Meeting report

Modeling Mammary Cancer in Mice Conference, Jackson Laboratory, Bar Harbor, Maine, 5–8 October, 1999

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Introduction

This was the second meeting held at the Jackson Laboratory devoted to developing improved preclinical mouse models for breast cancer. The previous meeting was held in October of 1997, and there has been substantial progress made in the field in the past 2 years. This year's meeting was preceded by a workshop coordinated by Gertraud Robinson [National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, Bethesda, Maryland, USA] designed to provide hands-on training in the many specialized techniques that have been developed to study the development of the mouse mammary gland. These biologic techniques, coupled with the use of transgenic and knockout mouse models, provide a powerful approach to dissect the mechanisms by which specific genes regulate normal mammary gland development and to identify the genetic alterations that are involved in the initiation and progression of breast cancer. Robert Cardiff (University of California at Davis, California, USA) also ran a pathology tutorial that discussed the consensus terminology and recommendations of a group of pathologists from a National Cancer Institute sponsored workshop held in Annaopolis, Maryland, USA in March 1999, entitled 'Comparative Pathology of Animal Models for Mammary Cancer'. Representative histology images can be accessed at http://mammary.nih.gov/atlas/histology/jaxworkshop/index.html.

The Keynote Address by Charles Daniel (University of California at Santa Cruz) provided an excellent historic perspective on the development of the normal mouse mammary gland and the pioneering efforts of a number of investigators at the Cancer Research Genetics Laboratory at the University of California at Berkeley who helped develop these techniques, which are so important for studying mammary gland biology. In addition, he emphasized the areas that require increased study in the future. These include studying the development of the embryonic mammary gland, developing techniques to manipulate the mammary stroma, incorporating information learned from other model systems such as *Caenohabditis elegans* and *Drosophila* into mammary gland biology, and finally investigating the biology of mammary stem cells and cell senescence. This year's meeting was especially timely because it coincided with a program just initiated by the US National Cancer Institute: The Mouse Models of Human Cancer Consortium.

Tumor suppressor genes

Bruce Ponder (University of Cambridge, Cambridge, UK) discussed the usefulness of mouse models in the study of genes that predispose humans to the development of breast cancer. He used the example of the BRCA2 exon 11 mutant mouse [1] to highlight the use of models in functional studies of known genes, and discussed strategies for the identification of modifiers of known genes in mouse models.

Barbara Weber (University of Pennsylvania, Philadelphia, USA) presented an overview of BRCA1 and BRCA2 structure, function, and roles in various cancers, with particular emphasis on the role played in DNA repair, and discussed the development of a model in which p53 mutation is required for cells to tolerate the loss of BRCA1. She also described new model systems for the study of BRCA1 function in yeast, animals, and human cell lines, and discussed risk factors affecting penetrance of BRCA1

C/EBP = CCAAT/enhancer binding protein; ETR = engineered transferrin receptor; ER = estrogen receptor; MION = monocrystalline iron oxide nanoparticles; MMTV = mouse mammary tumor virus; MRI = magnetic resonance imaging; NIDDK = National Institute of Diabetes and Digestive and Kidney Diseases; PrlR = prolactin receptor; rtTA = reverse tetracycline-controlled transactivator.

mutation and potential modifying genes such as GST-θ and NAT2. Dan Silver (Dana-Farber Cancer Institute, Boston, Massachusetts, USA) described reconstitution of BRCA1 function in a BRCA1-null cell line, and also studies detailing the subnuclear localization of the protein. Chuxia Deng (NIDDK) described a tissue-specific mutation of BRCA1 in mammary epithelium [2], leading to chromosome abnormality and tumorigenesis at low frequency after a long period. Introduction of a p53-null allele into these mice overcomes growth defects and promotes tumorigenesis, further indicating that BRCA1 mutation leads to development of a genetically unstable environment in which other mutations can occur and promote tumor development. Jean Feunteun (Institut Gustave-Roussy, Villejuif, France) presented data implicating BRCA1 and BRCA2 in the cellular response to oxidative DNA damage. Transcription-coupled repair of this damage is impaired in BRCA1- or BRCA2-deficient cells, suggesting another mechanism by which BRCA1/BRCA2 pathway defects may lead to genomic instability.

Daniel Medina (Baylor College of Medicine, Houston, Texas, USA) described studies in which p53-null mammary epithelium was transplanted into syngeneic wild-type mammary fat pads so that the effect of p53 absence in mammary tumorigenesis could be examined. Intact p53-null mice develop lymphomas and die very early. These studies showed that p53 absence is sufficient to promote mammary tumorigenesis, and that this process is enhanced by hormonal stimulation of the animals. Furthermore, most p53-null tumors are aneuploid, but this aneuploidy does not appear to result from aberrant centrosome regulation. This is inconsistent with observations in p53-null fibroblasts, highlighting the need to use the correct cell type in these mechanistic studies.

Angiogenesis and metastases

Judah Folkman (Children's Hospital, Boston, Massachusetts, USA) discussed the use of mouse models to identify new human angiogenesis inhibitors. Human tumors were grown in mice and those that were found to be inhibitory were then cultured to allow purification of the antiangiogenic factor. In this way, a new factor was discovered [3]. aaAT-III is a 53-kDa fragment of antithrombin and is a member of the serpin family of serine protease inhibitors. When four other serpins involved in clotting were analyzed, all were found to be cleaved to antiangiogenic factors.

The tumorigenicity of mouse mammary tumor virus (MMTV) varies depending on the strain of the virus. Tatyana Golovkina (Jackson Laboratory, Bar Harbor, Maine, USA) showed that the C3H/HeN strain has a low tumor incidence and is not metastatic, whereas the C3H/HeJ strain is both tumorigenic and metastatic. The difference between the strains was mapped to a region of the gag gene by creating hybrid viruses. In the C3H/HeJ metastatic tumors, increases in E-cadherin, β-catenin and α-catenin at either the messenger RNA or protein level were observed. This suggests that the gag region of MMTV may be involved in deregulating cellular signaling pathways to promote tumorigenesis and metastases.

Toshiyuki Yoneda (University of Texas Health Sciences center, San Antonio, Texas, USA) described a mouse model for studying bone metastases of breast cancer. Injection of MDA-231 breast cancer cells into the left ventricle of the hearts of nude mice leads to either osteolytic or osteoblastic bone metastases. Bisphosphonate, a specific inhibitor of osteoclast bone absorption, was able to suppress bone lysis by inducing apoptosis of the osteoclasts and breast cancer cells. Treatment with either a dominant negative transforming growth factor-β_2 receptor or an antiparathyroid hormone-related protein antibody also inhibited the growth of the bone metastases, demonstrating that growth factors in bone are important determinants of breast cancer metastases.

Carol MacLeod (University of California at San Diego) discussed the role of nitric oxide in tumor progression using the PyV-mT mouse model. By crossing these mice with $Nos2^{-/-}$ mice, tumor growth was delayed in a mixed background only. In an inbred background, the tumor burden did not change, but the number of metastases decreased. Cat2 is an arginine transporter that is important for production of nitric oxide by Nos2. A cross between the PyV-mT and $Cat2^{-/-}$ mice resulted in fewer and smaller lung metastases than $Cat2^{+/+}$ controls. Further work will characterize the role of Cat2 in metastasis and transplantation studies will determine whether the nitric oxide effect is autonomous in epithelial cells.

Heparanase degrades heparan sulfate proteoglycans and its levels are found to be elevated in the serum and urine of cancer patients. Yael Friedmann (Hadassah-Hebrew University Hospital, Jerusalem, Israel) described the cloning and functional characterization of murine heparanase. A 65-kDa precursor is cleaved to generate the 50-kDa active form of the enzyme. Heparanase was overexpressed in nonmetastatic T-lymphoma cells and then injected into mice subcutaneously, resulting in a highly metastatic phenotype.

Hormonal determinants

The role of steroid and peptide hormones in normal mammary gland development and prevention of breast cancer was discussed by several investigators. Paul Kelly (INSERM, Paris, France) presented an overview of the multiple functions of prolactin and the prolactin receptor (PrlR) that were elucidated by studying the PrlR knockout mouse. Mammary gland development and lactation were severely impaired in $PrlR^{+/-}$ and well as $PrlR^{-/-}$ mice. Interestingly, the heterozygote phenotype was much more

severe in a C57Bl/6 background than in 129SV mice, which could be rescued after a first pregnancy. Using mammary transplants and progesterone implants it was determined that progesterone can re-establish side branching in early pregnancy, but not lobuloalveolar development. This suggests that progesterone acts on ductal cells to stimulate side branching, and that prolactin may act later on alveolar precursors to facilitate lobuloalveolar development.

Jeff Rosen (Baylor College of Medicine) discussed how deletion of the transcription factor CCAAT/enhancer binding protein (C/EBP)-β also resulted in a severe inhibition of lobuloalveolar development and how C/EBP-β may regulate cell fate determination during mammary gland development. During normal mammary gland development the cellular distribution of the progesterone and prolactin receptors was unexpectedly altered from a nonuniform to a relatively uniform pattern in the ductal epithelium of C/EBP-$\beta^{-/-}$ mice. Unexpectedly, this coincided with a marked reduction in cell proliferation after acute stimulation with estrogen and progesterone. Steroid receptor-positive cells did not colocalize with proliferative cells, suggesting that in the normal mammary gland steroid hormones act via paracrine or possibly juxtacrine mechanisms to stimulate cell proliferation. These results in the mouse mimic those found in the normal human breast by Clarke *et al* [4].

Malcolm Pike (University of Southern California, Los Angeles, California, USA) discussed the mitogenic effects of progesterone on the human breast as reflected by breast density determined by mammography, and the need to modify the level and route of progestin administration currently used in hormone replacement therapy to decrease the risk of breast cancer. He also discussed a possible prevention regimen using a GnRH agonist and a low dose of ethinylestradiol.

Ken Korach (National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA) described the estrogen receptor (ER)-α and ER-β knockout mice and the crosstalk between growth factor and steroid receptor signaling pathways. ER-β knockout mice exhibited no significant differences in mammary gland development, whereas the ER-α knockout mice displayed severely inhibited ductal development. Differences were observed in the uterus and mammary gland with respect to the requirement for the presence of ER-α to facilitate mitogenic effects of growth factors. Proliferative effects of epidermal growth factor and insulin-like growth factor were not observed in the uterus of ER-α knockout mice, but epidermal growth factor and transforming growth factor-α still stimulated terminal end bud proliferation in the mammary gland. The ER-α knockout mice were capable of responding to progesterone, resulting in increased branching, and still developed mammary tumors when crossed with Wnt-1 transgenic mice. Crosses of ER-α knockout with *neu* transgenics displayed more dramatic effects on tumor latency and incidence than those seen with the Wnt-1 crosses [5].

Ruth Keri (Case Western Reserve University School of Medicine, Cleveland, Ohio, USA) presented a new mouse model of ovarian hyperstimulation due to overexpression of leutinizing hormone that resulted in ductal hypertrophy, but infrequent tumors. Finally, Lewis Chodosh (University of Pennsylvania, Philadelphia, USA) described the application of gene arrays containing known genes for the potential identification of those genes involved in parity-induced protection from breast cancer using the mouse as a model. This powerful approach has identified a number of gene clusters with temporal and spatial patterns of expression that make them excellent candidate biomarkers for parity-induced protection.

New approaches for modeling breast cancer in mice

Mouse models with immune defects have been created mainly for acquired immune deficiency research, but these mice can also be used as recipients for mammary gland or breast tumor transplants, as presented by Leonard Shultz (Jackson Laboratory, Bar Harbor, Maine, USA). By crossing existing knockout mice, it is possible to create more humanized mice with lowered levels of major histocompatibility complex class I, natural killer cells and complement, and increase the potential level of engraftment of human tumors. These engrafted mice can then be used to test new drugs or immunotherapies, study mechanisms of growth and metastasis, and identify modifiers of tumorigenesis.

A mouse model commonly used for mammary gland transplants and tissue-recombination experiments is the nude mouse (Whn$^{-/-}$). Ruth Baxter (Massachusetts General Hospital, Boston, Massachusetts, USA) presented data characterizing the mammary gland defect that results from a loss-of-function mutation in the Whn gene, a member of the forkhead/winged-helix class of transcription factors. The mammary glands of nude mice are only one-third the size of a normal gland during pregnancy, and nude mice are unable to lactate. Crossing nude mice with transgenic mice expressing Whn under the control of the promoter for the differentiation marker involucrin was able to rescue the lactation defect.

In order to delete genes in the mouse mammary gland specifically, two lines of mice have been created that express Cre recombinase under the control of mammary-specific promoters in the laboratory of Lothar Hennighausen (NIDDK). Two lines of MMTV-Cre mice, one of which is a germ-line integration, give mosaic expression in multiple tissues. A WAP-Cre mouse line, however, gives

mammary-restricted expression during late pregnancy that continues through involution. Both the MMTV-Cre and WAP-Cre mice were crossed with a mouse containing loxP sites flanking exon 11 in the BRCA1 gene to create a successful conditional mutation of BRCA1 in mammary epithelium, as already described [2].

Eran Andrechek (McMaster University, Hamilton, Ontario, Canada) discussed recent developments concerning the effects of erbB2/neu oncogene on mammary gland development and tumorigenesis. A knock-in of the neu complementary DNA into exon I of the deleted neu gene was able to rescue embryonic lethality of the erbB2 knockout, even though only 10% the level of wild-type neu protein was expressed. MMTV-Cre mice were then crossed with a floxed neo stop neuNT line to generate mice that conditionally express activated neu under the transcriptional control of the endogenous promoter. Surprisingly, elevated levels of the activated neu were observed in resulting mammary tumors, which contained amplified copies of the activated neu relative to the wild-type allele. Thus, this mouse model mimics for the first time the situation in human erbB2-positive breast tumors, in which amplification of erbB2 has been observed.

Lewis Chodosh (University of Pennsylvania, Philadelphia, USA) described another approach to generate a conditional transgenic model in which to study the effects of the c-*myc* oncogene on mammary tumorigenesis. His laboratory has successfully developed a tissue-restricted MMTV-driven reverse tetracycline-controlled transactivator (rtTA) transgenic mouse line that, when crossed with a TetO-c-*myc* mouse line, was able to generate mammary tumors when activated with doxycycline. This system was reversible, and upon removal of doxycycline established tumors regressed and displayed markedly decreased rates of proliferation, but there was little change in apoptosis. In previous studies using the MMTV-tet system in mice [6] the main pathology observed with the SV40 T antigen was in the salivary gland, and not in the mammary gland. Therefore, this MMTV-driven rtTA line developed by Chodosh *et al* should be a very useful tool for temporally regulating the expression of genes in the mammary gland.

Experimental prevention and therapeutics

Ronald Lubet (National Cancer Institute, Bethesda, Maryland, USA) discussed the efficacy of tamoxifen in chemoprevention trials, but stressed the need for development of agents effective against ER-negative lesions. He described successful chemoprevention studies in the C3(1) Tag mouse model, demonstrating that both dihydroepiandrosterone and 2-(difluoromethyl)-dl-ornithine are effective chemopreventative agents. Jolene Windle (Cancer Therapy and Research Center, San Antonio, Texas, USA) highlighted the importance of p53 status in response of tumors to different chemotherapeutic agents, and discussed the relative roles of cell cycle arrest and apoptosis in the tumor response. In the MMTV-*myc* mouse model, she showed that chemotherapeutic agents that induce cell cycle arrest are sufficient to cause tumor response, provided that some measure of apoptosis is naturally occurring in the tumors.

Richard Heyman (X-Ceptor Therapeutics, San Diego, California, USA) showed that in a carcinogen-induced breast cancer model, retinoid X-receptor agonists ('rexinoids') are as effective as tamoxifen in the prevention of mammary tumors and, furthermore, that rexinoids can cause regression of tamoxifen-resistant mouse tumors. This regression appears to be the result of altered proliferation and differentiation pathways. Powel Brown (Baylor College of Medicine) also demonstrated the efficacy of retinoid-selective retinoid compounds in chemoprevention studies in the C3(1) Tag mouse model, and emphasized that reduced toxicity can be achieved through the use of RXR-selective retinoids.

The use of photodynamic therapy in the treatment of mammary and salivary tumors arising in WAP-*ras* mice was described by Heinrich Walt (University Hospital, Zurich, Switzerland). Synthetic porphyrin-based photosensitizers are injected into the mice, and are more selectively taken up by tumor cells. After photosensitizer treatment, tumors are irradiated using a laser, which results in early apoptosis in the tumor, followed by necrosis in irradiated areas after 96 h. This presents a potentially new approach for tumor therapy.

Mario Colombo (Istituto Nazionale Tumori, Milan, Italy) discussed the utility of generating tumor vaccines using a syngeneic MMTV–neuT mouse model backcrossed into BALB/c mice in order to test for the presence of common tumor-associated antigens. Some cross-reacting antigens were observed that induced *in vivo* protection, but not a cytotoxic T-lymphocyte response.

Emerging technologies

Two presentations covered the use of magnetic resonance imaging (MRI) in visualizing a number of cellular processes, with an emphasis on the effective delivery of a molecular probe. James Basilion (Massachusetts General Hospital, Boston, Massachusetts, USA) outlined the use of MRI as a method to detect tumors or potentially monitor gene therapy. Use of an engineered transferrin receptor (ETR) complexed with monocrystalline iron oxide nanoparticles (MION) allows for sensitive detection by MRI, with the level of ETR expression correlating with MRI intensity. Injection of ETR + MION into the mouse tail vein allowed visualization of a tumor *in vivo*, due to preferential uptake of ETR by tumor cells. This application should be useful in helping tumor diagnosis by MRI. Alan Koretsky (National Institutes of Health, Bethesda, Maryland, USA) discussed

a number of novel uses for MRI, such as analyzing the embryonic phenotype of knockout or transgenic mice, measuring regional blood flow, determining the estimated mass of organs, and track tracing in excitable and olfactory cells. In addition to using MIONs in visualization, the use of manganese as an enhancing agent was also presented. An aerosol spray of $MnCl_2$ was used in conjunction with amyl acetate or pheremones to determine the regions of the olfactory bulb that were responsive to these signals.

A delivery system with great potential in the mammary gland field described by Margaret Neville (University of Colorado Health Sciences Center, Denver, Colorado, USA) is that of intraductal injections. A fine glass pipet with a tip of 60–75 μm in diameter can be easily made in the laboratory. The uses for up-the-teat or intraductal injection include the introduction of contrast or imaging agents for MRI, transduction of mammary epithelial cells with adenoviral constructs, or measurement of transmembrane permeability. Injected fluorescent probes can be directly visualized by confocal microscopy in the living mouse, allowing for time course studies to be performed. Introduction of an adenovirus-GFP or adenovirus-LacZ construct by this method has given promising results.

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