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TITLE: Identification of Novel Genes Affected by Gamma Irradiation Using a Gene-Trapped Library of Human Mammary Epithelial Cells

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Identification of Novel Genes Affected by Gamma Irradiation Using a Gene-Trapped Library of Human Mammary Epithelial Cells

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We propose that the expression of several unknown genes is affected by gamma radiation. Abnormal expression of these genes maybe one of the early steps in breast carcinogenesis induced by radiation. We plan to establish an assay that will allow us to screen for breast cells that contain a single mutation by gene trapping. We will be able to detect changes in the expression of a gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends procedure and sequenced. Cells that are affected by radiation will be isolated and analyzed to see if the changes can lead to transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression.

The 3’RACE protocol has recently been completed and thirty one genes potential genes were sequenced. Of these, six candidate genes were found. They include: human creatine kinase gene, human androgen receptor, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2 (EEF1B8), human ribosomal protein L27, and human DNA clone epithelial cells as discussed in the statement of work.

G protein, G protein-coupled receptor, extracellular signal-regulated kinase, Lysophosphatidic acid, pertussis toxin

Unclassified

Unclassified

Unclassified
INTRODUCTION

We propose that the expression of several unknown genes is affected by gamma irradiation. The subject and purpose of our research is that the abnormal expression of these genes may be one of the early steps in breast carcinogenesis induced by radiation. We plan to establish an assay that will allow us to screen for breast cells that contain a single mutation by gene trapping. We will be able to detect changes in the expression of a gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified and analyzed to see if the changes can lead to transformation of the normal breast epithelial cell into a neoplastic cell. This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression.

BODY

RESEARCH TRAINING

Ongoing training is very important throughout my predoctoral period. My department, Environmental and Radiological Health Sciences, places an important focus on training. Weekly, I attend Advanced Radiation Biology journal meetings where faculty and students interact and discuss current and relevant papers in breast cancer research and radiation effects. Each attendee presents one journal article every semester and leads the discussion. Weekly there is a Cell and Molecular Biology seminar where invited visiting speakers give a 50 minute presentation and discussion about relevant topics such as breast cancer research, cell signaling, and many more. There are also two departmental seminars I attend weekly where visiting speakers, as well as graduate students present their research. It is very important for my training that I keep current with the latest research techniques and discoveries by attending these meetings. My mentor, Dr. Robert Ullrich, is currently the Oncology Chair of the Veterinary Teaching Hospital here on campus, so I am also exposed to more clinical cancer research seminars and meetings that I attend there as well.

On July 11th through the 14th I attended the 94th annual meeting of the American Association for Cancer Research meeting in Washington D.C. This contributed a great deal to my overall predoctoral training by exposing me to cancer research scientists from all over the world. I was able to attend numerous oral and poster presentations and learn about the latest advances being made in breast cancer and radiation research.

RESEARCH PURPOSE & GOALS

We plan to identify novel genes affected by gamma irradiation and to characterize their function using a gene-trapped library of human mammary epithelial cells. We hypothesize that the mutation of these novel genes or its abnormal expression is one of the causes of early breast carcinogenesis. Mounting evidence suggests that gene products may function differently depending on cell type, developmental stage, or species. Thus, to identify novel gene(s) critical for the initiation of breast cancer, we need to study the irradiation effects of “loss of function” of a gene product in human breast epithelial cells.

The issue of how low dose gamma radiation may lead to breast cancer will be addressed by studying the genes affected by low dose gamma irradiation. We will focus on the trapped genes whose expression are immediately changed by a single dose of gamma irradiation, determine if this is a dose-dependent effect and further analyze whether this effect can lead to transformation of the breast cells.
The following are specific aims as outlined in the approved statement of work:

Specific Aim 1: To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones.
Specific Aim 2: To determine the effect of gamma irradiation on expression of reporter protein GFP (green fluorescent protein).
Specific Aim 3: To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells.
Specific Aim 4: To identify the trapped genes affected by gamma irradiation.

RESEARCH PROGRESS

Currently, specific aim 1 and specific aim 2 are completed. Attached in the appendices are color representations of the completed construction of the gene-trapped MCF10A clonal library as seen under a fluorescent microscope. This is included in one of my PowerPoint presentations. It is clearly observed in the pictorials, that the bright green fluorescence luminating from the cells is due to the retrovirus pRET being incorporated into the genome.

A total of 192 gene-trapped clones were analyzed by the construction of a single cell assay in 96-well plates. This was done to obtain single cell clones, hopefully each representing a different trapped gene. One 96-well plate contained the pooled gene-trapped MCF10A cells sorted by flow cytometry into a GFP positive pool and the other 96-well plate contained the pooled gene-trapped MCF10A cells sorted by flow cytometry into a GFP negative pool. Graphical representations of the flow cytometry data are included in the appendices in one of my power point presentations. Replica plating was then done from both of the original single cell assay plates for the following GFP expression levels to be measured at: basal, control, master, store at -80°C, 0.5 Gy, and 2.0 Gy gamma irradiated. GFP measurements were made with a microplate reader by the way of a sandwich ELISA assay. The sandwich ELISA assay was accomplished by first expanding the 96-well plates with the single cell clones into 24-well plates. These 24-well plates were then expanded further to allow for 2 wells for each single cell assay clone. This was done so that one well could be further expanded and frozen for later use and the other well would be utilized to collect the cell lysate from for the ELISA assay. All of the 24-well plates were then irradiated with 2.0 Gy from a $^{137}$Cs source. The following antibodies were used for the sandwich ELISA assay: anti-GFP (Mouse) was the primary antibody and peroxidase IgG mouse (Rabbit) was the secondary antibody. The entire protocol for the sandwich ELISA assay is included in the appendices.

Graphical representations of the gene expression of GFP after 2.0 Gy gamma radiation dose from a $^{137}$Cs source is attached as well. Here, clones that were up- or down-regulated at least 2-fold from basal readings were expanded for further analysis. The basal GFP readings of the gene-trapped clones are included in the appendices for comparison to the 2.0 Gy GFP readings. Out of the 192 clones analyzed, 92 were up- or down-regulated at least 2-fold in comparison to basal GFP readings. These clones were expanded in culture and RNA was collected for gene analysis.

There was a slight change in the order of the approved statement of work next. Specific aim 4 was undertaken prior to the characterization of the effect of gamma irradiation on the transformation of the MCF10A cells. This was felt to be an important substitution due to the fact that the gene that had been trapped should be identified before transformation assays were
undertaken. Transformation assays are very tedious and time consuming. If, for example, the gene trapped was an artifact, then the process of analyzing for transformation could be skipped.

Specific aim 4 dealt with the characterization of the trapped genes that were causing either an up- or down-regulation upon treatment with 2.0 Gy. To analyze this, the gene-trapped clones were expanded and RNA was collected by using Qiagen’s RNeasy kit. The protocol for this procedure is attached in the appendices. The RNA was then reverse transcribed into cDNA and amplified by the use of the Advantage-GC cDNA polymerase kit from BD Biosciences and the 3’RACE protocol from Invitrogen. Both protocols are included in the appendices. Gene specific primers for the neomycin marker found on our pRET retrovirus and against the polyA tail of the endogenous gene were designed. The sequences of the primers are given in the appendices. After each step, reverse transcription, first strand cDNA synthesis, and second strand cDNA synthesis, agarose gels were run to verify that the gene products were of the correct size.

When a gene product was of the correct size they were PCR purified by Qiagen’s PCR purification kit and transformed into One Shot competent E. coli cells via a TOPO Cloning kit from Invitrogen. The transformed clones were then added to LB media and grown overnight. Clones were growth had occurred were then subjected to Qiagen’s mini prep kit to harvest the DNA. Protocols for both of these procedures can be found in the appendices. The mini prep clones are then subjected to PCR with M13 primers and run on 1.5% agarose gels. The above mentioned M13 primer sequences and PCR reaction conditions are listed in the appendices. Gel electrophoresis images are provided in the appendices in one of my PowerPoint presentations to illustrate which clones were selected to be sequenced.

Sequencing of the positive mini prep clones was completed at Davis Sequencing which is located at the University of California at Davis. A total of 31 clones were sent off for sequencing and six yielded positive results. The six genes were determined by plugging the sequences of my clones into BLAST and searching for homologous genes. The other clones were determined to be artifacts of the cloning vector. The genes that were trapped were: human creatine kinase gene, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation elongation factor 1 beta 2 (EEF1B2), human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20.

The gene-trapped clones that had yielded these sequencing results were expanded and RNA was collected from them for real-time PCR analysis. This was done to look at the gene expression of the trapped genes in the gene-trapped clones. These expression levels could then be compared to the parental cell line, MCF10A and a breast cancer cell line, MCF7. In the appendices I have included my real-time PCR protocol and the sequences for the primers and probes that were utilized. Also, graphical representations of the relative gene expression of my genes of interest are included.

**KEY RESEARCH ACCOMPLISHMENTS**

- A pooled library of gene-trapped of human mammary epithelial cells was established and frozen for future use.
- 92 out of 192 single cell assay gene-trapped clones were up- or down-regulated at least 2-fold in response to a 2.0 Gy radiation dose.
- Out of the 31 clones that were sequenced, six clones were found to be homologous to known genes through a BLAST search. These genes include: human creatine kinase gene, human DORA reverse strand protein 1 (DREV1), human eukaryotic translation
elongation factor 1 beta 2, human androgen receptor, human ribosomal protein L27, and human DNA clone RP11-290F20 on chromosome 20.

REPORTABLE OUTCOMES

- The development of a gene-trapped cell library of MCF10A cells was accomplished with the retrovirus pRET.
- I was invited to give a poster presentation at Colorado State University for the Cell and Molecular Biology Interdisciplinary Graduate Program Graduate Student and Post Doc Poster Competition on February 21, 2003. My poster abstract is found in the appendices.
- I was invited to give a 20 minute oral talk and a poster presentation at the Minority Trainee Research Forum sponsored by NIH-National Institute of Diabetes and Digestive Kidney Diseases, NIH-National Institute of Allergy and Infectious Diseases, NIH-Office of Research on Women’s Health, and Merck and Company. It was held March 14-17, 2003 at the Westgate Hotel in San Diego, California. My abstract can be found in the appendices.
- On April 17, 2003 I gave a 20-minute oral presentation on my research to the faculty and students of the Department of Radiological and Environmental Health Sciences. The department has doctoral students give oral presentations every semester on how their research is progressing and any new findings. The PowerPoint slides from my presentation are given in the appendices.
- On November 21, 2002 I gave a 20-minute oral presentation on my research to the faculty and students of the Department of Radiological and Environmental Health Sciences.
Appendices

Primer sequences used for 3’RACE & Sequencing:
AD Poly (T): 5’-CGTAGCTCTAGACTCCGCTGCAACTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’
AD (T): 5’-CGTAGCTCTAGACTCCGCTGCAACT-3’
NEO1.5: 5’-GCCATGGGCTGACCGTCATTCTCCTGTCG-3’
AD: 5’-CGTAGCTCTAGACTCCGCTGCAAC-3’
NEO2.0: 5’-TACGGATATCGCCGCTCCGATTCGCAG-3’
AD PLUS: 5’-CGTAGCTCTAGACTCCGCTGCAACTTTTT-3’
NEO SEQ: 5’-TGACGAGATTTCTTCTGAGGGGATCC-3’
M13 Forward: 5’-GTAAAAACGACGGCAGC-3’
M13 Reverse: 5’-CAGGAAACAGCTATGAC-3’

Primer and Probe sequences for real-time PCR:
Androgen F1: 5’-CCCTGGCCGCGATGGT-3’
Androgen F2: 5’-ACCTGTGCGGGCATGGT-3’
Androgen F3: 5’-TACCTGGCCGCGATGGT-3’
Androgen R1: 5’-CCCATTGCTTATTGACACA-3’
Androgen R2: 5’-CCCATTGCTTATTGACACA-3’
Androgen R3: 5’-GCCCATTTCTGCTTTTGACACA-3’
DORA F1: 5’-GAGGCAAGTGGCATCTCTTGC-3’
DORA F2: 5’-GAGGCAAGTGGCATCTCTTGC-3’
DORA F3: 5’-GCCAAGTGGCATCTCTTGC-3’
DORA R1: 5’-CCCATTGCTTATTGACACA-3’
DORA R2: 5’-CCCATTGCTTATTGACACA-3’
DORA R3: 5’-CCCATTGCTTATTGACACA-3’
ANDROGEN PROBE: 6FAM-ACGCGATGGCCATATCCGCAATGCTCA-TAMRA
DORA PROBE: 6FAM-CTTGGATCCTCCCTTTCCCTCATTTGTTGGAATGTTG-TAMRA
CK F1: 5’-TGCTTACATGCCGCTGCTAAGTG-3’
CK F2: 5’-TGCTTACATGCCGCTGCTAAGTG-3’
CK F3: 5’-TGCTTACATGCCGCTGCTAAGTG-3’
CK R1: 5’-GCCATGTTGCGGCTGCTGCTGCT-3’
CK R2: 5’-GCCATGTTGCGGCTGCTGCTGCT-3’
CK R3: 5’-GCCATGTTGCGGCTGCTGCTGCT-3’
L27 F1: 5’-GCCCTCACAGCCATGGCTCT-3’
L27 F2: 5’-CATGGGCGCTGCTTCTTCTCCTGTATCAGCAATG-3’
L27 F3: 5’-CATGGGCGCTGCTTCTTCTCCTGTATCAGCAATG-3’
L27 R1: 5’-CATGGGCGCTGCTTCTTCTCCTGTATCAGCAATG-3’
L27 R2: 5’-CATGGGCGCTGCTTCTTCTCCTGTATCAGCAATG-3’
L27 R3: 5’-CATGGGCGCTGCTTCTTCTCCTGTATCAGCAATG-3’
EEF1B2 F1: 5’-CACAATTTGCGGCTGTCTCTTG-3’
EEF1B2 F2: 5’-CACAATTTGCGGCTGTCTCTTG-3’
EEF1B2 F3: 5’-CACAATTTGCGGCTGTCTCTTG-3’
EEF1B2 R1: 5’-ACCATGTTGCTGCTGCTGCTGCT-3’
EEF1B2 R2: 5’-ACCATGTTGCTGCTGCTGCTGCT-3’
EEF1B2 R3: 5'-AACCCATGGTGCAGCTGTA-3'
CK PROBE: 6FAM-TCCTGACCACCCGTTACCTGCTG-TAMRA
L27 PROBE: 6FAM-TGGCTGGAATGGACCCTACCC-TAMRA
EEF1B2 PROBE: 6FAM-TCTGCTGCTCCCAGCTCTGC-TAMRA

For the protocols used from Qiagen, Invitrogen and BD Biosciences, please visit the following websites:
http://www.invitrogen.com/content/sfs/manuals/topotaseq_man.pdf (TOPO TA Cloning)
http://www.invitrogen.com/content/sfs/manuals/18373019.pdf (3'RACE)
http://www1.qiagen.com/literature/handbooks/PDF/DNACleanupAndConcentration/QQ_Spin/1021422_HBQSpin_072002WW.pdf (PCR purification)
http://www1.qiagen.com/literature/handbooks/PDF/RNAStabilizationAndPurification/FromAnimalAndPlantTissuesBacteriaYeastAndFungi/RNY_Mini/1016272HBRNY_06201WW.pdf (RNasey Mini Kit)

General Protocol for the Sandwich ELISA method:

1. Before the assay, both antibody preparations should be purified and one must be labeled.
2. For most applications, a polyvinylchloride (PVC) microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for protein binding.
3. Bind the unlabeled antibody to the bottom of each well by adding approximately 50 m L of antibody solution to each well (20 m g/mL in PBS). PVC will bind approximately 100 ng/well (300 ng/cm2). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 m g/well. this is well above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again.
4. Incubate the plate overnight at 4°C to allow complete binding.
5. Wash the wells twice with PBS. A 500 mL squirt bottle is convenient. The antibody solution washes can be removed by flicking the plate over a suitable container.
6. The remaining sites for protein binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2hrs to overnight in a humid atmosphere at room temperature. (Note: Sodium azide is an inhibitor of horseradish peroxidase. Do not include sodium azide in buffers or wash solutions, if an HRP-labeled antibody will be used for detection.)
7. Wash wells twice with PBS.
8. Add 50 m L of the antigen solution to the wells (the antigen solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS with
0.02% sodium azide). Incubate for at least 2 hrs at room temperature in a humid atmosphere.

9. Wash the plate four times with PBS.
10. Add the labeled second antibody. The amount to be added can be determined in preliminary experiments. For accurate quantitation, the second antibody should be used in excess. All dilutions should be done in the blocking buffer.
11. Incubate for 2 hrs or more at room temperature in a humid atmosphere.
12. Wash with several changes of PBS.
13. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, optical densities at target wavelengths can be measured on an ELISA reader.

PCR conditions:
First strand cDNA synthesis:
94°C 3 minutes; 94°C 40 seconds, 72°C 4 minutes, 8 cycles; 94°C 40 seconds, 66°C 2 minutes, 72°C 2 minutes, 32 cycles; 72°C 4 minutes, 4°C overnight.
Second strand cDNA synthesis:
94°C 1 minute, 94°C 40 seconds, 72°C 4 minutes, 8 cycles; 94°C 40 seconds, 66°C 2 minutes, 72°C 2 minutes, 32 cycles; 72°C 4 minutes, 4°C overnight.
Mini prep PCR:
94°C 2 minutes; 94°C 1 minute, 55°C 1 minute, 72°C 1 minute, 25 cycles; 72°C 7 minutes.
Real-time PCR:
50°C 2 minutes; 95°C 10 minutes; 95°C 15 seconds, 60°C 1 minute, 60 cycles.
GFP negative sort population
GFP readings with microplate reader both before & after 2.0 Gy irradiation.
GFP positive sort population
GFP readings with microplate reader both before & after 2.0 Gy irradiation.
GFP positive Lanes A1 through A16
GFP negative Lanes A17 through A32

GFP readings from microplate reader for both before & after 2.0 Gy irradiation.

Each sample listed to the right is one individual clone of the 192 clones sampled.

Those clones up- or down-regulated at least 2-fold were expanded for further analysis.
Gene Expression after 2.0Gy irradiation dose

Ct

DORA

CK

L27

EEF1B2

GAPDH average

-30
-20
-10
0
10
20
30

2hr

4hr

8hr

12hr

24hr

30hr

MCF10A (no IR)
Cell and Molecular Biology
Interdisciplinary Graduate Program

Graduate Student & Post Doc
Poster Competition Abstracts

Spring 2003
IDENTIFICATION OF NOVEL GENES AFFECTED BY GAMMA IRRADIATION USING A GENE-TRAPPED LIBRARY OF HUMAN MAMMARY EPITHELIAL CELLS

Jennifer Malone and Robert Ullrich
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malones41500@hotmail.com, 491-7497 office, 491-5771 lab, classification: graduate student

Objective/Hypothesis: In this study, we plan to establish an assay to identify novel genes that are affected by gamma irradiation and to characterize their function and role in early breast carcinogenesis. We hypothesize that the mutation of these genes or their abnormal expression in response to gamma irradiation is one of the causes of breast carcinogenesis.

Specific Aims: The specific aims of this study are:
1. To establish a high throughput assay for detection of variation in gene expression in human mammary epithelial cells using gene-trapped MCF10A clones; 2. To determine the effect of gamma irradiation on the expression of reporter protein green fluorescent protein (GFP); 3. To characterize the effect of gamma irradiation on transformation of human mammary epithelial cells; 4. To identify the trapped genes affected by gamma irradiation in breast epithelial cells.

Methods: We plan to establish an assay that will allow us to screen for breast cells that contain a single gene mutation using a technique called gene trapping. We will be able to detect changes in the expression of a specific gene upon treatment with different doses of radiation. These radiation-responsive genes will be identified through the rapid amplification of cDNA ends (RACE) procedure and sequenced. Cells that are affected by radiation will be isolated and further analyzed to see if the changes can lead to the malignant transformation of the normal breast epithelial cell into a neoplastic cell.

Results: The MCF10A gene-trapped library has been established. Basal GFP levels have been measured from the replica plates of single cell clones in 96-well plates. Gamma irradiation of the single cell clones at 2.0 Gy has been performed. Clones that were either up or down-regulated in response to the radiation treatment will be expanded for further analysis.

Study Design: Using the poly-A trap retrovirus vector RET, we have established a gene-trapped library of clones from human mammary epithelial cells (MCF10A). It provides the strong base for the identification of novel genes that may be involved in essential signaling pathways in human mammary epithelial cells. We propose to establish a detection assay using a reporter gene GFP that has been incorporated into the genome of the cells, whose expression is regulated by endogenous promoters of the trapped genes. We will compare basal GFP expression before and after exposure to varying low dose gamma radiation (0-2 Gy) using replica plates of MCF10A gene-trapped clones. Next, we will further characterize the clones that are affected by gamma irradiation by performing colony formation assays (to determine survival), anchorage-independent growth and tumorigenicity assays on transformed clones that grow in soft agar. We will then identify the gene(s) involved by using a polymerase chain reaction protocol and sequencing analysis.

Conclusions: This assay may prove to be a powerful tool in the identification of novel genes that are affected by gamma irradiation in the early stages of breast cancer progression. This study will provide new information on the effects of radiation-responsive genes that can lead to breast cancer as well as to identify new markers for early detection of breast cancer. This study will focus on the identification of novel genes that are potential targets of gamma irradiation. It will provide essential information on the immediate and long-term effects of gamma irradiation of breast cells that may be the key to further understanding of the mechanism of radiation-induced breast cancer.
MINORITY TRAINEE RESEARCH FORUM

Invitational Scientific Presentations by Minority Biomedical Trainees from Academic, Industry, and Government Labs

MARCH 14-17, 2003
THE WESTGATE HOTEL
SAN DIEGO, CA

INVITEES:
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Graduate Students
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SPONSORED BY:
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• NIH – Office of Research on Women’s Health
• Merck & Company
7:00 a.m. – 5:00 p.m.

7:00 a.m. – 8:25 a.m.
Temple University

7:00 a.m. – 8:25 a.m.
UNC/MEK

8:30 a.m. – 11:30 a.m.

8:30 Jill White (IL)
Ph.D. Trainee
Northwestern University

8:45 Wayne Liljestrom (CO)
Ph.D. Trainee
University of Colorado Health Sciences Ctr.

9:00 Crystal Johnson (AL)
Ph.D. Trainee
University of Alabama-Birmingham

9:15 Fabrício Rojas (CA)
Ph.D. Trainee
California State University, Northridge

9:30 Jennifer Malone (CO)
Ph.D. Trainee
Colorado State University

9:45 Tyisha Williams (MD)
Ph.D. Trainee
Howard University

10:00 Angelica Lockett (IN)
Ph.D. Trainee
Indiana University & Purdue University

10:15 Kevin Hadley (CA)
PostDoc
Children’s Hospital of Oakland Research Institute

10:30 Lee Wilson (Canada)
PostDoc
University of Saskatchewan

10:45 Melissa Gonzales (CO)
PostDoc
University of Colorado Health Science Ctr.

11:00 Jesus Salvador (MD)
PostDoc
National Cancer Institute

11:15 Laundette Jones (MD)
PostDoc
Lombardi Cancer Center,
Georgetown University

Foyer
Regency
Preparing for Medical/Graduate School

Le Fontainebleau Restaurant
Science Education Initiatives

Versailles Ballroom
“Differential Induction of Gliarial-activated Inflammation by Oligomeric Versus Fibrillar Aβ1-42”

“Biochemical and Structural Characterization of SV40 Large-T Antigen”

“Genetic Relatedness of Levofloxacin-Resistant Streptococcus Pneumoniae Isolates from North America”

“Characterization of the Carboxylate Hydrolase Promoter in Achromobacter”

“Identification of Novel Genes affected by Gamma Irradiation using a Gene-trapped Library of Human Mammary Epithelial Cells”

“Mutation Analysis of Glutathione Reductase a Candidate Gene for Skin Color”

“Is LPS Induction of NFKB Activity Indirect and Due to TNFα?”

“Effect of α-thalassemia, Sickle-Cell Disease and Dietary Iron on Iron Storage in Transgenic Mice”

“Interaction of Inhalation Anesthetics with Supramolecular Biomimetic Structures”

“Nordihydroguaiaretic Acid Inhibits UVB-induced AP-1 Activation in Human Keratinocytes”

“A Role for the p53-effector Gene Gadd45α as an Autoimmune Disease Suppressor Gene”

“Cellular and Molecular Alterations Associated with Early and Late Stages of Mammary Tumor Progression in Conditional Bcr/ Axl Deficient Mice”
IDENTIFICATION OF NOVEL GENES AFFECTED BY GAMMA IRRADIATION USING A GENE-TRAPPED LIBRARY OF HUMAN MAMMARY EPITHELIAL CELLS

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Using a gene-trapped library of MCF10A cells to identify novel genes affected by gamma IR

Jennifer Malana
Colorado State University
November 21, 2002

Overview of presentation

- Introduction
- Experimental Design
- Future Directions
- Summary
- Acknowledgements

Introduction

Breast Cancer

- Most common malignancy in women in the US and second leading cause of cancer death in women (193,746 new cases in 2001 among women in the United States and about 40,600 deaths)
- Both genetic and environmental components associated with disease
- Up to 10% of breast cancer cases are due to genetic mutations in such genes as BRCA1, BRCA2, and ATM

Gamma Irradiation and Breast Cancer

- Induced with high frequency by radiation
- Tumor induction is one of the main treatments used to manage & kill cancer cells
- Breast cancer risk seen in women exposed to total dose of more than 1 Gy
- Exposure prior to age 10 leads to increased incidence of breast cancer, but exposures after menopause doesn't affect risk
- Radiation treatments for breast cancer linked to increased risk of secondary breast cancers among women exposed prior to the age of 45

What is Gene Trapping?

- A method of random insertional mutagenesis that uses a fragment of DNA coding for a reporter or selectable marker gene as a mutagen to specially disrupt gene function by producing intragenic integration events
- Gene trapping allows the simultaneous identification, sequencing, in vivo expression analysis, and phenotyping of the genes of interest

Gene Trapping

- The sequence of the 'trapped' gene can be identified using techniques that are based on the polymerase chain reaction (PCR), and this can lead to the isolation of novel genes regardless of their level of expression in vivo

Methods for introduction of vectors into cells

- Electroporation: used for DNA transformation. Relatively inefficient
- Retroviral vectors: high efficiency of gene transfer and integrate as a single copy

Retroviral gene trapping vectors

- The vectors used in this approach typically include a splice acceptor site upstream of a promoterless reporter gene and the selectable neo gene driven by an autonomous promoter
- A retroviral vector is introduced into packaging cell lines by transfection. Viral supe is then used to infect MCF10A cells
- Reporter gene activity reflects the activity of the endogenous gene into which the vector integrated
Removable Exon Trap (RET)
- A pol-yA trap retrovirus vector constructed by Ishida and Leder uses a combination of a very strong splice acceptor, an effective polyadenylation signal and a promoterless green fluorescent protein cDNA that allows the expression pattern of the trapped gene to be monitored in living cells.
- The integrated provirus can be removed from the genome of infected cells by excision using Cre homologous recombination due to its loxP sites.

Cell Sorting of Infected pool of GFP clones
- Cells sorted through flow cytometer in Dr. Fox's Laboratory.
- MCF10A clones were sorted by GFP expression into positive and negative pools.
- The positive GFP expression pool was then further sorted into high, medium, and low levels of GFP expression.

Positive GFP expression in MCF10A-1023 l, clone 109 from Replica Plating

Detection of basal GFP expression using microplate reader
- Boehringer microtiter plates will be used to quantitate GFP expression.
- 96-well plates scanned at 450 nm to detect GFP by the use of TMB substrate.
- Absorbance quantitated using Microplate manager software.

The Process of Vector Integration
- The vector is randomly integrated into the genome in regions where functional genes are found.
- The random insertion of a retroviral vector is designed to signal its presence via the activation of the reporter gene, which both marks the expression of the endogenous gene and potentially inducible the locus.
- Endogenous gene locus is usually but not always inactivated by vector integration, leading to a loss of function.

Experimental Design

Replica Plating
- Pooled clones of gene-trapped MCF10A clones plated by limiting dilution
- 1.0 × 10⁶/well in 96-well plates
- Grow overnight in presence of G418
- Once confluent, split replica plate into 96-well plates with each GFP and GFP + sort clonal cell population
- Triplicate wells will be for basal GFP detection, master, store at -90 degrees, 2° Gy, 0.5 Gy, and control

Future Directions
**Exposure to gamma irradiation**
- MCF10A clones will be subjected to varying low doses (0-2 Gy) of gamma irradiation by a sealed 32P-Ge source.
- Gamma irradiation is a known mutagenic agent.
- GFP expression quantified with microplate reader immediately following radiation exposure.

**3' RACE continued**
- Permits capture of unknown 3' mRNA sequences that lie between the exon and poly(A) tail.
- PCR products cloned into sequencing vector using Zero Blunt TOPO Cloning kit from Invitrogen.
- Nucleotide sequence of purified PCR fragments determined by sequencing with the M13 universal primer.
- Search BLASTX & BLASTN databases for homologous genes.

**Soft Agar Assay for Anchorage Independence**
- In 6-well plate: bottom layer contains 3 ml 1% agarose. Top layer contains 6 ml 0.5% agarose and 1 x 10^6 cells.
- Both layers contain appropriate media.
- Incubate at least 2 weeks or more to detect colonies to detect transformation.

**Summary of Soft Agar Assay**
- **Control Clone**
- **MCF10A (+/−)**
- **MCF10A-D14**
- **MCF10A-transfected**

**Chemoinvasion & Chemotaxis Assay**
- Determine invasiveness using Boyden Chamber assay.
- Cells seeded in upper chamber & incubated 9 hrs.
- Conditioned media in lower chamber used as chemotactic agent.
- Cells that crossed filter will be fixed & counted.
- Chemotaxis assay will serve as a control for invasion assay.

**Tumorigenicity in nude mice**
- To determine if anchorage-independent clones are fully malignant, MCF10A transformed cells will be injected subcutaneously into the subcutaneous area of 3-week-old irradiated athymic female nude mice (BALB/c background).
- Tumors monitored weekly.
- Sites of injection & palpation.
- Tumors will be examined histologically.

**Summary**
- Focus on identification of novel genes that are potential targets of IR.
- Provide essential info on immediate & long-term effects of IR on breast cells, which will lead to further understanding of the mechanism of radiation-induced breast caignogenesis.
- This assay can be used to test other potential environmental risk factors.
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- My committee members:
  - Dr. Sue Lana, Dr. Mike Fox, and Dr. Bill Hanneman
- All members of the Ullrich lab
**Gene Trapping: A Tool to Identify Novel Genes Affected by Gamma Irradiation in Breast Cancer**

Jennifer Malone  
Colorado State University  
April 17, 2003

**Hypothesis**
- Mutation of novel genes or their aberrant expression in response to single-dose gamma radiation is one of the causes of early breast cancer.

- **Specific aim 1:** Identification of genes affected by gamma radiation in human mammary epithelial cells using gene trapping with YACs.

- **Specific aim 2:** Subsequent analysis of the effect of gamma radiation on the expression of genes in the mammary epithelial cells.

**Radiation-Induced DNA Damage**
- The response to ionizing radiation involves the activation of signaling pathways, cell-cycle arrest, mutations, transformation, and cell death.

- Growth factors, cytokines, and genes involved in the cell cycle, apoptosis, and DNA repair are all known to be affected.

- Response to radiation: general response to cellular injury (indirect) - specific to radiation-induced damage (direct)

**Known radiation-inducible genes**
- BR works via DNA damage and ROS generation, which can induce the transcription of specific genes through the activation of P53, NF-κB and AP-1.

- Also known to be induced: GADD45, CACNA1A (G protein subunit), MDM2, AT3, BAX.

- Many known radiation-inducible genes have been found to be early response genes.

**Breast Cancer & Radiation**
- Breast cancer may be initiated with relatively high frequency by radiation.

- Induction of cancer is one of the main treatment modalities used in the management of cancer.

- Radiation has an impact in the treatment of breast cancer. Molecular and biologic responses to the treatments are different.

- Breast irradiation and drug administration have been known to activate signaling breast cancer.

- Radiation dose-related increase in the incidence of breast cancer has been observed.

- When a woman receives significant radiation prior to the age of 30, she becomes more likely to develop breast cancer.

**Breast Cancer Info**
- 2nd leading cause of cancer death after lung cancer.

- There are both genetic and environmental components associated with breast cancer.

- Up to 10% of breast cancer in western countries is due to genetic predisposition.

- Breast cancer incidence increases strongly with age.

**What genes might account for familial breast cancer?**

**Gene Trapping**
- Forms of transgenic integration.

- Homologous gene function by homologous integration.

- A gene is transferred from a selectable marker gene by homologous integration. The rodent endogenous gene is replaced by the selectable marker gene.

- The position of the reporter gene is determined by the homologous sequence.

- Homologous gene insertion occurs independently of the expression of target genes regardless of the expression.

- A successful gene insertion under conditions that are not critical for the expression of the target gene is needed to the isolation of novel genes regardless of their level of expression in vivo.

**RET retrovirus**
- Enhanced poly A trap for progenitor selection of wild integration.

- Strong splice acceptor and efficient poly A signal sufficient for the complete disruption of the trapped gene.

- Expression of trapped gene analyzed by G418 expression.

- Mutant phenotype verified by removing virus with Crelox-mediated homologous recombination.
Overview of Library Construction
- The process starts with the generation of transgenic animals where transgenes are inserted.
- Cells from these transgenic animals are harvested and used to isolate and clone transgenic mice.
- The isolated clones are then screened for the presence of the transgene.

The Process of Vector Integration
- The vector is linearized in the genome to integrate into the target site.
- DNA transfection is performed in vivo to deliver the vector.
- The transgene is then integrated into the genome.
- Expression is monitored to confirm successful integration.

Cell Sorting of Infected Pool of GFP Clones
- Cells are sorted by FACS into three equal populations.
- The sorted populations are then further sorted into high, medium, and low expression levels.

Flow Cytometry Analysis
- Initial sort of clone trapped, MCF10A clones, and GFP+ clones.
- FACS gates: gate 1 = GFP-low and low gate 2 = high GFP.
- Sorted cell mixture into high, medium, and low GFP populations.

Replica Plating
- Plate cell colonies from MCF10A clones on microplates, one per well.
- Incubate at 37°C for 24 hours.
- GFP expression is monitored.

GFP expression of replica plated gene-trapped MCF10A clones.
- GFP expression is monitored in each plate.
- Plates are monitored for GFP expression.
- Plates are then sorted into high, medium, and low expression levels.
**GFP detection via microplate reader**
- Bioluminescent microplate reader will be used to quantitate GFP expression.
- 96-well plates scanned at 450 nm to detect GFP by the use of TMB substrate.
- Absorbance quantitated using Microplate Manager software.
- Readings taken both before & after ionizing radiation.

**3' RACE Assay**
- Rapid Amplification of cDNA ends amplifies multiple cDNA sequence from a single sample between a defined internal site and either the 3' or 5' end of the mRNA.
- Uses universal poly A tail found in mRNA as a generic primer site for PCR.
- cDNA is converted to cDNA using reverse transcriptase and an oligo-dT adapter primer.
- cDNA amplified by PCR using a gene specific primer that anneals to a known exon sequence and an adapter primer that targets the poly-A tail region.

**3' RACE continued**
- Permits capture of unknown 3' mRNA sequences that lie between the exon and polyA tail.
- PCR products cloned into sequencing vector using Zero Blunt TOPO Cloning Kit from Invitrogen.
- Nucleotide sequence of purified PCR fragments determined by sequencing with the M13 universal primers.
- Search BLASTX & BLASTN databases for homologous genes.

**Gel Electrophoresis: PCR of Miniprep DNA**

**Sequencing Results**
- 33 samples submitted in Davis Sequencing at UC at Davis.

**Future Directions**
- Transformation Assays
- Repeat Assay
- Different Doses
In Summary

- HOPEFULLY, radiation-induced genes will be mapped.
- Both known & unknown radiation-induced genes will be discovered.
- Moving on to transformation assays.

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- My committee members: Dr. Sue Lane, Dr. Mike Fox, and Dr. Bill Hanneken
- All members of the Ulrich lab