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PRINCIPAL INVESTIGATOR: Carlos Sonnenschein, M.D.

CONTRACTING ORGANIZATION: Tufts University
Boston, MA 02111

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Carlos Sonnenschein, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tufts University
Boston, MA 02111

E-Mail: Carlos.sonnenschein@tufts.edu

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This application should be considered in the context of the competing theories of carcinogenesis. The first is *the somatic mutation theory* which is based on two main premises: the first, claims that the default state of cells in metazoa is *quiescence*, and the second posits that cancer is the result of the multistage process where successive mutations accumulate in a single target cell. Much was learned about gene expression under a variety of conditions. However, this aggressive effort failed to provide either an explanation for carcinogenesis or a rationale for effective therapies. The second theory is the *tissue organization field theory of carcinogenesis.* This theory has adopted two basic premises: the first postulates that proliferation is the default state of all cells (prokaryotes to metazoa), and the second states that tissues (stroma or epithelium, or both) are the targets of carcinogens.

The first task tested whether the primary target of the carcinogenic agent nitrosometylurea is the epithelium, the stroma or both. Recombinants were made where vehicle-treated stromal cells were recombined with epithelial cells from vehicle-treated and carcinogen-treated animals, and stromal cells from carcinogen-treated mammary fat pads were recombined with epithelial cells from vehicle-treated and carcinogen-treated animals. The second task tested the ability of the stroma to normalize tumor cells. Finally, the third task documents molecular changes in epithelial and stromal pericellular matrices.

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INTRODUCTION:

This research project had three specific tasks. The first was to determine which tissue is the target of the chemical carcinogen N-nitrosomethylurea in the rat mammary gland. This task was scheduled to occupy the first 18 months of support. The second task included screening and counting lesions and performing morphometric data analysis of whole mounts (branching pattern, relative abundance of the different ductal and alveolar structures). These studies aimed to identify the changes occurring between the time of exposure and the appearance of neoplasias. We also planned to analyze BrdU incorporation, and identify changes in components of the extracellular matrix. Finally, during years two and three we were supposed to explore the specific roles of hyaluronan and emmprin, two molecules that are enriched in tumors and involved in tumor-stromal cell interactions as mediators of neoplastic initiation and progression.

BODY:

Task #1. HYPOTHESIS: Is the target of the carcinogen the genomic DNA of epithelial cells, the stroma, or both? We anticipated that the first task of our research project would require 18 months to be completed. We are now at the end of the 24 months of support and we have accomplished the following:

Task #1 was completed. The results were published in the Journal of Cell Science, (The stroma as a crucial target of chemical carcinogens in the rat mammary gland. Maricel V. Maffini, Ana M. Soto, Janine M. Calabro, Angelo A. Ucci and Carlos Sonnenschein, 117, 1495-1502, 2004). Details are therein.

We observed that only those animals whose stroma was exposed to NMU developed neoplasias, regardless of whether or not the transplanted mammary epithelial cells were exposed to the carcinogen (Maffini et al 2004, Fig. 3A). The Ha-ras mutation was also assessed in DNA isolated from mammary epithelial cells, mammary fibroblasts, and mammary pre-adipocytes collected from intact virgin rats and grown in vitro. The presence of the mutation did not correlate with cell type, culture conditions or carcinogen treatment.

These results suggest the need to explore the roles that the stroma components, i.e. the cells (fibroblasts, adipocytes, mast cells, etc.) and the extracellular matrix, play in rodent mammary carcinogenesis. Efforts should also be directed at exploring the role of the stroma in experimental models for carcinogenesis involving organs other than the mammary gland (i.e., skin, prostate, liver, bladder). To accommodate a novel perspective on the role of the stroma in carcinogenesis, a rigorous analysis of concepts, definitions and experimental approaches is now needed. This will facilitate the identification of the mediators responsible for the altered tissue phenotype in cancers and of ways to reverse their effect by adopting a solid epigenetic perspective.

Task #2. To establish a pattern of the dynamic response of the stroma and the epithelium of the different combinations of tissue involved in mammary carcinogenesis as outlined in **Task #1**.

As stated in the FIRST YEAR REPORT we observed that documenting the time-course of repopulation of the cleared mammary gland could not be accomplished as proposed, because it took at least 30 days for the formation of a noticeable ductal tree. By 150 days, 1/3 of the "cleared" fat pad was covered with the tree-like epithelial growth. We decided, instead, to explore a related phenomenon that will shed light on the role of the stroma in carcinogenesis. Given that mammary glands are most vulnerable to chemical carcinogenesis at puberty and become resistant as the animal ages, we asked the question: Are these properties due to changes in the stroma? We performed a pilot study to assess whether the ability of the cleared mammary gland fat pad (CFP) to normalize cancer

cells varies in diverse physiological states, for example, the age of the host. This pilot study revealed that epithelial carcinoma cells (ECCs) formed tumors when injected in CFPs of "young" (24 and 50 day-old) hosts. In contrast, tumor formation was substantially decreased or absent when tumor cells were injected into CFP of "adult" (80 and 150 day-old) or multiparous hosts (after 2 pregnancies). Most remarkably, these data suggest a parallel to the phenomenon of age-dependent susceptibility and resistance to chemical carcinogens. This experiment will be completed during the third year of funding.

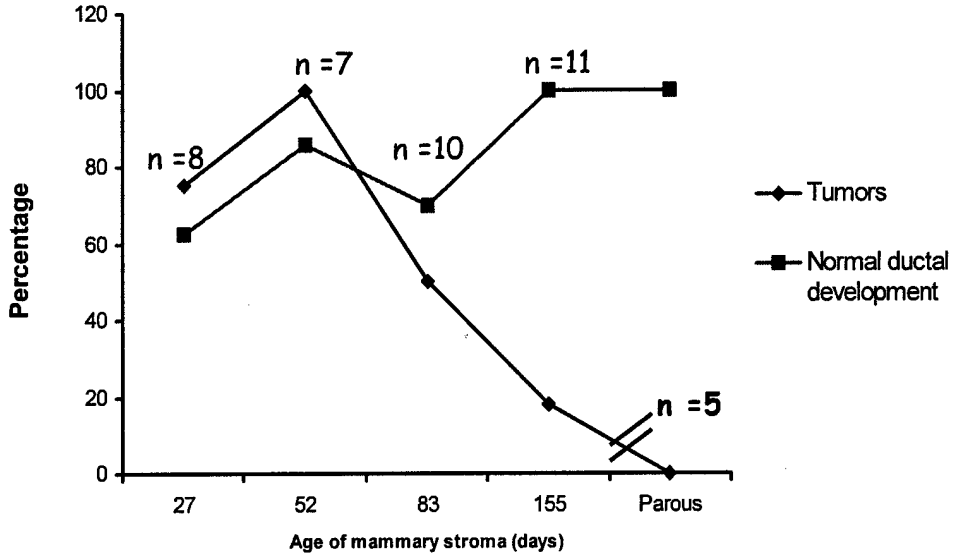

Fig.1: Development of ducts and tumors from ECCs transplanted into CFP in recipients of different ages. Note: Animals were sacrificed 6 months after transplantation. In young recipients, ductal growth did not reach 100% incidence simply because in some animals the tumor encompassed the entire fat pad.

Hence, susceptibility to chemical carcinogens and the ability to reprogram the neoplastic behavior seem to be linked to aging: as susceptibility to carcinogenesis decreases, the ability of the stroma to reprogram neoplastic epithelial cells increases. This observation strongly supports the notion that the neoplastic phenotype is context-dependent and, hence, it offers the intriguing possibility that the process of carcinogenesis is amenable to normalization or "cure" once the mechanisms of stroma-mediated "normalization" are elucidated.

Task #3. Define the relationship between early carcinogenic events and peri- and extracellular markers that are known to affect the proliferative and invasive behavior of cancer cells.

We have optimized the protocols to characterize EMMPRIN and hyaluronic acid expression. We are now optimizing the measurement of hyaluronic acid by means of an ELISA assay. We anticipate that this assay will become available in 4 months. Preliminary data gathered in normal mammary glands indicates that hyaluronic acid is expressed most abundantly in the epithelial cells of terminal end buds. We are starting to test the specific roles of hyaluronan and emmprin, two molecules that are enriched in tumors and involved in tumor-stromal cell interactions, as mediators of neoplastic initiation and progression.

KEY RESEARCH ACCOMPLISHMENTS:

• We have completed **Task #1**:the results clearly establish that the stroma is a main target of chemical carcinogens and suggest that carcinogenesis is a tissue organization-based problem.

• The new **Task #2** is revealing important findings: as stated during the previous Progress Report, we found that it was technically unfeasible to record the formation of pre-neoplastic lesions in the tissue recombinants due to 1) the timing of repopulation of the mammary gland by the inoculum (consisting of 50,000 mammary epithelial cells), and 2) a low but significant percentage of "no takes". In other words, pre-neoplastic lesions are detected at 15 days after NMU injection in intact animals, whereas a ductal tree is detected in the recombinants stating at 30 days only in a few animals; takes amounted to 60% in the pilot study. Hence, it would have been necessary to double the number of time points and animals per group. These modifications could not be accomplished due to budgetary restrictions (i.e., cost of animals and increased workforce).

However, the new Task #2 has shed light on the dual role of the stroma on promoting neoplastic behavior at puberty and of curtailing it once the gland reaches maturity. This experiment will be completed during the third year of funding.

• The histochemical techniques needed to explore **Task # 3** have been optimized and the hyaluronic acid ELISA will be optimized in the next 4 months.

REPORTABLE OUTCOMES:

Preliminary data was presented at the 12th International Conference of the International Society of Differentiation. (See enclosed abstract) A paper was published reporting the results of **Task # 1**: (see enclosed JCS paper).

CONCLUSIONS:

Our theory neutral experimental design tested whether the primary target of the carcinogen is the epithelium, the stroma, or both tissue compartments. We observed that neoplastic transformation of mammary epithelial cells occurred only when the stroma was exposed *in vivo* to N-nitrosomethylurea, regardless of whether or not the epithelial cells were exposed to the carcinogen. Mutation in the Ha-ras-1 gene did not correlate with initiation of neoplasia. Our results suggest that the stroma is a crucial target of the carcinogen and that mutation in the Ha-ras-1 gene is neither necessary nor sufficient for tumor initiation.

Further, we have found evidence that the ability of the stroma to induce and to curtail neoplastic behavior is age dependent.

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Soto AM, Maffini MV, Calabro JM, Wieloch C, Sonnenschein C. Mammary gland stroma is responsible for epithelial cell neoplasia. Differentiation 70:321, 2002

Maricel V. Maffini, Ana M. Soto, Janine M. Calabro, Angelo A. Ucci and Carlos Sonnenschein, The stroma as a crucial target of chemical carcinogens in the rat mammary gland. Journal of Cell Science, 117, 1495-1502, 2004.

APPENDICES:

Soto AM, Maffini MV, Calabro JM, Wieloch C, Sonnenschein C. Mammary gland stroma is responsible for epithelial cell neoplasia. Differentiation 70:321, 2002

Maricel V. Maffini, Ana M. Soto, Janine M. Calabro, Angelo A. Ucci and Carlos Sonnenschein, The stroma as a crucial target of chemical carcinogens in the rat mammary gland. Journal of Cell Science, 117, 1495-1502, 2004.

MAMMARY GLAND STROMA IS RESPONSIBLE FOR EPITHELIAL CELL NEOPLASIA

Ana M. Soto, Maricel V. Maffini, Janine M. Calabro, Carise Wieloch, Carlos Sonnenschein
Tufts University School of Medicine, 136 Harrison Ave. Boston, MA 02111, USA.

Mammary gland development is driven by a network of signals between stroma and epithelium. The tissue organization field theory of carcinogenesis (TOFT) proposes that altered reciprocal interactions between stroma and epithelium initiate the neoplastic process. We assessed whether the primary target of the carcinogen N-nitroso-methylurea (NMU) in mammary glands of Wistar-Furth rats is the epithelium, the stroma or both. The 4^{th} and 5^{th} mammary gland fat pads were cleared of epithelium (CFP) at 21 days of age. One month later, these animals were treated with NMU (Groups 1 and 2) or vehicle (3 and 4). One week later, vehicle-treated epithelial cells were transplanted into the CFP of Groups 1 and 4 while NMU-treated epithelial cells were transplanted into the CFP of Groups 2 and 3. Also, positive and negative controls consisting of intact virgin rats injected respectively with NMU (Group 5), and vehicle (Group 6) were included. Tumors appeared in Group 1 (92.8%), 2 (75%) and 5 (100%) and were absent in Groups 3, 4 and 6. Whole mount preparations and histology confirmed the mammary tumor origin of the palpable lesions. Our results suggest that only the stroma is the target of the carcinogen. This novel concept in carcinogenesis should provide for a more rational study of breast cancer.

The stroma as a crucial target in rat mammary gland carcinogenesis

Maricel V. Maffini[1], Ana M. Soto[1,*], Janine M. Calabro[1], Angelo A. Ucci[2] and Carlos Sonnenschein[1]

[1]Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA
[2]Department of Pathology, Tufts-New England Medical Center, 750 Washington Street, Boston, MA 02111, USA
*Author for correspondence (e-mail: ana.soto@tufts.edu)

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Summary

A complex network of interactions between the stroma, the extracellular matrix and the epithelium drives mammary gland development and function. Two main assumptions in chemical carcinogenesis of the mammary gland have been that carcinogens induce neoplasia by causing mutations in the DNA of the epithelial cells and that the alterations of tissue architecture observed in neoplasms are a consequence of this primary mutational event. Here, we use a rat mammary tissue recombination model and the chemical carcinogen *N*-nitrosomethylurea (NMU) to determine whether the primary target of the carcinogen is the epithelium, the stroma or both tissue compartments. Mammary epithelial cells were exposed in vitro either to the carcinogen or vehicle before being transplanted into the cleared fat pads of rats exposed to carcinogen or vehicle. We observed that neoplastic transformation of these mammary epithelial cells occurred only when the stroma was exposed in vivo to NMU, regardless of whether or not the epithelial cells were exposed to the carcinogen. Mammary epithelial cells exposed in vitro to the carcinogen formed phenotypically normal ducts when injected into a non-treated stroma. Mutation in the Ha-*ras*-1 gene did not correlate with initiation of neoplasia. Not only was it often found in both cleared mammary fat pads of vehicle-treated animals and intact mammary glands of untreated animals, but it was also absent in some tumors. Our results suggest that the stroma is a crucial target of the carcinogen and that mutation in the Ha-*ras*-1 gene is neither necessary nor sufficient for tumor initiation.

Key words: Mammary carcinogenesis, Stroma, Neoplasms, *N*-nitrosomethylurea, NMU, Ha-*ras*-1 mutation, Tissue architecture

Introduction

A comprehensive understanding of carcinogenesis in general, and in the rat mammary gland in particular, has been delayed because of epistemological issues. It has been obvious to many of us working in the field of carcinogenesis that we lack a consistently reliable set of premises on which we can base a solid rationale to conduct research (Sonnenschein and Soto, 1999a; Sonnenschein and Soto, 2000; Moss, 2003). For almost a century, a majority of researchers have followed the lead provided by Theodor Boveri in 1914, favoring the notion that carcinogenesis occurs at the cellular level of biological organization (Boveri, 1929). After a number of course corrections to accommodate lacks of fit, Boveri's ideas have coalesced into what is now generally accepted as the Somatic Mutation Theory of carcinogenesis (Hanahan and Weinberg, 2000; Mastorides and Maronpot, 2002). Throughout the twentieth century, this theory has been challenged by others, who proposed instead that carcinogenesis takes place at the tissue level of biological organization (Orr, 1958; Smithers, 1962; Hodges et al., 1977; Sonnenschein and Soto, 2000). In the past decade, attempts to find a synthetic position that would incorporate claims from both theoretical approaches have also been advanced (Folkman et al., 2000; Bissell and Radisky, 2001; Thiery, 2002). Objectively, however, the identification of the target(s) upon which the carcinogenic agents act in order to initiate neoplastic transformation has, so far, remained elusive.

The development of mammary cancer in susceptible rodent strains following administration of *N*-nitrosomethylurea (NMU) is a widely accepted model for the study of chemical carcinogenesis (Gullino et al., 1975). The majority of NMU-induced rat mammary tumors are carcinomas or adenocarcinomas, that is tumors of presumed epithelial origin (Thompson, H. J. et al., 2000a). According to the Somatic Mutation Theory, a neoplastic outcome would result from accumulated NMU-induced mutations in one of the epithelial cells of this gland (Guzman et al., 1992; Gould, 1995). Although these carcinomas show an altered organization of both the epithelium and the stroma, when examined through a light microscope, changes observed in the stroma have been assumed to be a secondary effect of the primary mutational events in the epithelium.

An alternative theory considers that carcinogenesis is a process akin to development gone awry (Pierce et al., 1978; Sonnenschein and Soto, 1999a). The Tissue Organization Field Theory proposes that carcinogens alter stromal-epithelial interactions and that proliferation is the default state of all cells (Sonnenschein and Soto, 1999b; Sonnenschein and Soto, 2000). Carcinogenesis would therefore be an emergent phenomenon that takes place at the tissue level of biological organization. As mentioned above, several authors have proposed synthetic approaches that straddle both theories as applied to mammary carcinogenesis (Bissell and Radisky, 2001; Wiseman and Werb, 2002; Thiery, 2002).

In an effort to deal comprehensively and simultaneously with all the competing theories, we decided to use a rat mammary tissue recombination model. This model affords an easy surgical separation of stroma and epithelium such that each compartment might be exclusively exposed to the carcinogen. We chose NMU because it is a direct carcinogen in that it does not need to be metabolized in order to form DNA adducts and has a very short half-life (Swann, 1968). This minimizes the risk of inadvertent indirect exposure of epithelial cells to the carcinogen when recombining them with the stroma. The outcome of the proposed experimental design would determine whether the primary target of NMU is the epithelium (as suggested by the Somatic Mutation Theory), the stroma (as implied by the Tissue Organization Field Theory), or both tissue compartments.

Materials and Methods

Chemicals and cell culture reagents

NMU (CAS #684-93-5), insulin, penicillin, progesterone, prolactin, fatty acid-free fraction V bovine serum albumin (BSA), hydrocortisone, human transferrin, ascorbic acid, gentamicin, aluminum potassium sulfate and methyl salicylate were purchased from Sigma-Aldrich. Human epidermal growth factor (EGF) and Matrigel™ were obtained from Becton Dickinson. Phenol red-free DMEM/F12 medium and trypsin were obtained from Gibco. Collagenase was purchased from Worthington Biochemical Corporation and pronase from Calbiochem. Percoll™ was obtained from Amersham Pharmacia Biotech and Carmine from Fisher Scientific.

Animals

Wistar-Furth rats were purchased from Harlan and housed with food and water ad libitum. Animals were maintained on a 14:10 hours light:dark cycle and care was in accordance with the Guidelines for the Care and Use of Animals and the Tufts-New England Medical Center Institutional Animal Care and Use Committee. When the animals were 21 days old, the mammary epithelium was surgically removed from the 4th and 5th inguinal mammary glands according to procedures outlined previously (DeOme et al., 1959). In each of the animals used in these experiments, the excised epithelium was whole-mounted and observed microscopically to assure that the ductal tree was removed in its entirety and that only a small portion of the fat pad remained attached to it (Fig. 1A).

Tissue recombination experimental design

The animals with cleared fat pads were distributed into experimental Groups 1-4. At 52 days of age, animals from Groups 1 and 2 received a single intraperitoneal dose of 50 mg NMU/kg body weight dissolved in warm 0.85 g/l NaCl solution (vehicle), pH 5.0; by contrast, Groups 3 and 4 were exposed to just the vehicle. Five days later, 50,000 mammary epithelial cells were injected into the cleared fat pads according to the following experimental design: animals from Groups 1 and 4 received vehicle-treated mammary epithelial cells, and animals from Groups 2 and 3 received NMU-treated mammary epithelial cells. Positive and negative control groups were used. These control groups were intact virgin animals that were age-matched to the animals in Groups 1 to 4. They were not subjected to any surgical manipulation. These animals were treated at 52 days of age with NMU and vehicle, respectively. They were injected at the same time with the animals used in Groups 1 to 4. Intact animals injected with NMU were considered as the positive control for tumor incidence and histopathology of the tumors (Group 5). Animals injected with vehicle were considered as the control for spontaneous tumors and for the normal architecture of the mammary gland (Group 6). Four experiments were performed where all the experimental groups were represented. Animals were excluded from the analyses when no epithelial outgrowths were found in the whole mounts ('no takes') or if they died as a result of surgical complications. The initial (i) and final (f) sample sizes at 9 months after the NMU injection were as follows: Group 1, i=14, f=13; Group 2, i=10, f=8; Group 3, i=12, f=10; Group 4, i=11, f=6.

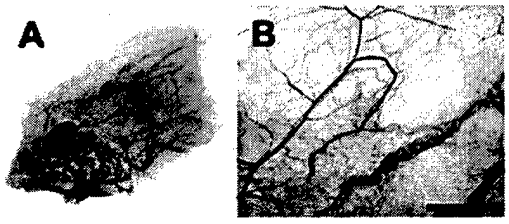

Fig. 1. (A) Whole-mount preparation of an intact mammary gland from a 21-day-old rat showing the ductal tree and lymph nodes. (B) Mammary gland fat pad cleared of epithelium at 21 days of age and excised at the end of the experiment, 11 months later. Bar, 4 mm.

Cleared fat pad repopulation

A second set of animals with cleared fat pads was transplanted with 50,000 mammary epithelial cells at 52 days of age. The recombinants were inspected 30, 60 and 90 days later.

Mammary epithelial cell culture

Mammary epithelial cells were isolated from 50-60-day-old virgin female Wistar-Furth rats using a combination of two previously described protocols (Hahm and Ip, 1990; Imagawa et al., 2000). Briefly, the 4th and 5th inguinal mammary glands were bilaterally excised, minced and digested in phenol red-free DMEM containing 0.15% collagenase III at 37°C for 2 hours with agitation. This digest was centrifuged and the pellet was then treated with 0.05% pronase for 30 minutes at 37°C with agitation. This suspension was filtered through a 530 μm-pore Nitex® filter (Sefar America) and the filtrate was centrifuged at 100 ***g*** for 3 minutes (Hahm and Ip, 1990). The pellet was resuspended in 1-2 ml of serum-free medium (SFM) containing phenol red-free DMEM/F12 plus 10 μg/ml insulin, 1 μg/ml progesterone, 10 ng/ml EGF, 1 μg/ml prolactin, 1 mg/ml BSA, 1 μg/ml hydrocortisone, 5 μg/ml human transferrin, 0.88 μg/ml ascorbic acid and 50 μg/ml gentamicin (Hahm and Ip, 1990). This cell suspension was layered over a pre-made Percoll™ gradient (Imagawa et al., 2000) and centrifuged for 20 minutes at 800 ***g***. Single epithelial cells and organoids were recovered from the gradient, diluted in SFM and similarly centrifuged. The pellet was resuspended in SFM and plated on Matrigel™-coated (100 μg/cm^2) 6-well plates (Becton Dickinson). This layer was enough to promote cell attachment but insufficient to facilitate three-dimensional growth. Non-epithelial cells were successfully removed by treating the plates with a 0.025% trypsin and 0.01% EDTA solution. Five days before being transplanted into recipient animals, the mammary epithelial cells were treated with SFM containing either vehicle or 50 μg/ml NMU for 1 hour at 37°C (Miyamoto et al., 1988). The cells were then rinsed twice with SFM and fresh SFM was added. NMU was used within 5 minutes of preparation. A different batch of mammary epithelial cells prepared following this protocol was used for each of the four experiments. The dose of NMU used in the in vitro experiments was selected following Miyamoto et al. (Miyamoto et al., 1988).

Epithelial cell transplantation

After harvesting by trypsinization, the cells were counted in a Coulter

Counter Apparatus (Model ZM, Coulter Electronics). The volume of the cell suspension was adjusted in order to inject 50,000 cells in 10 µl into the CFP using a Hamilton syringe. All rats receiving a cell transplant were palpated weekly, starting one month after the mammary epithelial cell inoculation. Thoracic glands were used as internal controls for the carcinogen and were equally palpated. Animals were sacrificed when inguinal tumors reached 1 cm in diameter or 9 months after cell transplant, whichever came first.

DNA extraction and analysis of Ha-*ras*-1 gene mutation

DNA was extracted from mammary neoplastic lesions, fat pads and whole mammary glands from virgin rats using the DNeasy kit (Quiagen), following the manufacturer's instructions. We used the mismatch amplification mutation assay (MAMA) described by Cha et al. (Cha et al., 1996) with some modifications. The MAMA is specific for the codon 12 GGA to GAA mutation in Ha-*ras*-1 gene. Briefly, this method uses two sets of primers; one targets the mutation and the other a control area in the genomic DNA. The mutant-specific mismatch primer PAA (5′-CTTGTGGTGGTGGGCGCTGAA-3′), the Pmnl2 (5′-ACTCGTCCACAAAATGGTTC-3′) and the control primers (P1: 5′-CCTGGTTTGGCAACCCCTGT-3′; and Pmnl2: 5′-ACTCGTCCACAAAATGGTTC-3′) were used at a 40 ng/µl concentration. The PCR was performed using Platinum Supermix (Invitrogen). The PCR products were run in a 2% agarose gel (Gibco). The expected size of the non-mutated Ha-*ras*-1 gene is 128 bp, whereas the mutated Ha-*ras*-1 gene is 74 bp.

Whole mounts and histology

Whole mounts were prepared following protocols described by the Laboratory of Genetics and Physiology at the National Institute of Diabetes, Digestive and Kidney Diseases within the National Institutes of Health (http://mammary.nih.gov), and Thompson et al. (Thompson et al., 1995). The mammary glands were removed and spread on a 75×50×1 mm glass slide (Fisher Scientific), fixed overnight in 10% phosphate-buffered formalin, dehydrated in 70%, 95% and 100% alcohols, cleared in toluene, rehydrated and stained with Carmine Alum. After staining, the whole mounts were dehydrated as described above, cleared in xylene, and bagged in Kpak® SealPak heat-seal pouches in methyl salicylate. The whole mounts were analyzed under a stereomicroscope for microscopic lesions. Tumors larger than 0.5 cm were removed before whole mounts were prepared and separately fixed as described above. Microscopic lesions were removed and embedded in paraffin for histological analysis. Images were captured with an AxioCam HR color digital camera (Carl Zeiss) attached to a Stemi 2000 stereomicroscope (Carl Zeiss).

Immunohistochemistry

An antigen-retrieval method based on microwave pretreatment and 0.01 M sodium citrate buffer (pH 6) was used as previously described (Maffini et al., 2001). Mouse monoclonal anti-pan cytokeratin (Sigma-Aldrich), anti-vimentin (Novocastra) and anti-desmin (Novocastra) were used at 1:700, 1:100 and 1:100 dilutions, respectively. The antigen-antibody reaction was visualized using the streptavidin-peroxidase complex, with diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as the chromogen. Counterstaining was performed with Harris' hematoxylin. For the double-staining immunofluorescence technique, cytokeratin and vimentin were detected using a previously described technique (Maffini et al., 2002). The primary antibodies were used at 1:100 dilutions in 4% BSA supplemented with 10% normal goat serum. Secondary antibodies and streptavidin-Alexa 594 and 488 (Molecular Probes) were used at 1:100 dilutions. Cell nuclei were counterstained with Hoechst 33258. Images were captured with an AxioCam HR color digital camera (Carl Zeiss) attached to an Axioskop 2 plus microscope (Carl Zeiss).

Statistics

Statistical significance of the incidence of neoplastic lesions and Ha-*ras*-1 gene mutation were determined using the χ^2 Test. The Mann-Whitney and Kruskal-Wallis tests were used to compare the latency periods and the number of lesions in inguinal and thoracic mammary glands between groups. To compare the latency of pectoral and inguinal lesions in the same animal within each treatment group, we used the Wilcoxon signed ranks test, and treated the pectoral and inguinal latency for each animal as a pair.

Results

Normal ducts developed from cultured mammary epithelial cells

The tissue recombination components were mammary gland stroma (cleared fat pad) and mammary epithelial cells grown in vitro (Fig. 2A). We observed the phenotype of the ductal outgrowth and the repopulation dynamics in the cleared fat pads after transplantation of 50,000 mammary epithelial cells. The ductal outgrowths were phenotypically normal and, 90 days after mammary epithelial cell transplantation, the ductal tree covered a third of the fat pad (Fig. 2B-E).

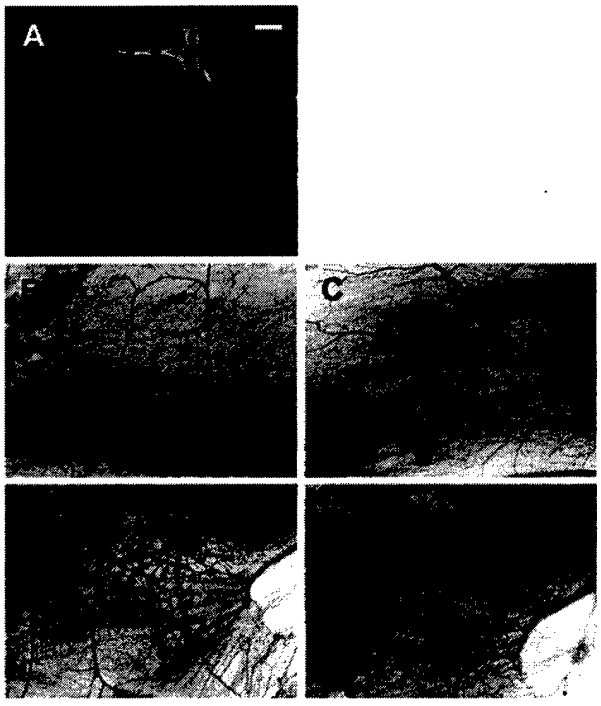

Fig. 2. Repopulation of the mammary gland. (A) Mammary epithelial cells grown in culture showing the expression of cytokeratin (red) and vimentin (green). Mammary epithelial cells averaged 90% of the total cell population transplanted into cleared fat pads. Counterstaining, Hoescht 33258 (blue). Mammary epithelial cells were injected into cleared fat pads and the recombinants were harvested at 0 (B), 30 (C), 60 (D) and 90 (E) days after cell injection. Bars, 20 µm (A); 2 mm (B-E).

Neoplastic transformation of mammary epithelial cells

We observed that only those animals whose stroma was exposed to NMU developed neoplasms, regardless of whether or not the transplanted mammary epithelial cells were exposed to the carcinogen (Fig. 3A). The incidence of neoplastic lesions in Groups 1 and 2 was 76.9% (10/13) and 75% (6/8), respectively (Fig. 3B). The positive control Group 5 had 100% incidence (6/6). There were no significant differences in neoplastic incidence between Groups 1 and 2 (P=0.920) or between Groups 1 or 2 and Group 5 (P=0.200 and P=0.186, respectively). By contrast, the animals whose stroma was exposed to vehicle developed no neoplasms, regardless of whether the mammary epithelial cells were exposed in vitro to NMU (Group 3, 0/10) or to vehicle (Group 4, 0/6). The negative control Group 6 had 0% incidence (0/6). Group 3 was significantly different from Group 1 (P<0.001) and Group 2 (P=0.001). Group 4 was also significantly different from Group 1 (P=0.002) and Group 2 (P=0.005).

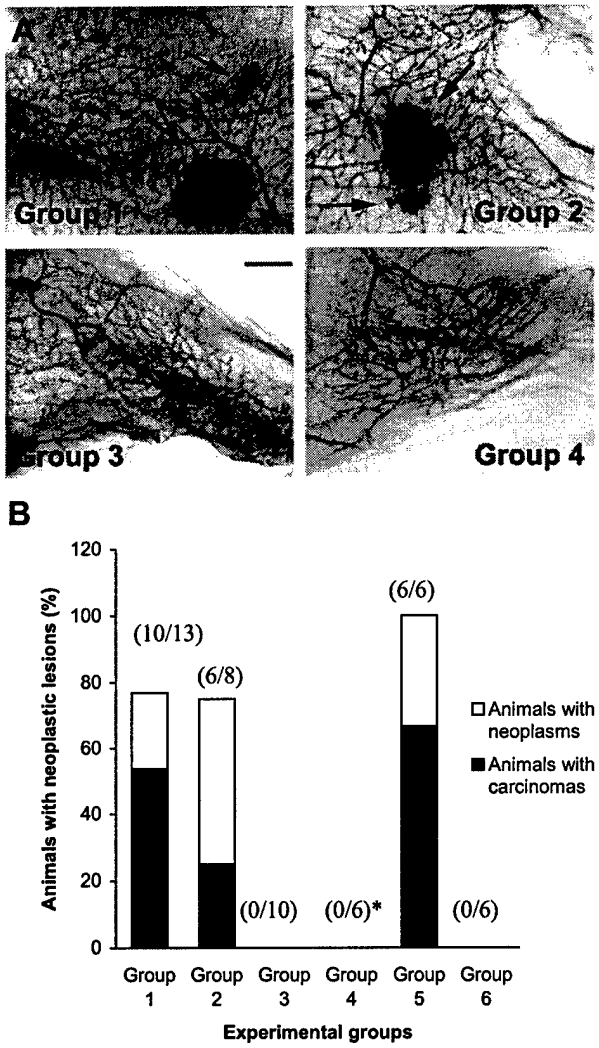

Fig. 3. Neoplasms developed in NMU-treated stroma only. (A) Mammary gland whole-mount preparations show abnormal outgrowths in animals whose cleared fat pads were exposed to NMU prior to recombination with vehicle-treated mammary epithelial cells (Group 1) or NMU-treated mammary epithelial cells (Group 2). Neoplastic lesions (arrows) were confirmed histologically. Groups 3 and 4 developed normal-like ductal outgrowths. Bar, 2 mm. (B) Percentage of neoplastic lesions and incidence of carcinomas per experimental group. The number of rats with neoplastic lesions out of the total number of animals in each group is indicated in parenthesis. *See Materials and Methods for further details.

Multiple neoplastic lesions were found

Multiple lesions were observed in the inguinal mammary glands of rats in Groups 1, 2 and 5 (Fig. 4A), suggesting that the neoplasms found in these groups were not a consequence of mechanical injury resulting from the injection procedure. The inguinal glands of Group 5 had twice as many lesions as those in Groups 1 and 2 (P=0.013 and P=0.001, respectively) (Fig. 4A). This difference might have been owing to the fact that the intact mammary glands in Group 5 had a full complement of epithelium whereas Groups 1 and 2 had an

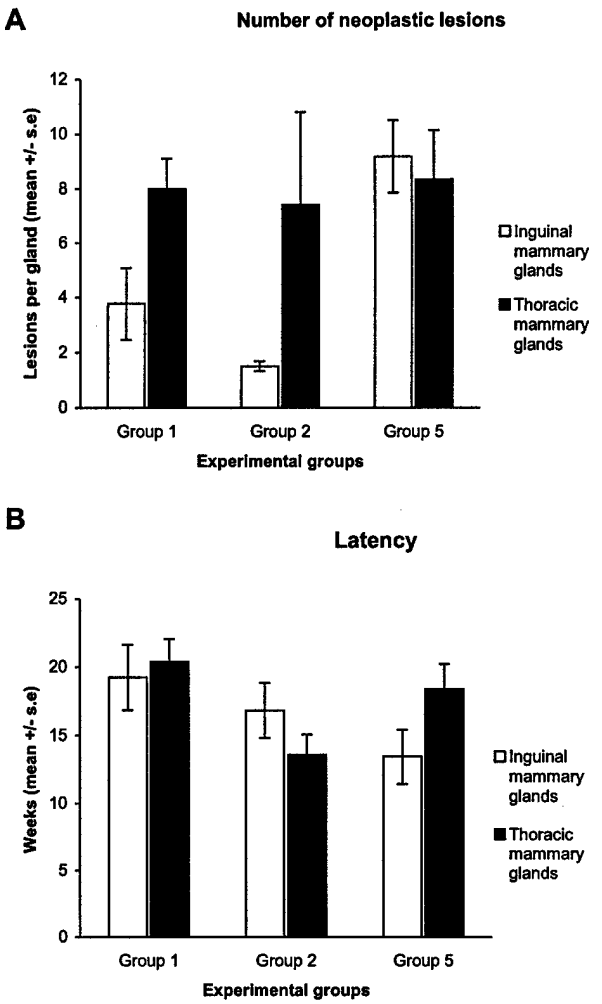

Fig. 4. Incidence of neoplasms and latency period. (A) Number of neoplastic lesions in inguinal and thoracic mammary glands (mean±s.e.). (B) Latency of neoplastic lesions in inguinal and thoracic mammary glands expressed in weeks (mean±s.e.).

initial population of only 50,000 mammary epithelial cells. The incidence of neoplastic lesions in the thoracic mammary glands of NMU-treated rats from Groups 1, 2 and 5 was comparable (P=0.622) (Fig. 4A). There was no significant difference among Groups 1, 2 and 5 regarding inguinal tumor latency periods (P=0.147). The latency period was similar in the thoracic and inguinal mammary glands within the same experimental groups (Group 1: P=0.276; Group 2: P=0.414; Group 5: P=0.684) (Fig. 4B).

We performed the histopathological analyses of the neoplastic lesions following the classification described by Russo et al. (Russo et al., 1990). Carcinomas were seen in 53.8% of the animals from Group 1, 25% of Group 2 and 66.7% of Group 5 (Fig. 3B), and represented 70%, 33% and 66.7% of the neoplasms found in these groups, respectively (Table 1). The most frequent type of neoplastic lesion was papillary carcinoma (Fig. 5B, Table 1). All the tumors were of epithelial origin; the neoplastic cells were cytokeratin positive, and vimentin and desmin negative (Fig. 5C). Regardless of whether or not the mammary epithelial cells had been exposed to NMU, the tissue-recombined mammary glands of animals that did not develop tumors appeared histologically similar to a normal mammary gland (Fig. 5A).

Mutated Ha-*ras*-1 does not correlate with neoplasia

We analyzed the DNA of neoplastic lesions from Groups 1 and 2 and observed that 2 out of 11 neoplasms from Group 1, and 1 out of 6 from Group 2, lacked the G-A mutation in the codon 12 of the Ha-*ras*-1 gene. Similarly, DNA taken from the neoplastic lesions in the positive control (Group 5) showed that 1 out of 7 lacked the point mutation (Fig. 6). No statistically significant difference was found between the groups (P=0.977). In order to test whether any correlation existed between the presence of the mutated Ha-*ras*-1 gene and the initiation of neoplasia, we analyzed DNA extracted from the stroma of animals treated with vehicle (i.e. Groups 3 and 4). All stroma samples from Groups 3 (7 out of 7) and 4 (6 out of 6) showed the mutation. Thus, we now report that this Ha-*ras*-1 gene mutation was present in the mammary gland fat pad of rats exposed to vehicle. Moreover, DNA harvested from whole mammary glands of intact rats randomly taken from our colony (4 out of 4) also showed the mutation, which agrees with previous findings (Cha et al., 1996). The incidence of mutated Ha-*ras*-1 gene was not significantly different between animals that were or were not exposed to NMU (P=0.604). Finally, the mutation was also assessed in DNA isolated from mammary epithelial cells, mammary fibroblasts, and mammary pre-adipocytes collected from intact virgin rats and grown in vitro. All these different types of cells were collected at different times during the course of 2 years. DNA was extracted from frozen cells, vehicle-treated cells and NMU-treated cells. The presence of the mutation did not correlate with cell type, culture conditions or carcinogen treatment (data not shown).

Table 1. Incidence of mammary neoplastic lesions in groups exposed to NMU

Experimental group	Histopathological classification	Incidence (%)*
Group 1	Carcinomas	70
	Papillomas	10
	Cystoadenomas	10
	Adenomas	10
Group 2	Carcinomas	33.3
	Papillomas	16.7
	Fibroadenomas	33.3
	Fibroma	16.7
Group 5 (Positive control)	Carcinomas	66.7
	Adenomas	16.7
	Cystoadenomas	16.6

*The number of neoplastic lesions in each histological category was divided by the total number of lesions observed in each experimental group

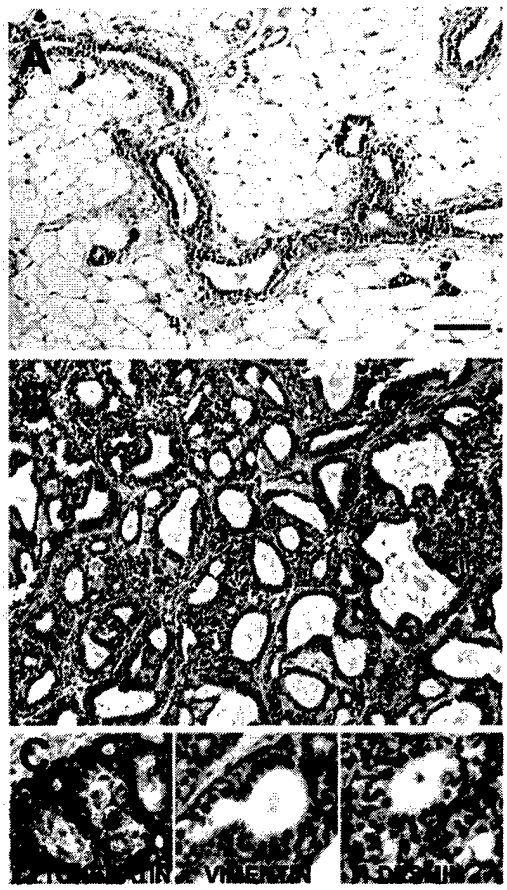

Fig. 5. Sections of recombinant tissues. (A) Section from a recombinant of vehicle-exposed stroma and NMU-exposed mammary epithelial cells. The histoarchitecture resembles a normal mammary gland. (B) Papillary carcinoma from a recombinant of NMU-exposed stroma and vehicle-exposed mammary epithelial cells. Hematoxylin and eosin staining (A,B). (C) Immunohistochemistry for cytokeratin, vimentin and desmin in sections of the tumor shown in B. Counterstaining: Harris' hematoxylin. Bar, 100 μm.

Discussion

Our results regarding the role of histoarchitecture in carcinogenesis are consistent with previous findings stemming from the use of diverse rodent models. Barcellos-Hoff and

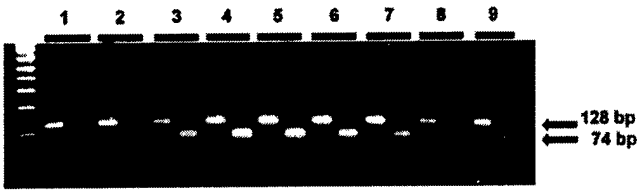

Fig. 6. Analysis of the presence of point mutation in the Ha-*ras*-1 gene using the MAMA. The mutant-specific amplification product is 74 bp whereas the normal product is 128 bp. Lanes 1, 5, 6 and 7: mammary tumors from Group 5. Tumor in lane 1 lacks Ha-*ras*-1 gene mutation. Lane 2: mammary tumor from Group 1. Note absence of Ha-*ras*-1 gene mutation. Lanes 3 and 4: mammary tumors from Group 2. Lanes 8 and 9: normal mammary tissue from intact animals taken randomly from the colony. Note: the smaller bands in lanes 1 and 2 correspond to dimers of the primer.

Ravani showed that radiation-induced changes in the stromal microenvironment contributed to the neoplastic progression of non-irradiated, quasi-normal, established COMMA-1 mammary epithelial cells (Barcellos-Hoff and Ravani, 2000). Sternlicht et al. observed that overexpression of the matrix metalloproteinase stromelysin-1 can induce carcinogenesis in mouse mammary glands (Sternlicht et al., 1999). Also, using tissue recombinant techniques, Olumi et al. concluded that 'primary, phenotypically normal fibroblasts associated with a human epithelial malignancy can stimulate progression of a nontumorigenic (prostate) epithelial cell' (Olumi et al., 1999). Thompson et al. have also used a tissue recombination model, the mouse prostate reconstitution model system, and observed that 'intrinsic properties of the BALB/c mesenchyme can arrest the progression of ras+myc-initiated C57BL/6 epithelium from benign hyperplasia to malignant carcinoma' (Thompson et al., 1993).

Our experiments, designed to explore simultaneously the competing theories mentioned in the introduction, suggest that the stroma is a target of NMU in mammary carcinogenesis. We were concerned, of course, that inadvertent technical mishaps might have influenced our data. For instance, epithelial cells might have remained in the fat pads after the clearing procedure and could have been exposed in vivo to NMU. We addressed this possibility by microscopically examining the tissue containing the ductal tree after clearing the fat pads at 21 days of age and verifying that the margins contained no epithelial cells (Fig. 1A). In addition, we also cleared the 5th mammary gland to prevent the migration of indigenous epithelial cells into the 4th cleared fat pad. Therefore, we consider it unlikely that epithelial cells were present after clearing. It was also reassuring to observe that cleared fat pads not injected with mammary epithelial cells remained free of epithelium at the end of the experiment (Fig. 1B).

Several research groups have used experimental rodent models to explore the concept that epithelial cells are the targets of carcinogens, as implied by the Somatic Mutation Theory. Miyamoto et al. reported tumor formation after mammary epithelial cells were exposed to NMU in vitro and injected into cleared fat pads (Miyamoto et al., 1988). These authors used a cell inoculum one order of magnitude higher than the one we used and a different cell purification method. Also, they added the NMU when the cultures were 3 days old and made no reference to the degree of fibroblast contamination. It is conceivable that, in their experiments, fibroblasts exposed to NMU in vitro could have played a role in the carcinogenic process. On the contrary, we repeatedly trypsinized and subcultured the mammary epithelial cells to enrich this pool of cells and reduce fibroblast contamination. In essence, we exposed a highly enriched mammary epithelial cell population to NMU (Fig. 2A). Furthermore, Miyamoto et al. injected the mammary epithelial cells into fat pads immediately after clearing, while in the midst of wound healing (Miyamoto et al., 1988). In this context, it has been shown that carcinogenesis is promoted by a wounded stroma (Konstantinidis et al., 1982; Sieweke et al., 1990). Their data and those by Guzman et al. do not suggest a positive correlation between tumor yield and either NMU concentration or the number of exposures to this carcinogen in vitro. Moreover, normal epithelial outgrowths were observed at all NMU doses (Guzman et al., 1987; Miyamoto et al., 1988). Using yet another protocol, Kamiya et al. showed that NMU- or radiation-exposed mammary epithelial cells yielded mammary carcinomas when grafted into rat fat pads that were 'cleared' by injecting 70% ethanol (Kamiya et al., 1995). They interpreted these data as evidence that tumor formation was due to undefined epigenetic factors rather than to mutations. They also observed that tumor incidence diminished as the number of cells injected increased, an outcome inconsistent with the Somatic Mutation Theory.

These experiments dealing with in vitro exposure to NMU were based on the premise that NMU acted directly on the epithelial cells and, therefore, under this rationale, no attempt was made to evaluate the role of the stroma in tumor formation (Greiner et al., 1983; Guzman et al., 1987; Miyamoto et al., 1988; Delp et al., 1990). The novelty of our observations stems from the fact that a carcinogen-treated stroma was able to transform vehicle-treated cells into neoplastic tissues comparable with those seen in intact NMU-exposed rats (positive control Group 5) (Fig. 5 and Table 1).

The prevalent hypothesis that NMU exposure results in carcinogenesis because of NMU-induced point mutations in the codon 12 of the Ha-*ras*-1 gene of mammary epithelial cells (Zarbl et al., 1985) has been challenged. As shown in our results and in the literature (Cha et al., 1994; Cha et al., 1996; Swanson et al., 1996; Shirai et al., 1997; Thompson, T. A. et al., 2000b), not all NMU-induced mammary neoplasms express this mutation. Also, Korkola and Archer have observed comparable results in NMU-induced pre-neoplastic lesions (Korkola and Archer, 1999). Equally important, this mutation is present in mammary glands from non-exposed animals (Cha et al., 1996). Here, we confirm these findings and show that the frequency of tumors expressing mutated Ha-*ras*-1 is statistically similar in the positive controls (Group 5) and recombinants from NMU-exposed stroma (Groups 1 and 2). Moreover, we also observed that mutated Ha-*ras*-1 is also present in the cleared mammary fat pad of vehicle-exposed animals. Furthermore, Zhang et al. demonstrated that increasing the dose of NMU increased total tumor yield but reduced the frequency of mammary tumors expressing mutated Ha-*ras*-1 (Zhang et al., 1990). In sum, these data suggest that the Ha-*ras*-1 gene mutation appears to be neither necessary nor sufficient for neoplastic transformation and that it is not exclusively present in the epithelial cells.

The concept that altered tissue architecture is at the core of carcinogenesis was pioneered by Waddington (Waddington, 1935), Orr (Orr, 1958) and, more recently, by Bissell and Radisky (Bissell and Radisky, 2001) and others (Sonnenschein and Soto, 2000; Moss, 2003, Weaver and Gilbert, 2004). Altogether, our data and those of others challenge the long-held notion that carcinogens induce mammary cancer by causing mutations in the DNA of an epithelial cell (Fearon and Vogelstein, 1990; Mastorides and Maronpot, 2002). These results suggest the need to explore the roles that the stroma components [i.e. the cells (fibroblasts, adipocytes, mast cells, etc.)] and the extracellular matrix play in rodent mammary carcinogenesis. Efforts should also be directed at exploring the role of the stroma in experimental models for carcinogenesis involving organs other than the mammary gland (i.e. skin, prostate, liver, bladder). To accommodate a novel perspective on the role of the stroma in carcinogenesis, a rigorous analysis of concepts, definitions and experimental approaches is now needed. This will facilitate the identification of the mediators responsible for the altered tissue phenotype in cancers and of ways to reverse their effect by adopting a solid epigenetic perspective.

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