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Overview

Contributions of Mouse Biology to Breast Cancer Research

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Introduction

Comparative medicine is based on the concept that animals and humans often share the same disease. This concept is perhaps best exemplified in mammary biology where the rodent has been the primary animal model for human breast cancer. The following section reviews some of the key concepts in breast cancer research from the last five decades in the form of "epitomes" based on the second annual UC Davis Mouse Biology Program Symposium, sponsored by the UC Davis Center for Comparative Medicine. Taken together, these epitomes place the current molecular, genomics and pre-clinical research in breast cancer into a historical context, demonstrating the biological basis for modern research.

The symposium brought together many of the investigators who pioneered mammary cancer research for a day of scientific presentations and discussions with some of the current breast cancer researchers. The participants were all directly or indirectly connected to the "West Coast Mammary Biology Group," centered at the UC Berkeley Cancer Research Genetics Laboratory (CRGL), led by Drs. Kenneth B. DeOme and Howard A. Bern, and the event coincided with the 50th anniversary of the founding of the CRGL by DeOme. The assemblage also cel-

ebrated the retirement of one of the participants, Lawrence J. T. Young, who had been involved in the key experiments through the five decades represented at the symposium.

All participants submitted a summary of their symposium comments and of their research. The following sections are presented in the "epitome" style; that is, short summaries of a field or concept. The first group of epitomes represents a historically significant review of the origin of many of the key concepts in mammary biology, frequently presented by the pioneering investigators themselves. The concluding epitomes provide some specific examples of how current biological and pre-clinical research continues to be influenced by the earlier work.

As indicated in the historical summary by Bern, the three major contributions of the CRGL under DeOme were in mammary preneoplasia, hormone regulation and the role of the mouse mammary tumor virus (MMTV) in mouse mammary tumorigenesis. The development of the gland-cleared fat pad transplantation techniques (Faulkin) led to operational definitions of senescence (Daniel), stem cells (Smith), and preneoplasia (Faulkin) in mouse mammary biology. Preneoplasia is a focal area of atypia with immortalized cells that are at high risk of undergoing malignant transformation. Senescence is the inability of a cell population to sustain growth on serial transplantation. Stem cells are those cells in the population that are capable of regenerating the entire mammary gland. These concepts were expanded upon by many students, led by Medina, who developed the hyperplastic outgrowth (HPO) transplant lines that were used to characterize preneoplasia (Young and Medina).

The concepts from the mouse mammary gland were applied to the study of precancer in humans and became the basis for current concepts of ductal carcinoma in situ (DCIS) (Wellings). Although operational proof by transplantation proved to be impossible, Wellings and Jensen found that the human mammary gland has focal atypical lesions that are associated with breast cancer. Their work is the foundation for the extensive studies of Page, his colleagues, and others that have provided incontrovertible demographic evidence that the lesions identified by Wellings are premalignant. Wellings and Cardiff have pointed out that the atypical lesions found in the mouse and the human are in the terminal units of the breast (lobular) and are morphologically identical.

The concepts and techniques developed in the 1960s are currently being applied to genetically engineered mice (Medina, MacLeod, Oshima, Sawai, Gregg, Cheung, and Lau) for detailed molecular analysis of genetic modifiers of cancer (Oshima), preneoplasia (Medina and MacLeod), metastases (Sawai and Gregg), angiogenesis (Cheung) and therapeutic anti-angiogenesis (Lau). Although current studies focus on the modern molecular biology and molecular medicine, they are based on the concepts of preneoplasia embodied in the hyperplastic alveolar nodule (HAN) and the hyperplastic outgrowth (HPO) and on the transplantation techniques developed by DeOme and his students, Faulkin, Daniel and Medina.

Another major CRGL contribution came in the area of hormone regulation. This group was led by Howard Bern and his student Satyabrata (Ranu) Nandi. They defined the hormones regulating mouse mammary gland development (Shyamala). The CRGL group established the roles of estrogen, progesterone, prolactin and insulin in mammary gland growth and development. Current investigators are now using genetically engineered mice to focus on the hormone receptor rather than the hormone. This has permitted a more detailed molecular dissection of hormone regulation (Shyamala). However, the estrogen hormone is still a key factor in breast cancer. Rodent models are now being used to study how alteration in hormone balance may lead to early chemo-intervention, preventing breast cancer (Guzman and Nandi).

Figure 1. Dr. Kenneth B. DeOme the founder of Cancer Research Genetics Laboratory at the University of California, Berkeley and leader of the West Coast Mammary Biology Group.

The symposium was designed to trace the background history of modern breast cancer research. These epitomes illustrate the origins of the key concepts and how they are being applied in current research. Each epitome is intended to stand on its own, with limited references to the key reviews and primary articles. The reader is invited to browse the epitomes to obtain an overview or use the individual epitome as a source for further in-depth study.

Robert D. Cardiff
University of California, Davis

The University of California Origins of Experimental Breast Cancer Research

This symposium coincided with the 50th anniversary of the founding of the Cancer Research (Genetics) Laboratory (CR(G)L) at the University of California, Berkeley, under the directorship of Kenneth B. DeOme, professor of zoology (Fig. 1). The California state legislature had provided funds to establish a colony of mice of reliable genetic background to be made available to cancer researchers throughout the state and elsewhere. DeOme, a mammalian geneticist and comparative histopathologist, was

the first director, who was followed by the tumor endocrinologist Satyabrata (Ranu) Nandi and now by the tumor molecular immunologist James Allison. Since its inception, the primary focus of the laboratory was on experimental mammary cancer, and this remains a major domain of research to this day.

DeOme attracted a variety of collaborators to the laboratory, including this author and Nandi as endocrinologists, Dorothy Pitelka and Morgan Harris as cell biologists, and David Weiss and Phyllis Blair as tumor immunologists. Accompanying these academic staff members were technicians, graduate and postdoctoral students (including MDs seeking the PhD degree and research careers), and visiting investigators. Many of the former predoctoral and postdoctoral students are now distinguished professors in their own right, both in the U.S. and abroad.

In the central program of the laboratory, devoted to understanding the genesis and progression of mammary cancer, significant understanding was attained early on of the role of the hyperplastic alveolar nodule (HAN) as a precancerous lesion, on the endocrine control of normal and abnormal mammary gland development, and on the nature and role of the mouse mammary tumor virus and its variants. A variety of techniques were developed, including recognition of the living hyperplastic alveolar nodule and its transplantation into the gland-free mammary fat pad, ultrastructural analysis of mammary cells in various growth and functional states, organ culture and cell culture, and hormone receptor biology. The laboratory was and still is characterized by the diversity of its personnel and its students, the diversity of its approaches and its fields of investigation, as well as by its ability to introduce unorthodox concepts and interpretations.

The first session of the symposium was composed of papers by former graduate students from the laboratory's younger days. These still-vigorous investigators address some of the fundamental issues arising from the focus of the laboratory on experimental breast cancer in rodents and their clinical implications. The later epitomes deal with older issues outside the purview of the CR(G)L, which remain relevant in understanding the basic biology of breast cancer. The remaining papers bring to the fore the most recent technology, largely molecular, used in the analysis of mammary cell transformation and progression that results in the emergence of the cancerous state.

Howard A. Bern
University of California, Berkeley

1) The Mammary Gland-Free Fat Pad Transplantation Systems

A) The Gland-Cleared Fat Pad: The Foundation for Mammary Immortality and Preneoplasia.

In the early 1950s, K.B. DeOme returned to the Berkeley campus with several strains of mice, each with a different incidence of mammary tumor. His goal was to study the neoplastic process in a laboratory animal that would permit multiple generations in a relatively short period of time. The hyperplastic alveolar nodule (HAN) had been identified in the mammary glands of strains of mice that had a high incidence of mammary tumors (Fig. 2). There was a correlation between the HAN and tumors but no direct evidence that tumors arose from HAN. We began experiments to demonstrate that nodules developed into mammary tumors.

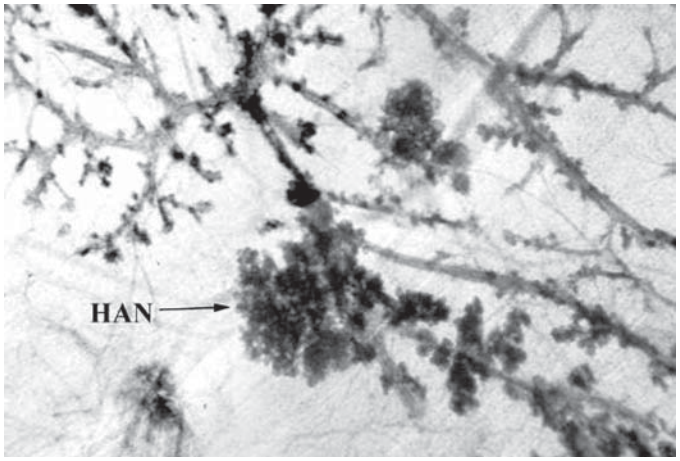

Figure 2. Photomicrograph of a hematoxylin-stained whole mount from a MMTV-infected mouse mammary gland showing the classical sub-gross picture of a HAN. Note how it stands out from the surrounding mammary gland.

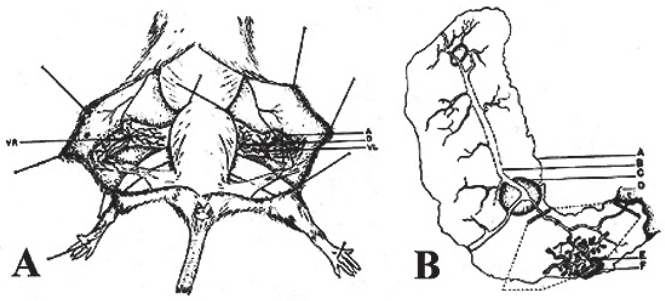

Figure 3. A drawing originally done for Dr. DeOme at CRGL to illustrate the technique for finding the number 4 mammary fat pads and identifying the key anatomic landmarks for clearing the fat pad of glands. Note that this represents a three-week-old mouse with the developing mammary just beyond the nipple.

For my master's thesis, a series of preliminary experiments were done that could best be described as "mapping" and "marking" of the HAN in situ and then waiting for tumor development. For a number of reasons, the techniques were not satisfactory, and we moved on to other methods. We had learned to recognize HAN in vivo and decided to place them in a site free of mammary tissue, so that when tumors developed, they could only have arisen from the HAN. The loose connective tissue of the back was chosen, away from any mammary glands. Two nodule transplants were placed in the backs of about 20 mice, and they were dutifully checked each week for about six months. No tumors were observed. The animals were terminated, and the transplants checked. Each transplant could be seen as a discrete lesion, but there was no growth of the lesion, and no tumors had developed. Back to the drawing board!

The murine mammary gland develops postnatally by the proliferation of ducts into the mammary fat pad associated with each nipple. This proliferation of ducts is just beginning at three weeks of age. If the developing gland is excised, the remaining mammary fat pad, with its circulation intact, can be used as a site for transplants. Experiments were done in which HAN and normal mammary tissue were transplanted to gland-free fat pads (Fig. 3). By 20 weeks following transplantation, eight tu-

mors had developed from the HAN, and no tumors had developed from the normal transplants. What was more surprising was that the transplants—both normal and HAN—had grown to fill the fat pads.

A series of experiments was performed to investigate the relationship of normal mammary tissue and nodule outgrowth tissue. As was concluded in a paper in 1960:

> "These experiments demonstrated the presence of a growth regulating system within the intact mammary gland which determines both the spacing of the parenchymal elements and the extent of mammary-gland growth. This growth-regulating system was shown to be effective between normal gland elements and hyperactive outgrowth, between hyperactive outgrowths, and, to a limited extent, between normal ductal elements and tumors. The ability to override this growth-regulating system appears to be the principal characteristic of neoplastic tissue" (L. J. Faulkin, Jr. and K. B. DeOme).

The ability to isolate and transplant selected pieces of mammary tissue led to a series of experiments that used the technique to perform "tissue culture" experiments in the mouse.

> "The in vivo life span of normal and preneoplastic mammary gland was studied by serial transplantation in gland-free mammary fat pads of young isogenic female mice. The growth of transplants at each generation was used as an index of aging. The growth rate of normal gland declined with time, and the oldest transplant line was lost after seven generations and two years of serial passage. In contrast, the growth of preneoplastic gland was not time-dependent, and two transplant lines were growing vigorously after more than 30 generations and eight years in passage. It is concluded that normal mammary gland has limited ability to proliferate in vivo even under favorable conditions, but that preneoplastic gland, like mammary tumors, has an apparently unlimited life span when similarly propagated" (C. W. Daniel, K. B. DeOme, J. T. Young, P. B. Blair, and L. J. Faulkin, Jr.)

By the time this paper appeared in 1968, the technique was well established and being used in a variety of experiments around the world. These experiments continue to provide the operational definition of preneoplasia and immortality. The theme of immortality and senescence is discussed in the next epitome.

Leslie J. Faulkin
University of California, Davis

B) Senescence of Mouse Mammary Epithelial Cells.

It is now widely accepted that most normal vertebrate tissues display a limited life span when continuously propagated either in vivo or in vitro, whereas malignant tissues have generally been found to be immortalized. In the early days of cell culture, however, the opposite conclusion, that normal cells were capable of limitless propagation, was put forward on the basis of serial passage studies. It now appears that these conclusions were in error, due primarily to the primitive techniques used for in-vitro cell propagation in which fresh cells, contained in embryo extract, were unknowingly added to the cultures. Using more rigorous techniques, Hayflick and Moorhead showed that embryonic human fibroblasts could be propagated for only about 50 population doublings, after which the cells displayed a distinctive senescence phenotype and the culture was eventually lost. Transformation into a cancerous phenotype permitted the cells to be serially passaged without apparent limit. This conclusion had a profound impact on biological thinking.

All of these studies suffered from difficulties in interpretation resulting from the methods employed, in which the conditions of monolayer culture, which used by necessity an artificial chemical and physical environment. A completely satisfactory method for controlling for the effects of these drastic artificial environmental changes was not available, nor is it to this day. Thus it can always be argued that the phenomenon of cell senescence in culture is to some extent an artifact of the methods employed.

As a result of this lively debate, a number of methods were used to serially transplant cells and tissues in inbred mice. The data from these experiments appeared to confirm the notion that normal cells inevitably displayed a limited replicative life span; yet again, technical difficulties imposed by the non-physiological design of the experiments made interpretation difficult and the results inconclusive.

With the development of the mouse mammary gland transplant system developed at CRGL by Faulkin and DeOme, the way was open to test the replicative life span of normal mammary tissues under conditions that were completely physiological. A number of such experiments were carried out with many experimental variations, and showed conclusively that phenotypically normal mammary epithelial tissue could be serially passaged in gland-free fat pads for only five to eight transplant generations before replicative potential was irreversibly lost. This result differed from cell culture findings not only with regard to the cell type (epithelium versus fibroblasts) and very different technology used, but also that the mammary tissue lost only its ability to grow and failed to show other senescent traits. Thus, this phenomenon is more correctly termed "replicative senescence."

An important advantage of the fat pad transplantation method was its ability to show that senescent changes could not be attributed to epithelium-stromal tissue interactions. Using genetic markers, subsequent studies demonstrated that the stromal component of the mammary outgrowth was always derived from the young host and not from stromal cells carried over during transplantation. Thus, the senescent gland was an "age-chimera," with progressively older epithelium and perpetually young stroma, leading to the conclusion that mammary senescence was intrinsic to the epithelium.

The flexibility inherent in the mammary transplant system permitted the design of numerous experiments that answered questions of both practical and theoretical importance. One such question was whether the observed replicative senescence was due to the passage of time or rather to the accumulated number of cell divisions. By transplanting at intervals of 12 months rather than the usual 2-3 months, the gland was held in static phase for most of each transplant generation, with only a small amount of cell turnover and in the absence of highly mitotic end buds. Under these conditions, the tissue could be maintained in excellent condition for longer than six years, or three times the life span of the average mouse. Appropriate controls, in which sublines were broken off at various times and transplanted at the short interval, where growth was continuous, showed the expected rate of loss of growth potential. In this

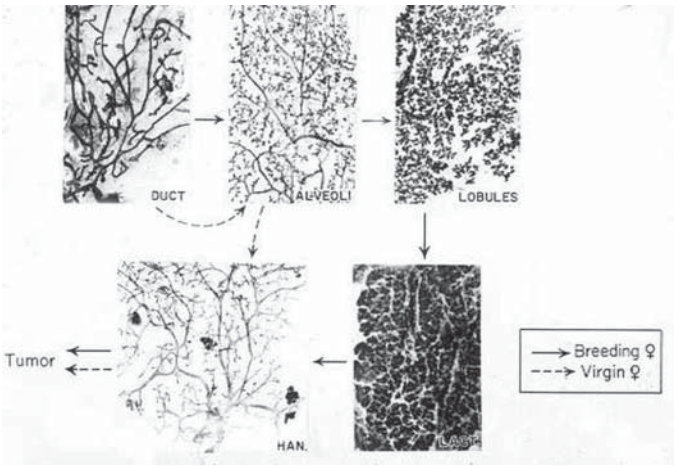

Figure 4. Graph showing the pregnancy cycle of the mouse mammary gland with the development of the HAN and tumors. This chart was first developed by the CRGL faculty and used by almost every student to explain the HAN.

way it was possible to demonstrate that the senescent phenotype was not a response to the passage of chronological time, but rather to the number of cell divisions. This number was estimated to be roughly 500, considerably more than that projected for cultured fibroblasts.

It had been known for many years that mammary tumors, as well as cancers of other types, could be serially propagated without limit in histocompatible or immunocompromised mice. Similar studies had not been carried out with the HAN, shown by DeOme and others in elegant studies to be premalignant to varying degrees. Because the HAN could be serially passaged in cleared fat pads, this led to the intriguing opportunity to determine whether immortalization was linked not only to malignancy, but to the premalignant state as well. These simple but time-consuming experiments showed without question that the HAN was immortalized, like the cancer which it had the potential to become, but remained more restrictive with respect to site of growth, providing the operational definition of preneoplasia. In recent years Medina has shown that this coupling is not without exception, but the exceptions are few and may be of the type that proves the rule. It is interesting that many more recent studies with cultured immortalized cell lines have demonstrated this same association; cancer is at least a two-step process, and immortalization is associated with an earlier rather than a later step.

Several related studies have shown other interesting features of mammary senescence. For example, serially passaged mammary ducts which have lost their ability to divide can be made to do so when exposed to the hormones of pregnancy (Fig. 4). A tiny, aged outgrowth bursts into bloom with alveoli and lobules that are indistinguishable from the host. At the time, this result was difficult to explain, but it now appears the development of a stem cell model for mammary development (see Smith epitome) may present a reasonable interpretation, in which serial passage in the non-pregnant animal irreplaceably depletes the store of ductile progenitor cells while leaving unaffected the alveolar progenitors. The stem cell is discussed by Smith in the next epitome. In any case, these experiments carried out in the 1960s and 1970s have now been repeated in laboratories throughout the world and have laid a solid foundation for further studies on cell senescence and its relation to both organismal aging and cancer.

Charles W. Daniel
University of California, Santa Cruz

C) Mammary Epithelial Stem Cells.

The gland-cleared fat pad transplantation technique, introduced in the previous epitomes, provided the operational definitions of preneoplasia and aging in mammary epithelium. As suggested by Daniel, these techniques opened the way to mammary stem cell research and the demonstration of multipotent tissue-specific stem cells. The elusive stem cell has now been demonstrated to exist in experiments showing that the progeny from a single cell may comprise the epithelial population of a fully developed lactating mammary outgrowth in mice. Serial transplantation of epithelial fragments from this clonally derived gland demonstrates that the subsequently generated outgrowths are also composed of progeny from the original antecedent. All epithelial cell types were found to be present within these clonal normal populations, including luminal, myoepithelial, ductal, and lobule-committed epithelial progenitors and fully competent mammary epithelial stem cells . These observations demonstrate the presence of multipotent tissue-specific epithelial stem cells among the parenchyma of the murine mammary gland. Genetic analysis of contiguous portions of individual human mammary ducts within the same breast also indicates their clonal derivation. So, what are the characteristics of these mammary stem cells?

The prevailing view is that stem cells are cells with the capacity for prolonged, if not unlimited, self-renewal that can produce at least one type of fully differentiated descendant. Often, an intermediate population of committed progenitors that have a restricted differentiation potential and a limited capacity for self-renewal may be identified between the stem cell and its differentiated progeny. Thus, the mammary gland may have as many as three types of stem cells: multipotential stem cells, oligopotent progenitor stem cells, and lineage-committed stem cells.

Tissue-specific stem cells have the capacity both to self-renew and to generate all the differentiated progeny represented in the tissue. While this definition applies to all stem cells, the potential of a tissue-specific stem cell differs depending on the tissue and the kind of population dynamics required to maintain tissue function. In many organ systems, the immediate progeny of a stem cell have also been shown to be division-competent. Therefore, a hierarchy of division-competent cells exists in all these tissues: primary tissue-specific stem cells, which can produce all the cell types constituting the tissue including new stem cells; progenitor cells, which are oligopotent, (i.e., produce only a subset of the cell types of an organ); and lineage-committed progeny, which can reproduce only equivalent cells in a specific lineage subset.

Multipotent cells have been demonstrated in mouse, rat and human mammary glands. Mammary epithelial stem cells possess special properties that allow them to persist in the tissue and to propagate new cells without differentiating. One strategy used to increase their reproductive lifetime is to generate cell diversity through the creation of surrogate oligopotent progenitors. Limiting dilution transplantation experiments provided evidence for the presence of three distinct multipotent epithelial cell types in the mouse mammary gland, one capable of produc-

ing all of the epithelial cell types present in the fully functional lactating gland (the multipotent stem cell) and two oligopotent downstream progenitors. The oligopotent progenitors have limited ability to produce either secretory lobulogenesis or branching ductal morphogenesis in pregnant hosts. All three mammary epithelial cell types arise from a single antecedent.

Several recent studies have demonstrated that the multipotent cells in mammary epithelium reside among the luminal cell population in human and mouse. However, no specific molecular signature for mammary epithelial stem cells has been found. Nevertheless, stem cells are expected to be distinct from their differentiated progeny—that by virtue of their ultrastructural morphology they will exhibit a rudimentary, undeveloped cytoplasm sparsely populated by organelle development (mitochondria, endoplasmic reticulum). Epithelial cells with these ultrastructural attributes are found in mouse, rat, ovine and human mammary tissue. These are the only cells that entered mitosis when mouse mammary explants were cultured. These rudimental mammary epithelial cells are found to represent a small percentage of the mammary population, but are present at all stages of normal mammary development and function and within hyperplastic and malignant mammary tissue.

Transplantation studies in mouse and rat indicate that stem cells capable of regenerating an entire functional gland are distributed throughout the epithelium and are present with undiminished vigor in aged mice regardless of reproductive history. Growth senescence of these cells may be reached but only after serial transplantation through multiple generations. We can therefore predict that growth senescent populations will have an absence of stem cells. This has been found to be correct; cells with the requisite ultrastructural morphology were not found within senescent mammary epithelial outgrowths.

The oligopotent stem cells can be detected by several types of experiments. The main line of evidence again comes from transplantation experiments. Individual implants taken randomly from a single outgrowth may show different patterns of growth senescence when propagated in pregnant hosts. For example, one implant may produce a complete branching mammary ductal system but fail to develop any secretory lobules in the pregnant host. Another may show the inability to develop branching ducts but produces fully functional secretory lobules. This illustrates that multipotent stem cells independently lose their capacity to originate the progeny necessary for lobular or ductal morphogenesis during growth senescence. Consequently, the property of producing progeny committed to either ductal or lobular morphogenesis is not only intrinsic to the mammary stem cell, but also subject to independent regulatory control.

Our observations in the mouse mammary gland suggest that multipotent progenitors can strictly limit the behavior of their progeny within a given environment and instruct, quite specifically, the extent to which these daughters can react to the local surroundings. This capability is demonstrated in experiments showing that progenitors from the same clonal population can produce, within the same host, vastly different mammary ductal structures. Subsequent serial transplantation indicates that the progeny found in these disparate mammary ductal populations retain the capacity to reproduce their specific ductal pattern. Therefore, local mammary stem cells have the capacity to control the specific patterns of the branching ducts they produce and to convey this pattern to their stem cell progeny.

The mammary stem cells (clonogens) produce the cellular and tissue diversity of the gland. As discussed above, much of this configuration may be cell autonomous; i.e., programmed by its progenitor (the stem cell). This concept becomes more fundamental when applied to the appearance of mammary hyperplasia and other parenchymal irregularities. The local tissue derangement is often demonstrably monoclonal in humans and rodents. We hypothesize that these cellular lesions exhibit cell autonomous characteristics conferred upon them by the transformed local stem cell. In support of this speculation, local regions of serially transplanted epithelial clones occasionally manifested focal regions of hyperplastic lobular development. These lesions proved to be clonal in origin and repeatedly produced hyperplastic lobular mammary outgrowths upon transplantation. One normal fragment, a fourth generation transplant, generated a very aggressive mammary neoplasm in situ. This growth was also composed of cells derived from the original clonogen and spawned several metastases to the lung of the tumor host. The lung lesions, as well, proved to be composed of cells descended from the primary founder. It therefore appears that premalignant and malignant clones may also constitute a lineage potential of aging mammary epithelial stem cells.

Gilbert H. Smith,
National Cancer Institute

D) Preneoplasia.

a. The Hyperplastic Outgrowth (HPO) in Mammary Tumor Biology.

The development of the gland-free (cleared) mammary fat pad transplantation system by DeOme and Faulkin led to many experiments that defined the current knowledge of the biology of the mammary gland. Regulatory factors confined growth to the limits of the fat pad and the distance between ducts and end buds. The clearing and transplantation technique demonstrated that normal mammary gland elements could reproduce the entire mammary tree, which in turn was able to respond to the hormones of pregnancy and lactation. Growth regulatory limits established that normal mammary epithelial cells contained multipotential properties, which led to the hypothesis of mammary stem cells (See Smith epitome). The serial transplantation of normal ductal elements demonstrated senescence (See Daniel epitome). It was demonstrated that normal mammary epithelial cells required the fat pads and not the brown fat lobes for growth. Normal mammary tissue could be maintained but would not grow in subcutaneous sites.

The transplantation of hyperplastic alveolar nodules (HAN) into cleared fat pads gave rise to immortalized hyperplastic outgrowths (HPO) (Fig. 5). While growth of the HPO was restricted to the fat pad and was not able to grow in subcutaneous sites, tumors frequently arose in the HPO. These tumors were immortalized growths that were able to overgrow normal mammary tissue, grow subcutaneously, and metastasize, fulfilling the criteria of autonomy. The development of tumors defines the HAN and HPO as preneoplastic tissues with a high risk of malignant transformation.

Medina and others have used the cleared fat pad transplantation technique to develop many HPO lines. Both high and low tumor incidence HPO lines with well defined morphological and biological characteristics were established; some have been maintained by serial transplantation for decades. The clearing

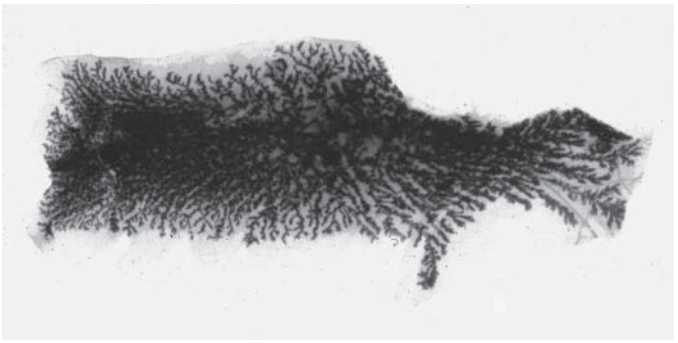

Figure 5. A whole mount showing the appearance of a hyperplastic outgrowth that was developed by clearing the mammary fat pad and transplanting a HAN into the gland free fat pad.

and transplantation technique provided the means to generate large amounts of well-characterized HPO tissues for biochemical, molecular and immunological studies. Studies using HPOs became the foundation for experimental studies of neoplastic progression in breast cancer. Mammary neoplastic progression was defined as at least a two-step process: 1) transformation of normal tissue to HAN (noduligenesis); and 2) transformation of hyperplastic tissue to malignant tissue (tumorigenesis). Since many of these tumors metastasize, metastasis is now recognized as a further step. These studies laid the foundation for the studies of premalignant lesions in the human breast (See Wellings epitome).

The HAN can be induced by a wide variety of agents (MMTV, hormones, chemical carcinogens, non-specific genetic damage). Expansion of the premalignant HAN by transplantation to yield the HPO determined that they were immortal and this permitted large scale molecular and nucleic acid analyses. The universal properties of immortalization, alveolar hyperplasia, and tumorigenicity are independent characteristics. Further, the main cellular pathway deregulated in HPOs is associated with the proliferation process, primarily the process involved in the G1 cell cycle. Alterations in other cellular pathways, such as S phase cell cycle progression, apoptosis, growth factor-mediated signal transduction, RNA transcriptional and translational regulators, appear with the progression to the malignant state.

The HAN/HPO transplantation system has been used to define many of the biological, cellular, and molecular characteristics of mammary epithelial cells. HANs were described in feral mice free of the endogenous mouse mammary tumor virus. Studies in the GR/2A and BALB/cfC3H strains demonstrated that HPO lines were clonal—the HPOs not only differed from normal tissue, but the tumors which arose in the HPOs clearly emerged as a clonal subset of cells that were subclones of the HPO. The role of the *h-ras* gene in tumorigenesis was investigated using transfection studies of the D1 HPO. Reconstitution studies of the mammary gland, determination of "occult" hyperplastic cells in normal mammary glands, and the development and characterization of the BALB/cNIV strain, were all done using various manipulations of the HAN/HPO transplantation system.

The lobuloalveolar HPO represents one of two general cellular pathways for mammary tumorigenesis. Ductal hyperplasias are preneoplastic cell populations that exhibit several properties distinct from those of the lobuloalveolar HPO. Immortal ductal outgrowths are aneuploid, hormone-dependent, and at a high risk to develop tumors. A comparison of the two pathways leads to the

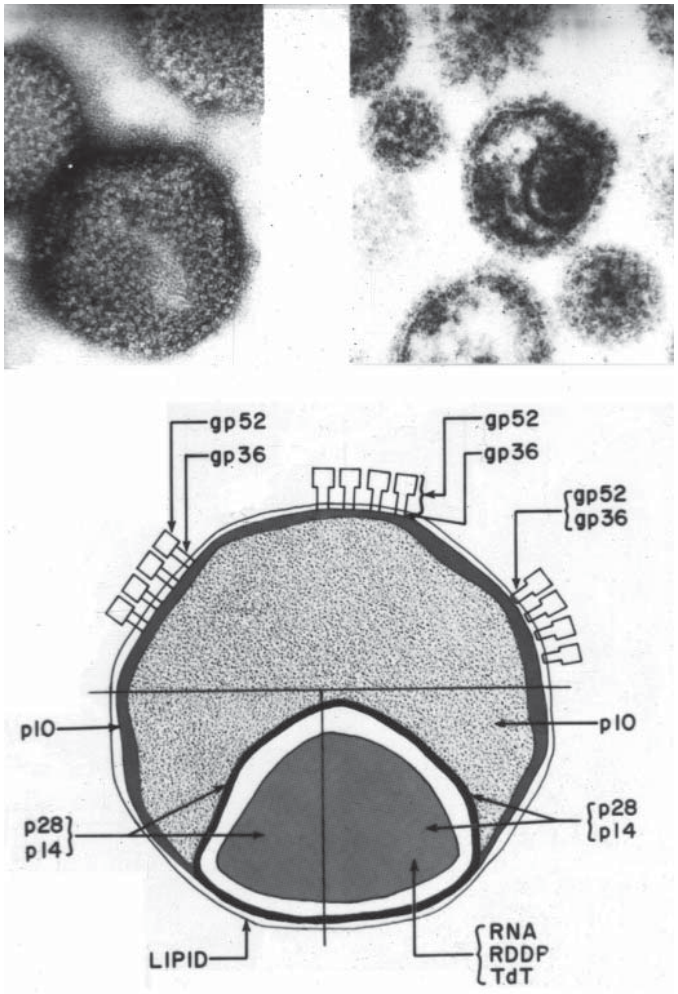

Figure 6. The top panel shows the ultrastructure of the mouse mammary tumor virus using negative staining techniques (left) and thin sections (right). The lower panel is a cartoon of MMTV that shows the location of the major proteins.

hypothesis that each is derived from a different type of progenitor cell.

Lawrence J. T. Young,
University of California, Davis

Daniel Medina
Baylor College of Medicine

b. The Mouse Mammary Tumor Virus and Mammary Tumorigenesis.

The mouse mammary tumor virus (MMTV) was first detected as an oncogenic agent, called the "milk agent," by The Jackson Laboratory staff. Further studies proved that the agent was a filterable particulate that induces mammary tumors in mice; it became known as the Bittner agent. Subsequently, MMTV was found to be an RNA tumor virus or a retrovirus with DNA-producing reverse transcriptase and with the ability to integrate into the host genome as a DNA genetic element (Fig. 6).

In the early 1960s the CRGL group, led by Nandi, found that MMTV was the agent causing the preneoplastic HAN and developed the "nodulogenesis" assay for infectious particles. Mo-

lecular proof came from the discovery of clonal integration of MMTV in the HAN and HPO.

The viral structure had been thoroughly characterized by the 1980s, and the advent of advanced molecular analysis techniques allowed the genomes of many different strains of MMTV to be characterized. While several types of laboratory mouse strains have characteristic patterns of endogenous MMTV genes, some feral mice were found to carry neither endogenous nor exogenous MMTV, clearly demonstrating that the endogenous viral DNA is not required for growth and development of the mouse. The genome of the endogenous MMTV can be distinguished from the milk-borne exogenous virus. Further, the insertion of the exogenous MMTV sequences proved useful in establishing that both hyperplasias and tumors are the result of virus-related clonal selection. Thus, both could be classified as neoplasms. The insertion of the virus also led to the identification of oncogenes, primarily in the *Wnt* pathway, that were mutationally activated by insertion of the virus.

Although the viral genome primarily codes for structural genes, the MMTV Long Terminal Repeat (LTR) sequences provided surprises that have become the foundations of current research. One surprise was the superantigen that stimulates the destruction of specific clones of T-cells. The superantigen transcript originates in the open reading frame (ORF) of the LTR and splices in several different elements, including a portion of the envelope gene, to form an *ORF* gene product. The resulting product binds non-specifically to specific classes of T-cell receptors, leading to the rapid destruction of the cells carrying that variant and the release of cytokines.

The second surprise comes from the LTR itself. The LTR has hormone-responsive elements that control transcription of the viral genes. These elements facilitate the production of infectious virus during lactation. They are also involved in the insertion activation of oncogenes leading to mammary tumors. The hormone sensitivity of the MMTV LTR has been used to target expression of oncogenic transgenes to the mammary gland and other hormone sensitive tissues. As a result, virtually every potential mammary oncogene has been placed behind the MMTV LTR promoter. Although the MMTV LTR is a somewhat leaky promoter, resulting in tumors in other organs, it does target the mammary gland and is the foundation for mammary tumor biology in genetically engineered mice. The studies discussed in other parts of this symposium were based on transgenes promoted by the MMTV LTR.

Since an infectious human counterpart to MMTV has never been convincingly demonstrated, interest in MMTV as a virus has waned. However, the MMTV genome has continued to be useful to the endocrinologist, immunologist, molecular biologist, cell biologist, and oncologist. Different parts of the virus are still central to mammary tumor biology. They have been instrumental in characterization of genes found in human cancer.

Robert D. Cardiff
University of California, Davis

c. Mammary Tumor Viruses in Wild Mice and Humans.

In the 1970s the National Cancer Institute's Virus Cancer Program sponsored a concerted effort to determine the role, if any, of retroviruses in human cancer. The scientific rationale for this program was largely based on the proven etiologic role of the mouse mammary tumor virus (MMTV) and murine leukemia virus (MuLV) in laboratory mice (*Mus musculus domesticus*). Little was known, however, about the biology of these retroviruses in feral, outbred wild mice, the progenitor of the lab mouse. I was given the opportunity to pursue this research, and it proved to be a fascinating scientific adventure. I will summarize here the major conclusions from our study of MMTV in wild mice.

Using electron microscopy to detect MMTV extracellular Type B particles and intracellular Type A particles in prelactating breast and milk, we found evidence of MMTV infection in about 50% of pregnant wild mice. Sucrose gradient fractions of wild mouse milk containing Type B particles were also positive for 70S RNA and reverse transcriptase activity. MMTV virions were also seen in non-mammary tissues such as salivary gland, seminal vesicle, and certain non-mammary tumors: hepatoma, pulmonary adenoma, and lymphoma. MMTV virions were detected in about 50% of the infrequent spontaneous breast tumors in wild mice. Immunoperoxidase staining of MMTV envelope (gp52) antigen confirmed the presence of the virus in prelactating mammary glands and spontaneous breast tumors in wild mice. In addition, this immunohistochemical technique revealed a marked variability in the extent of positively staining cells, ranging from about 1% to greater than 50%, in normal and malignant mammary tissues. These surveys confirmed that MMTV did indeed exist in feral mice, with an extreme variability in degree of quantitative expression. However, MMTV could not be detected in at least half of the normal wild mice or in about half of their breast tumors. This approximately 50% prevalence of detectable MMTV was equally distributed among the wild mice from all trapping areas sampled in Southern California, regardless of the extent of MuLV expression in the same mice.

In experimental transmission experiments, MMTV purified from wild mouse milk proved only weakly pathogenic in susceptible laboratory mice. In the natural host, the MMTV was also weakly pathogenic, because spontaneous breast tumors developed in just 1%-3% of aging wild mice, regardless of the level of expression of MMTV or MuLV in those individuals. Control of MMTV expression in wild mice was not due to a strong immune response, because MMTV antibodies were rarely detectable. Genetic resistance to MMTV pathogenesis was apparent in wild mice because they were quite refractory to the experimental induction of mammary cancer after introduction of the pathogenic MMTV from C3H laboratory mice.

In the real world of wild mice, MMTV thus appeared to be a relatively weak carcinogen whose presence was neither essential nor sufficient for the induction of mammary gland tumors. But the possibility remained that low-level undetectable expression of MMTV genes, either exogenous or endogenous (inherited), might be critical to mammary gland development or tumorigenesis. The fortuitous discovery that some wild mice, about 5% of those sampled, lacked any exogenous or endogenous MMTV proviral DNA helped to establish that these genes were not essential for mammary gland development or tumorigenesis. Furthermore, MMTV DNA-negative mice were a valuable resource for selective inbreeding of single MMTV genomes from laboratory mice to better understand the phenotypes of specific MMTV alleles.

In addition to these discoveries of MMTV biology in wild mice, we used the same techniques to look without success for Type B particles in human milk, including high-risk breast can-

cer families. These results helped to invalidate claims, prevalent at that time, that MMTV-like agents were associated with human breast cancer.

Murray B. Gardner
University of California, Davis

d) Comparative Pathology of the Mammary Gland.

The identification of the mouse hyperplastic alveolar nodule as a focal atypical growth associated with the development of malignancy led us to seek focal atypical growths with premalignant potential in the human breast. Using our experience at CRGL, we adapted the mouse whole-mount technique to the human breast, so that entire breast could be studied at the "subgross" level. At the time, "fibrocystic disease" (now referred to as fibrocystic change) was a known risk factor in human breast cancer, but the early lesions had not been clearly delineated. Since we could not perform transplant experiments in humans, we relied upon statistical and epidemiological evidence to support the hypothesis. Our studies prove that epithelial hyperplasia arising in the terminal ducts and lobules (terminal ductal-lobular units, or TDLU) is the major precancerous lesion of the human breast. Cancers do not commonly arise in the larger duct systems as previously thought.

Each TDLU consists of a lobule with its entering terminal duct. The lobule is a globular group of terminal, blindly ending secretory ductules (i.e., acini or alveoli) arising from a central axial space, sometimes referred to as the *intralobular terminal duct*. The lobule with its ductules is embedded in a loose and edematous intralobular stroma. The lobule is often likened to a bunch of grapes suspended on a single stem. In this analogy, the ductules (acini) are the grapes and the stem is the entering terminal duct. The terminal duct connects the lobule to an arborizing system of progressively larger ducts, constituting a lobe. The final ductal termination is the galactophore, which serves one or more of the radiating triangular lobes, of which there are six to 12 per human breast.

Based on our observations of serial whole mount sections of the breast, the smallest and therefore the earliest lesions are found in the TDLU. Thus all of the common infiltrating scirrhous and lobular carcinomas, fibroadenomas, and the various lesions of fibrocystic change (mammary dysplasia) arise in the TDLU. Fibrocystic disease includes simple epithelial cysts, sclerosing adenosis, florid adenosis, aprocrine metaplastic cysts, radial scars and, most importantly, precancerous epithelial hyperplasia (epitheliosis).

Precancerous epithelial hyperplasia of the TDLU progresses by a linear, sequential series of changes that are characterized by increasing nuclear atypicality and anaplasia, ending in carcinoma-in-situ of the classical ductal or lobular cytological types. While these lesions clearly involve the TDLU, the exact cellular origin of the atypia is uncertain. Possible sites include the extralobular terminal duct, the intralobular terminal duct, the ductules (acini), or any one of the three. These concepts are derived from the study of more than 200 sliced whole human breasts using a subgross sampling technique with histological confirmation.

Briefly, the whole breasts were thoroughly fixed in 10% formalin, serially sliced at a thickness of 2-3 mm, defatted with acetone, stained with hematoxylin, dehydrated, and cleared and stored in methyl salicylate. Hematoxylin stains only nuclei when applied at low pH. Therefore, the bluest portions of the sections have the greatest concentrations of cells. Individual lesions were identified, photographed at the subgross level (2-10 X with a dissecting microscope) and cut out of the specimen for histological diagnosis and correlation.

The data was tabulated as differential counts of lesions on a case-by-case-basis with clinical correlation. The total number of lesions per breast varies from 2 to over 500. No breasts are without lesions. Following are examples of differential counts from three breasts.

Case #1. 29-year-old nulliparous female, died of traumatic injuries. No known previous diseases.

Fibroadenoma (FA)	4
Atypical lobules, type A (ALA), low grade	5
Hyperplastic terminal duct (HTD), low grade	4
Sclerosing adenosis (SA)	2
Aprocrine metaplastic cysts (APO cyst)	8
Total	23

Conclusion: Presence of ALA, HTD and APO cysts suggests high risk.

Case #2. 79-year-old multiparous female with no history of breast disease. Died of congestive heart failure. Atrophic breasts with very few lobules.

Epithelial cysts (EP cyst)	2
ALA, low grade	1
Total	3

Conclusion: Low risk.

Case #3. 46-year-old female with a history of five previous breast biopsies for "lumpy breasts." Diagnosis in each instance: fibrocystic disease with focal aytpia.

EP cyst	25
SA	10
APO cyst	38
ALA, grade 1	10
ALA, GRADES 2 AND 3	31
ALA, grade 4	10
Ductal carcinoma-in-situ (DCIS), arising in ALA	3
Infiltrating duct carcinoma, 0.8 cm diameter	1
Total	128

Conclusion: Very high risk breast.

Statistical analysis of the differential counts indicates that the presence of numerous atypical lobules correlates with coincident carcinoma in the same breast. There is a strong correlation between the presence of ALA, DCIS and infiltrating carcinoma. There is evidence that fibrocystic lesions in general identify breasts with phenotypic instability of precancerous potential. These studies have been confirmed and expanded by more detailed epidemiological studies of many investigators, including

the studies of Page and his group.

Finally there are similarities between the hyperplastic alveolar nodules of the mouse model and the atypical lobules of the human:

1. Both are characteristic of high-risk strains, or families.
2. Both increase in number with age during reproductive life.
3. Both persist after the loss of endocrine support.
4. Both show morphological intermediates suggesting precancerous potential.

However, the classical HAN, defined by DeOme's group, is primarily MMTV-induced. Thus far no convincing evidence of a comparable human virus has been found. Further, the oncogenes in MMTV-related tumorigenesis are rarely associated with human breast cancer, and the resulting tumors do not resemble the common human breast cancers. With the development of genetically engineered mice (GEM), genes such as *ras*, *myc*, *neu* and others have been inserted into the mouse genome. The mammary tumors arising in these GEM have remarkable morphological similarity to their human counterparts, suggesting that the genotype of the tumor has a profound effect on the phenotype. Human genes produce "human-like" preneoplasias and tumors.

Sefton R. Wellings
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2) Hormone Regulation

A. Ovarian Hormones in Mammary Growth and Development.

Howard Bern and S. Nandi, working at CRGL in the early 1960s, used a combination of classical experiments employing endocrine ablation and supplementation with exogenous hormones to help define the hormonal regulation of the mouse mammary gland. Briefly, they found that estrogen was needed for ductal elongation. Estrogen was required for duct growth, and the combination of estrogen and progesterone was required for branching morphogenesis and mature development. Full lobuloalveolar development and lactation requires insulin, corticosteroid, prolactin, and estrogen. Much more rigorous, controlled experiments in organ culture by their students Elias, Rivera, and Wellings characterized the basic hormones of lactation and focused attention on the cellular biology of the mammary gland. The early experimental era also demonstrated that even though MMTV production is under the influence of hormones, the MMTV-induced tumors are hormone independent.

With the discovery of estrogen receptors alpha (ERα) in the early 1970s, it became apparent that these receptors may play a key role in mammary development and carcinogenesis. Shyamala and Nandi identified ERα in normal mouse mammary glands and mammary tumors. At present, steroid receptors of all types are known to play key roles in mammary growth and development and in breast cancer. An extensive body of literature documents the participation of ER and progesterone receptor (PR) in mammary gene expression. However, the regulation of the mammary gland by the two key ovarian hormones, estrogen and progesterone, and the precise roles of their cognate receptors has turned out to be quite complex, particularly with the discovery of ERβ and the existence of two isoforms of PR.

To a large extent this difficulty is because of the over-reliance on in vitro systems and biochemical and molecular approaches. However, as discussed in previous epitomes, mammary glands are composed of several cell types with different developmental potentials. Further, their mutual interactions dictate the behavior of mammary epithelial cells. Therefore, resolution of the respective roles of ER and PR in normal mammary growth, differentiation and carcinogenesis, required analyses of the expression of these receptors at the level of individual cell types. Since the relative proportion of stromal versus epithelial cells can vary as a function of mammary development and carcinogenesis and also in response to hormones and/or growth factors, biochemical assays need to be complemented by immunolocalization assays. Fortunately, with the advent of genetically engineered mice and increasing use of the immunolocalization techniques, considerable progress has been made in our understanding of the relative roles of these various types of receptors in mammary function. This new understanding is summarized here.

The two forms of ER are coded by distinct genes. Both forms are expressed in mammary tissues. Studies on ERα null mutant (ERKO) mice have clearly established that this receptor is essential for ductal morphogenesis accompanying puberty. Mammary glands of ERKO mice do express low constitutive levels of PR. Upon appropriate stimulation, these glands exhibit lobuloalveolar development. Mammary glands of ERβ null mutant mice (BERKO) do not exhibit any impairment in mammary development. In PR null mutant mice (PRKO), ductal morphogenesis is not impaired while lobulo-alveolar morphogenesis, accompanying pregnancy, is severely impaired. Taken together, these observations establish that the ERβ is not needed for normal mammary development. ERα, but not PR, is essential for ductal morphogenesis while PR is essential for alveolar morphogenesis.

PR exist in two molecular forms, A and B forms, and unlike ER, the two isoforms arise from a single gene. The relative levels of expression of the two isoforms are critical for appropriate tissue responsiveness to progesterone such that an imbalance in the native ratio of the two isoforms can lead to alterations in PR signaling. Indeed, in transgenic mice carrying additional A form, (PR-A transgenics), mammary development is abnormal, characterized by presence of ductal hyperplasias, extensive side branching, loss of basement membrane components, and loss of cell-cell adhesion. In transgenic mice carrying additional B form (PR-B transgenics), the mammary glands display acinar hyperplasias, and stunted ductal elongation and the ductal cells undergo premature senescence. Never-the-less, these mammary glands are able to undergo alveolar proliferation upon pregnancy. However, they consist of compact acini with many abnormal mitotic figures and are frequently surrounded by dense connective tissue. These structures are also somewhat disordered with limited secondary and tertiary ductal branching. These observations emphasize that, during mammary gland morphogenesis, PR-A and PR-B have different activities and that altered expression of PR isoforms can lead to abnormal mammary development (Fig. 7). The fact that the diminished growth potential of the mammary glands of PR-B transgenics is restricted to ductal elongation and not alveolar proliferation also indicates that PR-B is restricted to only a subset of epithelial cells, which in turn, is tied to the developmental fates of mammary epithelial cells.

Immunolocalization studies have clearly documented that in rodent and human tissues, both ER and PR are heterogeneously distributed in the mammary epithelium. The ERα positive cells

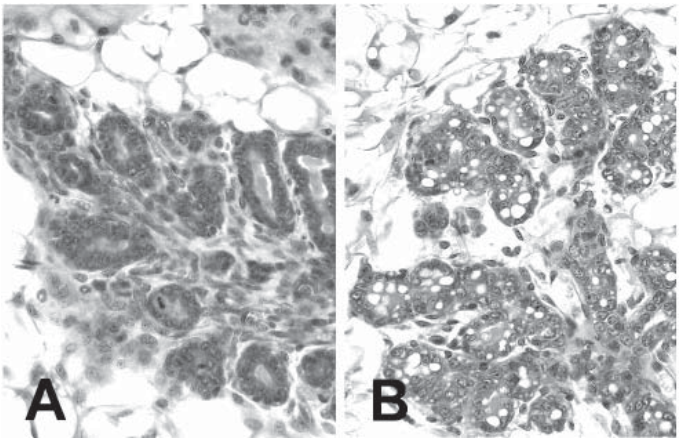

Figure 7. Photomicrographs showing mammary glands from pregnant PR-B (A) and normal FVB (B) mice. Note that the PR-B acini are surrounded by fibrous connective tissue and are not as well developed as the normal acini.

appear to be distributed singly and surrounded by ERα negative cells. Studies attempting to correlate ER expression with cell proliferation have shown that there is almost mutual exclusion of ERα expression and cell proliferation in estrogen-stimulated normal human breast epithelium.

However, after menopause, the number of proliferating ERα positive epithelial cells increases dramatically (from 0.01% to 11.0%). This suggests that the older breast either fails to downregulate ERα as cells enter the cycle or fails to suppress division of ERα positive cells. Studies on mouse mammary glands also indicate that aging increases the estrogenic sensitivity of mammary glands. Also, in nulliparous rat mammary glands, there is an age-dependent increase in the proportion of proliferating cells also positive for ERα. Taken together, these observations suggest that, at menopause, there may be a critical transition in the epithelial cells with regard to their estrogenic sensitivity.

Menopause results from changes in the hypothalamic-hypophyseal axis leading to permanent changes in the endogenous hormonal milieu. As such, changes in ERα expression and estrogenic sensitivity of mammary epithelial cells in postmenopausal females emphasize, once again, that circulating levels of pituitary hormones can impact the proliferative status of mammary epithelial cells. In this context, it is significant to note that in one of the rodent experimental models, which recapitulates the pregnancy dependent protection of mammary carcinogenesis (See Guzman and coworkers epitome), the endocrine basis for the protection appears to be due to a resetting of the hypothalamic-hypophyseal axis.

G. Shyamala
Lawerence National Laboratory, Berkeley

B. Preventing Breast Cancer with Estrogen: Mimicking the Protective Effect of Pregnancy.

Epidemiological, clinical, and experimental studies have implicated hormones in the genesis of breast cancer, and exposure to estrogen is the main hormone associated with increased risk. A causal relationship between estrogen and breast cancer is based on duration of use, dosage, and the time at which exposure occurred. Estrogen-induced carcinogenesis is likely a complex process requiring many changes, including receptor-mediated and genotoxic events, involving different signal-transduction pathways. These findings suggest that treatment of breast cancers of diverse or unknown etiologies is likely to be extremely complex.

One area of interest has been the effects of pregnancy on breast cancer risk. Women who have undergone a full-term pregnancy before 20 years of age have one-half the risk of developing breast cancer compared with nulliparous women. This protective effect of early pregnancy is universal, occurring worldwide among women of all ethnic groups, and is clearly of major consideration in devising strategies for the prevention of breast cancer. Rats and mice that undergo a full-term pregnancy also have a greatly reduced susceptibility to mammary carcinogenesis compared to nulliparous animals and are used as experimental models to study the protective effect conferred by pregnancy. During pregnancy a complex of ovarian, pituitary, and placental hormones and growth factors act to cause proliferation and differentiation of the mammary gland in preparation for lactation. After parturition, under the influence of lactogenic hormones, the mammary gland becomes fully lactational. After weaning, involution of the differentiated lobuloalveolar structures occurs, characterized by apoptosis of the majority of the mammary epithelial cells due to a decrease of lactogenic hormones.

The mechanisms for the protective effect of pregnancy have not been defined. The most widely accepted explanation attributes the protective effect to the pregnancy-induced differentiation of the target structures for carcinogenesis, the terminal end buds and terminal ducts (TDLU). In essence, pregnancy may result in the permanent removal or modification of a population of cells that are highly susceptible to the development of breast cancer.

Exogenous hormonal treatments have been used to mimic the protective effect of pregnancy. Human chorionic gonadotropin and treatments with estradiol (E) plus progesterone (P) induce protection from mammary carcinogenesis. Although all of these experimental findings could be explained on the basis of death or modification of the target cells for mammary cancer or preneoplastic cells, alternate explanations are possible. Previous studies in parous rats have shown that an altered hormone environment changed the sensitivity of mammary epithelial cells to hormones and to shifts in receptor levels. Pregnancy in rats results in the long-term decrease in serum level of growth hormone that might be involved in a decrease in the promotional environment for mammary carcinogenesis.

Like pregnancy, short-term treatments with E or E+P that confer protection from mammary carcinogenesis result in a persistent decrease in the blood levels of the mammogenic hormones. We have found that E was the most effective hormone in inducing protection. Any short-term treatment with E that results in blood levels of E equivalent to those of pregnancy results in protection from mammary carcinogenesis. Treatments with E that are equivalent to the lowest levels of E during pregnancy confer protection but do not result in full lobulo-alveolar differentiation.

Parous rats are refractory to mammary carcinogenesis. However, long-term treatment of carcinogen-treated parous rats with ovarian hormones results in the induction of a high incidence of overt mammary cancers. Similar long-term treatment of rats protected from mammary cancer by short-term hormonal treatment also results in a high incidence of mammary cancer. Taken

together, these studies indicate that the target cells for mammary carcinogenesis persist and are not lost due to differentiation. Short-term continuous treatment of nulliparous rats with pregnancy levels of E or E+P results in long-term protection against mammary cancer, more that an 80% reduction in mammary cancer incidence, more than a 90% reduction in mammary cancer multiplicity, and an increase in the average age of cancer latency. The protective treatment is non-toxic and is as effective as full term-pregnancy or ovariectomy, or long-term treatment with Tamoxifen with no loss of reproductive function.

We hypothesize that protection against mammary cancer induced by pregnancy or short-term hormonal treatment is due to the effects of the pregnancy levels of E. These high levels of hormone permanently reset the hypothalamic-hypophyseal axis, resulting in a permanent reduction in growth hormone and prolactin secretion and thus reducing the promotional environment for mammary carcinogenesis. We further hypothesize that the protective effect of pregnancy is not due to a loss of target cells for carcinogenesis but is simply due to inadequate mammogenic hormones for the promotion/progression of initiated cells to frank carcinomas. These studies have resulted in a new paradigm for the role of a full-term pregnancy at an early age in the protective effect against mammary cancer.

Raphael C. Guzman, Lakshmanaswamy Rajkumar, Jason Yang, Gudmundur Thordarson, and Satyabrata Nandi
University of California, Berkeley

3) Genetically Engineered Mice and Neoplastic Progresson

A) Genetically Engineered Mice.

As suggested in several of the previous epitomes, the development of genetically engineered mice (GEM) has had a dramatic effect on the study of mouse mammary biology and tumor biology. While the mouse has always been considered a convenient model for the study of mammary biology, MMTV-induced tumors do not resemble human breast cancers. The mouse mammary tumors are morphologically distinct with relatively low-grade cytology, have pushing rather than invasive borders, and metastasize almost exclusively to the lungs. Further, the genes that are activated by MMTV are rarely activated or amplified in human breast cancer. However, insertion of genes identified in human breast cancer, such as *src*, *myc* and *erbB2*, into the mouse genome has provided tumor phenotypes that resemble human breast cancer in startling morphological detail (Fig. 8). The mouse has now become the test animal for the verification of oncogenes and tumor suppressor genes.

In spite of the development of numerous mouse models of human breast cancer, little attention has been devoted to the early events in tumorigenesis in these genetically engineered tumors. In general, investigators have documented the presence of tumors and the presence of potential precursor lesions, but few studies have used transplantation techniques and the operational definitions of preneoplasia developed at the CRGL. The following epitomes discuss current work, much of it in press, that applies the key concepts of mammary tumor biology to GEM. Medina discusses the development of preneoplasia using the tumor suppressor gene *p53*. MacLeod demonstrates that GEM with the Polyoma Virus middle T transgene (PyV-mT) have preneoplastic lesions. The PyV-mT system has also been

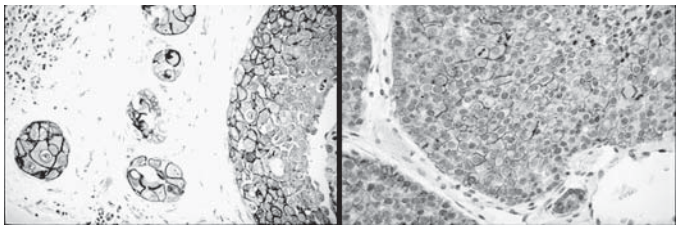

Figure 8. Photomicrographs comparing the immunohistochemical distributions of Erb-B2 in human (A) and mouse (B) mammary tumors.

exploited to study the transition between tumorigenesis and malignancy discussed in the last section.

Robert D. Cardiff
University of California, Davis

B) Premalignancy.

a) The Preneoplastic Phenotype in the P53 Null Mammary Gland.

Mammary tumorigenesis in rodents and humans progresses through intermediate stages termed preneoplastic or premalignant lesions. The preneoplastic stage has been extensively studied in a variety of murine models. The most frequently studied lesion is the hyperplastic alveolar nodule or HAN. HAN can be induced by MMTV, chemical carcinogens, or reproductive hormones, or arise spontaneously. Regardless of the mode of origin, HAN have similar biological properties. HANs and their transplanted outgrowths are hormone-independent, immortal, high-risk lesions that are often genetically stable and give rise to invasive tumors that are frequently metastatic. Despite the valuable information about the cellular and molecular basis for preneoplasia that has been learned from the study of these lesions, they appear to represent a cellular pathway that is infrequently observed in human breast cancer. In order to gain insight into the preneoplastic stage in human breast cancer, we have examined animal models where the genetic lesion is one that is frequently observed in human breast cancer. One such model is the *p53* null mammary gland. The *p53* null mouse has an early lethal phenotype, as most of the mice die within four months due to lymphosarcomas. To circumvent this problem, we utilized transplantation of the *p53* null mammary gland into the mammary fats pads of *p53* wild-type syngeneic mice (BALB/c). We have examined both normal and tumorigenic mammary development. Normal mammary development is essentially unimpaired in *p53* null mammary cells, with the exception of a transient delay in early involution. However, the *p53* null mammary cells are at high risk for tumorigenesis. In untreated mice, 40% of the transplants will develop tumors by 60 weeks after transplantation. The mammary tumors are metastatic and genetically unstable, with high levels of aneuploidy and chromosomal imbalance by Competitive Genomic Hybridization.

The preneoplastic lesion in the *p53* null mammary cells is a ductal hyperplasia that progresses through ductal carcinoma in situ to invasive breast cancer. In addition, the ductal hyperplasias are immortal, exhibit genetic instability, and are hormone dependent. The cells also exhibit elevated levels of cyclin D1 protein. The biological, cellular, and molecular properties of these lesions suggest that they mimic a subset of premalignant lesions that are found in human breast cancer. Thus, this model system would be an excellent system to examine secondary events that are essential

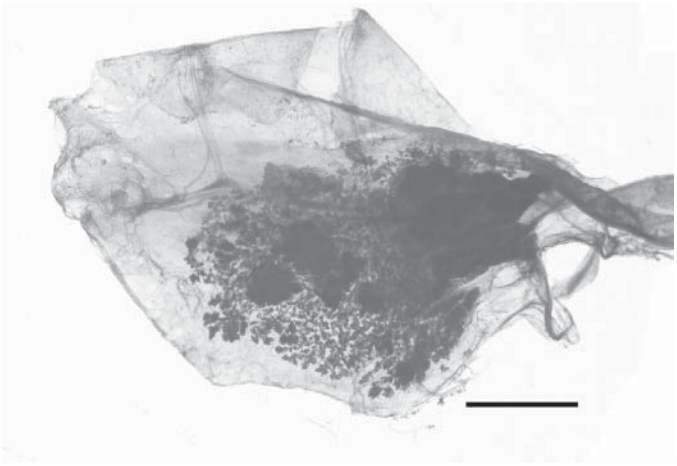

Figure 9. A whole mount preparation showing a hyperplastic outgrowth from a PyV-mT mammary gland. Note that the outgrowth is a mass of cystic and solid dysplastic masses.

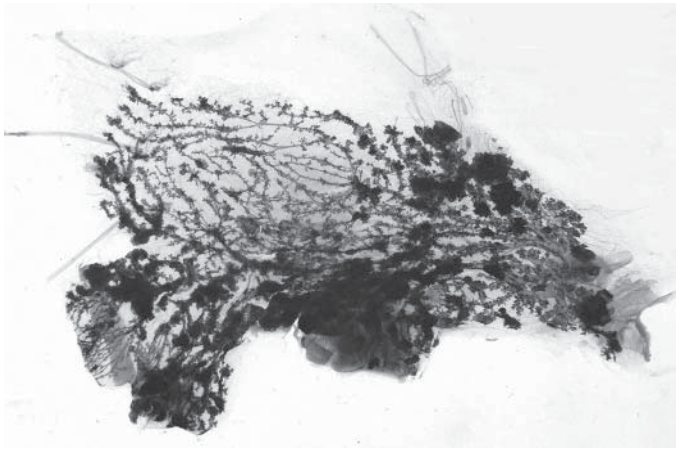

Figure 10. A whole mount preparation showing the mammary gland from a PyV-mT transgenic mouse. Note the numerous masses.

for premalignant progression.

It is important to note that these types of sophisticated studies in GEM would not be possible without the development and perfection of transplantation techniques in the 1960s and 1970s.

Daniel Medina
Baylor College of Medicine

b) A New Model for Mammary Premalignancy.

The unique experimental biology of the mouse mammary gland described in the previous epitomes and the development of transgenic models of human breast cancer have opened new opportunities to systematically investigate the biology of early proliferative mammary disease. Although the existing transgenic mouse models of mammary cancer have provided important insights into the pathways that are disrupted in tumorigenesis, these models have not been widely used to examine the crucial early stages of hyperplasia that increase the risk of breast cancer in humans. To address the need for a more comprehensive and uniform analysis of mammary lesions in transgenic models, a panel of expert pathologists was convened by the National Institutes of Health at Annapolis. The panel, led by Robert Cardiff, recommended that diagnostic nomenclature of transgenic lesions in mice be based on similar morphological, immunohistochemical, and biological criteria so that direct comparisons among mouse models and with humans would be possible. The characterization of the biological growth potential of hyperplastic lesions, urged by the panel, is founded upon the pioneering mammary gland transplant work of Ken DeOme and Larry Young, with important applications for this technology developed further by Daniel Medina and others. In addition, Robert Cardiff, Sefton Wellings and their colleagues established essential guidelines for the comparative histopathological and biological analysis of mammary lesions over several decades. These pioneers, among many others, have enabled the development of a new model system of mammary premalignancy that is specifically designed to investigate the early events in the pathology of breast cancer.

To develop a readily available and reproducible model of premalignancy, we chose the well-studied transgenic model of mammary tumorigenesis that expresses the polyoma virus middle-T antigen (PyV-mT) under MMTV control (Figs. 9 and 10). A long-standing collaboration of Robert Cardiff and Lawrence Young with my research team resulted in the detailed characterization of premalignant lesions from PyV-mT glands. We assessed the histological characteristics of these lesions and examined by immunohistochemistry several important markers of mammary differentiation and cell growth. We demonstrated via transplantation experiments that the early proliferative lesions in these mice are truly premalignant and consistent with a multi-step model of tumorigenesis. We also determined that the cells found within these lesions are heterogeneous in their potential for transformation to frank malignancy. Armed with this information, we established five hyperplastic outgrowth lines from these lesions that are now available for detailed analysis These lines are currently in the 8th transplant generation. Their growth without regression documents that they were immortalized. Their malignant potential was tested by subcutaneously transplanting HPO tissue into syngeneic recipient animals. These ectopic transplants did not progress to tumors, providing strong evidence the cells were not malignant. However, all lines gave rise to areas of atypical hyperplasia and eventually developed tumors within the recipient fad pad, indicating that the HPO lines represent premalignant tissues. The tumors that developed within these HPOs grew in ectopic sites and metastasized, confirming that they were malignant.

While all isolated HPO lines expressed PyV-mT protein, their potential for malignant progression and their subgross morphology were quite heterogeneous. Two lines had a high incidence of malignant transformation after 14 weeks and the other three had a low incidence. Additionally, pulmonary metastases were detected in the lungs of tumor-bearing mice. The difference in tumor latencies confirms the heterogeneity among the premalignant lesions that arise within the PyV-mT mammary gland.

These PyV-mT transgenic HPO lines were developed to meet a need for stable, premalignant mammary tissue with reproducible tumor latencies and metastatic properties. We anticipate that these lines might serve several useful purposes:

1. To identify the genetic changes that lead to transformation to malignancy using microarray and other new technologies;
2. To test the effect of different genetically engineered stromal environments on tumor progression;
3. As a test system to identify chemoprevention or intervention strategies;

4. As a means to identify potential biomarkers for detection and evaluation.

The development of the HPO lines, described in this epitome, depended heavily upon the pioneering work of numerous scientists over the past 50 years. Several of those key investigators participated in the Mouse Biology Symposium.

Carol L. MacLeod
University of California, San Diego

Lawrence J. T. Young
University of California, Davis.

B. Malignancy and Metastasis.

a. The MMTV-LTR:PyV-mT Transgenic Mouse System and Metastasis.

Transgenic female mice with the MMTV LTR-promoted polyoma virus middle T (*PyV-mT*) develop palpable mammary tumors within five weeks of birth and 100% metastases. As such, the PyV-mT system is the most explosive tumor and metastases system available for the study of neoplastic progression in the mammary gland. Like many other tumors in the *erbB2* pathway, *PyV-mT* tumors are morphologically different from MMTV-induced mammary tumors and are often indistinguishable from human breast cancers. PyV-mT is a 65 kd membrane-anchored cytoplasmic protein that disrupts the *c-erbB2* signal transduction pathway by interacting directly with PI3-kinase, src, shc2, and PP2a and indirectly with other members of the *c-erbB2* signal transduction pathway. It is a molecular surrogate for *c-erbB2*.

Muller and associates have developed a number of modifications of the *MMTV-LTR:PyV-mT* system. The Db mouse is one such modification that has site-directed mutagenesis at sites 315 and 322. These double base (Db) mutants prevent the interaction of the PI3-kinase with middle T. The Db animals develop mammary tumors. However, the tumors do not metastasize.

In order to study the phenomenon of metastases, serial transplant lines of PyV-mT and of Db tumors were developed. As predicted, the wild-type PyV-mT tumors (dubbed Met-1) grew rapidly and were 100% metastatic to the lungs 60 days post transplant. In contrast, the Db tumors (Db7) grew rapidly, but less than 10% of the tumor-bearing mice developed metastases. For subsequent studies, pulmonary metastases from the Met-1 lines and the Db lines were isolated and transplanted. The tumor lines from Met-1 (pMet) and from Db (pD) metastases were 100% metastatic.

Detailed morphological studies demonstrated that the Met1 lines were better differentiated than the Db lines. Angiogenesis was the only predictive variable. Additional studies using the resources from the PyV-mT system appear in other epitomes.

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b. Genetic Modification of Tumors: An Ets2 Restriction of Mammary Tumors in Mice.

Epithelial–mesenchymal interactions are fundamental opportunities for molecular instruction during development. Thus perhaps it is not surprising that epithelial–stromal interactions are important for tumor development. Recently, one focus of cancer research has been to understand the interaction of the cancer cell with its surrounding cellular environment. Epithelial-stromal interactions provide new opportunities for altering the fate of transformed epithelial cells. Our research has arrived at the carcinoma-stromal interface as a result of investigating the role of a specific transcription factor, Ets2, in development and cancer. The PyV-mT transgenic mouse tumor system has provided a convenient model for these studies.

Ets2 is one of 25 members of the *Ets* family of transcription factors found in the human genome. All *Ets* members use the same protein motif to recognize and bind DNA. *Ets1* and *Ets2* were identified as the endogenous counterparts of oncogene fusion genes created by the recombination of retroviruses with cellular genes. Both Ets1 and Ets2 also share an N-terminal protein domain, named after a Drosophila gene that mediates growth factor-initiated developmental signals. Epidermal growth factor homologs activate mitogen-activated protein kinases that in turn activate the pointed-P2 Ets gene product by phosphorylation of a specific threonine residue. Ras signaling activates mammalian Ets2 by phosphorylation of the homologous threonine. Thus, Ets2 acts downstream of Ras and MAP kinase signaling to induce the transcription of particular growth factor-regulated genes.

We have inactivated the Ets2 gene in mice by gene-targeting methods. Fibroblasts that are deficient in Ets2 fail to respond to Fibroblast Growth Factor (FGF) or Epidermal Growth Factor (EGF) by increasing Matrix Metalloproteinase 3 (MMP3) or MMP13 RNA unless exogenous Ets2 is provided. MMP3 was one of the first genes to be shown to be activated by oncogenic Ras expression. Ets2 is also essential for MMP9 expression in trophoblast cells. Thus Ets2 is limiting for the expression of several MMPs in a tissue-dependent manner.

We have tested the importance of Ets2 for mammary tumors by generating bigenic animals that carry the *MMTV-PyV-mT* transgene and a targeted *Ets2* allele. The inclusion of a single targeted Ets2 allele ($Ets2^{db1}$) resulted in a slower appearance of rapidly growing mammary tumors. After 80 days, tumors arising in $Ets2^{db1/+}$ mice were approximately 50% of the size of wild type animals. However, once the tumors arose, the rates of growth were indistinguishable in the two types of mice. In early stages of development, histopathological analysis revealed that tumors arising in $Ets2^{db1/+}$ mice were more differentiated than those found in wild-type mice. RNA analysis revealed that the expression of PyV-MT RNA was the same in tumors arising in both types of mice. In addition, mammary gland development was the same in females of both Ets2 genotypes. Thus the difference in tumor development was not due to differential expression of PyV-MT.

As the Ets2 dependence of PyV-MT tumor development could be due to Ets2-dependent processes in either the transformed epithelial cells or in the stroma, we have performed transplantation studies in $Ets2^{db1/+}$ mice. Preliminary results indicate that the DB-7 tumors serially transplanted into fat pads grew more slowly in FVB/N $Ets2^{db1/+}$ mice than in wild-type littermates. In contrast, DB-7 tumors transplanted subcutaneously grew more slowly than in the fat pad and no difference in size was evident in $Ets2^{db1/+}$ mice. These results indicate that the mammary fat pad environment has an Ets2-dependent stimulatory effect on transplanted mammary tumors.

Further evidence that an Ets2-dependent stromal effect may limit tumor development was provided by the construction of a transgenic mouse line that expressed a dominant negative inhibitor of Ets2 in the mammary epithelium. The Ets2-dominant

negative RNA was expressed in considerable excess of endogenous Ets2 in the mammary and salivary glands as expected from the tissue specificity of the MMTV promoter. However, when crossed with the MMTV-PyV-MT transgenic mouse line, no effect of the Ets2-dominant negative protein was found on tumor appearance or growth. These preliminary results are consistent with a stromal rather than epithelial site of action for Ets2 in mammary tumors.

Multiple cell types compose the stromal environment of the mammary fat pad. These include fibroblasts, endothelial cells, and inflammatory cells derived from the circulation. Many of these cell types communicate with transformed mammary epithelial cells. Ets2 and Ets1 have been implicated in the regulation of potentially key target genes of endothelial cells, fibroblasts, macrophages, and neutrophils. It remains to be determined which of these cell types may be responsible for the Ets2-dependent stromal effect on mouse mammary tumors. However, the techniques and concepts developed by DeOme and his colleagues are available to assist in the analysis.

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c. Signal Transduction and Metastatic Potential.

As suggested in the previous epitomes, neoplastic progression of tumors is a complex, multifactorial process. For example, metastasis involves various steps such as cellular migration, implantation, neovascularization, and tissue colonization. The molecular sequence of events that are necessary for metastatic conversion is poorly understood. However, it is recognized that the dysregulation of multiple genes is a necessary prerequisite for this process. Accordingly, an analysis of alterations in signal transduction pathways should provide important new information that will further our understanding of mammary tumor metastasis.

To achieve this goal, we are using the Met-1/Db-7/pD transgenic mouse model for metastatic breast cancer to dissect the intracellular activation pathways that are critical for metastatic conversion. In this transgenic mouse model, mammary cells have been transformed with a wild-type or mutant form of the PyV-mT antigen which is under the control of the estrogen-inducible mouse mammary tumor virus promoter. Two transplantable mammary tumor lines, Met-1 and Db-7, have been developed with significant differences in their metastatic potential. Upon transplantation, the Met-1 tumors, which express wild-type PyV-MT, produce pulmonary metastatic tumors in 100% of transplanted nude mice. Whereas, the Db-7 tumors, which express a PyV-MT mutant that is defective for phosphatidylinositol-3-kinase (PI3K) binding and activation, develop fewer pulmonary metastases (8.8%). Importantly, highly metastatic tumors that were derived from the low metastatic Db-7 tumors have been characterized; these metastatic tumors have been designated pD. PD tumors represent metastatic revertants that could shed crucial insight into the mechanism of metastatic progression. This system has been used to determine whether the metastatic potential of the mammary tumors is defined by activation of the PI3K signaling pathway.

A combined biochemical and genetic approach was taken to determine whether PI3K and/or downstream-signaling intermediates in the PI3K activation pathway are important for regulating metastatic progression. Our initial studies have shown that PyV-MT activates PI3K, protein kinase B (Akt), and p21-activated kinase (PAK) in transient expression experiments. Both Akt and PAK are highly conserved serine-threonine kinases that are important for cellular regulation. Akt activation is important for inhibiting apoptotic signals and promoting signals for cellular survival. PAK is activated through interactions with Ras-like G-proteins (Rac and Cdc42). These kinases also interact with guanine nucleotide exchange factors (Pix and Cool) and adapter proteins (Nck). PAK proteins have been shown to mediate growth factor-induced morphological changes involving actin-based cellular structures (i.e., formation of membrane ruffles and peripheral filopodia). These changes are critical for cellular mobility and locomotion. PAKs also modulate gene expression through activation of the Jun kinase (Jnk) signaling pathway.

Although Akt has previously been shown to be a downstream signaling intermediate of PI3K, it is not clear whether PAK activity is influenced by PI3K. Because PAK activation is important for influencing cellular morphology and motility, it is possible that this phenotype is important for the increased ability of metastatic cells to migrate. Analyses of Met-1, Db-7, and pD tumors for PAK and Akt activation revealed that both Akt and PAK were found to be activated in the metastatic Met-1 and PD tumors but not in the low metastatic Db-7 tumors. Immunoblot analysis reveals that a form of PAK may also be differentially expressed in the Met-1 and pD tumors but not in the Db-7 tumors. Other markers of cellular signaling or transformation that have been previously implicated in tumor metastasis were examined by immunoblot analysis. Results from these experiments revealed that CD44v3, SEK, Rock-1, PTEN, or Tiam were not differentially activated or expressed in the metastatic tumors (Met-1 or pD). These results indicate that the activation of PI3K and its downstream-signaling intermediates, Akt and PAK, appear to be linked to metastatic progression in the transgenic model for breast cancer metastasis. Further work must be performed to elucidate the mechanism(s) by which PI3K is activated in the metastatic tumors derived from Db-7 (pD). Additional studies must be performed to determine whether PI3K, Akt, or PAK activation is sufficient for metastatic progression.

It is important to note that the mutations of PyV-MT that abrogate PI3K association and activation have not changed or reverted in the highly metastatic pD lines. Thus, some cellular mutations must have occurred that have enabled the Db-7 cells to become metastatic, and this conversion is linked with the activation of Akt and PAK without mutating the PyV-MT lesions. Thus, the cellular signaling pathways that are activated in the pD tumors have bypassed the PyV-MT oncogene and made an end-run using an as-yet-unidentified pathway to activate PI3K. These results demonstrate the importance of the PI3K pathway for metastatic progression. Identification of the pathways upstream of PI3K that are activated in the pD tumors should shed light on how metastatic reversion can occur. Finally, by characterizing the pathways that are important for metastatic conversion, specific signaling molecules in these pathways could represent important chemotherapeutic targets for the development of novel compounds to inhibit tumor dissemination.

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d. Mouse Models and Genomics.

Over the last decade, mouse biology has become the modern vehicle for human disease studies, and over the last few years genomics has become the way we view the pathology of disease. The intersection of these two disciplines offers an unprecedented opportunity to observe the remarkable changes that can occur at the DNA and RNA level. But maybe more important, the mouse gives us the ability to systematically study these changes with the use of transgenic and knockout technology. The focus of our laboratory has been on metastatic mammary carcinoma, with the specific hypothesis that the PI3 kinase (PI3-K) pathway is intimately involved. In order to investigate this hypothesis using transcript profiling, we have employed the use of two related transgenic mouse models of mammary carcinoma (termed Met and Db) that have differential activation of PI3-K and display significant differences in metastatic potential described in previous epitomes.

The site-directed mutation at 315 and 322 interferes with the interaction of PyV-mT with the p85 subunit of PI3-K, and therefore this cell-signaling pathway is not activated. This subtle difference in the mT gene significantly affects the metastatic phenotype and gives us the opportunity to ask these questions: what are the transcriptional differences between these tumors; could these changes be the downstream targets of PI3-K; and are these changes necessary for metastasis?

Until recently, the study of the abundance of mRNA or expression of genes in a cell or tissue sample has been limited to the ability to analyze only a few genes at the time, with the consequence of having an incomplete representation of the behavior of all the genes (~30,000) within the sample. With this limited view of the cell or tissue, it is difficult to fully understand its biology. With the advent of microarray technology, it has now become possible to analyze the expression levels of thousands of genes in parallel, in a highly reproducible and rapid, albeit expensive, fashion. Oligonucleotide microarrays are created by attaching hundreds of thousands of short oligonucleotides (15-25 base pairs), representing genes of interest, to a solid support by directly synthesizing the oligonucleotide onto the surface. RNA from the cell or tissue sample of interest is then transcribed into cDNA, linearly amplified and biotin-labeled, and hybridized to the array. The array is then scanned on a high-resolution scanner after being stained with fluorescently labeled streptavidin and washed. The amount of fluorescence detected at each oligonucleotide represents the abundance of that mRNA species in the specimen. In our experience, this strategy provides highly reproducible data, with coefficients of correlation greater than *r2=0.99*, and is correlative with standard Northern blot and quantitative RT-PCR measures.

We studied the Met and Db tumors by using Affymetrix GeneChip oligonucleotide arrays that contain about 12,000 murine genes and ESTs. Not surprisingly, we found many changes. One particular group of genes that was upregulated in the Met tumors were genes associated with lobular differentiation. This may be counterintuitive, as dogma states that aggressive tumors are associated with less differentiation, but nevertheless, this transcript data is correlative with the histopathology of the Met tumors. These tumors show a more lobular and glandular appearance, while the Db tumors show a more undifferentiated solid growth pattern.

We decided to concentrate our studies on one gene that was in this group of upregulated differentiation genes: osteopontin (OPN). Our hypothesis was that OPN is necessary for metastasis, and therefore, if it was silenced in the Met tumors, there would be less metastasis; conversely, if it was turned on in the Db tumors, there would be increased metastasis. To test this hypothesis, Met cells were transfected with antisense OPN, and Db cells were transfected with full-length st*OPN*. The resulting migration assay showed that Met cells transfected with OPN anti-sense showed decreased in vitro invasiveness, while the OPN-transfected Db cells showed increased invasiveness. With this data, a genetic study can be used to rigorously test the hypothesis by genetically crossing the PyV-mT with *OPN* knockout mice.

As illustrated, there is a unique synergy of genomics and mouse biology. The mouse gives us the ability to test and validate the genomic information. In human studies, genomic information can be used to form causal correlations, which, albeit important, do not offer us the ability to rigorously test our hypothesis. With mouse models, however, we have the ability to intimately and rigorously integrate the genomic information and dissect it in order to understand the inherent in vivo biology. This data then can be translated and used to understand the biological phenomena in humans.

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e. Metastasis and Angiogenesis.

Angiogenesis is another key factor in the stromal-epithelial interaction in tumorigenesis. It plays a vital role in the development of solid tumors. Since metastatic disease is the major source of mortality in human breast tumor patients, one is led to assume that there is a direct relationship between angiogenesis and metastasis. Indeed, current concepts, including the seminal work of Folkman, suggest a strong correlation between angiogenesis and prognosis in human breast tumor patients, although there has been no direct experimental evidence to link angiogenesis with metastasis.

Since it is neither ethical nor feasible to experiment directly on human patients, the development of a metastatic tumor model and in vivo technologies to objectively study angiogenesis and metastasis was warranted. Mice bearing the MMTV-LTR-promoted PyV-mT transgene were used, since the tumors develop very rapidly, and metastatic and non-metastatic variants were available for study. As previously described, two PyV-mT tumor lines have been developed.

1. Met-1 (Metastatic Line). Breast tumor cells containing the *PyV-MT* transgene (from Dr. Muller's laboratory at McMaster University, Hamilton, Ontario, Canada) was used to initiate this transplantable metastatic (> 90%; > 35 days post-transplantation) breast tumor line in nude mice.
2. Db-7 (Non-metastatic Line). This transplantable non-metastatic breast tumor line was also derived from the *PyV-MT* transgene—this line was the result of a double base mutation at positions 315 and 322. This line was utilized as a reference (non-metastatic) control to compare with Met-1. Db-7 has a low incidence of metastases (< 10 %; > 60 days).

For studies on angiogenesis, samples of Met-1 and Db-7 tumors were transplanted into the 4th inguinal mammary fat pads of recipient nude mice and analyzed using computer-assisted intravital microscopy. At various times post-transplantation, animals were anesthetized and the respective tumors were

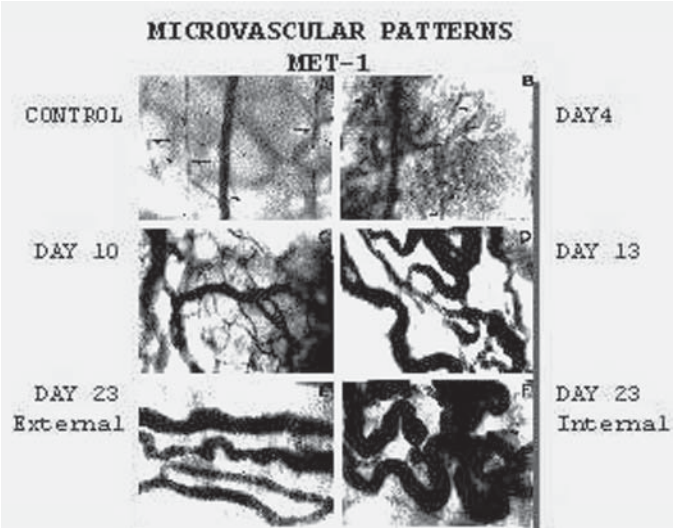

Figure 11. A comparison of the microvessels as visualized by the Cheung intravital angiography technique. Note the vessels (black tubes) and their relative tortuosity.

exposed and studied. Using reverse illumination intravital microscopy, videotapes were made of the intratumoral microcirculation of both tumor lines. The videotapes were analyzed and quantified for angiogenic (distribution and morphometric) characteristics, using imaging software developed in-house. The angiogenic parameters were correlated with metastatic outcome in the same animal.

After videotaping of the intratumoral microcirculation, each tumor was excised, processed, sectioned and immunostained for α-smooth muscle actin. Each stained slide was scanned and scored under light microscopy at 100× and 200× for microvascular density (MVD) "hot-spots." The results were correlated with intravital angiogenic parameters and metastatic outcome.

Angiogenesis started two to three days post-transplantation in both Met-1 and Db-7 tumors. Angiogenesis and intratumoral vascular development progressed rapidly for six to seven days. The external (surface) microcirculation of Met-1 and Db-7 was similar throughout the study. However, significant differences in the intratumoral (internal) microcirculation between Met-1 and Db-7 started to appear six to seven days post-transplantation. Angiogenesis developed more rapidly in Met-1, and the intratumoral microcirculation became complex and heterogeneous. The intratumoral microvessels in Met-1 became extremely tortuous (Fig. 11). The Db-7 microcirculation, though also heterogeneous, was less dense and poorly organized. The intratumoral microvessels in Db-7 were relatively non-tortuous in morphometry.

A tortuosity index (TI) was developed to quantify the degree of curvature of the intratumoral vessels. The actual meandering path of the vessel between the two points was measured. TI for each vessel was computed as the ratio between the straight-line distance and the actual meandering distance. Theoretically, the TI for a straight line is close to 1 (0.7-0.9) and for a curved line is close to 0.1 (0.1-0.6). The TI of the intratumoral vessels in Met-1 was 0.44 ± 0.06, and differed significantly ($P < 0.001$) from the TI (0.77 ± 0.04) of the relatively non-tortuous intratumoral vessels in Db-7.

The histological microvessel density (MVD) inside Met-1 and Db-7 tumors was heterogeneous. MVD "hot-spots" were scored. The MVD was significantly ($P < 0.001$) higher in Met-1 (20.8 ± 9.9 microvessels/200× field; 33.4 ± 12.6 microvessels/100× field) than in Db-7 (7.8 ± 4.1 microvessels/200× field; 12.8 ± 5.6 microvessels/100× field).

This study demonstrated that angiogenesis is a significant predictive marker for metastases. Microvessel tortuosity is a unique intratumoral parameter. The TI for a tumor (e.g. Met-1) correlates with intratumoral MVD, vascular distribution, and metastasis in the same animal. The microvessel tortuosity in Met-1 also explains the high MVD in metastatic solid tumors, since the high density of vessels and vessel cross-sections in the "hot-spots" may represent the result of histological sectioning across highly tortuous (instead of straight) vessels. This study provides quantitative validation of MVD and angiogenesis as prognostic indicators for mammary tumor metastasis in the nude mice and experimental evidence supporting the use of MVD in human breast cancer.

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f. Application of Met-1 Transgenic Breast Cancer: Delineating Antiangiogenic Property of Paclitaxel (Taxol).

The previous epitomes describe the evidence that the mouse mammary gland is a useful model for the analysis of key concepts in mammary biology. With the introduction of genetically engineered mice (GEM) with mammary cancer, certain hypotheses could be readily tested. We have learned that GEM tumors model human preneoplasia, metastasis, gene expression, signal transduction, and angiogenesis. If all these are accurate statements, the mouse should also be useful for preclinical trials. We have used the highly metastatic MET1 line to test the efficacy of paclitaxel (Taxol).

Paclitaxel, a diterpene extracted from the bark of the Pacific yew tree (*Taxus brevifolia*), is a potent radiosensitizer. The radiosensitizing and cytotoxic action of paclitaxel is believed to be a result of inhibition of microtubule depolymerization and blockade of cell division in the G2/M phase of the cell cycle. In addition, paclitaxel appears to inhibit normal neovascularization such as that of chick allantoic membrane and VEGF-treated cornea in mice. Inhibition of angiogenesis may in part contribute to the anticancer activity of paclitaxel. However, direct evidence of paclitaxel-induced antiangiogenesis in tumor is lacking.

To test the intrinsic anti-angiogenic property of a cytotoxic agent, one must exclude the non-specific inhibition of neovascularization resulting from its cytotoxic effect. In preliminary studies, we identified a threshold dose of paclitaxel at 6 mg/kg injected intraperitoneally daily for five days, which did not inhibit the growth of Met-1 in nude mice. Therefore, paclitaxel, at doses of 0, 3 and 6 mg/kg, was injected intraperitoneally daily for five days into three groups of four nude mice, each bearing Met-1 tumors 4 to 6 mm2 in size. Tumor size was measured in two perpendicular dimensions every two days until the mice were sacrificed 12 days after the first paclitaxel injection.

Intratumoral microcirculation was studied in situ under computer-assisted intravital microscopy as previously described by Cheung et al. The tortuosity index, which was an indicator of degree of meandering of a microvessel, was calculated as the ratio of the shortest distance to the actual length between two points of a meandering vessel, as defined previously.

Microvessels were identified with immunohistochemical staining for α-actin. Microvessel density was determined as reported by Cheung et al. Microvessel density was expressed as the average number of microvessels per microscopic field.

Immunostaining for vascular endothelial growth factor (VEGF) was performed by applying, at a dilution of 1:10 in PBS, a rabbit polyclonal antibody raised against a peptide of the first 191 amino acids of human VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). A biotinylated goat anti-rabbit antibody (Bio-Rad, Hercules, Calif.) was used as the secondary antibody. For each slide, number of positively stained tumor cells was counted in three different microscopic fields.

The tissue culture of Met-1 was exposed to 0, 2.2, 4.4 or 8.8 nM paclitaxel for 48 h. Level of VEGF expression in the culture medium was quantified by ELISA (Oncogene Research Products, Cambridge, Mass.).

The tumors in each group grew rapidly with the mean growth rates ± standard deviation (S.D.) of 13 ± 0.8, 15 ± 4.2 and 11 ± 2.2 mm2/day, respectively, for the control, 3 mg/kg, and 6 mg/kg paclitaxel-treated groups. There was no significant difference ($P > 0.05$) in the growth rates or doubling times among the groups as tested by analysis of variance. The mean tortuosity index + S.D. for the microvessels in the paclitaxel-treated tumors was 0.66 ± 0.10, which was significantly ($P < 0.05$) different from that of 0.39 ± 0.07 in the untreated tumors. The mean microvessel density ± S.D., expressed as number of microvessels per microscopic field, was 33 ± 8 in the control group, which was significantly ($P < 0.05$) higher than that of either 17 ± 2 in the 3 mg/kg or 12 ± 6 in the 6 mg/kg paclitaxel-treated group. The number of VEGF-expressing tumor cells per microscopic field was 31 ± 16 (mean ± S.D.) in the control group (Fig. 3a), which was significantly ($P < 0.10$) higher than that of 20 ± 12 in the 3 mg/kg and 12 ± 8 in the 6 mg/kg paclitaxel-treated group. With non-cytotoxic concentrations of 2.2 to 8.8 nM, paclitaxel induced a dose-dependent suppression of VEGF expression in the Met-1 cell cultures. Paclitaxel, at concentrations of 4.4 and 8.8 nM, significantly ($P < 0.05$) decreased the level of VEGF secreted by Met-1 into the culture medium by 20% and 25%, respectively, as compared to that of the control.

The Met-1 transgenic breast cancer model is highly vascularized and the intratumoral microcirculation can be readily studied by intravital microscopy and immunohistochemical analysis. This breast cancer is an appropriate model for identifying inhibitors of angiogenesis, such as paclitaxel, reported in this study. We demonstrated that paclitaxel at non-cytotoxic doses, given by daily intraperitoneal injection for five days, inhibited microvascular proliferation in this breast tumor model. The extent of inhibition of microvascular proliferation was indicated by a significant decrease in tortuosity index measured by intravital microscopy and a reduction in microvessel density detected by immunostaining of α-actin. Thus, it appears that paclitaxel, at relatively low doses, possesses an anti-angiogenic property independent of its anti-proliferative action. We speculate that the anti-angiogenesis action of low-dose paclitaxel may in part contribute to long-term control of cancer growth observed in the clinic. This hypothesis is being tested by studying various dosing and timing schedules of paclitaxel administration in the transgenic breast cancer model described in this study.

In summary, we have demonstrated that, in addition to its well-characterized microtubule stabilizing effect, anti-angiogenesis by down-regulation of VEGF is another mode of action of paclitaxel on tumor cells. The transgenic breast cancer model will allow us to further characterize the mechanism of paclitaxel-induced anti-angiogenesis as well as its relationship to tumor growth and metastasis.

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Summation

These epitomes were introduced with the thought that comparative medicine is based on the concept humans and animals often share the same disease. Although the concept has been suggested in the past, the development of genetically engineered mice (GEM) has brought this concept into breathtaking reality. The sometimes rather vague morphological and biochemical similarities between diseases of humans and animals described by previous generations of scientists were frequently obscured by the differences between the various species. However, with the advent of GEM, the similarities have been emphatically confirmed by the current scientific generation at the genetic and molecular level. The molecules that cause diseases in humans cause the same diseases in mice. There is truly One Medicine!

Increasing reliance on the electronic PubMed abstract leaves the gullible graduate student with the impression that history began three years ago. Science is in danger of substituting sophisticated molecular technique for sound biology. Before becoming too enamored with their marvelous ability to unravel the molecular mysteries of disease, the current generation needs to pause to appreciate the insights of the previous generations of biologists.

These short essays end with increasingly sophisticated descriptions of the molecular biology of breast cancer. Modern scientific disciplines of signal transduction (Sawai), genomics (Gregg), bioengineering (Cheung), pharmacogenetics (Lau and Guzman and Nandi), gene discovery (Gregg), genomics (Gregg), host transcription factors (Oshima), GEM premalignancy (Medina and MacLeod) are all invoked for their great promise for understanding and curing breast cancer. Implicit in all of our optimism is the One Medicine concept of comparative medicine. Surely, an understanding of the effects of molecules on any animal will lead to insight into the human disease. In breast cancer, the genetically engineered mouse leads the way. However, the understanding we seek is based on the foundation of experimental biology presented in the initial epitomes by Bern, Faulkin, Daniel, Shyamala and Smith.

These basic concepts of causation, preneoplasia and progression have been tested in humans (Wellings) and other species (Gardner and Guzman and Nandi). They have been applied to strategies for treatment (Lau), for prevention (Guzman and Nandi) and for intervention (MacLeod). Although a comparable virus has not been found in human (Gardner), the mouse mammary tumor virus has been useful for gene discovery, understanding of clonality in neoplastic progression, and as the promoter for targeting oncogenes to the mammary gland (Cardiff and Gardner).

These epitomes and this symposium brought the current generation that concentrates on molecular biology together with many of the biologists who pioneered the field of mammary tumor biology. It gave all concerned an appreciation for the historical roots of modern breast cancer research. We hope that

sharing these epitomes with the general reader will enhance their appreciation of the value of reviewing and understanding biological as well as the molecular basis of disease.

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