001, **332:**233-244
18. Shields JM, Der CJ and Powers S **Identification of Ras-regulated genes by representational difference analysis.** *Methods Enzymol* 2001, **332:**221-232
19. Habets GG, Knepper M, Sumortin J, Choi YJ, Sasazuki T, Shirasawa S and Bollag G **cDNA array analyses of K-ras-induced gene transcription.** *Methods Enzymol* 2001, **332:**245-260
20. Brem R, Certa U, Neeb M, Nair AP and Moroni C **Global analysis of differential gene expression after transformation with the v-H-ras oncogene in a murine tumor model.** *Oncogene* 2001, **20:**2854-2858
21. Sers C, Tchernitsa OI, Zuber J, Diatchenko L, Zhumabayeva B, Desai S, Htun S, Hyder K, Wiechen K, Agoulnik A, Scharff KM, Siebert PD and Schafer R **Gene expression profiling in RAS oncogene-transformed cell lines and in solid tumors using subtractive suppression hybridization and cDNA arrays.** *Adv Enzyme Regul* 2002, **42:**63-82
22. Diaz-Guerra M, Haddow S, Bauluz C, Jorcano JL, Cano A, Balmain A and Quintanilla M **Expression of simple epithelial cytokeratins in mouse epidermal keratinocytes harboring Harvey ras gene alterations.** *Cancer Res* 1992, **52:**680-687
23. Hiwasa T, Yokoyama S, Ha JM, Noguchi S and Sakiyama S **c-Ha-ras gene products are potent inhibitors of cathepsins B and L.** *FEBS Lett* 1987, **211:**23-26
24. Contente S, Kenyon K, Rimoldi D and Friedman RM **Expression of gene rrg is associated with reversion of NIH 3T3 transformed by LTR-c-H-ras.** *Science* 1990, **249:**796-798
25. Shields JM, Rogers-Graham K and Der CJ **Loss of transgelin in breast and colon tumors and in RIE-1 cells by Ras deregulation of gene expression through Raf-independent pathways.** *J Biol Chem* 2002, **277:**9790-9799
26. Harvey M, Sands AT, Weiss RS, Hegi ME, Wiseman RW, Pantazis P, Giovanella BC, Tainsky MA, Bradley A and Donehower LA **In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice.** *Oncogene* 1993, **8:**2457-2467
27. Serrano M, Lin AW, McCurrach ME, Beach D and Lowe SW **Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.** *Cell* 1997, **88:**593-602