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1
Mouse models of human breast cancer: evolution or convolution?

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The remarkable generation of scores of increasingly sophisticated mouse models of mammary cancer over the past two decades has provided tremendous insights into molecular derangements that can lead to cancer. The relationships of these models to human breast cancer, however, remain problematic. Recent advances in genomic technologies offer significant opportunities to identify critical changes that occur during cancer evolution and to distinguish in a complex and comprehensive manner the key similarities and differences between mouse models and human cancer. Comparisons between mouse and human tumors are being performed using comparative genomic hybridization, gene expression profiling, and proteomic analyses. The appropriate use of genetically engineered mouse models of mammary cancer in preclinical studies remains an important challenge which may also be aided by genomic technologies. Genomic approaches to cancer are generating huge datasets that represent a complex system of underlying networks of genetic interactions. Mouse models offer a tremendous opportunity to identify such networks and how they relate to human cancer. The challenge of the future remains to decipher these networks in order to identify the genetic nodes of oncogenesis that may be important targets for chemoprevention and therapy.

2
Estrogen receptor alpha-positive and negative mouse mammary tumors through somatic mutations of *p53* in mammary carcinogenesis

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Approximately 70% of human breast cancers are estrogen receptor alpha (ERα)-positive, but the origins of ERα-positive and ERα-negative tumors remain unclear. Most mouse models produce only ERα-negative tumors. In addition, these mouse tumors metastasize at a low rate relative to human breast tumors. We report that somatic mutations of *p53* in mouse mammary epithelial cells lead to ERα-positive and ERα-negative tumors. *p53* inactivation in pre-pubertal/pubertal mice, but not in adult mice, leads to the development of ERα-positive tumors, suggesting that developmental stages influence the availability of ERα-positive tumor origin cells. These tumors have a high rate of metastasis that is independent of tumor latency. An inverse relationship between the number of targeted cells and median tumor latency was also observed. The median tumor latency reaches a plateau when targeted cell numbers exceed 20%, implying the existence of saturation kinetics for breast carcinogenesis. Genetic alterations commonly observed in human breast cancer including c-*myc* amplification and Her2/Neu/erbB2 activation were seen in these mouse tumors. Since it is feasible to isolate ERα-positive epithelial cells from normal mammary glands and tumors, molecular mechanisms underlying ERα-positive and ERα-negative mammary carcinogenesis can be systematically addressed using this model.

3
Mouse models for BRCA1-associated breast cancer

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Breast tumor suppressor gene 1 (BRCA1) is a well-known transcription regulator, mutations of which cause tumor formation in a tissue-specific manner. In the past years, we have studied functions of Brca1 in mouse models carrying a number of different mutations. We showed that impaired Brca1 function causes chromosome damages, failure of the G2/M cell cycle checkpoint, and centrosome amplification, leading to p53-dependent lethality. Our further analysis revealed that Brca1 also plays an important role in spindle checkpoint through regulating Mad2. We showed that mice carrying a targeted disruption of Brca1 in mammary epithelium developed mammary tumors at low frequency after long latency and the tumorigenesis was significantly accelerated in a $p53^{+/-}$ genetic background. Mammary tumors were highly diverse in histopathology and displayed extensive genetic/molecular alterations, including overexpression of ErbB2, c-*myc*, p27 and cyclin D_1, and downregulation of p16. The most noticeable change is expression of estrogen receptor alpha (ERα). We showed that the absence of Brca1 resulted in increased expression of ERα in epithelial cells at pre-malignant stages and initiating tumors. However, expression of ERα was diminished in tumors of more advanced stages. This observation suggests that ERα-mediated signals are involved in tumorigenesis. Finally, we provided evidence that BRCA1 affects the MAPK pathway through interacting with estrogen/ERα signals, which may account for tissue-specific tumorigenesis.

4
Integrin-mediated signal transduction in transgenic mouse models of human breast cancer

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The regulated growth and development of the mammary epithelium depends on the interaction between the epithelial cells with the adjacent extracellular matrix. This interaction is primarily mediated through the integrin receptor family. One of the primary signaling effectors on the integrin class of receptors is the integrin-linked kinase (ILK). To explore the importance of integrin coupled signaling pathways in mammary tumor progression, we have used transgenic mouse models to elucidate the role of the beta-1 integrin and ILK in mammary tumorigenesis and metastasis. First, we demonstrated that mammary epithelial expression of ILK is capable of inducing focal metastatic mammary tumors. Interestingly these mammary tumors exhibited evidence of epithelial mesenchymal transition. These observations provide direct evidence that mammary epithelial-specific expression of ILK can result in the direct induction of mammary tumors. To further explore the importance of the beta-1 class of integrin receptors and ILK in mammary tumorigenesis, we have generated mammary-specific knockouts of either beta-1 integrin or ILK using the Cre/LOXP recombination approach. Preliminary analyses of the mammary ductal outgrowth in these strains has revealed that a functional ILK is required for normal mammary gland development whereas a functional beta-1 integrin appears to be dispensable for normal mammary gland development. Although beta-1integrin receptor function is not required for normal mammary gland development, mammary-specific ablation of beta-1 results in dramatic inhibition of mammary tumors in transgenic mice expressing the Polyomavirus middle T oncogene. Taken together, these observations suggest that a functional beta-1 integrin receptor is required for efficient mammary tumor progression.

5
Reversibility and progression in conditional transgenic mouse models of breast cancer

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A cardinal feature of human cancers is the progressive selection and outgrowth of cells that possess increasingly aggressive properties. This process ultimately leads to resistance to therapeutic agents, distant metastasis, and tumor recurrence. Together, these three manifestations of tumor progression are responsible for the vast majority of cancer deaths. Nevertheless, while tumor progression constitutes a problem of unrivaled clinical importance, the mechanisms underlying it are largely unknown. As such, elucidating the molecular, cellular, and pathophysiological events that contribute to tumor progression is a critical priority in cancer research. To better define the genetic, cellular, molecular, and physiological events that contribute to breast cancer progression, we have created a series of novel inducible bitransgenic mouse models in which the oncogenes c-*myc*, Neu, Wnt1, v-Ha-Ras, and Akt1 can be conditionally expressed in the mammary epithelia of animals treated with tetracycline derivatives. Tumor formation in each of these models is highly penetrant, mammary-specific, and absolutely dependent on transgene induction by doxycycline. The properties of this model system permit the direct visualization and analysis of each stage of mammary tumorigenesis from normal mammary tissue in the uninduced state to hyperplasias, atypical hyperplasias, invasive carcinomas, and distant metastases that arise as a consequence of oncogene activation. The inducible nature of the transgenic models that we have developed permits essentially complete downregulation of an oncogenic stimulus within an intact tumor. Remarkably, we have found that–following transgene deinduction by doxycycline withdrawal–many of these oncogene-induced primary mammary tumors rapidly regress to a clinically undetectable state. However, despite this dramatic regression behavior, a substantial fraction of tumors that have previously regressed to a non-palpable state recur spontaneously in the absence of transgene expression over periods of up to 1 year. This finding suggests that many animals in whom tumors have regressed still harbor residual cancerous disease and that additional genetic events may occur in these remaining cells that lead to the recurrence of actively growing tumors. In addition, we have further demonstrated that a subset of primary mammary tumors fail to regress fully following doxycycline withdrawal, and instead acquire the ability to survive and grow in the absence of oncogene overexpression. In some cases, this behavior is associated with identifiable spontaneous genetic events that are tightly linked to the ability of tumors to progress to transgene independence. In aggregate, the tendency of mammary tumors in this system to metastasize, to develop resistance to oncogene downregulation, and to recur spontaneously with long latency suggests that these models mimic critical aspects of the natural history of human breast cancer. As such, this system may represent a valuable new means to understand the biology of tumor progression and to identify the molecular mechanisms by which mammary tumors escape their dependence on particular oncogenic pathways for growth.

6
Genetic manipulation of the mammary gland by transplantation

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Mammary epithelium can be reconstituted *in vivo* by transplanting fragments of mammary epithelium, or suspensions of mammary epithelial cells, into the 'cleared' mammary fat pad of a syngeneic recipient mouse. A 'cleared' mammary fat pad is one from which the natural epithelium has been removed at 3 weeks of age. Genes can be introduced into the epithelium before transplantation using retrovirus vectors, or the epithelium can be taken from a knockout mouse [1]. The applications of transplantation, and its advantages and disadvantages compared with transgenesis will be surveyed, including the ability to use hormone-insensitive promoters; to introduce genes into clones of cells rather than whole tissues; the ease of studying early preneoplastic change; and the use of transplantation with transgenic knockouts, to rescue embryonic lethals and to distinguish systemic from local effects [2].

References

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7
The Mutant Mouse Regional Resource Center Program

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The Mutant Mouse Regional Resource Center (MMRRC) Program serves as the National Institutes of Health (NIH) premier repository of

spontaneous and induced mutant mouse lines. The MMRRC Program was established in 1997 through a U01 funding mechanism from the NIH National Center for Research Resources. The purpose of the MMRRC Program is to ensure the continued availability of scientifically valuable, genetically engineered mice and to distribute these mice to qualified researchers studying human and animal biology and disease. The MMRRC Program is made up of a national network of regional breeding and distribution centers, in which UC Davis participates as the West Coast regional Center. The facilities and personnel of the MMRRC regional Centers cryopreserve and distribute mouse strains with potential value in the research of human disease and biology. These strains are then made available for distribution to qualified researchers. Mice are supplied either from a production colony or from a colony recovered from cryopreservation. MMRRC Centers also offer cryopreserved material from some strains for resuscitation at the recipient scientist's institution. The MMRRC at UC Davis is made up from contributions of a number of UC Davis campus resources and units, including the UC Davis Mouse Biology Program, the Center for Comparative Medicine, and the Center for Laboratory Animal Sciences. We provide a host of services in support of the MMRRC Program, including importation of mouse strains by rederivation, cryopreservation and reanimation of frozen embryos and germplasm, assisted reproduction techniques (*in vitro* fertilization, intracytoplasmic sperm injection, intracytoplasmic nuclear injection), and comprehensive genotyping (including speed congenics) and phenotyping (pathology, behavior, clinical pathology, etc.) capabilities. As part of its participation in the MMRRC Program, the MMRRC at UC Davis provides a comprehensive list of services and procedures to insure safe and expedient importation, maintenance, archiving, genotyping, phenotyping, and distribution of mutant mouse lines. A number of these services and procedures are provided at no charge to the Donating Investigator, while others are offered for a fee to Requesting Investigators. In addition, using the type 3 submission mechanism, a Donating Investigator can negotiate with the MMRRC at UC Davis to perform selected services and procedures on their mouse strain. For more information on the MMRRC National Program, or to donate mice to, or request mice from, the MMRRC please visit the website (www.mmrrc.org) or go to the MMRRC at UC Davis website (http://mbp.compmed.ucdavis.edu/modules.php?name=mmrrc).

8
Genetically altered mice and cancer research: effects of the other 99.999% of the genome and other variables upon phenotype

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The modern laboratory mouse was derived from a diverse gene pool of multiple species of the *Mus musculus* genospecies complex from throughout Europe and Asia. The progenitors of laboratory mice were thus hybrid species, but they subsequently underwent a genetic bottleneck at the beginning of the last century. Thus, the genomes of inbred strains of mice, although homozygous, contain variable allelic contributions from this diverse origin, including endogenous retrovirus and retrovirus-like elements. Domestication of the mouse through international trading has also introduced over 60 infectious pathogens that can cause overt disease, as well as more subtle effects upon research. Burgeoning mouse populations and exchange among scientists have led to the re-emergence of many of these agents. Genetic alteration of the mouse often leads to atypical outcomes of these infections which confound research. Diet, bedding, water, behavior, and other factors also contribute to variable research results. Effective cancer research should take advantage of the opportunity to utilize the mouse as a genetically, microbially, and environmentally standardized animal model system, but this requires global awareness of the biology of the laboratory mouse.

9
Advanced immunophenotyping: more is better!

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Recent advances in multicolor flow cytometry have made possible the simultaneous analysis of up to 12 distinct cell surface molecules. This technical advance increases sensitivity and specificity for more accurate diagnosis and classification of various malignancies. In turn, this allows for a better assessment of disease prognosis and the establishment of a treatment regimen. In addition, multicolor flow cytometry provides a sensitive technique for investigating the occurrence of minimal residual disease. Studies of leukemias and lymphomas are particularly suitable for investigation by flow cytometry. Studies on solid tumors such as breast cancer, or on tumor infiltrating immune cells, are also possible if appropriate processing techniques are used to generate single-cell suspensions without altering cell surface receptor expression. In addition to analytical immunophenotyping, isolation of even very small (malignant) cell populations by multicolor flow cytometry provides a highly pure source for DNA and RNA analyses. Such combined sorting and molecular approaches provide a comprehensive basis for studying the molecular mechanisms underlying malignant transformation. Importantly, they also enable distinct diagnoses to be made when cell surface marker immunophenotyping alone is inconclusive. To exemplify the importance of combining various immunophenotyping tools, such as multicolor flow cytometry and gene expression analyses, with the classical tools of histopathology and differential blood count, we provide data on the analysis of a mouse model of human chronic B-cell leukemia (B-CLL): the New Zealand Black mouse. We relied for this study on gene expression databases generated by the National Cancer Institute for various human B-cell malignancies. B-CLL is one of the most common forms of leukemia in the Western world. No therapies currently exist against this usually slow-progressing but always fatal malignancy, making the development of a good animal model an important task. The data show that although the New Zealand Black mouse shows a spontaneous expansion of a $CD5^+$ B-cell population (a hallmark of human B-CLL), it does not fit the classification of B-CLL by either phenotypic or genetic analysis criteria. In conclusion, increasing the number of simultaneous measurements for immunophenotyping and cell sorting by flow cytometry and combining it with comprehensive gene expression studies supports and expands the power of classical analysis tools.

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10
Whole Slide Imaging and telepathology

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Accurate diagnosis of lesions in genetically engineered mice requires a level of experience and expertise that is not available in all institutions. As a result, there is an unmet demand for the services of a limited number of expert pathologists. In order to meet this demand, we have developed several electronic, web-based educational and consulting tools to expand the availability of expert pathologists.

Image Galleries The immediate need has been for publication quality images that are connected to a robust database. This has been accomplished using a shareware program that is connected to our SQL Mutant Mouse Pathology Archive database. This program develops Image Galleries 'on the fly' that can display still images with demographic, genetic, and diagnostic information stored in the database. The application includes a dialog box that permits the viewer and the pathologist to exchange information and comments. However, the images are limited to adynamic jpeg and tiff files that illustrate limited fields selected by the photographer and do not display or annotate the image in full context.

Whole Slide Imaging To provide the digital view of the complete slide in context, several new technologies have been merged to the Image Archive using Whole Slide Imaging. Whole Slide images are digital images of the entire block face or slide. These digitized images are captured, compressed and viewed on any browser over the internet. The digital image allows users to view entire microscopic images at any magnification on their monitor. The images are captured with the ScanScope (AperioTech Inc., Vista, CA, USA) in approximately 3–5 min at a resolution of 50,000 dpi, producing 8–20 Gb of raw data. To make the images accessible to the community, the images are processed with the Zoomify compression algorithm which results in a 200–300 Mb image that can be viewed with a standard web browser using a Flash6 plug-in. The plug-in only downloads the pixels required to render the view requested by the user (approximately 75 kb/view).
The images can be annotated with layers that point to specific features in the image allowing users to easily navigate to selected points of interest within a slide while still being able to maneuver the entire slide. Examples of these images can be viewed online (http://imagearchive.compmed.ucdavis.edu).
Telepathology The full capabilities of the Zoomify system, have been exploited by combining the Whole Slide Imaging with a Telepathology Conferencing Tool that allows multiple simultaneous users to view the same slide in session becoming a virtual multi-headed microscope. A number of users can view, manipulate, and annotate a single image and share their perspective utilizing the application's voice conferencing system (voice over). A preselected Moderator controls the conference using a baton-passing utility that limits the number of users who can manipulate the image at any given time. This system allows pathologists to practice within a virtual world-wide group, gathering expert opinions from widely dispersed locations. Every investigator will have access to appropriate experts.

11
Mammary pathology of the genetically engineered mouse

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Human breast cancer has been modeled in well over 100 types of genetically engineered mice (GEM). Many of the mutated or overexpressed genes found in human breast cancer also initiate mammary cancer in GEM. The histopathology of GEM models of breast cancer have proved to be unique. Most GEM tumors do not resemble the spontaneous tumors induced by the mouse mammary tumor virus (MMTV) or by carcinogenic agents. The pattern of some GEM tumors closely resembles that seen in some human breast cancers. The most provocative and most thoroughly studied GEM group belongs to the ERBB2 (HER2) signal transduction pathway. GEM bearing ERBB2, various forms of mutated ERBB2 and ERBB2-related genes produce tumors via similar molecular mechanisms, and also have a remarkable morphological resemblance to some forms of human breast cancer. Tumors associated with tumor suppressor genes, such as *Brca1* and SV40 Tag (that suppresses expression of Rb and p53) tend to resemble poorly differentiated tumors and, in some cases, medullary carcinoma of the human breast. Many of the GEM develop mammary tumors with characteristic or unique gene-specific 'signature' phenotypes that readily can be identified microscopically. The principle that genotype predicts phenotype can be applied to other GEM and extended to include entire molecular pathways. Studies of tumor kinetics in bi-transgenic mice suggest that some combinations of genes are synergistic while others are inhibitory. Although the majority of breast cancer models have been induced using the MMTV-LTR as a promoter, C3(1), WAP, and BLG promoters frequently have been used. Tumors retain the phenotype characteristic of the oncogene, for the most part, regardless of promoter. In some cases, inserting the transgene behind the native promoter or 'knocking-in' an oncogenic variant at the normal gene locus has also resulted in tumors characteristic of the oncogene. GEM with inactivation of tumor suppressor genes generally die with tumors other than mammary tumors. The mammary tumors that do develop tend to be more poorly differentiated, aneuploid tumors with a wider range of phenotypes. However, when crossed with GEM bearing activated transgenes, the tumors are most likely to resemble the phenotype of the transgene. The tumors in bigenic mice do, however, have more pleomorphic and aneuploid cells. The great majority of mouse mammary tumors are hormone independent. This is in contrast to human breast carcinomas, 50% of which express the receptors for estrogen and progesterone, and are dependent (at least partially) on these hormones for growth. The biology of metastasis is also quite different with mouse carcinomas displaying a hematogeneous spread almost exclusively to the lung and with human carcinomas displaying regional lymph node involvement with preferential systemic spread to the bone, brain and liver.

12
Positron emission tomography and X-ray computed tomography: tools for mouse phenotyping?

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Significant advances in imaging technology now permit non-invasive imaging of mice with high spatial resolution and high sensitivity to biochemical and molecular alterations. Two imaging technologies will be discussed. X-ray computed tomography uses transmission of X-rays through a mouse to create high-resolution anatomic images, which can be useful for detecting phenotypic-related alterations in morphology. Positron emission tomography utilizes trace amounts of radiopharmaceuticals to measure biologic function, for example glucose metabolism, receptor binding and gene expression. Screening of genetically engineered mice with positron emission tomography and a radiopharmaceutical that correlates with glucose metabolism has demonstrated sensitivity to phenotypic changes. Ultimately, the merger of these two complementary imaging modalities, providing spatially registered images of anatomy and function, may provide a powerful tool for whole-body phenotypic analysis, although a number of challenges must be addressed to realize a high-throughput imaging tool for these applications.

13
Genetically modified cancer models: the value of luciferase-tagged tumors and *in vivo* luciferase-based reporter assays in oncology drug discovery

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Recent technological advances in visible light imaging allowed us to use this technique to non-invasively detect and quantify tumor cells, and to follow responses to drug candidates at molecular level in mouse models of human cancers. Human prostate tumor cells tagged with a firefly luciferase (PC-3M2AC6) were used in an orthotopic model, and in a model of experimental bone metastasis in athymic nude mice. Upon intravenous injection of D-luciferin these cells emitted light, that could be non-invasively registered with a charged-coupled device camera. Light emission was proportional to the tumor burden. The assay had sufficient throughput to allow for screening of advanced drug candidates for efficacy. Several compounds demonstrated significant and reproducible activity against tumors growing in prostates and in bones. These findings would not be possible without the non-invasive method of quantitation of bone tumor burden. A reporter gene, where luciferase is expressed from the p21waf-1 promoter, was introduced into the H1299 human lung cancer cell line. So constructed reporter cells were encapsulated in hollow fibers and the fibers were implanted subcutaneously into athymic, nude mice. The walls of hollow fibers are permeable to molecules up to 0.5 MDa in size, but not to cells. The animals were then treated intravenously with various histone deacetylase inhibitors twice (24 and 32 hours post implantation); the fibers were retrieved from the animals 18 hours after

the last treatment, and were processes for light emission *ex vivo*. Histone deacetylase inhibitors induced expression of luciferase and thus light emission in the presence of D-luciferin. Results of this pharmacodynamic 40 hour assay were predictive of anti-tumor activity of histone deacetylase inhibitors in mouse xenograft tumor models. Therefore 5 week efficacy studies could be replaced with 2 day pharmacodynamic assays. Preliminary results for the *in vivo* Caspase 3 induction assay will be presented.

14
Herceptin-sensitivity of HER2-transgenic mouse mammary tumors

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The long-term goal of our work is to better understand the mechanisms of Herceptin action and resistance. Toward that end, we have produced transgenic mice that express HER2 under the control of the mouse mammary tumor virus (MMTV) promoter. Transgenic mouse models offer the advantage of having immune system components that may be important in the action of antibodies but are lacking in immunodeficient hosts. ErbB2 transgenic mice have been produced by Muller and colleagues using the rat homolog *neu*, which does not bind Herceptin. Therefore, we made mice using the human version, HER2. These mice express HER2 in the mammary gland at very high levels and develop HER2-positive mammary tumors within 6–8 months. Several tumors have been propagated as allografts in wild-type mice by implanting a small piece of tumor into the mammary fat pad. Cohorts of mice bearing the same tumor line were then treated with 4D5, the murine version of Herceptin, or vehicle. Using this approach, we found that several tumor lines are completely Herceptin resistant, others are growth inhibited, and one regresses completely. As observed previously in tumors from *neu* transgenic mice, all of our HER2 tumors contain small deletions in the juxtamembrane region of the HER2 transcript. Within a tumor line, each mutation is unique and remains stable on continued passage. It is unclear, however, whether such mutations are responsible for determining Herceptin sensitivity. Gene expression profiling between the highly sensitive tumor line (F2-1282) and a Herceptin-resistant line (Fo5) revealed a large number of differences that could contribute to resistance. Gene expression profiling is also being used to identify changes that take place in F2-1282, the Herceptin-sensitive tumor, when it is exposed to Herceptin/4D5. In summary, like individual breast cancer patients, tumors from MMTV-HER2 mice display the entire spectrum of responsiveness to Herceptin/4D5, from resistant to complete regression, and therefore may help provide insights into cofactors that are important for Herceptin activity.

15
Role of animal models in oncology drug discovery

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Tumor xenografts have been used for over three decades in oncology drug development with little predictive value. A case has been made for use of orthotopic xenograft models as well as transgenic tumor models as a better reflection of tumor biology. However, their use in drug development has been limited, thus far, due to technical challenges of monitoring tumor growth. Recent advances in small animal imaging are addressing some of those challenges; however, the predictive ability of these models with regards to clinical response is still questionable. This presentation will focus on the utility of animal models in oncology drug development and a paradigm shift in their value as tools to evaluate biological effects of new agents as well as understanding the pharmacokinetic/pharmacodynamic relationship to aid in better clinical development.

16
Use of mouse models to validate and therapeutically target transforming growth factor beta as an important player in breast cancer progression

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Transforming growth factor betas (TGF-βs) play key roles in embryogenesis, maintenance of adult homeostasis and response to injury. In epithelial carcinogenesis the TGF-β play complex roles, functioning as tumor suppressors early in the process, but as pro-oncogenic factors in late-stage metastatic disease, when TGF-β ligands are frequently overexpressed. To probe this complexity *in vivo*, and determine whether TGF-β might be a viable therapeutic target, we developed a transgenic mouse overexpressing a soluble TGF-β antagonist. This antagonist ('SR2F') consisted of the extracellular domain of the type II TGF-β receptor fused to the Fc domain of human IgG_1. The SR2F was secreted into the circulation and distributed to all organs except the brain. To determine the effect of this TGF-β antagonist on breast carcinogenesis, the mouse mammary tumor virus (MMTV)-SR2F and wild-type control mice were crossed with the MMTV-*neu* transgenic mouse model of metastatic breast cancer. The *neu*/SR2F bigenic mice showed a significant threefold decrease in the incidence of lung metastases compared with mice expressing *neu* alone. A similar suppression of metastasis was seen in using a tail vein injection model of metastatic melanoma. Importantly, the SR2F did not accelerate primary tumorigenesis, despite the fact that TGF-β has been shown to function as a tumor suppressor in the MMTV-*neu* model [1]. Furthermore, none of the pathology that is usually associated with TGF-β loss, such as autoimmune disease and increased spontaneous tumorigenesis, was observed on prolonged exposure to SR2F. The mechanistic basis for the unexpected selectivity of the SR2F in antagonizing the pro-metastatic effects of TGF-β while sparing effects on tumor suppression and normal homeostasis is currently not clear, but it does not seem to be a dosage effect. Overall, our data suggest that high molecular weight TGF-β antagonists might have promise in the clinic for prevention of metastasis. This study demonstrates the utility of a transgenic approach for testing expensive protein-based therapeutics in long-term realistic models of cancer progression.

Reference

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17
Development of a transgenic mouse line for the evaluation of the androgen receptor activity *in vivo*

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Androgens control a broad range of physiological functions by binding and modulating the activity of the androgen receptor (AR), a member of the nuclear hormone receptor superfamily. The AR is a ligand-dependent transcription factor that is widely distributed among reproductive and non-reproductive tissues. It is predicted that, as in the case of the estrogen receptor, different AR ligands will promote distinct pharmacological activities in different cell types depending on the specific transcriptional environment, that is the presence or the absence of specific co-activators and co-repressors. In order to test this prediction *in vivo*, we generated a transgenic mouse line (ARLUC) that expresses the luciferase cDNA downstream of an androgen-responsive promoter containing two copies of the 11-base pair DR-1 (5′-GAACG-GAACA-3′) oriented as an overlapping direct repeat. This arrangement demonstrated a strong preference for AR binding and transactivation when compared with the glucocorticoid receptor [1]. Tissues with an

active AR emit light when tested for luciferase activity that is detected by the use of a cooled charged-coupled device camera or by direct measurements of enzymatic activity in tissue extracts. Experiments through biochemical analysis of tissue extracts showed expression of luciferase in the testis, seminal vesicles, quadriceps, brain and bone marrow. The luciferase expression was observed to be androgen-dependent since it was dramatically diminished in castrated animals and in animals treated with the anti-androgen bicalutamide compared with intact animals. The use of such a model in the evaluation of AR modulators will be discussed.

Reference

1. Zhou *et al.*: *J Biol Chem* 1997, **272:**8227-8235.

18
Clinical breast cancer and estrogen

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Endocrine therapy, targeting estrogen production or the estrogen receptor, is a crucial component in the treatment of breast cancer. This has been recognized for over a century. Ovarian ablation is the oldest form of endocrine therapy, first used in 1896. This presentation will review: the history of endocrine therapy in the treatment of breast cancer; endocrine strategies in premenopausal and postmenopausal women; data on the use of endocrine therapy in early breast cancer; new directions in the endocrine therapy of metastatic breast cancer; and clinical implications of HER2-neu status and endocrine therapy.

19
Pregnancy levels of estrogen prevents breast cancer

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Pregnancy and lactation with breast feeding at an early age are the only natural phenomena known to drastically reduce the risk for breast cancer in women of all ethnicities worldwide. Parous rats and mice (lacking mammary tumor virus) are also highly protected against chemically induced breast carcinogenesis. Our research goals during the past 10 years have been to define the reason for the highly reduced risk of the parous phenotype to breast cancers as well as to develop safe, inexpensive, mechanism-based hormonal intervention procedures mimicking the protective effects of pregnancy in rats. The first part of our presentation will describe the development of two short-term treatments, modified from Huggins and colleagues' 1962 procedure, with estradiol (E) and progesterone (P) in silastic capsules for 7–21 days. We have acronymed these procedures as short-term E treatment (STET) and short-term E + P treatment (STEPT). These treatments containing late pregnancy levels of E, with or without P, for 7 days are sufficient to reduce the breast carcinoma incidence by over 80% and multiplicity by 90% in rats exposed to the potent carcinogen, *N*-methyl-*N*-nitrosourea (MNU). Non-pregnancy or low early pregnancy levels of E were ineffective in protecting rats against MNU-induced breast carcinogenesis. This has been the first demonstration suggesting that the late pregnancy levels of estrogen may be the reason for the protective effect of pregnancy in breast cancer risk reduction. The second part of our presentation will focus on experiments characterizing the parous phenotypes: Why are they protected against breast cancers? Our results show that parous rats are not fully protected; they are susceptible to MNU-induced initiation and develop microscopic latent mammary cancers. These rats are, however, protected from further promotion-progression from developing into overt palpable cancers. Our results also indicate that parous rats have persistently reduced mammogenic hormones, growth hormone and prolactin as well as reduced levels of the receptors for estrogen, progesterone and epidermal growth factor in their mammary epithelial cells. The final section concludes by suggesting that our treatment procedure for breast cancer prevention in carcinogen-treated rats requires only 7 days of treatment (rat gestation 21 days) with no more than 1 μg E/day and is as effective as full-term pregnancy or ovariectomy or prolonged treatment with tamoxifen, without any loss of ovarian function. Our results also suggest that breast cancer protective effects of full-term pregnancy and short-term treatments (STET/STEPT) are due to persistently decreased hormonal environment for promotion-progression of the latent mammary cancers to overt cancers.

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20
Steroid regulation of breast cancer cell proliferation

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Estrogens are potent mitogens in a number of target tissues including the mammary gland where they play a pivotal role in the development and progression of mammary carcinoma. The demonstration that estrogen-induced mitogenesis is associated with an increased rate of progression through the G_1 phase of the cell cycle has focused attention on the estrogen regulation of molecules in the cyclin/CDK/pRb pathway that controls G_1 to S phase progression. Steroid-responsive breast cancer cells pretreated with a pure estrogen antagonist arrest in quiescence (i.e. G_0) and respond to estrogen treatment with synchronous progression into the S phase. Entry into the S phase is preceded by increased expression of c-*myc* and cyclin D_1, activation of cyclin D_1–Cdk4 and cyclin E–Cdk2 complexes and phosphorylation of the retinoblastoma gene product, pRb. Activation of cyclin D_1–Cdk4 is due predominantly to estrogen-induced transcriptional activation of cyclin D_1. In contrast, cyclin E–Cdk2 activation does not involve major changes in cyclin E expression but rather redistribution of the p21 CDK inhibitor away from cyclin E–Cdk2 complexes. This is mediated by two distinct mechanisms: sequestration into newly formed cyclin D_1–Cdk4–p21 complexes and transcriptional inhibition of p21 gene expression. In the same model progestins are growth inhibitory and arrest cells in the G_1 phase. Growth arrest is accompanied by decreased expression of both cyclin D_1 and cyclin E and induction of the CDK inhibitor p18INK4C. These changes lead to reassortment of cyclin–CDK–CDK inhibitor complexes and increasing availability of p27 to form inhibitory cyclin E–Cdk2–p27 complexes. Thus, both cyclin D_1–Cdk4 and cyclin E–Cdk2 activities are inhibited, resulting in decreased pRb phosphorylation and arrest in the G_1 phase. These data indicate that steroid hormones stimulate or inhibit cell cycle progression through effects on multiple targets in the pRb pathway. The aberrant expression of several of these targets in breast cancer, i.e. overexpression of c-*myc*, cyclin D_1 and cyclin E and loss of expression of p27, potentially contributes to the loss of steroid sensitivity and endocrine resistance associated with the progression of breast cancer.

21
The ErbB receptor tyrosine kinases and their roles in cancer

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The involvement of the ErbB receptor tyrosine kinases in human cancer, as well as their essential role in various physiological events during normal development, have motivated a high interest in this receptor family. Approaches taken to block the activity of ErbB receptors in cancer cells have not only proven that they drive *in vitro* tumor cell proliferation, but have also become clinically relevant for targeting tumors with deregulated ErbB signaling. The mechanisms and downstream effectors through which the ErbB receptors influence processes linked to malignant development, including proliferation, cell survival, angiogenesis, migration and invasion, are, however, only now becoming apparent. We are very interested in how ErbB receptors, in particular ErbB1 and ErbB2, contribute to processes linked to cancer

progression. I will discuss the role of ErbB receptors in tumor cell proliferation, migration, and induction of tumor vasculature.

22
Predicting breast cancer behavior by microarray analysis

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In the treatment of breast cancer, patient-tailored therapy is becoming increasingly important. Decisions on optimal treatment include the choice between mastectomy and breast-conserving treatment; dose of radiotherapy; and decisions on adjuvant chemotherapy and hormonal therapy. Gene expression profiling by microarray analysis allows the study of the level of expression of large numbers of mRNAs in a single experiment. Gene expression analysis can be used to subclassify tumors on the basis of hierarchical cluster analysis in specific subgroups; supervised cluster analysis can be used to directly link gene expression profiles to clinical characteristics, including prognosis and response to various forms of treatment. We have used microarray analysis, first on a series of 117 breast carcinomas and more recently on a series of 295 breast carcinomas. We have defined a gene expression profile of 70 genes that is predictive for a short interval to distant metastases.

23
The molecular biology of mammary intraepithelial neoplasia outgrowths

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Genetically engineered mice have been used extensively to model human breast cancer. Yet few have been developed and characterized to model the progression from hyperplasia to cancer. We have developed a transplantable model for mammary hyperplasia that progresses to invasive carcinoma, effectively mimicking human ductal carcinoma *in situ* (DCIS). As such, this provides a unique model for chemoprevention trials for high-risk premalignant breast lesions. These transplantable lines, also known as mammary intraepihelial neoplasia outgrowths (MIN-Os), were developed by our group and are derived from the mammary hyperplasia of mouse mammary tumor virus-polyomavirus middle T (mT) transgenic mice. The polyomavirus transgene provides an attractive model for human mammary carcinoma, as it is capable of transforming cells by triggering signal transduction pathways that have been implicated to be activated by erbB2, through interactions between its mT gene product and key cellular signaling proteins—such as c-Src, Shc, and phosphatidylinositol 3-kinase, which have all been implicated as important in human breast cancer. These transplanted lines are heterogeneous; however, within a line, through multiple generations, the MIN-Os show consistent growth rate, histopathology, and latency to tumor formation. The lines and the tumors that arise from them maintain their defining characteristics in 'tests by transplantation'. Histopathologically, the MIN-Os resemble human DCIS, and the resulting mammary invasive carcinoma resembles human invasive ductal carcinoma. With gene expression studies, we have found dysregulated genes and pathways that have been shown to be similarly altered in human DCIS, suggesting that our model is related to DCIS not only at a histopathological level, but also at a molecular level. These gene expression studies suggest the importance of stromal–epithelial interactions, the extracellular matrix proteoglycan-mediated regulation of cell proliferation signaling, actin cytoskeleton organization, and the insulin-like growth factors and their effectors in the transition from hyperplasia to transformed invasive carcinoma. We have begun using this human DCIS model for developing chemoprevention strategies for high-risk breast lesions. With *in vitro* assays, we have ranked inhibitors for effectiveness and inclusion in *in vivo* studies. We have demonstrated that inhibitors to phosphatidylinositol 3-kinase and related downstream mediators are effective in inhibiting growth. This mouse model provides an attractive platform that is amenable to interventional studies and chemoprevention preclinical trials, with easily measurable end-points for testing effectiveness of agents while providing tissue for correlative molecular studies.

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24
The comparative genetics and genomics of cancer: of mice and men

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Human tumors accumulate a remarkably diverse spectrum of recurrent genomic abnormalities thought to reflect functional reprogramming of the cancer cell phenotype. However, the causes and consequences of many of these abnormalities are unknown. We describe here several mouse-model-based approaches to functional interpretation of these aberrations. Specifically, we demonstrate that integration of information on recurrent aberrations in human breast tumors with information on regions of susceptibility in mice and/or recurrent genomic abnormality in breast tumors that arise in transgenic mice indicates regions of particular importance in human tumors. We also present evidence from analyses of genomic abnormalities in tumors that arise in 'RIP-Tag' transgenic mice that both the genetic background and the temporal dynamics of the initiating oncogenic event significantly affect the spectrum of abnormalities that arises during tumorigenesis.

25
Proof-of-concept *in vivo* evaluation of targeted therapeutics: lessons from immunoliposomes

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As part of a longstanding collaborative project within the Bay Area Breast Cancer Translational Research Program (UCSF Breast SPORE), we have continued to use nude mouse human breast cancer xenograft models to test our hypothesis that more effective and less toxic delivery of potent anticancer drugs can be achieved using immunoliposomes (ILs), consisting of receptor-internalizing monoclonal antibody fragments covalently linked to drug-encapsulated long-circulating liposomes (Ls). Our lead agent, anti-HER2 immunoliposomes containing doxorubicin (anti-HER2 ILs-dox), now approaches clinical testing after preclinical evaluation and optimization against multiple breast cancer xenograft models expressing high (+3), moderate (+2), or low (+1) levels of the HER2/ErbB2 growth factor receptor. The enhanced *in vivo* therapeutic index achieved by anti-HER2 ILs-dox over immunoliposomes containing doxorubicin (+/– Herceptin/trastuzumab) or free doxorubicin was found to be due to the greater intracellular drug delivery achieved by receptor internalizing ILs. A new bioassay measuring HER2 receptor internalization by ILs and performed *ex vivo* on breast tumor cells or explants appears capable of identifying a subset of HER2 overexpressing breast tumors that may not respond to some HER2 receptor-targeted therapeutics. Other standard breast cancer chemotherapeutics (e.g. vinorelbine, camptothecins) and investigational agents (e.g. ellipticine, hydroxamic acid inhibitors of histone deacetylase) have been similarly encapsulated and evaluated against these tumor xenograft models, all showing enhanced therapeutic efficacy by the targeted ILs>nontargeted Ls>free drug. Likewise, the modular versatility of this drug delivery platform has been proven by linking drug-encapsulated Ls with an epidermal growth factor receptor (EGFR)/HER1 targeting/internalizing antibody and demonstrating the significantly improved efficacy and specificity of anti-EGFR ILs against EGFR overexpressing human breast cancer xenografts.

26
Synergy between the erbB and transforming growth factor beta signaling networks: implications for molecular therapeutics in human neoplasia

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The overexpression and aberrant function of the epidermal growth factor receptor (EGFR) (HER1, erbB1) and its ligands in several human carcinomas have provided a rationale for targeting this signaling network with novel treatment approaches. Based on the structure and function of the EGFR, two anti-receptor strategies have been developed. The first strategy uses humanized monoclonal antibodies generated against the receptor's ligand-binding, extracellular domain. These antibodies block binding of receptor-activating ligands and, in some cases, can induce receptor endocytosis and downregulation. The second approach uses small molecules that compete with ATP for binding to the receptor's kinase pocket, thus blocking receptor activation and the transduction of post-receptor signals. In addition, these are effective in blocking ligand-independent intracellular signals that laterally activate the receptor. Data will be presented in support of the merits of using antibodies and small molecules in combination. The transforming growth factor beta (TGF-β) signaling pathway is also associated with metastatic tumor progression. Antibodies against TGF-β ligands, small molecule inhibitors of the TGF-β type I receptor (TβRI) serine/threonine kinase, and soluble TβRII:Fc fusion proteins are anti-signaling approaches in development. Data suggest that both the erbB and TGF-β signaling networks can synergistically contribute to tumor progression. For example, signaling by the Ras/MAPK pathway, downstream erbB receptors, has been reported to abrogate the antiproliferative effect of TGF-β in epithelial cells. Therefore, we have examined whether overexpression of HER2/*neu* (erbB2), a potent inducer of Ras/MAPK signaling, modifies the inhibitory effect of TGF-β against MCF-10A human breast epithelial cells. MCF-10A stably transfected with a HER2 expression vector retained TGF-β receptors. Exogenous TGF-β inhibited MCF-10A/HER2 cell proliferation and still induced both Smad2 translocation to the nucleus and pCAGA-Lux reporter activity. In wound closure and transwell assays, exogenous TGF-β induced lamellopodia and actin stress fiber formation and motility of MCF-10A/HER2 but not of control cells transfected with vector alone. These effects were blocked by addition of the phosphatidylinositol 3-kinase inhibitor LY294002, the p38Mapk inhibitor SB202190, and the MEK1/2 inhibitor U0126. The HER2 antibody Herceptin blocked TGF-β-induced motility but not Smad-dependent reporter activity. Infection with an adenovirus encoding a constitutively active TβRI mutant (T204D) induced motility of MCF-10A/HER2 but not control cells. In HER2-overexpressing cells, Rac1 and Pak1 were constitutively associated with HER2. TGF-β enhanced this association as well as MCF-10A/HER2 Rac1 activity as measured by Rac1 binding to a GST-Pak binding domain fusion protein. Thus, overexpression of HER2 unmasks the ability of TGF-β to induce epithelial cell motility. This effect is not limited to HER2 in that treatment of EGFR-amplified A431 squamous cancer cells with TGF-β also induces motility which is blocked by the EGFR tyrosine kinase inhibitor ZD1839. To follow these results, we have generated mouse mammary tumor virus (MMTV)/*neu* × MMTV/TGFβ1S223/225 bigenic mice. TGFβ1 delayed mammary ductal extension in the bigenics compared with MMTV/*neu* mice but mammary tumor latency was similar. Although the bigenic tumors were smaller and less proliferative, they exhibited a higher histological grade and were more metastatic than MMTV/*neu* tumors. Finally, TGF-β accelerated tumor cell intravasation in MMTV/*neu* × MMTV/TGFβ1 bigenic mice compared with MMTV/*neu* mice. These data suggest, first, cooperation between the erbB receptor and TGF-β signaling in promoting the metastatic phenotype of human breast cancer cells. Second, they imply that combined inhibition of multiple signaling networks in human cancer cells might be required in order to meaningfully alter their natural progression.

27
The mouse in preclinical trials: transgenic, carcinogen-induced, or xenograph models – which to use?

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Animal models have been extensively used to test promising new agents for the treatment and prevention of cancer. Many different animal models are available for preclinical testing, and the choice of the specific model to use is often a critical step in successful drug development. Many different mouse models of human breast cancer have been developed that have been used to test promising anti-cancer drugs. These include mouse models that spontaneously develop mammary tumors, carcinogen-treated mouse models, xenograft models, transgenic mice and gene knockout mice that develop mammary tumors. The particular strengths and weakness of these models for testing therapeutic agents will be reviewed. The most widely used preclinical models for testing agents for the treatment of cancer are xenograft models. Xenograft studies using human cancer cell lines are easily conducted, are relatively rapid and, importantly, are recognized by the Food and Drug Administration as providing evidence of preclinical anti-tumor activity against human cancer. However, certain studies cannot be done using xenografts of human cancer cell lines. These include testing of immune-based therapies or testing of cancer preventive agents. For such studies, other models are needed. Testing of novel vaccines against cancer requires an immunocompetent host, and thus may require vaccination against murine tumors or studies to be done in 'humanized' mice. Studies of cancer preventive agents requires xenografts of human normal breast tissue or carcinoma-*in-situ* lesions, or alternatively mouse or rat models that develop tumors, either spontaneously or after carcinogen treatment. One of the most commonly used models for testing chemopreventive agents has been the carcinogen-treated rat model. This model has been successfully used to demonstrate the chemopreventive activity of many agents, including selective estrogen receptor modulators (SERMs) such as tamoxifen, raloxifene, and idoxifene, and retinoid compounds, and is particularly useful for testing agents for the prevention or treatment of estrogen receptor-positive mammary cancer. More recently, transgenic mouse models have been used to study the activity of chemopreventive agents, particularly for the prevention of estrogen receptor-negative breast cancer. We have used two such transgenic mice (C3(1)-SV40 T antigen, and mouse mammary tumor virus [MMTV]-erbB2 mice) to investigate the preventive activity of receptor-selective retinoid compounds. Both of these transgenic mouse lines develop premalignant lesions that then evolve into invasive mammary tumors that eventually metastasize. We have found that 9-*cis*-retinoic acid, which binds both retinoic acid receptors and retinoid X receptors (RXRs), and the RXR-selective retinoid, LGD1069 (bexarotene, Targretin), suppresses the development of non-invasive and invasive mammary tumors in both C3(1) SV40 T antigen mice and MMTV-erbB2 mice. These retinoids interfere with tumorigenesis by suppressing proliferation of normal and premalignant mammary epithelial cells, ultimately suppressing the development of invasive cancer. Based on these results in these mouse models, we initiated a human clinical trial using retinoids for the prevention of human breast cancer, which is now ongoing. These results demonstrate the utility of genetically engineered mouse models for the testing of molecularly targeted agents for the prevention of breast cancer.

28
Transgenic models are predictive: the herceptin and flavopiridol experience

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Intact cyclin D_1 functions are essential for transformation by erbB2. We have used transgenic models, tissue culture experiments and human tumor samples to particularly address the role of cyclin D_1–cyclin

dependent kinase interactions in transformation by erbB2. The p16 tumor suppressor specifically blocks cyclin dependent kinase 4 (Cdk4) activity and blocked tumorigenesis by erbB2, demonstrating that deregulation of the cyclin dependent kinase partner of cyclin D_1 is an essential target of erbB2. We then investigated the uses of a candidate drug that inhibits Cdk4 in erbB2-mediated tumorigenesis. Individual drugs active against ErbB2 and cyclin D_1 (Herceptin and flavopiridol) were synergistically cytotoxic against ErbB2-positive breast cancer. The addition of flavopiridol to Herceptin synergistically lowered erbB2 levels in these cells. Our data suggest the potential use of combinations of cyclin dependent kinase inhibitors and Herceptin in breast cancer and form the basis for an ongoing trial of this drug combination.

29
Modeling breast cancer in the rat: a method to produce knockout rats

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The rat is an important murine model for biomedical research. It is a key model for many diseases including cardiovascular diseases, diabetes, neurological diseases, and certain cancers such as breast cancer. Through much work the rat genomic toolbox is almost full and includes dense genetic maps, an approximately sevenfold genomic sequence and transgenic technologies. A key missing tool was the ability to produce knockout rats. This was in part due to the fact that despite over a decade of work embryonic stem cells could not be made to go germline. In order to approach this problem we developed a unique technology that combined the use of germline mutagenesis with a yeast truncation gap repair assay to select gene-specific knockout rats. We first developed the protocols to efficiently mutagenize the rat genome with ethyl nitroso urea. In order to establish knockout rats for specifically chosen genes, we bred ethyl nitroso urea mutagenized males with untreated females to produce F1 pups. These pre-weanling pups were screened for functional mutations (e.g. nonsense mutations, out of frame-shifts mutations, etc.). This was accomplished using a highly efficient and economical yeast gap-repair truncation assay. For this assay either genomic DNA or cDNA is produced from tail clips of rat pups. This DNA is then used as a PCR template to amplify the targeted gene sequence. This unpurified PCR product together with our reporter vector is cotransformed into yeast that does not express ADE2. The yeast through homologous recombination clones the PCR product into the non-integrated ADE2 reporter plasmid. In scoring the yeast plates, a negative plate has mostly white large colonies and a plate positive for a knockout allele has one-half red colonies (rat) and one-half white colonies. We have used this technology to knockout two breast cancer suppressor genes, B*rca1* and *Brca2*. The technology described can be applied to most rapidly breeding species ranging from zebra fish to mice.

30
Genetically engineered mouse models of human breast cancer

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This presentation will discuss the strengths and weaknesses of currently available mouse models for mammary cancer. The major underlying premise is that we cannot understand, investigate, and hopefully cure breast cancer without animal models and that for a variety of biological, scientific, economic, and ethical reasons rodents represent the best available model systems for mammary cancer. This being said, how accurately do rodent models reflect the human condition and what are the characteristics of an ideal model system? Do they reflect the molecular complexity and stochastic nature of human breast cancer and do they represent an appropriate format for testing of newly developed agents that specifically interact with designated molecular targets? In addition, a case will be made for understanding the natural history of mammary cancer following treatment and that too often the development of a tumor is seen as an endpoint and not as a beginning. Finally, it is clear that we have a very poor understanding of precursor lesions in rodents and it will be critical to understand how these relate to fully developed malignant tumors before the full potential of these models can be realized.

31
Role of differentiation in carcinogenesis and cancer prevention

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Ductal carcinoma is the malignant disease most frequently diagnosed in American women. This cancer type originates in Stem Cells 1, epithelial cells that are the main components of the undifferentiated mammary gland. The susceptibility of Stem Cells 1 to become transformed has been attributed to their high rate of cell proliferation, their ability to bind and activate the carcinogen and their low DNA reparative activity. Epidemiological evidence indicates that a woman's lifetime risk for developing breast cancer is decreased by a full-term pregnancy at a young age. The protection conferred by pregnancy is the result of the differentiation of the organ. The reproduction of the hormonal milieu of an early full-term pregnancy by administration of human chorionic gonadotropin (hCG) to virgin rats induces lobular development, completing the cycle of differentiation of the breast that converts the highly susceptible Stem Cell 1 into a resistant Stem Cell 2 through a process of induction of a specific genomic signature in the mammary epithelium. Both pregnancy and hCG exert a protective effect on the mammary gland by reducing the incidence of 7,12-dimethyl-benz(a)anthracene (DMBA)-induced carcinomas. Although differentiation significantly reduces cell proliferation in the mammary epithelium, the differentiated Stem Cell 2 remains capable of responding with proliferation to given stimuli, such as a new pregnancy; it is also capable of metabolizing the carcinogen and of repairing DNA damage more efficiently than the Stem Cell 1. The basic biological concept is that the conversion of the susceptible Stem Cell 1 to a refractory Stem Cell 2 after pregnancy or hCG treatment of virgin rats results from the differentiation of the mammary gland, a phenomenon manifested at morphological, cell kinetic and functional levels. Morphological changes consist of a progressive branching of the mammary parenchyma and lobule formation, which in turn result in a reduction in cell growth fraction and lengthening of the cell cycle. The functional changes comprise increased synthesis of inhibin, β-casein and other milk-related bioactive peptides. In addition, pregnancy or hCG also increases the expression of programmed cell death genes, including TRPM2, ICE, p53, c-*myc*, and bcl-XS, inducing as well apoptosis, and downregulation of cyclins. Programmed cell death genes were activated through a p53-dependent process, modulated by c-*myc*, and with partial dependence on the bcl-2 family-related genes. Data generated using the cDNA microarray techniques have allowed us to demonstrated that while lobular development regressed after the cessation of pregnancy or hormone administration, programmed cell death genes remained activated, and new sets of genes reached a peak of maximal expression while others became downregulated, creating a genomic signature that is specific for both pregnancy and hCG treatment, but significantly different from those induced by the ovarian steroid hormones estrogen and progesterone. These mechanisms play a role in the protection exerted by hCG from chemically induced carcinogenesis, and might be even involved in the life-time reduction in breast cancer risk induced in women by full-term and multiple pregnancies. In addition, hCG inhibits the progression of DMBA-induced mammary tumors on the early phases of tumor progression (i.e. intraductal proliferation, *in situ* carcinomas and invasive carcinomas). These observations led us to infer that hCG, like pregnancy, induces early genomic changes that lead the mammary gland to full differentiation, and that these changes result in a permanently imprinted genomic signature that regulates the long-lasting refractoriness of the mammary gland to carcinogenesis. The permanence of these changes makes them ideal

surrogate markers of hCG effect in the evaluation of this hormone as a breast cancer preventive agent.

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32
Genetic determinants of susceptibility to estrogen-induced mammary cancer in the rat

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Estrogens are inextricably linked to the etiology of breast cancer. We have demonstrated that the ACI rat strain exhibits a unique propensity to develop mammary cancer when treated continuously with 17β-estradiol (E2). Treatment of ovary intact ACI rats with E2 results in virtually a 100% incidence of mammary carcinoma with a median latency of approximately 140 days. These mammary cancers are dependent upon exogenous E2 and exhibit genomic instability, a hallmark of breast cancers in humans. In contrast, the Copenhagen (COP) and Brown Norway (BN) rat strains are resistant to E2-induced mammary carcinogenesis. Susceptibility to E2-induced mammary cancer behaves as an incompletely dominant or dominant trait in crosses between the ACI strain and the COP or BN strains. Genetic linkage analyses of several hundred phenotypically defined F2 progeny from reciprocal crosses between the ACI and COP or BN strains revealed a total of seven genetic determinants of susceptibility to E2-induced mammary cancer on chromosomes 2, 3, 4, 5, 6, 7 and 18. The chromosome 5 locus, designated Emca1, determines susceptibility to E2-induced mammary cancer in reciprocal crosses between the highly susceptible ACI strain and the resistant COP or BN strains. Potential candidate genes residing within the Emca1 locus include Cdkn2a, Cdkn2b and Cdkn2c. Studies on Cdkn2a indicate that expression of the p16Ink4a protein product of the Cdkn2a locus is downregulated at an early stage of E2-induced mammary carcinogenesis in the ACI rat. We are currently evaluating these genes further to determine whether and how they contribute to mammary cancer etiology in this model.

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33
Transforming growth factor β's role in mammary gland development and carcinogenesis

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The pluripotent cytokine transforming growth factor β 1 (TGF-β) can inhibit epithelial proliferation, induce apoptosis and modulate stromal composition. Our studies indicate that epithelial TGF-β production and activity are differentially regulated by estrogen and progesterone during normal mammary gland development and that, in turn, TGF-β regulates epithelial apoptosis and proliferation in a hormone-dependent manner. Our observation that TGF-β activity co-localizes in estrogen receptor-positive cells and that TGF-β depletion increases their frequency and proliferation supports the conclusion that it is a functional determinant of cell fate decisions in response to hormones. Although TGF-β is characterized as a classic tumor suppressor, it can also promote cancer progression. We have shown that the TGF-β signal pathway is rapidly activated as a consequence of ionizing radiation exposure, which is a known breast carcinogen; its role in DNA damage response is not known. We found that mice heterozygous for deletion of the TgfB1 gene fail to undergo cell apoptosis and cell cycle delay in response to DNA damage. Furthermore, TgfB1$^{+/-}$ mammary epithelial cells fail to appropriately activate p53, indicating that the TGF-β ligand is essential for induction of rapid molecular responses to DNA damage that determine cell fate decisions. Thus, TGF-β action during DNA damage response supports its role as a tumor suppressor. Its loss during carcinogenesis would contribute to genomic instability and promote tumor progression, and in particular may be relevant to the genesis of estrogen receptor-positive tumors.

34
Hormonal interactions during mammary gland development

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Mammary morphogenesis is the result of the complex interplay of prolactin (PRL), estrogen (E), progesterone (P) and growth factors. The spatio-temporal patterns of hormone and growth factor action on the epithelial and stromal compartments during development and differentiation of the mammary gland give vital clues to cell fate. Concurrent with the morphological changes in the gland during puberty, progesterone receptors (PR) localize at early branch points. During peripubertal morphogenesis PR distribution shifts from a homogeneous to a heterogeneous pattern The Hox-related, homeobox containing gene, Msx2, is highly expressed during branching morphogenesis where our studies *in vivo* and *in vitro* show that its expression is regulated by P in the presence of E. The overexpression of Msx-2 in stable transfectants of the 'normal' mouse mammary epithelial cell line, NmuMg, results in a highly branched phenotype compared with control cells transfected with the empty vector (EV) when grown in collagen gels. The NmuMg-Msx2 cells constitutively overexpress cyclin D_1 and form multiple large colonies when grown in soft agar. When the NmuMg-Msx2 were implanted into nude mice either subcutaneously or in the mammary fat pad, rapidly growing tumors arise within 8 weeks in 97% of the mice compared with small, slow-growing tumors in 42% of animals given the NmuMg-EV cells. PRL, in concert with P, acts during ductal branching and alveologenesis within the mammary gland. As the animal matures, the distribution of the PRL receptor in the epithelium, like that of the PR, progresses from a homogeneous to a heterogeneous pattern, supporting our hypothesis that these hormones synergize to stimulate epithelial and stromal proliferation.

35
Function of LEF1 in early mammary development

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LEF1, a member of the LEF1/TCF transcription factors, is a component of the canonical Wnt-signalling pathway. The Lef1 knockout mouse

(Grosschedl) lacks hair, vibrissae, teeth, and mammary glands–all derivatives of the ectoderm. Experimental analysis of the role of LEF1 in tooth development has revealed an entirely non-cell-autonomous function–rather surprising for a component of a signal reception pathway. In fact, Lef1-deficient tooth development can be fully rescued by recombinant fibroblast growth factors, indicating that the sole function of LEF1 in this organ is to link Wnt- and fibroblast growth factor-signalling activities. Apparently, LEF1 is a crucial transcription factor regulating epithelial–mesenchymal interaction. Basically, a similar role of LEF1 was found in vibrissa (whisker) development but its function in mammary gland development remains enigmatic. Mammary buds do form in E11/12 mutant embryos, persisting (with variable penetrance) for 2–3 days, but no glands ever grow out. Although mutant mammary glands occasionally do develop in culture, no defined factors were found to be capable of rescuing mammary development. Our preliminary results indicate an additional function of LEF1 in mammary development, possibly in establishing mammary versus epidermal cell fate.

36
Expression profiling of breast cancer, influence of tumor genotype and patient genotype

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Breast cancers are heterogeneous and consist of several pathologic subtypes with different histological appearances of the malignant cells, different clinical presentations and outcomes, and the patients show a diverse range of responses to a given treatment. Furthermore, breast tumor tissue also shows heterogeneity with respect to its microenvironment including specifically the types and numbers of infiltrating lymphocytes, adipocytes, stromal and endothelial cells. The cellular composition of tumors is a central determinant of both the biological and clinical features of an individual's disease. We have performed expression studies of more than 300 breast carcinomas of different stages and histological subtypes, using high-density cDNA microarrays, aiming at novel tumor classification that can predict survival and treatment response. The expression patterns observed provided a remarkably distinctive molecular portrait of each tumor. The tumors could be classified into five novel subtypes (two luminal epithelial derived estrogen receptor-positive tumor subtypes, a basal epithelial-like, an ERBB2-positive group, and a normal breast-like group). Survival analyses showed significantly different outcome for patients belonging to the various subtypes, including a poor prognosis for the basal-like and a significant difference in outcome for the two luminal/estrogen receptor-positive subtypes. Differences in TP53 mutation frequency between the subtypes indicated an important role for this gene in determining the gene expression pattern in the various tumors. The frequency of the different genotypes of the codon 72 polymorphism of the TP53 genes was significantly different in the five subgroups identified by expression analysis, indicating that the TP53 genotypes also have an impact on the expression profile. Unequal distribution of the different genotypes of the CYP19 gene between the different subgroups was also observed. Cluster analyses of two published, independent data sets representing different patient cohorts from different laboratories, uncovered some of the same breast cancer subtypes. In the one data set that included information on time to development of distant metastasis, subtypes were associated with significant differences in this clinical feature. By including a group of tumors from BRCA1 carriers in the analysis we found that this genotype predisposes to the basal tumor subtype. Our results strongly support the idea that many of these breast tumor subtypes represent biologically distinct disease entities, and that both the patient's genotype and the tumor genotype have a strong influence on the expression pattern developed in a given tumor.

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37
Germline mutations in DNA repair and cell cycle checkpoint genes: consequential somatic gene alterations and genome instability

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Germline alterations in the BRCA1, BRCA2 and CHK2 genes were analysed in a series of breast cancer cases from the Icelandic population of 285,000 and risk was estimated. By screening 1172 cancer patients for the detected T59K sequence variant in the CHK2 gene, it was detected in a total of four breast cancer patients, two colon cancer patients, one stomach cancer patient and one ovary cancer patient, but not in 452 healthy individuals. The results suggest a tumor suppressor function for CHK2 in a small proportion of breast tumors and that the T59K CHK2 sequence variant is a low-penetrance allele with respect to tumor growth. A rare mutation was detected in the BRCA1 gene and a frequent mutation in the BRCA2 gene. The BRCA2 999del5 is classified as a founder mutation and the Icelandic population-based analysis gives an excellent opportunity to define the risk of this sequence variant for breast cancer and other cancer types. The BRCA2 999del5 mutation is present in 7% of unrelated breast cancer patients in Iceland, the prevalence increases with lower age of diagnosis and the estimated breast cancer risk in mutation carriers at the age of 70 years is 40%. A detailed analysis was done on the somatic events in tumors of 46 BRCA2 999del5 carriers with respect to genomic instability and gene alteration. Chromosome alterations were elevated and of specific architecture compared with sporadic cases, in line with the DNA/chromosome repair function of Brca2. An alternative mechanism of TP53 involvement seems to be specific to the pathogenesis of BRCA2 999del5 tumors. Furthermore, alterations at the FHIT gene located at the FRA3B locus with subsequent reduced expression seem to be of specific relevance in the BRCA2 tumors.

38
Using genomic similarities and differences to interpret mouse models of human development and cancer

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Comparative genomic approaches have been used extensively to identify DNA sequences involved in the regulation of conserved human genes. These approaches are indeed powerful, permitting the ~5% of the genome that is comprised of critical functional elements to be distilled from the background of non-conserved DNA. Using genome alignment tools, promoters, enhancers and other types of regulatory sequences can be identified, providing ready access to DNA sequence elements that are difficult to identify by other means. Comparative genomic alignments can also reveal the presence of novel genes. However, while most studies focus on sequences that are similar between the human and the mouse, the differences between the two genomes are also revealing, exposing different mechanisms of gene regulation and function and even gene-content differences in humans and rodents. Both the similarities and differences in genomic structure are critical to the interpretation of rodent models for human disease. We are using comparative genomic approaches to define genes and regulatory elements associated with imprinting and developmental disorders and susceptibility to cancer. In each case, comparative genomics approaches have been critical to identification of genes and regulatory elements that are central to development of disease-related symptoms in the animals. Differences in gene regulation and structure revealed by these studies will also aid in exptraloting results from these mouse models to similar diseases in human patients.

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A hybrid functional/anatomical imaging system for high-throughput planar projection mouse imaging

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Imaging the mouse has become a valuable adjunct to genomic and cancer research. Although tomographic techniques such as high-resolution positron emission tomography (microPET) and computed tomography (microCT) are extremely useful, there is also a need for the rapid assessment of mouse anatomy and function at very low radiation doses, with low cost and high throughput. A system was designed using a stimulable phosphor BaFBr imaging plate (commonly called computed radiography [CR]) as the detector. A planar emission image (I-125) is acquired onto one side of the 18 cm × 24 cm CR detector. Subsequent to the emission image acquisition, precise translation of the mouse platform over the CR plate allows the acquisition of an X-ray radiographic image onto the other side of the same CR plate. The image is then read out in a CR reader, which produces a 1770 × 2370 pixel digital image with 0.100 mm pixel pitch. The I-125 and X-ray images are extracted from the larger image, and are mechanically registered, allowing the functional I-125 emission data to be overlaid onto the anatomical X-ray image. Because the CR imaging plates and other required hardware are relatively inexpensive, it is possible that up to 20 mice could be imaged simultaneously using 20 imaging plates, limited only by how many mice can be safely anesthetized and monitored by the technician(s). Once the mice are safely back in their cages, the CR plates can then be read out and the images processed. The overall design of the dual imaging system will be discussed, and the results of a prototype system currently in our laboratory will be presented. Monte Carlo techniques were used to assess the X-ray-associated radiation dose levels with the hybrid imaging system, and the findings suggest that the radiation levels due to the X-ray procedure are far lower than for microCT. We conclude that for appropriate research applications such as monitoring tumor growth over time, or monitoring tumor regression due to therapy, the hybrid imaging system may provide useful tumor kinetic information with high spatial and temporal resolution, and using low radiation dose levels.

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Imaging mouse models of breast cancer with positron emission tomography

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Positron emission tomography (PET) is a nuclear imaging method that produces quantitative three-dimensional images of the distribution of positron-labeled radiotracers *in vivo*. Recent dramatic improvements in spatial resolution, and the development of dedicated small-animal PET scanners, now enable PET imaging studies to be carried out in mice. This allows the initiation, development and progression of cancer to be monitored longitudinally within individual animals. We will show examples of how PET is being used to track tumor development, and to detect early tumor response to chemotherapy. We will also show that potential therapeutics can be directly radiolabeled and how their biodistribution and concentration at the target site can be measured by PET imaging. The advantages and disadvantages of PET imaging compared with other imaging modalities will be discussed.

41
Imaging angiogenesis in early stages of breast cancer using a standardized radiolabeled adapter protein docked to vascular endothelial growth factor

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Tumor growth, local invasion, and metastatic dissemination are dependent on the formation of new microvessels. Angiogenesis is therefore a crucial event in tumor progression. In recent years anti-angiogenic agents have been developed as a novel approach to cancer treatment. Successful intervention with tumor angiogenesis can induce tumor vasculature regression, leading to a complete cessation of tumor growth. Clinically, however, anti-angiogenesis inhibitors have been used with marginal success. For the development of novel effective anti-angiogenic therapies it is of crucial interest, therefore, to be able to screen new treatments for both the effects on the tumor vasculature as well as the tumor burden itself. We have engineered the murine breast cancer cell line 4T1 to stably express the luciferase gene of the North American firefly. This allowed us to visualize tumor burden by *in vivo* bioluminescence imaging. The 4T1 mouse mammary carcinoma is derived from Balb/c mice and very closely models advanced stage (stage IV) human breast cancer in immunogenicity, metastatic properties and growth characteristics. Additionally, we have developed the adapter/docking tag system based on interactions between an 18–127 amino acid fragment of human RNase I and a 1–15 amino acid fragment of RNase I fused to a targeting protein. To visualize angiogenesis we applied to the docking system labeling vascular endothelial growth factor with ^{99m}Tc in 4T1 breast cancer tumor-bearing mice. In preliminary studies we were able to detect neovascularization in mouse breast cancer tumor nodules as small as 2–3 mm in diameter. We found that the ^{99m}Tc-labeled vascular endothelial growth factor complexes selectively and specifically bound to tumor neovasculature. We expect that ^{99m}Tc-Adapter, a broadly applicable and general humanized radionuclide imaging 'payload' module, can be readily employed for a non-destructive labeling of many targeting proteins armed with the docking tag. Availability of multiple imaging proteins might have tremendous implications for the development and evaluation of novel anti-cancer and, specifically, anti-angiogenic therapies.

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Ultrasound imaging of tumor perfusion

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Our goal is to evaluate the use of ultrasound to detect small regions of increased vascular density and altered blood flow and to quantify small changes in these parameters due to effects of new anti-angiogenic drugs. Regions containing intravascular contrast agents are identified using a new ultrasound strategy that combines subharmonic and phase inversion imaging. The contrast agent is repeatedly destroyed in order to estimate the time required for local replenishment. Parameters are estimated based on this strategy and include measures of the spatial extent of flow, the spatial integral of flow, and the time required for 80% replenishment. In a study of 25 tumors, we first demonstrate that regions of viable tumor as small as 1 mm, as verified by histology, can be detected and show similar morphology to images acquired with computed tomography (CT). The spatial mapping of vessels with ultra-

sound is superior to contrast enhanced computed tomography due to the intravascular distribution of ultrasound contrast agents. Estimation of the time to 80% replenishment was conducted on kidney and tumor data and is a robust parameter not altered by attenuation. Mean times to 80% replenishment of 1–5 s were estimated for the kidney cortex and mean times of 6–14 s were observed for viable tumor tissue. This broad range of replenishment times is indicative of abnormal tumor vascular density and tortuosity. Changes in flow parameters with anti-angiogenic therapy are significant beginning at 48 hours post-treatment.

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Advanced imaging and detection R&D in the Center for Biophotonics

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I will describe some of the activities taking place in our National Science Foundation-sponsored Center for Biophotonics Science and Technology. The mission of this center is the application of photonics to the grand challenges of biosciences and medicine. Some examples of these challenges include imaging of single biomolecules within living cells and development of non-invasive diagnostic and therapeutic medical devices. Our Center currently sponsors 20 different research projects that involve interdisciplinary researchers from 10 different collaborating institutions. These institutions are UC Berkeley, UC San Francisco, Stanford University, Mills College, Lawrence Livermore National Laboratory, UT San Antonio, Hampton University, Alabama A&M University, Fisk University as well as UC Davis. Projects focus on developing new technologies for imaging from the whole organism to the single molecule levels. These technologies include bioluminescence, non-linear optical, X-ray laser and free-electron laser microscopy. We are also developing optical sensors that can be used for monitoring the biochemistry of living cells as well as detecting diseases at the level of single molecule sensitivity. Of particular emphasis is our work on portable monitors for infectious biologic agents. We likewise are developing new medical device technologies based on photo-activated materials, laser fluorescence as well as scattering and absorption spectroscopy. I will also discuss our industry partnership programs as well as opportunities for new collaborations and partnering.

44
Preneoplasia in mouse models

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Premalignancy in mouse models has been studied for the past 50 years. These present as epithelial hyperplasia; several types of which occur in the mouse mammary gland depending on the initiating stimulus. Alveolar hyperplasias are frequently observed in models where mouse mammary tumor virus, hormones or chemical carcinogens are the initiating agents. Ductal hyperplasias are frequently observed in genetically engineered models where specific oncogenes have been deregulated. The premalignant nature of these lesions can be verified by transplantation into the mammary fat pads of either syngeneic or immunocompromised mice. The alveolar hyperplasias generate tumors that are frequently estrogen-receptor negative and diploid, although metastatic to lung. Estrogen receptor expression is downregulated early in premalignant progression, often when the hyperplastic lesion is first identified. The ductal hyperplasias progress through ductal carcinoma *in situ* and generate tumors that are frequently estrogen-receptor negative but the tumors are aneuploid. In recent years, premalignancy in genetically engineered mice has been extensively characterized at the biological level in p53 null mammary epithelium and in polyoma MT mammary gland, and to a lesser extent in the SV40LT antigen mammary gland. The molecular alterations in these lesions are beginning to be analyzed by array methodologies. These models provide the opportunity to identify early events causal in premalignant progression as well as to test the efficacy of agents that prevent progression.

45
Mammary intraepithelial neoplasia outgrowths (MINO): MINO and human ductal carcinoma *in situ*

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Mouse models of human mammary intraepithelial neoplasia have been described in a number of contexts. In general, descriptions of early stages of progression in genetically engineered mice that go on to develop invasive and even metastatic carcinomas are compared with human ductal carcinoma *in situ* (DCIS). In some instances, lesions with phenotypic similarity to human DCIS are compared, even without evidence of progression. The terminology for these processes in the mouse mammary gland requires precise definition. Otherwise, it is impossible to compare the variety of available models for study utility. Experimentally driven operational definitions are optimal. We have studied a series of transplantable mammary intraepithelial neoplasia outgrowths (MINOs) derived from the transgenic mouse mammary tumor virus promoter-driven polyoma virus middle T oncogene FVB mice. We have characterized their behavior experimentally and morphologically. Experimentally, these MINOs meet the most stringent criteria to be considered premalignant. They are capable of orthotopic growth in the gland cleared mammary fat pad, are without being capable of ectopic (subcutaneous) growth, and have a consistent transformation to the fully malignant behavior (capable of ectopic growth)—defined therefore as invasive carcinoma. Morphologically, there is a high degree of heterogeneity both within and between individual MINO lines. Nevertheless, all consistently transform to behaviorally and morphologically similar carcinomas. MINOs therefore compare closely with human DCIS. Human DCIS is similarly defined by its universal potential (if incompletely treated) to progress to invasive carcinoma, despite heterogeneity within a given breast or between individual patients. We extend the comparison using immunophenotypic analysis, and gene expression analysis. Overall, our goal is to better model human DCIS, in a reproducible and robust mouse model system suitable for interventional studies. By creating a serially transplantable series of MINOs we have removed much of the variability which would otherwise confound such study. Derivation of MINOs from a variety of genetically engineered mice backgrounds may provide additional critical reagents for the preclinical study of DCIS.

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Premalignancy in the human breast: how is it defined, is morphology good enough?

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With increasing sensitivity of methods of detection, microscopic lesions are being identified which produce a diagnostic dilemma for the pathologist. This is because their natural history is largely unknown. Some postmortem studies would suggest that what a pathologist would call a potential precursor lesion for invasive breast cancer may be present with an incidence as high as 40% in some age groups. Although we are now beginning to understand the spectrum of protein, RNA expression, gene mutations and epigenetic changes in breast cancer, this is a long way from being able to predict from a single lesion what its potential is to invade or metastasise. It is, however, reassuring that the recent advances in stem cell biology in the breast may assist in the identification of the potential target cells for carcinogenesis. These studies have relied on the use of classical markers for fully differentiated luminal and myoepithelial/basal cells. The current vogue to classify tumours on the basis of microarray data as 'basal-like' or lumenal may be leading us into

assumptions about histogenesis that are not proven. Whether these studies will assist in identifing preneoplasia will be discussed in relation to our current knowledge on the evidence for myoepithelial and lumenal cell differentiation in human breast cancer.

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In situ to invasive carcinoma transition: escape or release

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To identify molecular alterations involved in the initiation and progression of breast carcinomas in all cell types composing the breast, we generated Serial Analysis of Gene Expression (SAGE) libraries from purified epithelial, myoepithelial, endothelial cells, infiltrating leukocytes, and fibroblasts from normal breast tissue, and *in situ* and invasive breast carcinomas. Based on these SAGE data and follow-up studies using mRNA *in situ* hybridization and immunohistochemistry, we determined that dramatic gene expression changes occur not only in the epithelial (cancer) cells, but in various stromal cells as well. We found that the most consistent and dramatic gene expression changes during breast tumor progression occur in myoepithelial and myofibroblasts cells and the majority of aberrantly expressed genes encode secreted proteins or cell surface receptors. Interestingly the receptors for several chemokines upregulated in myoepithelial cells and/or myofibroblasts are expressed by epithelial cells. Moreover, we demonstrated that these stromal chemokines enhance the growth, migration, and invasion of breast cancer cells *in vitro*. Based on these data we propose that abnormal paracrine interactions between epithelial and myoepithelial/myofibroblasts cells, mediated in part by chemokines, may play a role in breast tumorigenesis and the progression of *in situ* carcinomas to invasive tumors.

48
Regulation of human mammary stem cells

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Breast epithelial stem cells are thought to be the primary targets in the etiology of breast cancer. Since breast cancers are predominantly steroid receptor-positive, we tested the hypothesis that normal human breast epithelial stem cells include a steroid receptor-positive population. Based on studies in hematopoietic and other tissues, we isolated a breast epithelial side population (SP) reported to be enriched in stem cells. Fluorescence-activated cell sorting analysis indicates that 5% of breast epithelial cells are SP in nature. Seventy per cent of SP cells lack markers of myoepithelial (CALLA) and luminal cells (MUC1), suggesting that they are undifferentiated. This is consistent with reports that breast stem cells are $CALLA^-/MUC1^-$ and express cytokeratin (CK) 19. In three-dimensional matrigel culture, SP (but not non-SP) cells produce branching structures similar to lobules. These structures comprise at least two cell populations expressing either CK14 (myoepithelial) or CK18 (luminal), suggesting that SP cells include a population with the potential to differentiate. Next, we analysed the relationship between steroid receptor expression, proliferation and CK19 expression. Results show that only 10–20% of breast epithelial cells contain receptors for estrogen and progesterone (ERa and PR), that these cells (70–80%) are in an intermediate/suprabasal position, are rarely proliferative and co-express CK19 providing evidence for ERa/PR expression by stem cells. These data are supported by the finding that SP cells are six times more likely to express steroid receptors than non-SP cells (60% versus 10%). Another population enriched for stem cells, the label retaining cells, express the putative stem cell markers Musashi-1 (Msi1) and p21CIP1, and are also ERa/PR-positive, although Msi1 and p21CIP1 are never co-expressed, suggesting that these molecules have separate functions in stem cell regulation. The data suggest that ERa/PR-positive human breast epithelial cells include a stem cell population and thus steroid receptor-positive breast cancers may arise from steroid receptor-positive stem cells present in the normal breast epithelium.

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Characterization, isolation and cultivation of distinctive human breast cell populations

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The adult human breast gland consists of a branched system of ducts and ductules essentially composed of an inner layer of luminal epithelial cells expressing keratins of simple epithelia and an outer layer of myoepithelial cells expressing keratins of stratified epithelia and a range of contractile filaments. With a view to understanding why some breast carcinomas appear as either basal-like, bimodal or stem cell-derived and others as strictly luminal epithelial-like, we set out to elucidate whether a hierarchy of epithelial differentiation could be demonstrated within the lineages of the human breast gland. For that purpose we have used primary cultures from reduction mammoplasties, immunomagnetic cell sorting and a set of markers for each of the major two lineages. Our initial observations on sorted primary cultures led to the conclusion that myoepithelial cells were lineage restricted in their differentiation repertoire while a subset of cells within the luminal epithelial lineage could convert to myoepithelial cells. Next, we included a three-dimensional reconstituted basement membrane assay in which luminal epithelial cells essentially formed spherical acinus-like structures while myoepithelial cells formed larger solid balls. In an effort to enrich for cells with a broader morphogenic potential we isolated and immortalized a cell type intermediate between luminal and myoepithelial cells, which could make relatively elaborate terminal duct-like structures and organize layers of both luminal-like and myoepithelial-like cells. Currently, we are expanding on our panel of cloned cell lines with normal cellular equivalents *in situ* as determined by immunochemical portraying. These cell lines are now being tested for their plasticity in culture to further approach a tentative hierarchical scheme for breast epithelial differentiation.

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Stem cells in normal breast development and breast cancer

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Although it has been postulated that the epithelial components of the mammary gland arise through differentiation of a stem cell compartment, the isolation and characterization of these cells have been limited by the lack of defined cell surface markers and the development of suitable *in vitro* culture systems able to maintain these cells in an undifferentiated state. We have developed an *in vitro* culture system in which primary human mammary epithelial cells, isolated from reduction mammoplasties, are cultured as 'mammospheres' on non-adherent surfaces. We have demonstrated that mammospheres are highly enriched in undifferentiated cells capable of both self-renewal as well as differentiation into the three lineages of the mammary gland: myoepithelial cells, ductal epithelial cells, and alveolar epithelial cells. Affymetrix-based gene arrays demonstrate a significant overlap between genes expressed in mammospheres and those previously described in hematopoietic, neuronal, and embryonic stem cells. Utilizing these systems, we have found that both leukemia inhibitory factor as well as NOTCH play a role in cell fate determination. Whereas leukemia inhibitory factor affects primarily stem cell self-renewal, Notch signaling affects both self-renewal of stem cells as well as lineage-specific commitment of mammary progenitor cells. We hypothesize that mammary stem cells or their immediate progeny are targets for transformation during carcinogenesis. Normal stem cells and carcinoma cells share

many characteristics, including self-renewal capacity, telomerase expression, ability to differentiate, resistance to apoptosis, and ability to home to specific sites. Important events in transformation may involve disregulation of pathways that control normal stem cell self-renewal, such as Notch, Wnt, LMO4, PTEN, and BMI1. In addition, the phenotypic heterogeneity found in human breast cancers best fits a stem cell model in which transformed stem or progenitor cells undergo aberrant differentiation. Using flow cytometry, we have identified a small population of cells within primary or metastatic human breast cancers that bear the cell surface phenotype $CD44^+CD24^-$/lowESA+$Lineage^-$ that have properties of tumor stem cells. As few as 200 of these cells are able to generate tumors in NOD-SCID mice, while the vast majority of cells in these tumors that lack this phenotype are incapable of tumor formation, even when tens of thousands of cells are injected. Consistent with a stem cell model, the tumorigenic stem cells generate tumors that recapitulate the phenotypic heterogeneity found in the original tumors. Current therapies have been developed by virtue of their ability to induce tumor regression and may selectively target more differentiated cells in tumors, while leaving the tumor stem cell population intact, accounting for treatment resistance and relapse. More effective therapies will require the targeting and elimination of the tumor stem cell population in breast cancer patients.

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Functional characterization of mammary stem cells in development and breast cancer

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Breast cancer is a genetically and clinically heterogeneous disease. Whether different target cells contribute to this heterogeneity, and which cell types are most susceptible to oncogenesis is still not well understood. Identifying mammary cell lineage markers is a prerequisite for elucidating the function of stem cells in mammary development and tumorigenesis, and especially for understanding preneoplastic progression. Our laboratory has used genetically engineered mice coupled with fluoresence-activated cell sorting analysis and transplantation into the cleared mammary fat pad, as a model system in which to isolate and characterize functional mammary progenitors and stem cells. Taking advantage of approaches similar to those employed to isolate and characterize hematopoietic and epidermal stem cells, we identified a population of self-renewing, label retention cells, which excluded Hoechst dye and were present on fluoresence-activated cell sorting analysis as side-population (SP) cells. The SP cells and label retention cells represented approximately 0.5% of mammary epithelial cells in the immature mouse. DNA microarrays have been employed to determine the gene expression profiles of these SP cells in comparison with the non-SP population. Approximately 75% of the SP cells expressed stem cell antigen-1 (Sca-1). Sca-1 cells have been localized to the terminal end buds of growing ducts and as few as 1000 $Sca\text{-}1^+$ cells were able to generate mammary gland outgrowths containing luminal, alveolar and myoepithelial cells. In addition, no outgrowths were observed in Sca-1–mammary epithelial cell transplants. In different genetically engineered mice mammary tumor models Sca-1 expression levels, as well as several other putative markers of progenitors, including keratin-6, have dramatically altered expression profiles. For example, Wnt-1 tumors express these markers and contain at least two populations of tumor cells, epithelial cells and myoepithelial cells that share secondary mutations such as loss of p53 and Pten, implying that they arose from a common progenitor. Mammary tumors arising in transgenic mice expressing β-catenin and c-*myc*, downstream components of the canonical Wnt signaling pathway, also contain a significant proportion of myoepithelial cells as well as cells expressing keratin 6; however, progenitor cell markers and myoepithelial cells are lacking in mammary tumors from transgenic mice expressing Neu, H-Ras or polyoma middle T antigen. Interestingly, K6 expression was also detected in the ductal epithelium of C/EBPβ null mice, suggesting that germline deletion of this bZIP transcription factor alters mammary epithelial cell fate. These data suggest that the genetic heterogeneity observed in breast cancer results from the activation of specific oncogene and/or tumor suppressor-regulated signaling pathways in specific mammary progenitors. Finally, recent studies in several other laboratories have identified a comparable population of multipotent stem cells in the normal human breast as well as a population of tumorigenic stem cells in some breast cancers. Understanding the differences between normal and cancer stem cells may provide new therapeutic targets for the treatment of breast cancer.

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Prevention of breast tumor angiogenesis and metastasis by cytostatic molecules in relevant mouse models

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Human breast cancer cells are capable of secreting a variety of growth factors which have autocrine and paracrine functions such as fibroblast growth factor 2, platelet-derived growth factor and transforming growth factor beta. Modulations of the synthesis of these paracrine growth factors by non-toxic therapeutic agents has not been fully investigated. Sodium phenylacetate (NaPa), a physiological metabolite of phenylalanine, has an antiproliferative effect against several human cancer cell lines. We have previously shown that NaPa exhibits powerful anti-tumor activity against breast cancer MCF7-ras xenografts in nude mice. Moreover, in this model, we have observed that NaPa can prevent tumor recurrence after tamoxifen treatment. Although glutamine depletion was proposed as a mechanism underlying NaPa growth inhibition, protein prenylation, regulating cellular function of p21/ras, may also be inhibited by NaPa. We showed that NaPa modifies the synthesis of growth factors secreted by MCF-7 and MCF-7ras tumor cells leading to cell proliferation inhibitions. This could explain *in vitro* and *in vivo* NaPa inhibition of MCF-7ras cells, which secreted higher levels of these growth factors. It was hypothesized that inhibiting tumor angiogenesis will halt tumor growth and decrease metastatic potential. Anti-angiogenic agents targeting the tumor vasculature are expected to block the neovascularization and thereby prevent metastasis. We previously showed that carbomethyl benzylamide dextran (CMDB7) prevents tumor growth and tumor angiogenesis by binding to angiogenic growth factors, thereby preventing them from reaching their receptors on tumor or stromal cells. We showed that CMDB7 inhibited neovessel formation within the fibroblast growth factor 2-enriched matrigel in mice, and its anticancer effect was then tested in a metastatic breast cancer model. Human MDA-MB435 cells were injected into the mammary fat pad of nude mice, and breast tumors developed within 1 week; all the mice had lung metastases at 12 weeks. CMDB7 treatment for 10 weeks reduced the incidence of the lung metastases to 12%. Histological analysis showed markedly less tumor neovascularization in the CMDB7-treated mice. We studied the uptake of CMDB7 labeled with ^{99m}Tc in MCF-7ras-tumor-bearing mice. The blood clearance of ^{99m}TcCMDB7is rapid and the liver, speen and kidney uptakes are weak. Our results confirm the non-toxicity of CMDB7 and the usefulness of CMDB7 in cancer therapy by targeting breast tumors. Associations of CMDB and NaPa show a synergestic antiproliferative effect on the MCF-7ras cell line. We have synthesized new molecule esters of CMDB with phenyl-acetic acid. These new molecules, called NaPaC, are 100-fold more efficient on the *in vitro* growth of these cells. NaPaC inhibits the MCF-7ras tumor growth in nude mice 10-fold more than the parental molecules. Moreover, NaPaC have a strong anti-angiogenic effect on the MCF-7ras tumors. This anti-angiogenic effect

is associated with a strong necro-apoptotic effect both *in vitro* and *in vivo*. Taken together, these results show that new cytostatic atoxic molecules, in associations, can be used as anti-tumoral, anti-angiogenic and anti-metastatic therapies in breast cancer.

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53
Immunotherapy of cancer

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The use of appropriate mouse models in tumor immunotherapy is a crucial part of preclinical studies; indeed, without convincing 'mouse data', clinical studies usually do not ensue. However, there are problems in extrapolating from the mouse to the human; these will be separately considered for antibody-mediated and cell-mediated immunotherapy.

Antibody-based immunotherapy Monoclonal antibodies can be passively given to Scid mice bearing human tumors; the monoclonal antibodies may require complement and Fc receptors to mediate their effect, or act by inhibiting or stimulating signaling, leading to apoptosis. If complement is crucial, the mouse is not always the best model and additional complement may need to be provided. In preclinical studies in cancer, active immunization to produce only antibodies is not often done; although we have an example where such antibodies were ineffective in murine tumor models, but are apparently effective in humans.

Cellular immunity In contrast to passive antibody administration, most efforts to examine cellular immunity involve direct immunization of mice, which examines: the antigen; the mode of delivery; and the immunogenicity of the antigen, including presentation by both class I and class II MHC molecules. Human antigens are 'foreign' to mice and the mouse H2 usually presents different peptides than does HLA. To overcome these problems, transgenic mice and tumors can be used if available—transgenic for tumor antigens and the MHC. If these are not available, mouse studies of an antigen are only a guide to immunogenicity. Adoptive transfer of human cells (either immune or non-immune T cells, dendritic cells, etc.) can be used to reconstitute mice, but this has inherent difficulties, as the infusion is a xenograft. In spite of all of the problems, useful preclinical data has emerged, and several examples of different modalities will be presented.

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Mouse models for pretesting of immunotherapeutic strategies for cancer patients

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There are two major reasons for using mouse models for preclinical testing of immunotherapetic strategies before proceeding to clinical trial. First, the requirements of regulatory authorities for toxicity testing and, second, the need of the investigator to convince himself/herself and the grant-giving bodies that proceeding with a clinical trial is scientifically justified. In both cases, the mouse model presents problems specific to a therapy depending on immune effector cells and their products. These are particularly evident in evaluating therapies for cancer patients, where analysis of cellular responses can often be better evaluated in *in vitro* studies with human peripheral blood leukocytes. However, where mouse models can show an effect on tumour growth, they can be extremely useful for evaluating the mechanisms underlying the effect, as we have found in evaluating tumour rejection of MUC1 expressing tumours. Moreover, strains carrying transgenes of human target antigens allow testing for auto-immunity.

Antibody-based therapies Preclinical testing in mouse models has been successfully translated into the clinic using a humanised version of the original mouse antibody against the c-erbB2 receptor (Herceptin). Clinical studies with a humanised MUC1 antibody have been approved and initiated, even though the data obtained in mouse models were equivocal. The use of antibodies to deliver toxic agents depends on the efficacy of delivery of the toxic material: when therapy depends on delivering high-dose radioactivity, imaging studies in patients can provide better preclinical testing than the mouse model.

Cell-based therapies Passive delivery of immune effector cells modified *in vitro* are in the pipeline for clinical testing. T cells modified with hybrid receptors (with extracelluar antibody sequences) to target tumour antigens, and dendritic cells loaded with tumour antigens are the focus of attention. Our own focus is on the MUC1 antigen in both cases. The use of the mouse model for the T-cell receptor studies may require the development of a parallel set of constructs appropriate to the mouse model, and *in vitro* studies with human peripheral blood leukocytes may be more useful. In the case of dendritic cells (DC), the mouse DC are taken from the bone marrow or spleen, whereas the source of human DC is either peripheral monocytes stimulated to differentiate into DC or $CD34^+$ cells isolated from patients treated with granulocyte–macrophage colony-stimulating factor. Nevertheless, much can be gained from the use of mouse models for evaluating DC-based approaches. Where specific antigens are being explored, mice transgenic for the antigen are preferred.

Active immunisation against tumour antigens This also includes the use of DCs loaded with specific antigens or tumour lysates. However, simpler delivery of the immunogen would be highly preferable since the good manufacturing practice facilities required for delivery of modified cells are expensive and sites are limited. DNA-based formulations provide several advantages. The sequences are easy to manipulate and production of immunogen can be relatively cheap. The studies in mouse models to be reported here have led to a small trial using MUC1 cDNA in breast cancer patients, and have allowed characterisation of important domains in the antigen and of the components of the immune system important for effective tumour rejection.

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Meta-analyses of transcriptional information: impact of data integration in understanding human breast cancers

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We have established a functional and comprehensive state-of-the-art expression array technology platform that supports large-scale investigations in human, mouse, rat, zebrafish, and *Schizosaccharomyces pombe* systems. Critical to its success is the establishment of a microarray database with high storage and computational capacity, development of standardized experimental protocols, novel analytical approaches, customized analytical tools, and design oligonucleotides for microarray expansion. The goal is that every expression array experiment performed can be cross-analyzed against another throughout the history of the institute and with data in the public domain. This data-dense platform has enabled a far-reaching and comprehensive genomics approach to the molecular and clinical characterization of human cancers. We have applied this approach to uncover underlying transcriptional control cassettes in human breast cancers, and to decipher potential regulatory pathways. Our results suggest that fundamental mechanisms can be inferred from these genomic observations that can be readily tested. Some of these examples will be discussed.

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VelociGene: a high-throughput approach for functionizing the genome via custom gene mutation and high-resolution expression analysis in mice

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Now that the genome has been sequenced, determining gene function presents the next major challenge. Many scientists agree that the most

powerful technologies for determining gene function involve genetic manipulations that knock out, replace, or overexpress gene products in mice so as to evaluate functional consequences. Unfortunately, even in the most sophisticated laboratories, such approaches still remain rather custom and low-throughput. I will describe a new set of technologies that allow for an unprecedented rate of generation of knockouts, knockins and transgenics—easily industrializable and scaleable to thousands per year. These approaches involve rapid manipulation of very large pieces of DNA (hundreds of kilobases in size), allowing the entire genome to be spanned by about 25,000 separate pieces of DNA. VelociGene has enormous flexibility, allowing the production of custom mutations with nucleotide precision, deletions of very large size, reporter knockins, transgenic overexpression, as well as conditional and complex alleles. Genetically modified mice produced via VelociGene are phenotyped using a variety of high-throughput approaches, ranging from high-throughput/high-resolution reporter gene analyses to localize the target gene with cellular resolution, to four-dimensional transcriptional fingerprinting via microarray. These approaches have identified and validated multiple targets in therapeutic areas such as obesity and diabetes, arthritis and cartilage growth, muscle atrophy, and angiogenesis and cancer.

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Genomic approaches to drug target discovery using mouse models

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Cancer results from the accumulation of somatic mutations of proto-oncogenes and/or tumor suppressor genes during carcinogenesis. An important source of somatic mutations in some animal cancer models is provirus insertion mutation. Such mutations can arise from either retrovirus infection or retrotransposition of active proviral elements present in the germline, and result in the disruption and/or dysregulation of genes at or near sites of new provirus integration. This phenomenon serves as the basis for an experimental strategy for identifying cancer genes, called provirus tagging, in which proviruses act as both mutagens and tags for the subsequent identification of mutated genes that participate in carcinogenesis. In the mouse, two retroviruses cause cancer by this mechanism; mouse mammary tumor virus and murine leukemia virus. The development of PCR-based methods for efficiently recovering host/virus junction fragments from new proviral integrants, as well as the availability of the mouse genome sequence and new bioinformatics tools, has recently led to a dramatic advance in the power of this experimental strategy. As a consequence, large-scale provirus insertion mutation screens are now ongoing in at least three biotechnology companies and two public research centers. These screens demonstrate that mouse provirus insertion mutation models are able to efficiently identify genes involved in human cancer. Moreover, they suggest that an unexpectedly large fraction of the genes in the mammalian genome can contribute to the process of carcinogenesis. Because provirus tagging allows the precise molecular discrimination between cause and consequence, provirus insertion mutation models provide a useful new filter for cancer drug target selection.

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Target discovery in the postgenomic era

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Genetic screens can reveal new pathway modifier genes that would be difficult to uncover using other experimental approaches. The conservation of biochemical pathways and ability to quickly screen large numbers of candidate target genes strongly supports the use of model system genetics. We have carried out large-scale genetic screens in *Drosophila melanogaster*, *Caenorhabditis elegans*, and cells to identify modifier genes of cancer-related pathways and phenotypes. Genetic screens can identify the function of novel genes, and establish functional links between genes that may have been previously identified, but whose role in a process was not understood. Invertebrate genetic screens are carried out in animals or cells with mutations in cancer genes that produce a measureable phenotype. In *Drosophila* we typically perform tissue-specific screens, in the eye or the wing, so that the cancer mutations do not affect viability or fertility of the organism. These sensitized genetic backgrounds are then screened to identify genes that modify the visible phenotype. Modifier genes can be identified through reverse genetic approaches, including transposon insertions and RNA interference. The availability of fully sequenced genomes and the use of reverse genetic tools such as RNA interference enables genetic screens to be focused on classes of proteins which are amenable to drug discovery, thus enhancing the efficiency of target identification. Genetic screens will help build a better understanding of signal transduction pathways and gene function on a large scale.

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Rational engineering of Fc/Fc receptor interactions to improve antibody potency

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Despite the growing clinical application of monoclonal antibodies as anti-cancer therapeutics, their ability to destroy targeted tumor cells is sub-optimal. We have leveraged our Protein Design Automation® technology to engineer antibodies with greater tumor killing power by augmenting the capacity of the Fc region to interact with the Fc gamma receptors (FcγRs) that mediate cytotoxic effector functions. A panel of Fc variants has been generated that display substantial enhancements over existing variants in both *in vitro* Fc/FcγR binding assays and cell-based ADCC assays. Our most promising candidates have been validated in the context of three approved anti-cancer antibodies, and characterized with regard to their specificity for activation versus inhibitory receptors, and their affinity for clinically relevant polymorphic forms of FcγRIIIa.

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From gene expression patterns to antibody diagnostics

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Applied Genomics Inc., through its collaboration with investigators at Stanford University, has targeted the production of over 650 polyclonal, affinity-purified, anti-peptide antibody reagents to candidate genes thought to distinguish biologically distinct subclasses of carcinoma. We have screened and validated these reagents by performing immunohistochemistry on proprietary tissue arrays constructed in-house using over 300 independent tumor samples from each of breast, lung, and colon carcinoma. We have used this database to identify proprietary antibody panels useful for distinguishing the diversity of cancer cases within tissue types and similarities between subclasses derived from different tissues. Individual reagents within these antibody panels are beginning to reveal staining patterns that distinguish novel subtypes of cells in normal tissues, and subclasses of tumors that transcend tissue origins. We are using our subclassification panels in collaboration with clinical trials groups and pharmaceutical companies to identify subclasses of patients that respond to existing therapeutics and novel therapeutics that are in development. The goal of this work is to identify biomarkers for patient selection both in late-phase clinical trials and for marketed therapeutics.

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Clinical applications of Affymetrix Genechip™ arrays for RNA and DNA analysis

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Scientists and clinicians are familiar with Affymetrix Genechip™ arrays being utilized predominantly in the areas of basic research, target dis-

covery, and target validation. Recently, however, studies are being conducted and published using this research use only platform in clinical research, including phase II and phase III clinical trials. During this presentation, two breast cancer studies will be reviewed. One study utilizes gene expression profiling for predicting therapeutic response to docetaxel [1]. The other study, loss of heterozygosity in breast cancer, utilizes our new DNA analysis array for mapping over 10,000 single nucleotide polymorphisms in the human genome.

Reference

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Combined hormonal therapy for advanced breast cancer in postmenopausal patients

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The primary therapeutic goal for patients with locally recurrent, locally advanced, or metastatic breast cancer is retarding the progression of cancer for as long as possible, while minimizing side effects and maintaining good quality of life. The majority of breast cancers have nuclear receptors for estrogen (ER) and/or progesterone (PR) and utilize estrogen as a major growth factor. Antagonizing estrogen to control tumor growth has been achieved in two ways: by competing for the binding site of the estrogen receptor with anti-estrogens (SERMS) or by removing estrogen by inhibiting its synthesis with aromatase (estrogen synthetase) inhibitors (AIs). Cancer cells develop resistance to SERMS such as tamoxifen (Nolvadex®) or toremifene (Fareston®) by increasing uptake of estrogens from plasma, by developing hypersensitivity to the residual level of ER stimulation and by increasing levels of estrogen synthesizing enzymes, such as aromatase, within the tumor cells as much as 3–10 fold. High *in-situ*, intra- and peri-tumoral estrogen levels may displace the SERM and cause clinical progression of the disease. In contrast resistance to AI therapy may also develop by causing hypersensitivity to residual and exogenous estrogens. Hence it has been hypothesized that a combination of a SERM and an AI (total estrogen blockade) might be the optimal way to treat hormone dependent breast cancer and prevent the emergence of resistance. Combining tamoxifen (SERM) with an AI (anastrozole) was one of three arms of the Phase 3 adjuvant ATAC study comparing tamoxifen to anastrozole and the combination. Interestingly the combination arm performed worst of all, the hypothesis being that in the low estrogenic environment created by the AI, tamoxifen exaggerates its known partial estrogenic signal. It has been hypothesized that if a SERM with less inherent estrogenicity than tamoxifen were combined with an AI the therapy would be more successful. Similar to letrozole and tamoxifen combined in another trial, a 27% reduction in plasma levels of anastrozole was also detected. BioMedicines is currently testing that hypothesis in a clinical Phase 3 trial combining the experimental and currently unapproved AI atamestane and the approved SERM toremifene and comparing that combination to the approved AI letrozole. Toremifene is clinically equi-effective against tamoxifen in treatment naïve patients, but is 40-fold less estrogenic in a low estrogen environment in preclinical models. Atamestane is a steroidal AI, which has shown a median time to progression of 8 months, with 10% of the patients progression-free at 36 months, in tamoxifen-resistant patients. It is well tolerated and may have advantages over the non-steroidal AI's in terms of efficacy and toxicity. Atamestane also does not interact pharmacologically with the toremifene. The control arm is letrozole, currently the most effective single agent endocrine therapy for postmenopausal women. Patients eligible for our study are required to have positive receptor status, have measurable tumor lesions and to have received the last dose of adjuvant hormonal therapy at least 12 months prior to enrollment. Our trial strategy is thus similar to the total androgen blockade approach to prostate cancer where this approach has been successful.

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CTLA-4 based therapy (MDX-010)

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CTLA-4 is a negative regulator of T cell activity. Antibodies to CTLA-4 have been shown to directly activate anti-tumor responses in a several syngeneic murine models. For less immunogenic murine tumors, anti-CTLA-4 treatment has anti-tumor activity when combined with vaccines or other therapeutic regimens. In order to explore the use of CTLA-4 blockade in human immunotherapy, we have developed and characterized an antibody to human CTLA-4 (MDX-010), derived from HuMAb mice, which blocks the binding of CTLA-4 to B7 ligands. MDX-010 was able to inhibit the growth of tumors in mice transgenic for human CTLA-4. Vaccination experiments and multiple dosing in primates were used to further define the activity and safety profiles of MDX-010. Several human clinical trials using MDX-010 have been completed and multiple trials are in progress; the clinical experience in these trials will be summarized.