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Isolation of Oncogenes from Rat Mammary Tumors by a Highly Efficient Retrovirus Expression Cloning System

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A majority of mammary tumors induced with *N*-methyl-*N*-nitrosourea in rats contain G to A transitional mutation of c-Ha-ras at the 12th codon. Additional oncogene activation is known to be necessary for further tumor progression. To isolate novel oncogenes, we used an expression cloning system utilizing the pMX retroviral vector in combination with BOSC23 packaging cells. First, we elucidated the sensitivity of this system in the NIH 3T3 focus assay; foci were detectable even after 10^{-6} dilution using v-Ha-ras, neuT, and β-galactosidase constructs in pMX vector. This system is sensitive enough to detect low copy number cDNAs. We used the pMX/BOSC23 expression cloning system to clone novel oncogenes from rat mammary tumors harboring an activated c-Ha-ras and isolated several candidate oncogenes that caused transformation of NIH 3T3 cells and/or generated tumors when transplanted to nude mice. © 1999 Academic Press

Cancer is recognized as a disease arising from accumulation of gene alterations. In human breast cancer, these include oncogenic activation of c-*erb*B2, cyclin D1 and c-*myc* by their overexpression and loss of function of p53 and BRCA 1 and 2 by their allele loss or mutations (1, 2). In rat mammary carcinogenesis induced with *N*-methyl-*N*-nitrosourea (MNU), G → A transition mutation of c-Ha-*ras* is frequently found at the 12th codon (3, 4). To analyze the effect of this mutation, Miyamoto *et al.* (5) transfected primary mouse mammary epithelial cells with human genomic H-ras with a 12th codon mutation. The transfected cells gave rise only to hyperplasia when transplanted to the mammary fat pads of mice. This result suggested that some other activated oncogene(s) must be required for further progression. To address this issue, we attempted to isolate other activated oncogenes from rat mammary tumors with activated c-Ha-*ras*.

Although the activated c-Ha-*ras* was cloned by transfection of genomic DNA from human bladder cancer using NIH 3T3 fibroblasts as recipients (6), transfection efficiency of genomic DNA is very low. Miki *et al.* (7) developed an expression cloning system by making a λ phage library in λpCEV27 vector to overcome this problem by the insertion of cDNA of interest under a strong MMLV-LTR (Moloney murine leukemia virus long terminal repeat) promoter flanked by λ phage arms. We isolated MAT1 oncogene from MNU-induced mouse mammary tumors (8) and found the insertion of a retrotransposon in a c-Ha-*ras* intron in a metastatic rat mammary adenocarcinoma using this method (9). But the transfection efficiency of phage DNA (about 40-48 kb in length) is still not very efficient because of its size constraint.

Recently, expression cloning systems using retrovirus vectors have been reported (10–12). They used a pBabe based retrovirus vector (13) in combination with NIH 3T3 based packaging cell line, GP + E86 (14) and isolated several oncogenes (15–18). Although they described that the infection efficiency of retrovirus is much higher than any transfection methods, the titer of the library was $1–4 \times 10^5$/ml. So rare genes might be missed using this method. To obtain higher titers, we developed a retrovirus expression cloning system using pBabeX in the combination of BOSC23 packaging cells (19, 20). Then we improved a retrovirus vector called pMX (21) which has MMLV and ϕMOV's longer hybrid packaging signal for higher efficiency than conventional retrovirus vectors. BOSC23 packaging cell line was produced (22) for more efficient transfection and was chosen for production of pMX retroviral library plasmids. An advantage of using BOSC23 is that drug

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selection of transfected cells is unnecessary thus avoiding imbalance of the library.

Here, we applied the pMX/BOSC23 system for isolation of transforming genes. It revealed a number of expressed proliferation-related genes and showed that some of them were able to transform NIH 3T3 cells *in vitro* with a focus assay and/or were tumorigenic in the nude mouse assay.

MATERIALS AND METHODS

Retroviral vector and constructs. High efficiency retroviral vector pMX (21) was used for library construction and for a negative control of NIH 3T3 focus assay and nude mouse assay. v-Ha-*ras* (23) and neuT, an activated form of rat *neu* (24), were cloned into the pMX vector at *Bst*XI and *Eco*RI/*Sal*I sites and designated as pMX-v-H-ras and pMX-neuT, respectively which were used for positive controls. β-galactosidase (25) cDNA cloned at *Hin*dIII/*Not*I site of pMX (pMX-β-gal) was used to check the efficiency of the system. Transfected or infected cells were stained with X-gal as described (26).

Tumor tissue preparation. 50–60 day-old Sprague–Dawley rats were injected with MNU and kept until tumors developed (27). Mammary tumors were excised and frozen on dry ice immediately. The presence of G35 to A35 mutation of c-Ha-*ras* protooncogene at codon 12 was analyzed with allele specific oligonucleotide hybridization as described (28), and a pool of tumors with c-Ha-*ras* mutation were chosen for cDNA library construction.

Construction of a cDNA library. The procedure is summarized in Fig. 1. Total RNA was isolated with UltraSpec RNA reagent (Biotecx, Houston, TX) and poly(A) RNA was isolated with PolyATtract (Promega, Madison, WI). cDNA was synthesized using a cDNA synthesis kit according to manufacturer's instruction (Stratagene, La Jolla, CA) with modifications. Briefly, first strand cDNA was synthesized with a linker-primer containing poly dT and the second strand was synthesized. After blunting the cDNA termini, 5′-phosphorylated *Bst*XI adapters consisted of 5′-CTTTCCAGCACA-3′ and 5′-GAA-AGGTC-3′ (Invitrogen, San Diego, CA) were ligated. Then, cDNA was size-fractionated with 0.8% agarose gel, and 0.9-9.4 kb cDNAs were recovered with QIAEX II (QIAGEN, Chatsworth, CA) and ligated into *Bst*XI sites of pMX vector (21) at 12°C for 17 hours. The ligation reaction was desalted using QIAEX II and eluted with 40 μl of 10 mM Tris-Cl, pH 8.4. Four-μl of ligation mixture was used for electrotransformation of 40 μl of XL1-BlueMRF' (Stratagene) with GenePulsar (Bio-Rad Laboratories, Richmond, CA) according to manufacturer's instructions. 960 μl of SOC was added immediately to each electrotransformed competent cells. Ten reactions were pooled, incubated at 37°C for 60 minutes with shaking, plated onto ten LB agar plates containing 50 μg/ml ampicillin in 15 cm dishes, and incubated at 37°C overnight. Next day, after the number of colonies were counted, bacterial colonies were harvested with LB media, and plasmids were prepared with QIAGEN columns (29).

Cell culture. NIH 3T3 cells (ATCC CRL 1658) (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle media (DMEM) with high glucose (GIBCO BRL, Cat. #12100-061, Gaithersburg, MD) + 10% calf serum (CS) (BioWhittaker, Walkersville, MD) and split before reaching confluence. BOSC23 cells (ATCC CRL 11270) (22) were purchased from American Type Culture Collection and maintained in DMEM + 10% fetal calf serum (FCS) (HyClone Laboratories, Inc., Logan, UT) + GPT selection reagent (Specialty Media, Lavallette, NJ) as described (19, 21). Media was changed to DMEM + 10% FCS without GPT selection reagent 2 days before transfection.

Screening of the pMX library with NIH 3T3 focus assay. Transfection and infection procedures were as described (19, 21). Briefly, 2×10^6 BOSC23 cells were seeded onto 60 mm dishes 1 day before transfection. Three μg pMX library plasmid were used for transfection with 18 μl Lipofectamine (GIBCO BRL) as suggested by the manufacturer. Medium was replaced with DMEM + 10% FCS after 5 hours, and changed to 3 ml DMEM + 10% CS on the next day. NIH 3T3 cells were seeded at 7.5×10^5 in 10 cm dishes. 24 hours later, medium containing retrovirus from one plate was filtered with a 0.45-μm syringe filter (Corning Glass Works, Corning, NY), and used for the infection of two 10 cm plates of NIH 3T3 cells in the presence of 8 μg/ml of polybrene (Sigma, St. Louis, MO). One plate of NIH 3T3 cells was split to four plates on the next day and fed with DMEM + 5% CS two days after infection, when they reached 70% confluence. Culture was maintained with DMEM + 5% CS twice a week until foci appeared. Foci that appeared after 2 weeks were classified as "strong foci" and after 3 weeks as "weak foci."

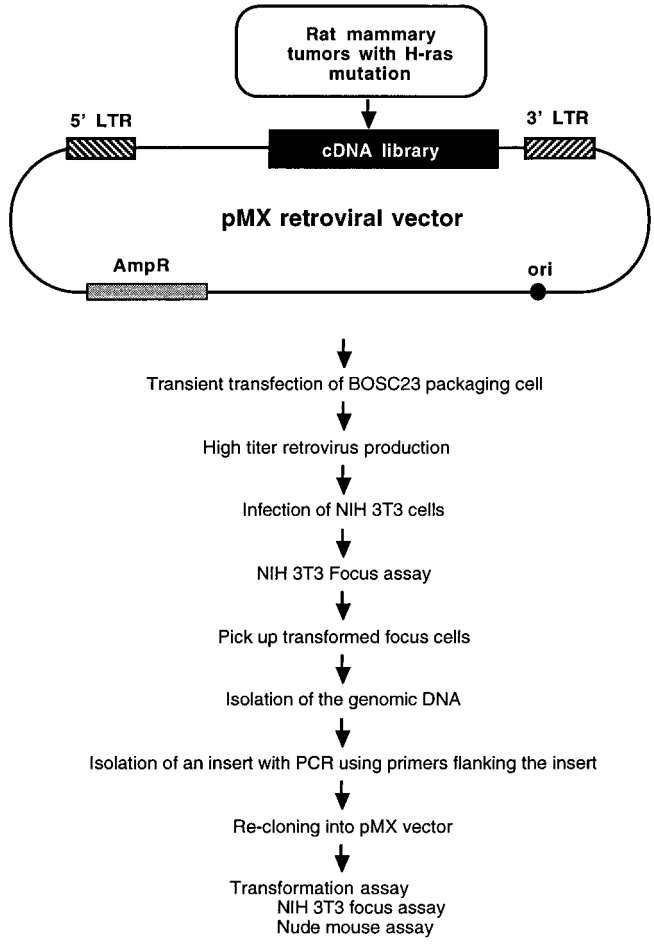

FIG. 1. Scheme of the procedure. A rat mammary tumor cDNA library was made in pMX retroviral vector. This library was used for the cloning of transforming genes with NIH 3T3 assay and nude mice assay.

Recovery of integrated cDNA inserts. The foci cells were isolated and expanded and genomic DNAs were isolated as described elsewhere (30). PCR was performed in 100 μl reaction mixture containing 1 μg genomic DNA, 20 pmol 5′ and 3′ primers, 20 nmol each dNTP, 2.5 units c*Pfu* DNA polymerase and its buffer provided (Stratagene). 5′ primer sequences were CTC AAA GTA GAC GGC ATC GCA (MX1) or GTC GAA TTC GGT GGA CCA TCC TCT AGA CTG C (*Eco*RI/MX3). 3′ primer sequences were CTA CAG GTG GGG TCT TTC ATT C (MX2) or TTA GCG GCC GCT ACA GGT GGG GTC TTT CAT TC (*Not*I/MX2). The PCR was run as follows: 94°C-1 min ×1,

94°C-30 sec: 60°C-30 sec: 72°C-10 min ×30, 72°C-10 min ×1 with DNA Thermal Cycler 480 (Perkin–Elmer Cetus, Emeryville, CA). In case c*Pfu* DNA polymerase could not amplify PCR products, r*Tth* DNA polymerase, XL (Perkin–Elmer, Emeryville, CA) was used. 100 μl PCR mixture contained 1 μg genomic DNA, 20 pmol 5′ and 3′ primers, 20 nmol each dNTP, 1.2 μmol $Mg(OAc)_2$, 2 units r*Tth* DNA polymerase, XL and its buffer provided (Perkin–Elmer). The condition was 94°C-1 min ×1, 94°C-30 sec: 65°C-5 min ×16, 94°C-30 sec: 65°C-5 min + increment 15 sec/cycle ×21, 72°C-10 min ×1. Amplified PCR fragments were purified with QIAquick PCR kit (QIAGEN), digested with *Bam*HI and *Sal*I (Boehringer Mannheim Biochemical, Indianapolis, IN). Digested PCR products were isolated with QIAEX II (QIAGEN) and cloned into *Bam*HI and *Sal*I sites of pMX vector.

Sequencing analysis. Either PCR products or cloned plasmids were subjected to sequencing analysis using sequencing primers, MX1 or GGT GGA CCA TCC TCT AGA CTG C (MX3) with AmpliCycle sequencing kit (Perkin–Elmer) with ABI automated sequencer (Perkin–Elmer) at the Automated Sequence Facility at University of California, Berkeley. Then homology of the sequences were searched with BLAST method (31).

Transformation assay and nude mouse assay. To confirm the transforming activity of the cloned PCR products, NIH 3T3 focus assay was performed. The transfection and infection procedure was basically as described in "Screening of the pMX library with NIH 3T3 focus assay." Briefly, the cloned plasmids were transfected to BOSC23 cells. Retroviruses were harvested 2 days later and infected to NIH 3T3 cells, which were maintained in DMEM + 5% CS. After 2–3 weeks, the cells were washed with phosphate buffered saline (PBS), stained with 2% crystal violet in 20% methanol for 1 min, washed with tap water, dried, and the number of foci were counted. This assay was repeated 3 times for each clone. The foci appeared within 2 weeks were judged as "strong foci" and ones formed at 3 weeks were considered as "weak."

For nude mouse assay, 1×10^6 infected NIH 3T3 cells were suspended in 100 μl media 199 (GIBCO BRL) and injected to a nude mouse subcutaneously. For each clone, two mice were used to inject four sites per mouse. Tumor formation was monitored biweekly.

RESULTS AND DISCUSSION

Titration of pMX-based vectors. Whitehead *et al.* (12) have reported a novel method for cloning of oncogenes using retroviral expression libraries. They used pBabe (13)-based retroviral vector, pCTV, with the combination of GP + E86 packaging cells (14). They obtained a titer of 10^5 cfu/ml with their system. Since the packaging cells amplify the transfected provirus to infectious retroviral particles, it means that the number of actually screened library clones was less than 10^5.

Whitehead *et al.* (12) analyzed the minimum detectable level of a transforming gene using N-ras or polyoma T antigen in the NIH 3T3 focus assay. Thirty to 70 foci appeared with 100 pg of those plasmids diluted in 1 μg of the vector plasmid ($1:10^4$ dilution). To compare the efficiency of our pMX/BOSC23 system (Table 1), 300, 30 or 3 pg of pMX-βgal, pMX-v-H-ras and pMX-neuT plasmids were diluted in 3 μg of pMX and revealed higher sensitivity of this system. The number of transformed foci induced with pMX-v-H-ras were 219, 39, and 6 at 10^{-4}, 10^{-5}, and 10^{-6} dilution, respectively. And with pMX-neuT, the numbers were 112, 15, and 4. Finally with pMX-βgal, it was still also detectable after 10^{-6} dilution and the number of blue-stained cells were

TABLE 1

Detection Limit of the Transforming cDNAs Diluted in pMX Vector in the BOSC23 Retroviral Transfer System

	Dilution in pMX[a]		
Plasmids	10^{-4}	10^{-5}	10^{-6}
pMX-v-H-ras	219	39	6
pMX-neuT	112	15	4
pMX-β-gal	many	many	positive

[a] Three hundred, 30, or 3 pg of each plasmid listed above were diluted in 3 μg of pMX for 10^{-4}, 10^{-5}, or 10^{-6} dilution. They were transfected to BOSC23 packaging cells and the produced retrovirus was used for the subsequent infection of NIH 3T3 cells. The numbers of transformed NIH 3T3 foci were counted. pMX alone was used for the negative control and produced only one small spontaneous focus.

4. The reason that the numbers were not 10-fold could be that two or more transformed foci might have been fused and counted as one focus. We concluded that this system was useful even for minimally expressed oncogenes.

Construction and titration of the cDNA library. A cDNA library from rat mammary tumor was constructed in a high efficiency retrovirus vector, pMX. The titer of the library was measured by counting the number of colonies on LB agar plates after electrotransformation. The titer was approximately 2.1×10^5. This relatively low titer was possibly due to size-fractionation which chose cDNAs above 0.9 kb. To evaluate how much plasmid was necessary to cover the whole library, we transfected 2×10^6 BOSC23 with 3 μg pMX-βgal and stained with X-gal substrate. Approximately 10–13%, i.e., $2–2.7 \times 10^5$ cells were stained blue. Therefore, only 5 plates (total 1×10^7 BOSC23 cells) were sufficient to cover 1×10^6 clones which was enough to screen a library with the titer of 2.1×10^5.

Screening of the library with NIH 3T3 focus assay. One transfected BOSC23 plate was used to infect two NIH 3T3 plates (7.5×10^5 cells in a 10 cm dish) which were further split to 8 plates (one to four), resulting in 40 NIH 3T3 plates. Fourteen days after infection, approximately 60 foci developed per plate (2400 foci in 40 plates). We chose 12 strongly transforming clones from each transfection (total 60 foci). At 18 days, an additional 6 foci from each (subtotal 30, and total 90 foci) were picked to isolate weakly transforming genes as well as strong ones. Genomic DNAs were isolated and subjected to PCR amplification. A control experiment using pMX vector alone induced no strong foci and a negligible number of weak foci. Thus, spontaneous transformation by insertion of a pMX retroviral vector was considered to be minimal.

The isolated inserts from 6 strong and 13 moderate/weak foci were partially sequenced using MX1 or MX3 primers for the sense orientation and MX2 primer for

TABLE 2

Isolated Clones from Rat Mammary Tumors Using the Retrovirus Expression Cloning

Categories	Description[a]	Insert size (kb)	Regions of cloned inserts[b]	GenBank Accession No.[a]	Identities[a]	3T3 focus assay[c]	Nude mice assay[d]
I	Transcription/translation						
	Elongation factor-1 alpha	0.9	C-ter 1/3 ORF + 3′-UTR	L10339 (rat)	identical	moderate	+
	Ribosomal protein L7a	1.0	full length	X15013 (rat)	identical	weak	−
	Ribosomal protein L3	0.9	C-ter 1/2 ORF + 3′-UTR	X62166 (rat)	identical	weak	−
	Poly(A) binding protein II	1.0	C-ter 4/5 ORF + 3′-UTR	X89969 (bovine), U94858 (rat)	380/437 (86%) to bovine	moderate	+
	Scaffold protein Pbp1 homologue	2.3	full length	AF003693 (mouse)	350/406 (86%)	moderate	+
II	Nucleotide biosynthesis						
	RBL-NDP kinase 18 kDa subunit (p18)	0.9	full length	M91597 (rat)	identical	weak	+
III	Cell/genome structure						
	Histone H3.3	1.3	full length	X73683 (rat)	identical	weak	−
IV	Signal transduction						
	mNck-beta (SH2/SH3 adaptor protein)	1.7	full length	AF043260 (mouse)	617/659 (93%)	weak	+
	c-H-ras (G → A mut. at 12th codon)	1.2	full length	M13011 (rat)	identical	strong	+
V	Miscellaneous						
	E46 (host cell factor 1)	1.2	C-ter 1/2 ORF + 3′-UTR	X61506 (mouse)	683/741 (92%)	weak	−

[a] Each clone was partially sequenced and BLAST-searched. Retrieved genes are listed with their GenBank accession numbers and identities.

[b] Regions of cloned inserts were deduced by comparison with the retrieved genes. C-ter, C-terminus; ORF, open reading frame; UTR, untranslated region.

[c] Transformation activity was confirmed by NIH 3T3 focus assay. Foci that appeared within 2 weeks were judged as "strong". Foci 2 mm or larger in diameter that developed by 3 weeks were termed "moderate" and "weak" when they were smaller than 2 mm.

[d] NIH 3T3 cells were infected with the transforming clones and transplanted to nude mice. The mice were monitored for the appearance of tumors (+) or not (−).

the antisense orientation and BLAST-searched (31) for their identification. All six strong foci consisted of activated H-ras. Thirteen moderate/weak foci included 4 unknown genes which were not homologous to any published genes including expressed sequence tags (ESTs) in GenBank. A panel of isolated clones which were homologous to known genes is listed in Table 2. They can be categorized as to their functions (I) transcription/translation, (II) nucleotide biosynthesis, (III) cell/genome organization, (IV) signal transduction, and (V) miscellaneous.

Category I includes elongation factor 1α (EF1α) (32), ribosomal proteins L7a (33) and L3 (34), poly(A) binding protein II (35) and scaffold protein Pbp1 homologue (unpublished, refer to Table 2 for the GenBank accession number). They are related to the basic transcription and translation machinery. Among them, EF1α has been reported to have oncogenic activity and the translational machinery may predispose cells to become more susceptible to malignant transformation (32). Others belong to the transcription machinery. Poly(A) binding protein II may stabilize oncogenes such as c-*myc* which have destabilization signals in their 3′-untranslated region (36). *PBP1* (Pab1p-binding protein gene) product (Pbp1p) has been reported to bind to poly(A)-binding protein (Pab1p) encoded by the *PAB1* gene and concluded that Pbp1p may act to repress the ability of Pab1p to negatively regulate polyadenylation (37). Continuous expression of the proteins in this category may promote the transcription/translation of proliferation-related genes and may transform cells.

Category II genes are related to nucleotide biosynthesis. It included nucleoside diphosphate (NDP) kinase 18 kDa subunit (p18) (38, 39). NDP kinases such as nm23 regulate a diverse array of cellular events including growth and development and are also implicated in the pathogenesis and metastasis of tumors (40).

Category III consists of histone H3.3 (41). Upregulation of histone gene expression is tightly coupled to the G1-S-phase transition of the cell cycle, and mRNA levels rise 30-100-fold during S-phase (42). Genetic activation involves the relaxation of the superstructure of chromatin, which could be modulated by its interaction with the nuclear matrix (43). According to the literature, it may be involved in the transformation of cells by a still unknown mechanism.

Category IV contains the rat homologue of a mouse Src homology (SH) 2/SH3 adaptor protein, mNck-β. The adapter proteins contain SH2 and SH3 domains and provide one of the principal ways by which signals are transduced in cells using protein-protein interactions between proline-rich motifs and SH3 domains and induced interactions between phosphotyrosine residues and SH2 domains. While Grb2 connects activated receptor tyrosine kinases with Sos and activates p21ras, recent evidence suggests that this may not be the major mechanism by which Crk and Nck (44) signal to downstream effectors (45). An attractive model for mammary carcinogenesis would be that Nck activates an unknown pathway which cooperates with the Ha-ras signal transduction pathway. Double transduction of Nck and Ha-ras could cause progression of primary mammary epithelial cells (5). We used rat mammary carcinomas having G $\rightarrow$ A transition mutation at the 12th codon of c-Ha-*ras*. This gene was re-cloned by our method, and provided confirmation of the usefulness of this system.

Category V is consisted of E46/host cell factor 1 (46) which mechanism has not been clarified yet.

Tumorigenesis in nude mouse assay. Nude mouse assay was performed to confirm the transforming activity of isolated clones with NIH 3T3 focus assay. One million infected NIH 3T3 cells were injected into four sites subcutaneously in nude mice, and tumor formation was monitored. Tumors formed from cells infected with c-Ha-*ras*, EF1α, Poly(A) binding protein II, scaffold protein Pbp1 homologue, NDP kinase and Nck-β. However, Ribosomal proteins, histone H3.3 and E46 host cell factor which possessed weakly transforming potential did not cause tumors. Although nude mouse assay was generally considered more sensitive than NIH 3T3 focus assay (47, 48), opposite results have been reported as well (47). They thought this discrepancy was an intrinsic difference between the nude mouse assay and the focus assay (48). Furthermore, they also suggested that the immune response to tumor cells in nude mice could be responsible for the failure of tumor formation. Our clones which could induce foci but failed to cause tumors might be classified to a different class of oncogenes, or tumors transformed with these oncogenes tended to evoke immune response stronger than the other ones.

In this report, we demonstrated the high sensitivity of our retroviral expression system using pMX in combination with BOSC23 and successfully isolated a number of candidate oncogenes. The next step will be analysis of the expression level and mutation status of these genes including unknown candidates in the original mammary tumors. In combination with the molecular analysis, transforming activity of these candidate oncogenes can be analyzed functionally by introduction of these genes using this retroviral system. Overexpressed or mutated and activated oncogene(s) may orchestrate transformation of primary mammary epithelial cells with mutated c-Ha-ras, although further progression possibly requires spontaneous inactivation of tumor suppressors. Our goal is to identify the mechanism of cellular transformation in both rodent and eventually in human cases for a better understanding of breast carcinogenesis.

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