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13. ABSTRACT *(Maximum 200 Words)*

Towards achieving our overall goal of generating ER knockout mice for studying mammary tumorigenesis, we had proposed to generate ER-α conditional knockout mice. Towards this end, we have faced some difficulty in constructing the targeting construct required for the generation of knockout mice. This delay in making the gene targeting construct for ER-α gene ablation was mainly due to the personnel problem: Dr. Fan Xu, a postdoctoral fellow on this grant, decided to quit only after working for 4 months. Since his departure, we are waiting for another Research Assistant to join the lab, whose H-1 visa has just been approved by the INS and he is expected to start work in my lab on February 1, 2001. In the mean time, as presented in this progress report, we have obtained exciting new data with regard to ERα and ERβ interaction and their target genes in breast cancer cell lines. Our major findings include the demonstration that ER-β forms the strongest homodimer followed by ERα/β heterodimer and ER-α homodimer. The second very important finding is the progress made in identifying target genes for ERα in the breast cancer. Knowledge of which genes are modulated by which dimer pairs of estrogen receptors will be of great importance in interpreting our results in the knockout mice, a central theme of our `original proposal.

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Sohaib A. Khan, P.D. Jan 01

PI - Signature Date

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INTRODUCTION

Estrogens play an important role in the normal physiology of the breast and have a well –established proliferative effect upon breast tissue. It is generally believed that estrogens play a significant role in the development of breast cancer. This contention is strengthened by observations that the estrogen receptor is a hormone dependent transcription factor that regulates the expression of growth factors and protooncogenes in breast tumor cell lines. Moreover, the growth and progression of many breast cancers are dependent upon estrogen, making measurement of ER-α standard in the treatment decisions for patients with breast cancer. The ER-positive breast tumors are generally associated with faster growing and more aggressive tumors than the ER-positive tumors, which can be controlled with antiestrogen therapy. Towards understanding the role of ER in breast tumorigenesis, we have initiated a program, using transgenic mouse technology to ablate ER-α gene in the mammary gland. This mouse model is expected to be of great use in addressing the role of estrogen receptor in mammary tumorigenesis.

BODY

Towards understanding the role of estrogen receptor in breast tumorigenesis, we have made significant progress. However, a few key developments in the estrogen receptor field required us to focus on following critical issues.

1. Description of a new ER-α mutant in breast cancer patients.

After several attempts by many labs to identify a meaningful mutation in estrogen receptor, Fuqua and colleagues (1) have succeeded in identifying a common (34%) somatic mutation in the estrogen receptor-α gene. This mutation (K303R) is observed at high frequency in a typical mammary hyperplasia (premalignancy). The other significance of this mutant is that it is hypersensitive, at very low doses of estrogen, it shows elevated ERE-reporter activity. In preparation for making a transgenic mice line overexpressing this ER mutant in the breast, we have created this mutation in ER-α by site-directed mutagenesis.

2. Study on human ER-K303R mutant:

The mutant was created through mutagenesis followed by sequencing verification. Its transcriptional activity on ERE-lacZ reporter construct was tested in yeast strain RS188N at 1, 10, 100 pM of estradiol. The result did not show obvious difference between the wild type ER and the mutant ER-K303R and the mutant alone did not confer hypersensitivity on low estradiol concentration. Because it has been shown that this mutant can recruit TIF2 at very low estradiol concentration (1), further experiments are designed to address whether the hypersensitivity of this mutation is conferred through the recruitment of TIF2. This will be tested in both yeast and human breast cancer cell lines such as MCF C4-12, MDA-MB231, MDA-MB435, and Hela. Meanwhile, generations of stable cell lines of this mutant in MDA-MB231 and 435 are under way.

3. Protein-protein interaction between ERα and ERβ.
As proposed, our objective is to generate a conditional ER-α knockout mouse model for use in mammary tumorigenesis. Gustafsson and colleagues, who discovered ERβ, have recently proposed a model, called Ying-Yang" model of ER action (personal communication). According to this model ER-α promotes cell proliferation and when needed, ERβ puts a break on ER action. With this model in mind, we have studied the interactions between different ERs using the yeast two-hybrid system. Our results shown in Figure 1, clearly shows that ERβ

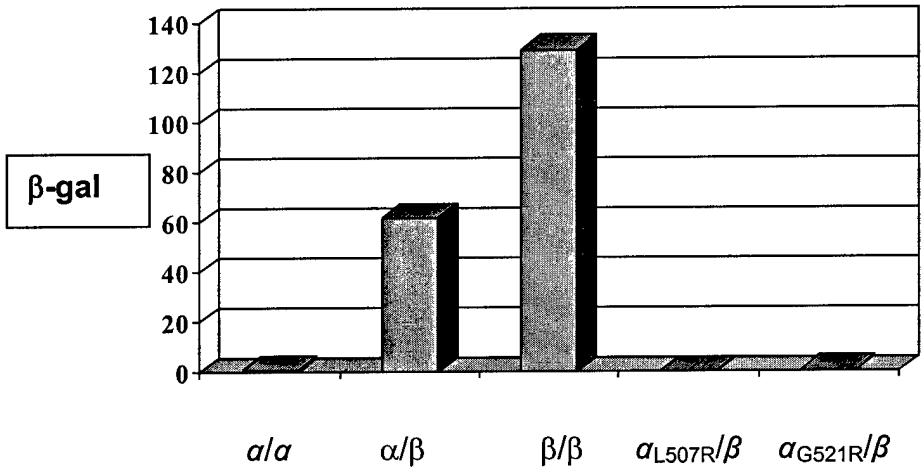

Figure 1. Yeast two-hybrid assay to measure interaction between ERs. The figure shows a "fold-induction" of β-galactosidase activity (a measurement of protein-protein interaction) in yeast transformed with different pairs of ERs (as shown on the X axis). Yeast was transformed with pairs of expression vectors harboring the ERs, and after estradiol challenge the β-galactosidase activity was measured. The higher the β-gal activity, stronger the dimer is.

homodimer is the strongest followed by ERα/β heterodimer. We also tested the ability of two ERα mutants (G521R and L507R) to heterodimerize with ERβ. These mutations are known to make the alpha-receptor homodimerization defective; our results show that they also could not heterodimerize.

4. Non-genomic action of estradiol in C4-12 breast cancer cells. To assess the estrogen receptor requirement for rapid E2 effects, an MCF-7 clone (C4-12) was utilized which does not express detectable levels of ER α or β. Cultures were serum deprived for 48 h prior to brief exposure to E2 (10^{-10} M). For comparison, a C4-12 stable transfectant, (ER-HA-4), which expresses ERα was also analyzed. Surprisingly, the ER null C4-12 cells exhibited marked activation

of ERK1/2 within 5 minutes. ER-HA-4 cells showed a largely reduce effect (Figure 2)

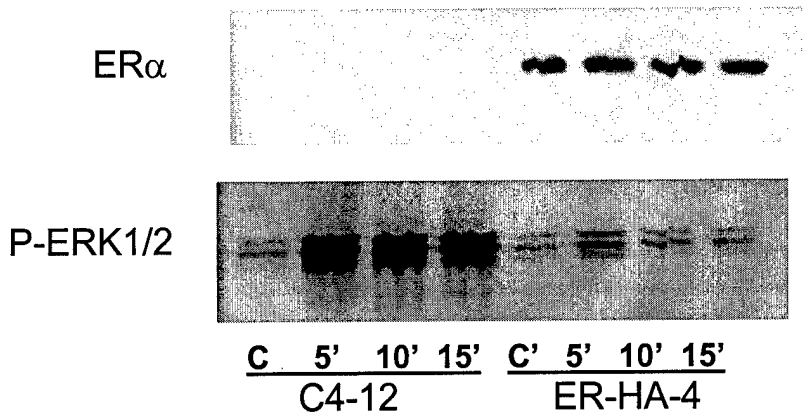

Figure 2. Western blot analysis of ER-α (top panel) and the activated MAPK activity in the breast cancer cells (bottom panel). Cells after a challenge with estradiol for 5, 10 15 minutes were either analyzed for ER-α expression or for the expression of activated ERK1/2. "C" control cells, which did not receive estradiol.

5. Estrogen receptor-α gene targeting vector:

A lambda clone harboring a 10 kb Bam H1 fragment of mouse ERα gene was obtained from Dennis Lubahn, (University of Missouri). Since it was an old preparation of the lambda DNA, we first determined the titer of the phage, which turned out to be 8.6 x 10^8 pfu/ml. We then made a large-scale preparation of the phage DNA. A restriction enzyme analysis revealed spurious results. We suspect that the clone, we received from Dr Lubahn may not be the right clone. There are two more clones that we need to analyze. However, simultaneously, we are also making plan to clone our own mouse ERα gene. This snag has delayed our plan but we are optimistic that the targeting construct will be ready for blastocyst injection in the next 2-3 months. One reason that contributed to this unexpected delay in the preparation of the targeting construct was the fact that the key person, Dr. Fan Xu quit only after 4 months of his employment in my laboratory. Although Dr. Xu came with previous experience in gene knockout technology and worked diligently, he decided to change career and switched to software programming field. Since his departure, I have offered the job to another experienced person in the field of molecular biology, who received his Master's degree from the Children's Hospital Medical Center at the University of Cincinnati. Although the offer was made in October 2000, we received the approval for his H-1 visa from the INS on 1/25/01. Mr. Feng is now expected to start on this project on February 1, 01.

Proposed Changes for the Future Work:
In light of the new developments in the field and our new observations, we propose the following changes. These changes will not affect the overall objectives of the original proposal. In fact the new experiments will be in addition to those proposed in the original application, and are expected to strengthen the final outcome of this proposal. We are not requesting additional funds for these experiments. Any additional funds needed, will be obtained from other sources.

1. Finish the microarray analysis of the breast cancer cell lines expressing either of the two ERs alone or both.
2. Using a mammary specific WAP promoter generate transgenic mice overexpressing dominant negative ER cDNA. The transgenic mice expressing the dominant negative cDNA are expected to be null for both ERα and ERβ activities. These transgenic mice will be useful in addressing the role of the two forms of ER in mammary tumorigenesis.

Key Research Accomplishments:

- Compared the dimerization potential between ERα and ERβ and found that the ERα/β heterodimer is the strongest. This information will be important in analyzing our results in the transgenic mice.
- Using microarray analyzed if different ER dimmers have unique target genes in the breast cancer cell line.
- Observed that the somatic ERα mutant did not possess elevated ERE-dependent activity.

REPORTABLE OUTCOMES
Abstracts:

Singleton, D., and Khan, S. "Estrogen receptor dependence of MAP-kinase activation in breast and uterine tumor cells Annual Meeting of the "ENDOCRINE SOCIETY", Denver, Co (2001)

Cell Lines:

We have generated yeast cell lines transformed with different pairs of the estrogen receptor.

CONCLUSIONS
Estrogen receptor plays a key role in breast cancer. However, little is known about the involvement of the receptor in the progression of breast tumorigenesis. Towards achieving our overall goal of generating ER knockout mice for studying mammary tumorigenesis, we had proposed to generate ER-α conditional knockout mice. Towards this end, we have faced some difficulty in constructing the targeting construct required for the generation of knockout mice. This delay in making the gene targeting construct for ER-α gene ablation was mainly due to the personnel problem: Dr. Fan Xu, a postdoctoral fellow on this grant, decided to

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Cancer Res 2000; 60:4026-9.