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The goal of this research training program is to produce highly qualified scientists for careers as independent investigators in the field of breast cancer. In the last 20 years, there has been a steady increase in the incidence of breast cancer. During the same period, there has been a fundamental revolution in the understanding of molecular and cell biological concepts related to cell growth, function and tumorigenesis. To utilize what has been learned and to continue future progress in the area of breast cancer requires the continued availability of well-trained, innovative and committed scientists. This program represents an interdepartmental training program involving thirteen investigators from six departments. Trainees are predoctoral fellows with backgrounds in biochemistry, cell and molecular biology, molecular genetics and molecular virology. The training program provides trainees with additional foundation in carcinogenesis and breast cancer. In addition to the core curriculum taken by the predoctoral fellows in their respective academic departments, program enhancement is provided through trainees' participation in a graduate course on "Molecular Carcinogenesis," a Breast Cancer Seminar Series, and participation at national meetings, and local seminars. Six predoctoral trainees are currently enrolled in the training program.

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Daniel Medina July 12, 1996

PI - Signature Date

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5. INTRODUCTION:

Breast cancer is a complex disease whose ultimate understanding will require the integration of facts resulting from a multidisciplinary approach. Continued basic science research will provide a fuller understanding of the basic mechanisms of breast cancer which is necessary to conquer the disease in humans. In order to have the scientific human armamentarium to further this understanding, this training grant focuses on producing qualified scientists for careers as independent investigators in the area of breast cancer. The rationale for a targeted training grant in breast cancer is based on the belief that the elucidation of how oncogenes, tumor suppressor genes, hormones and growth factors act at the molecular level and as developmental-specific agents are critical questions directly relevant to the etiology, prevention, diagnosis, treatment and prognosis of human breast cancer. The training program draws together individuals who have an established research and training background in the mammary gland with individuals who have a research and training background in cell biology, molecular endocrinology, molecular biology, molecular virology, viral oncology, molecular genetics and biochemistry. The strength of the program is two-fold. First, the program brings together members of diverse disciplines to focus on the training of predoctoral students for careers in an area which, by its biological nature, is multi-disciplinary. Second, the program brings new intellectual approaches and insights to the problem of breast cancer which will be continued by the next generation of research scientists.

The design of the training program provides for trainees to be exposed to clinical problems and recent advances as well as the multi-disciplinary approaches to answering fundamental questions related to breast cancer research. The familiarity and close proximity of the training faculty facilitate and encourage the development of a new generation of research scientists who will be able to understand the problem of breast cancer at a more complex level and from a multi-disciplinary orientation.

6. BODY

Six predoctoral students are supported by the training grant. The six students, their departmental affiliation, major advisor, thesis problem and an Abstract of their research is provided below:

a. Annette C. Hollman, Department of Molecular Virology, Dr. Janet S. Butel, "Wnt-1, int-2, MMTVLTR-ORF and p53 cooperatively in mammary tumorigenesis."

The working hypothesis of this research is that different oncogenes mediate discrete steps in mammary tumorigenesis and wild-type p53 counteracts those tumorigenic effects. The aims of this project are the following: (1) To transfect MMECLs of different p53 status with the mammary oncogenes *wnt* -1, *int* - 2, and MMTV-LTR-ORF, singly or in combination; (2) To determine if changes in outgrowth morphology or tumorigenicity correlate with the combination of oncogenes introduced or with the p53 status of the parental cell line; and (3) To determine if p53 mutations occur during the process of tumor formation by cells expressing mammary oncogenes.

During the past year, three experimental approaches were pursued.

Determination of the level of p53 expression in MMECL's: Most point mutations in p53 cause a conformational change which alters the immunoreactivity of the protein. Antibody Pab 240 binds only the mutant conformation and Pab 246 binds only the wild-type conformation. An immunoprecipitation/western blot assay was developed to quantitate both mutant and wild-type p53 protein. Recombinant GST-p53 protein expressed in *E.coli* was used as a standard on these Western blots to quantitate the amount of p53 present in cell lysates. SV40-transformed mouse embryo fibroblasts (B1a cells) were used, which overexpress wild-type p53, as a positive control. 40ng of p53 per mg total cellular protein can be precipitated from lysates of these cells with antibody Pab 246. No detectable p53 could be precipitated from lysates of FSK4 or FSK7 mouse mammary epithelial cells with either antibody. The limit of detection in this assay is 1ng p53 per mg total cellular protein; therefore, FSK4 and FSK7 cells contain at least 40-fold less p53 than B1a cells.

Preparation of mammalian expression vectors for transfection of MMECL's: The *int-2* cDNA was received in a sequencing vector from Dr. Harold Varmus, and subcloned into the mammalian expression vector pcDNA3. Restriction mapping and PCR analysis was used to confirm the identity of the new construct. Wnt-1 cDNA was also obtained from Dr. Varmus and is currently being subcloned into pcDNA3.

Optimization of transfection conditions for MMECL's: The conditions for transfection of FSK4, FSK7, and TM3 cells were optimized using a β-galactosidase expressing plasmid. The highest transfection efficiency, obtained with Lipofectin reagent, was between 0.1% and 0.2%. This is sufficient to perform the planned experiments.

b. Jeffrey M. Jones, Department of Molecular Virology, Dr. Lawrence A. Donehower, "The role of p53 in mammary tumorigenesis using a mouse model."

The progression of a normal cell or group of cells to what is defined as a tumor includes a wide variety of biological and genetic changes. Current tumor progression models describe this as a multi-step process whereby cells undergo a series of changes before becoming fully malignant. Among the most common genetic changes in all types of cancer is the loss or inactivation of the tumor suppressor gene, p53.

In order to specifically address the role of loss of p53 in breast cancer and how this affects tumor progression we have developed a mammary tumor specific mouse model. We have crossed our p53 deficient mice which are highly susceptible to a wide variety of early onset spontaneous tumors to *Wnt-1* transgenic mice. The *Wnt-1* transgenic mice carry a MMTV LTR driving mammary specific expression of a *Wnt-1* transgene. The ectopic expression of this growth factor results in the development of mammary tumors in these mice. The *Wnt-1* transgenic mice are a well established mammary tumorigenesis model. By crossing these two strains of mice we have established mammary tumorigenesis model. By crossing these two strains of mice we have established a new model in which we can specifically address the role of p53 in mammary tumorigenesis.

Our objective is to use these mice to address the role of p53 in the suppression of mammary tumor growth *in vivo*. We have already established that the loss or absence of p53 in our mammary tumors allows them to grow at a greater rate than those tumors which retain at least a single wildtype copy of p53. We are currently addressing two of the best known mechanisms by which p53 influences tumor growth and how each of these influences the rate of growth of our tumors. Specifically we are examining the role of p53 loss (1) in a reduction in the number of cells undergoing apoptosis in a tumor; (2) in increasing the genomic instability of tumor cells; (3) in increasing the proliferative capacity of tumor cells; (4) in increasing the invasiveness and metastatic potential of tumor cells; and (5) in increasing the angiogenic capacity of the developing tumor. The degree of apoptosis within the tumors is being assessed using both a DNA laddering and TUNEL assay. Proliferation is being measured by BrdU incorporation. Cells which have incorporated BrdU are being quantitated using both immunohistochemical and flow cytometric approaches. The degree of vascularization of our tumors is being assessed by immunohistochemical staining for Factor VIII. In addition to this we are looking at genes related to each of these phenomena and how their expression corresponds to p53 status, tumor growth and the degree to which a given mechanism may affect tumor growth.

Our data currently indicates that p53 deficient tumors have greater numbers of chromosomal abnormalities than those with functional p53 as determined by Comparative Genomic Hybridization (CGH). Our apoptotic analyses have indicated no significant difference in the percentage of apoptotic cells in our tumors in the presence or absence of functional p53. Initial estimates of proliferation within our tumors as indicated by BrdU incorporation suggest that there is a greater degree of proliferation in the absence of p53. This suggests that the primary factor for the acceleration of growth in the absence of p53 in this model tumor system is due to p53's role in inhibiting the cell cycle and not due to a decrease in apoptosis.

c. Sharon Bonnette, Department of Cell Biology, Dr. Daniel Medina, "Mechanism of TGFβ1 inhibition of mammary cell growth."

Transforming growth factor-β1 (TGF–β1) is widely known for its antiproliferative effects on most epithelial cells. The cellular growth advantage of some carcinomas, brain tumors, and melanomas is thought to derive, in part, from the resistance to the antiproliferative effects of TGF–β1. Thus in some systems, if TGF–β1 responsiveness of the normal or immortal epithelial cell is compared with the transformed counterpart, it is frequently noted that the former are growth-inhibition sensitive, whereas the latter are only partially or completely resistant to the antiproliferative effects. In our lab we have observed a similar finding in two cell lines generated from mouse mammary epithelial cells. An immortal cell line, FSK3, is sensitive to TGF–β1 growth inhibitory effects while its transformed counterpart, although still responsive, now responds in a growth stimulatory manner. Because of the differential responses in these two closely related cell lines, we are provided with a good system to study the changes in TGF–β1 signaling that occur during tumorigenesis.

The overall aim is to characterize the parental FSK3 cell line and its transformed derivative, TM3, in terms of the regulation of cell cycle molecules that may direct their noted differential responses to TGF–β1. One of my early objectives was to rule out whether differences in the levels of TGF–β1 type I, II, and III receptor levels in the two cell lines were responsible for the differential responses to TGF–β1. By ^{125}I- TGF–β1 radiolabeling experiments both cell lines exhibited the same receptor profile which led to the conclusion that the differential responses were most likely due to differences in the regulation of downstream molecules.

Downregulation of the synthesis of an early cell cycle molecule, CDK4, has been linked to growth inhibition by TGF–β1 in mink lung epithelial cells (Ewen et al. 1993). Thus, my second objective was to look at the regulation of CDK4 by TGF–β1 in both of these cell lines and see whether differential regulation of the protein levels of CDK4 and or CDK4 associated D-type cyclins could be mediating the individual responses. I have investigated CDK4 synthesis levels in TM3 and FSK3 cells treated with TGF–β1 by methods of ^{35}S methionine metabolic labeling and by western blot. Levels of CDK4 protein did not change in treated vs. nontreated cells.

In TM3 and FSK3, cyclin D1 synthesis was also unaffected by TGF–β1 treatment. Additionally, TGF–β1 treatment did not affect the levels of cyclin D1, D2, D3, or p27 association with CDK4. Although levels of the D-type cyclins did not change upon TGF–β1 treatment, TM3 exhibited a 4-fold higher amount of D1, an 11-fold higher level of D2, and a 2-fold higher level of D3 total protein and CDK4-associated protein. Last, CDK4 activities of treated cells did not change in response to TGF–β1. Since these results showed that CDK4 is probably not playing a role in mediating the TGF–β1 response, my next objective was to study CDK2 regulation.

Kinase assays were performed using histone H1 as a substrate for CDK2 immunoprecipitates. These experiments revealed no differences in kinase activity among treated and untreated cells. Since CDK2 associates with both cyclin E and A, it was hypothesized that perhaps total CDK2 activity was unaltered but the distribution of the associated activities of cyclin E and A could differ. Thus cyclin E associated kinase activity was also investigated. These experiments showed similar results of no alteration of cyclin E-associated-cdk2's ability to phosphorylate histone H1 in treated and untreated cells. Additionally, to be sure that the kinase assay could detect as little as a two-fold reduction in kinase activity, a titration of protein used in the cdk2 immunoprecipitations was performed. The results showed that the assay was able to detect a two-fold difference when the amount of protein was varied in the range of 0.25-1.0 mg.

A fourth objective has been to identify early genomic responses to TGFβ-1 in the FSK3 and TM3 MMEC. An early genomic response to TGF–β1 treatment in HaCaT and ovarian carcinoma cell lines is the upregulation of p21-cip mRNA. This response is p53 independent and is not seen in Mv1Lu cells. RPA analysis was performed to investigate this response. Results showed that there is no regulation of p21 at this level by TGF–β1. More experiments will be done to also investigate the expression of junB and c-myc mRNA in response to TGFβ-1. JunB upregulation and c-myc downregulation have been shown to be classic early responses to TGF–β1 in many different cell types.

From the data gathered in these studies, it seems that their are no detectable effects on various cell cycle proteins in TGF–β1 treated FSK3 and TM3 MMEC. It has been postulated by Alexandrow and Moses (Cancer Research 55, 3928-3932, 1995) that some cells may retain sensitivity to the effects of TGF–β1 just up to the G1-S transition, and that this sensitivity to the antiproliferative effects do not require stimulation or inhibition of gene expression. Instead TGF–β1's action may be mediated through posttranslational events that are directly involved in regulating the formation and activation of complexes required directly for the act of DNA replication.

d. Steven Chua, Department of Cell Biology, Dr. Ming-Jer Tsai, "Development of a bitransgenic mouse system to study oncogene function."

Transgenic mice technology have been utilized to study the tumorigenic potential of oncogenes in the mammary gland. However, the constitutive expression of these oncogenes does not allow for the detail analysis of the initiation or progression events in tumorigenesis. To render these studies possible, it is thus necessary to develop a regulatable system to control the expression of the oncogene(s).

Towards this goal, our lab began the generation of a novel bitransgenic system that is capable of inducing target gene expression. This system consists of two lines of mice, a transactivator and a target. The transactivator carries a regulatable chimeric transcription factor under the control of a tissue specific promoter. This regulator has three functional domains: a VP16 activation domain, a Ga14 DNA binding domain and a modified progesterone receptor ligand binding domain responsive to exogenous antiprogestins (i.e. RU486) but not to progestins.

The target carries a gene under the control of four Ga14 regulatory elements upstream of a minimal promoter. Both the monogenic transactivator and target lines should be silent by themselves. When these two lines are crossed to generate bitransgenic mice, the regulator can then induce the expression of the target only when RU486 is administered.

The regulator was placed under the control of the MMTV promoter to target expression to the mammary glands. After verifying the functionality of the regulators in transient transfection studies, transgenic mice were generated. Three transactivator lines were obtained that express the regulator in the mammary glands detectable by northerns. These lines are currently being mated to the *int-2* target mice kindly made available by Dr. Phil Leder. The role and timing of the expressed *int-2* target oncogene in mammary tumorigenesis in the resulting bitransgenic mice will be investigated. The ability of our regulatable system to control not only the timing but also the level of expression of the *int-2* oncogene will provide the means to generate a developmental and temporal specific model for mammary tumorigenesis.

e. Esha A. Gangolli, Department of Cell Biology, Dr. Bert W. O'Malley, "Generation of a progesterone receptor transgenic mouse model."

The role of estrogen and progesterone receptors in mammary gland development and carcinogenesis has been studied widely. However, since progesterone receptor (PR) is induced on estrogen treatment, the relative contributions of the estrogen receptor (ER) and PR are unclear. Null mutant mice deficient for ER show defects in ductal morphogenesis of the mammary gland, whereas mice that lack PR show reduced ductal branching and no lobuloalveolar development. Therefore it is hypothesized that PR plays a secondary role relative to ER in mammary development. It is also unclear as to the relative importance of PR localized in the epithelium versus the stroma. To clarify this issue, it is necessary to identify the temporospatial expression of PR protein in the mammary gland. Due to the structural complexity of this gland, conventional analysis by Western analysis or immunocytochemistry is not very efficient.

The generation of a PR-reporter gene transgenic mouse will enable cellular resolution of the pattern of PR expression. A transgenic construct consists of about 3 kb of the mouse (Balb/c) PR promoter and part of the coding region (upto exon 2) fused in-frame to the bacterial β-galactosidase gene (lacZ). This construct contains a nuclear localization signal derived from the SV40 T antigen, and will therefore be able to detect transgene expression against any cellular background staining. The transgene will be excised from the plasmid backbone and microinjected into the male pronucleus of a one-cell mouse embryo, and injected embryos will be transferred to a pseudopregnant foster mother for further development. Lines of mice will be bred for stable transgene integration. Transgene expression in these lines will be monitored by genomic analysis.

The generation of this transgenic mouse model will enable the precise localization of PR in the mammary gland (as well as other tissues such as uterus, ovary, brain, bone etc.) both during development and carcinogenesis. In future experiments, the PR null mutant mouse will

be bred with this transgenic mouse to determine if there are any subtle structural consequences of PR ablation in these tissues.

f. Deana Roy, Department of Cell Biology, Dr. Jeffrey M. Rosen, "Molecular markers for terminal end buds in mammary gland development."

The purpose of this study is to identify molecular markers of Terminal End Bud (TEB) cell populations and to uncover factors which are important for virgin mammary gland development in the rat. Differential Display PCR (DD-PCR) was used to identify candidate markers. DD-PCR was performed with tissue from 45 day old nulliparous Wistar-Furth rats. Total RNA from the End bud (E), Mid gland (M) and Stroma (S) of the mammary gland was isolated and reversed transcribed into cDNA. DD-PCR has been carried out on the three tissue mRNA samples (E, M and S) with 16 different primer sets.

Fourteen cDNAs have been subcloned, sequenced and compared to GenBank sequences. Five of the End bud Differential Display (EDD) clones; EDD-C6, EDD-G5, EDD-C3, EDD-C14, EDD-C15 are similar to expressed sequence tags from cDNA libraries (human fetal brain, fetal spleen, ovary, adult brain and fetal cochlea respectively). EDD-C17 is cytochrome C oxidase, a mitochondrial enzyme involved in energy metabolism. EDD-C16 is similar to calcium binding protein Cab45. EDD-G6, G7, C11 and C18 have no similarities to sequences in the databank.

EDD-C2 has 85-90% identity to p190-B, a new member of the RhoGAP family. RhoGAPs are GTPase Activating Proteins (GAP) which aid in the catalyzation of GTP to GDP by Rho GTPases. Work is in progress to determine the spatial and temporal expression pattern of p190-B. Preliminary immunofluorescence experiments with a polyclonal antibody suggest that p190-B is preferentially expressed in the TEBs, and stroma but not the alveolar buds of the virgin mammary gland. However, two additional antibodies against unique peptide sequences of p190-B are being generated to confirm this result.

EDD-C12 is rat adrenomedullin. Adrenomedullin is a 52 amino acid peptide factor which can induce vasorelaxation, bronchodilation and stimulate proliferation in an autocrine manner. Immunohistochemistry was performed with rat mammary glands from various time points of mammary development. Adrenomedullin was localized to the cytoplasm of epithelial and stromal cells of the virgin gland. Staining was most prominent in the epithelium of the TEB and in cells surrounding the ducts. However, staining was also detected in the alveolar buds, stroma, blood vessels and lymph node. Interestingly, in the 18 day pregnant gland adrenomedullin was localized to the nucleus and cytoplasm of alveoli and cells of the stroma. This nuclear and cytoplasmic staining was also observed at time points during lactation and involution but to a lesser extent.

The expression of these clones will be determined by RNAse Protection Assays (RPA) or by RT-PCR. Additionally, *in situ* PCR will be performed with clones which are found to be preferentially expressed in the TEB by RT-PCR or RPA. *In situ* PCR will be used to localize mRNA expression in the virgin mammary gland. Additionally, the newly generated

antibodies to P190-B will be affinity purified and used for immunoprecipitation experiments as well as immunohistochemistry and confocal microscopy to localize protein expression during mammary gland development.

7. CONCLUSIONS:

The training program in breast cancer is functioning as planned. Six students are currently enrolled and actively involved in their research phases of their training program. The breast cancer journal club meets every two weeks and it is anticipated that the involvement of additional laboratories will develop with the increased number of research projects on breast cancer due to new funding. In this past year, the laboratories of Dr. Wade Harper, Biochemistry and Dr. Peter Hornsby, Cell Biology have participated in our journal club. Both are recipients of research grants from DOD. Additionally, two outside guest speakers, Drs. Gilbert Smith, National Cancer Institute and Barbara Weber, University of Pennsylvania, presented seminars and met with our students. This expansion should provide even greater exposure to our students to emerging problems in breast cancer.