Award Number: DAMD17-01-1-0199

TITLE: Use of a Transgenic Mouse Model with a Regulatable Estrogen Receptor Alpha (ER) to Study the Role of ER in Mammary Gland Development and Cancer

PRINCIPAL INVESTIGATOR: Sonia L. Sugg, M.D.

CONTRACTING ORGANIZATION: Medical College of Wisconsin
Milwaukee, WI 53226-0509

REPORT DATE: September 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
### Abstract

Estrogen (E2) is required for the development of breast cancer, but there are few animal models to study its mechanism of action in vivo. We hypothesize that the timing of estrogen receptor α (ERα) action is crucial in normal mammary gland development and tumorigenesis. Therefore, a transgenic mouse model with a regulatable ERα is being generated. In vitro, a mutant ERα (525L) has a severely attenuated response to endogenous estradiol (E2) but a wild-type (WT) response to diethylstilbestrol (DES). Gene targeting was used to insert 525L into the WT ER gene. Positive clones were injected into mouse blastocysts and chimeras were generated. There has been no germline transmission so far. Additional injections and further breeding continues. The resultant transgenic mouse will enable us to study how the timing of ER activation modulates normal mammary maturation and the development of mammary cancers.
# TABLE OF CONTENTS

Cover........................................................................................................... 1
SF 298........................................................................................................ 2
Introduction............................................................................................. 4
Body......................................................................................................... 4-9
Key Research Accomplishments............................................................. 10
Reportable Outcomes............................................................................. 10
Conclusions............................................................................................ 10
References............................................................................................. 11
INTRODUCTION

Estrogen and its receptor are required for normal breast development and are important in the treatment and prevention of breast cancer. However, there are few suitable animal models to study the role of ER activation in the breast development and cancer. We developed a mutant ER having a severely attenuated response to endogenous estradiol while demonstrating a wild-type response to a synthetic estrogen agonist in vitro. This was due to a difference in binding affinity. We postulate that the timing and nature of estrogen action is crucial in normal mammary gland development and tumorigenesis. We are proposing a transgenic mouse model with a regulatable ER to test this hypothesis. Our specific aims are to 1) Develop transgenic mice using gene-targeting techniques expressing only the ligand discriminating mutant ER. 2) Define the contribution of ligand induced transcriptional activation of ER in the development of normal mammary gland. 3) Investigate how the timing of ER activation modulates the development of genetically and chemically induced mammary cancers. The mutant ER mice are uniquely suited to study the role of ligand independent ER activation in vivo, and will also be useful in studying reproductive tract development and behavior.

BODY

Task 1. Transgenic mice expressing only a mutant ER with ligand discrimination will be developed. (months 1 - 12)

a) Mouse ER genomic DNA will be cloned. A targeting construct (8 to 10 kb) will be generated.

Construct A (MCW): The 15 kb construct is composed of mouse genomic sequence from ERα (exons 8-9 and external downstream sequence), cloned into the pZero vector (Invitrogen) at the multiple cloning site, Xhol (5' end of insert) and HindIII (3' end of insert). A Neomycin resistance cassette, consisting of the PGK promoter, neo open reading frame and poly A tail sequence, is found approximately 1.2 kb downstream of exon 9 in reverse orientation at a BamH1 site found in the genomic DNA. For removal with Cre-recombinase, the Neo cassette was flanked by LoxP sites (Figure 1). This construct was provided by our collaborators Deborah Swope and Ken Korach. Approximate restriction enzyme sites and distances between sites were determined by sequencing and restriction enzyme/DNA gel analysis.
Construct B (Chicago): An 11.5 kilobase ERα construct containing the G525L mutation (purple bar in green exon 9 box), an 18 bp 6xHis-tag epitope, and an extra XbaI site, was engineered to facilitate homologous recombination into the mouse genome (Figure 2). The original construct was obtained from Dr. Kenneth Korach and the G525L mutation was inserted in our laboratory by site directed mutagenesis. We also inserted an ACN cassette into the targeting construct, obtained from Dr. Wondisford at the University of Chicago, which contained a testis-specific promoter (tACE), Cre structural gene, mouse RNA polymerase gene, and neo cassette, flanked at the 5' and 3' ends by loxP sites (Figure 2).

Mutagenesis: Mutagenesis was performed by PCR using Qiagen’s site directed mutagenesis kit. Three rounds of mutagenesis were performed following the kit’s instructions. Briefly, primers were designed using MacVector to 1) alter the coding sequence at codon 525 to convert glycine (G) to leucine (L). 2) Create a novel XbaI site in exon 9 while maintaining the amino acid sequence. 3) Remove a nucleotide insertion event (4G→3G) near the end of exon 9 that was discovered upon sequencing. Each round of mutagenesis was performed as directed, with the PCR extension time expanded to accommodate 15 kb.

Ligand binding characterization of G525L mutation: We hypothesized that the ligand discrimination of G525L is a result of differences in binding affinity between E2 and DES present in G525L and not in WT ERα. Charcoal assays were used to determine saturation binding characteristics. The G525L mutant receptor had a Kd of 0.03nm with DES, compared with a Kd of 12.5nm with E2. In contrast, the WT receptor had a Kd of 0.04 with DES, and a Kd of 0.12nm with E2.

Characterization of dominant negative effects of G525L mutation: We were concerned that our inability to obtain germline transmission using 2 different constructs could be due to a possible dominant negative effect of the G525L mutation on wild type ER, thus preventing normal fetal development, even in the heterozygous state. To examine if mutant G525L ERα will exert a dominant negative effect on the wild type receptor in the heterozygous...
mice, a standard luciferase dominant negative experiment with various ratios of wild type to mutant G525L ERα was performed (Fig 3). Results indicate a dominant negative effect is unlikely.

Treatment of a potential dominant negative effect. Luciferase assays with ER-negative Ishikawa cells, cotransfected with mutant G525L ERα and an ERE-reporter, showed transcription was not stimulated by low concentrations of 17β-estradiol (E2), but was stimulated by diethylstilbestrol (DES) or genistein (Fig 4). This demonstrates DES or genistein could be administered to heterozygous mutant G525L ERα mice to prevent a possible dominant negative effect.

Characterization of ligand-independent pathways of G525L ERα. We expect the classical ligand-dependent ERα signaling pathways to not be stimulated in G525L ERα mutant mice since the endogenous estrogens will not be able to bind to ERα. However, since the AF1 region could still be phosphorylated, we expect some of the ligand-independent and nongenomic pathways could still be active. Therefore assays were performed to examine the effects of the ligand independent and nongenomic pathways with mutant ERα. Luciferase assays with ER-negative Ishikawa cells, cotransfected with wild type or mutant G525L ERα and an ERE-reporter and treated with various concentrations of epidermal growth factor (EGF) in 2.5% serum, showed similar transcriptional responses (Fig 5). This preliminary study indicates the mutant G525L ERα ligand-independent pathways are functional.

b) Mice transgenic and homozygous for mutant ER will be generated.
A two-pronged effort, with 2 different targeting constructs, was started at MCW and the University of Chicago. The rationale was to increase the probability of, and decrease the time required to successfully generating a transgenic mouse.
1) **Work performed using targeting construct A.**

A 15 kb G525L mutant ER targeting construct was used to generate 18/380 (4.7%) positive ES cell clones, as detected by 5' probe Southern blot screening. The positive clones were further confirmed by 3' external probe Southern hybridization, PCR of exon 9 followed by restriction enzyme XbaI digest, G525L mutation sequencing, and PCR of the neo selection marker (see Table 1). Three positive clones 29, 200, and 362 were selected for blastocyst injection. The genomic DNA from these 3 clones was again confirmed by all of the above methods to be positive prior to blastocyst injection. Clones 200 and 362 had litters that were aborted or cannibalized. Clone 29 generated 6 chimeras with 60 to 90% coat color chimerism. Ten breeding cycles generated no germline transmission.

(2003-2004) Clones 200 and 362 were reinjected, clone 200 generated 5 chimeras with 35 to 60% coat color chimerism, clone 362 generated only one chimera. Six breeding cycles of clone 200 chimeras yielded no germ line transmission. Clone 29 was reinjected, and produced no chimeras.

2) **Work performed using targeting construct B.**

(2002-2003) The targeting construct was electroporated into ES cells. Positive clones were identified by 5' external probe Southern blots. A PCR strategy to identify 3' integration was also developed. PCR of exon 9 was used to detect the 6xHis-tag, XbaI site, and G525L mutation in the positive clones. Southern blots with an internal probe were also performed to demonstrate only one insertion event in each positive clone. Two positive clones, 55 and 71, were obtained. They were injected into mouse blastocysts and four chimeras, one female from clone 55 and two males and one female from clone 71, were generated. Thus far, germline transmission was not been achieved with any of the chimeras. Therefore, a new set of experiments using a RW4 ES cells and additional refinement of screening procedures were started.
The mutant G525L ERα targeting construct was electroporated into RW4 ES cells. Clones were initially screened by a Southern blot with a 5’ probe external to the targeting construct (Fig 6A). Four positive clones were initially identified. A Southern blot with an internal probe on the 3’ end of the targeting construct (Fig 6B), PCR with external 3’ sequence primers (Fig 6C), and PCR for identification of the mutant His-tag, added XbaI site (Fig 6D), and G525L mutation (Fig 6E), confirmed only two of these four clones, 42 and 71, had the targeting construct correctly inserted.

Clones 42 and 71 were injected into the blastocysts of pseudo-pregnant mice. Clone 42 produced eleven chimeras, eight males and three females, all with a high degree of chimerism. Clone 71 produced seven chimeras, three males and four females, with varying degrees of chimerism. Three agouti pups have been produced in total by two male clone 42 chimeras. Two male clone 71 chimeras are also currently being mated.
Southern blot and PCR strategies have been developed to genotype the F1 agouti pups (Fig 7).

**Future Directions:**

- To further confirm the mutant G525L ERα ligand-independent pathways are active in cell culture experiments.
  - The luciferase assay with EGF treatment will be optimized and repeated with the MEK inhibitor U0126 to determine if the effects of EGF on ERE-reporter expression are mediated through MAPK.
- To determine if the nongenomic pathways are still functional with the mutant G525L ERα in cell culture experiments.
  - Ishikawa cells transfected with wt or mutant G525L ERα will be treated with E2 or vehicle for several minutes and ERK1/2 phosphorylation will be analyzed by Western blotting. We anticipate nongenomic signaling pathways will be active in cells expressing mutant G525L ERα.
  - These nongenomic pathways should also not be sensitive to inhibitors of transcription, like α-amanitin and actionmycin D, and translation, like cyclohexamide.
- Clone 42 F1 agouti pups will be genotyped.
  - If germline transmission is successful, heterozygous mice will be bred so homozygous mice can be studied.
  - If germline transmission is not successful, mice will be given genistein or DES to avoid the possible dominant negative effect of the mutant G525L ERα. If this also fails, the His-tag will be removed from the target construct and the electroporation process will be repeated again.
KEY RESEARCH ACCOMPLISHMENTS:

1) Ligand binding characterization of the G525L mutant.
3) Preliminary investigation of ligand-independent pathways of G525L ERα.
4) Generation of mutant ER chimeras from 2 targeting constructs.

REPORTABLE OUTCOMES:

Abstracts:

CONCLUSIONS

Over the three years of this award, we have made substantial progress toward creating the transgenic mouse model with a regulatable ER that was proposed for this project. Germline transmission of the G525L mutant ER has been elusive, however. The parent construct had been successfully used in Dr. Korach’s laboratory to generate transgenic mice. Two different versions of the construct were able to generate chimeric mice, but no germline transmission has yet occurred despite numerous breeding attempts at two different institutions. Preliminary experiments demonstrate that this is not a result of dominant negative effects of the mutant ER, which could potentially inhibit normal development of the fetal heterozygote mouse. We are continuing to pursue creation of this model, and are awaiting the results of the current round of breeding attempts. Additional experiments have been designed in case of failure, which may be due to the effect of the mutant ER on fetal development, despite the assays showing no dominant negative effect. We are hopeful that the transgenic mice will soon be available. We will then be able to characterize the phenotype, followed by experiments to determine how the timing of ER action affects mammary gland maturation and modulates mammary tumorigenesis and latency in models of genetically and chemically induced mammary cancers. With the widespread clinical use of ER ligands, detailed understanding of ER action is increasingly important. This model will allow us to manipulate ER transcriptional activity in-vivo during different developmental stages, and will add significantly to current knowledge on ligand-activated and ligand-independent ER action. Results from our proposal on how the timing of ER activation modulates normal mammary maturation and the development of mammary cancers will likely lead to determining susceptible periods for treatment in the prevention of breast cancer in humans.
REFERENCES


List of personnel receiving pay from the research effort:

1) Sonia L Sugg
2) Geoffrey Greene
3) Jing Li
4) Donna McAllister
5) Bixia Xiang