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Progestins and glucocorticoids are two classes of steroid hormone with very distinct biological functions. Progestins have been shown to be related to the incidence and progression of breast cancer, but glucocorticoids have no direct relation with breast cancer to our knowledge. However, progesterone receptor and glucocorticoid receptor share many structural and functional similarities including identical DNA sequence recognition characteristics. In order to understand the role of progestins in breast cancer and how progesterone receptor mediates this hormone specific regulation, we proposed to identify genes that are differentially regulated by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2 using two systematic strategies: a retroviral promoter-trapping strategy and an oligonucleotide array analysis. A modified retroviral promoter-trapping system has been established. Several clonal cell lines that represent potential hormone differentially regulated genes were generated using this system. In the Affymetrix oligonucleotide array analysis, genes that are differentially regulated by progestins and glucocorticoids were identified. The findings from these studies provide us interesting study candidates for understanding the mechanisms of gene differential regulation by progestins and glucocorticoids in breast cancer as well as how progestins function specifically in breast.

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Yihong Wan 7/5/00

PI - Signature Date

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Introduction

Progestins and glucocorticoids are two class of steroid hormones with very distinct biological functions. The major physiological role of progestins in the mammal are to establish and maintain pregnancy in the uterus and ovary; to promote lobular-alveolar development of the mammary gland and to supress milk protein synthesis during pregnancy. Some classical biological actions of glucocorticoids are regulation of metabolism and supression of bone formation, inflammation and the immune system. In the pathology of breast cancer, it has been shown that progestin agonist can increase the incidence of spontaneous mammary tumors. Some studies indicated that physiological doses of progestin agonists can stimulate the growth of established tumors, while high pharmacological dose progestins can inhibit the growth of established tumors (1). However, there is no evidence of a role of glucocorticoids in breast cancer even though both progesterone receptor and glucocorticoid receptor are expressed in the mammary gland. Interestingly, progesterone receptor (PR) and glucocorticoid receptor (GR) share many similar structural and functional characteristics: similar functional domains; similar, if not identical, DNA sequence recognition specificity; associate with a similar complex of molecular chaperones in the absence of hormone and with a similar set of coactivators or corepressors in the presence of hormone. How can two receptors with such remarkable similarity mediate such dramatically different biological functions? We hypothesize that one way that progestins and glucocorticoids can exert different biological effects is through their different abilities to regulate the expression of certain target genes. In order to understand the role of progestins in breast cancer and how progesterone receptor mediates this hormone specific regulation, the purpose of this research is to identify genes that are differentially regulated by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2 using two systematic strategies: a retroviral promoter-trapping strategy and an Affymetrix oligonucleotide array analysis. Promoters of the genes identified will be cloned. The long term goal of this investigation is to understand the mechanisms of the differential hormone regulation. The genes identified in this investigation play an important role in hormone functional specificity. Knowledge gained from this investigation will be fundamental to the understanding of both hormone actions and their roles in breast cancer.

Body

AIM ONE: Develop a retroviral promoter-trapping strategy to identify promoters/enhancers differentially induced by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2.

To identify the promoters that can be differentially induced by progestins and glucocorticoids, a cre/lox retroviral promoter-trapping strategy was designed based on the elegant system originally designed by Dr. Harald von Melchner (2). Due to the difficulty of obtaining stable clonal cell lines from T47D/A1-2 cells, I have established a cre/lox retroviral promoter-trapping system in a mouse fibroblast cell line, 4F, that expresses GR from the endogenous gene and the B-isoform of PR from a stably transfected and integrated gene (3,4). This system will allow us to identify the promoters that can be induced by progestins and glucocorticoids as well as the promoters that can be differentially induced by these two hormones. The expression of the genes identified in this system will then be analyzed in the breast cancer cell line T47D/A1-2.

First, I constructed four double-selection reporter plasmids by modifying the original plasmid obtained from Dr. von Melchner. After considerable trial and error, the double-selection reporter "tkneo/hygro" turned out to permit the most desirable selection. In the tkneo/hygro double-selection reporter plasmid, the first selection marker, a fusion gene between herpes thymidine kinase and the neomycin resistance gene (tkneo) (5), was flanked by two direct-repeats of loxP sites. The second selection marker, a hygromycin resistance gene, is located behind the second loxP site (Fig. 1). When introduced into a cell, this tkneo/hygro double-selection reporter allows sensitive detection of the expression of Cre recombinase in the cell. In the absence of Cre, the first marker "tkneo" gene will be expressed from a constitutive mouse pgk promoter, the second marker "hygro" gene will not be expressed due to the lack of a second promoter in front of it and the presence of a pair of polyadenylation sequences upstream to ensure that translatable mRNA encoding the second marker cannot be produced. Therefore, the cells will be neomycin resistant but hygromycin sensitive. When Cre is expressed, it catalyzes recombination between the loxP sites deleting the sequences in between (6,7). Thus, the first selection marker is deleted, and the second selection marker is placed right behind the pgk promoter and is expressed. The cells now will be neomycin sensitive but hygromycin resistant (Fig. 1). Therefore, 4F cell lines with a stably integrated copy of the tkneo/hygro double-selection plasmid can report the expression of Cre in each cell by undergoing a drug-resistance-phenotype switch.

To generate a 4F cell line with stably integrated tkneo/hygro double-selection reporter, I transfected the tkneo/hygro plasmid into the 4F cell line by electroporation (8). Neomycin resistant cells were cloned and subcloned. Several experiments were performed to choose the clones that can detect Cre expression by a switch of drug resistance phenotype. First, these neomycin resistant cells were put under hygromycin selection to test whether there is any leaky expression of hygromycin resistant gene either due to transcription through the polyadenylation sequences or due to the integration of the hygromycin gene behind a constitutively active cellular promoter. The 4F clones that display hygromycin sensitivity were saved for further test. Second, in order to test whether the 4F clones will switch to a phenotype of hygromycin resistant and neomycin sensitive after Cre expression, I constructed an expression plasmid pCMVCre and transiently transfected it into the 4F tkneo/hygro clones by a calcium phosphate coprecipitation method. Cells were selected for hygromycin resistance. Then the hygromycin resistant cells

were pooled and put under neomycin selection to test neomycin sensitivity. Those original 4F-tkneo/hygro clones that gave rise to hygromycin resistant but neomycin sensitive cells after Cre expression were saved for further test. Third, genomic DNA Southern blot analyses were performed to detect the copy number and integrity of the tkneo/hygro insert. To test integration copy number, a restriction enzyme that cuts only once in the double-selection reporter construct (BamHI, see Fig. 1) was used to digest the genomic DNA from each 4F clone. To test insert integrity, a restriction enzyme that cuts the plasmid both in front of and behind the tkneo/hygro cassette (AlwNI, see Fig. 1) was used to digest the genomic DNA from each 4F clone. Digested DNA was separated by agarose gel electrophoresis and transferred to nylon membrane. The membrane was probed with either neomycin resistance gene or hygromycin resistance gene. The 4F-tkneo/hygro clones with low copy number and good integrity of integration were saved for further test. Out of 24 4F clones that I have screened, two clones (clone 21 and 29) can undergo clean drug-resistance-phenotype-switch once there is Cre gene expressed in the cells, thus, these two clones were chosen to be the best candidates for further tests.

In this gene-trapping system, a promoterless Cre gene will be randomly integrated into the genome of the 4F-tkneo/hygro target cell line and will function as a promoter trap (2). The promoterless Cre gene is contained in a self-inactivating retroviral plasmid vector [pGgU3creen(-)] obtained from Dr. von Melchner, and is inserted in the U3 region of an enhancer-deleted long terminal repeat. Viral replication and long terminal repeat-mediated duplication place the promoter-less Cre sequence just 30 nucleotides downstream of the flanking cellular DNA (Fig. 2) (9,10). Therefore, Cre is not expressed unless the virus integrated into a cellular promoter, a constitutively active promoter or an inducible promoter. In order to generate a cell line that can produce U3Cre virus, I stably transfected this retroviral plasmid into an amphitropic packaging cell line PA317 (11) obtained from Dr. Gail Harrison by a calcium phosphate coprecipitation method. Since the U3Cre retroviral plasmid lacks a selection marker, the plasmid pRSVneo was cotransfected at 1/10 amount of the retroviral plasmid. Neomycin resistant colonies were cloned. Clones were screened for the existence and integrity of Cre gene by PCR amplification of genomic DNA followed by Southern blot analysis. Out of 8 clones screened, #2, 3, 4 and 8 are Cre-gene positive. The ability of these packaging cell clones to produce U3Cre retrovirus was tested by infecting the target cells previously transfected with the double-selection reporter plasmid (4F-tkneo/hygro cells). The infected cells were put under hygromycin selection. The appearance of hygromycin resistant colonies, resulting from the integration of the U3Cre provirus behind a constitutively active cellular promoter, indicated that PA317-U3Cre clone #2, 3, and 4 can produce functional U3Cre virus, and virus generated by clone #4 had the highest titer. Viral stock were grown up and titered using 4F-tkneo/hygro clone #21 as target cells. Because the U3Cre provirus lacks a constitutively expressed drug resistance marker, virus titers were derived by multiplying the number of hygromycin resistant colonies by the average frequency of integrations that enable U3 gene activation of other similar gene-trap vectors (9,12). The viral stocks have high titer ($>10^7$ cfu/ml) and will be used for infection in the promoter-trap selection.

To further test the efficiency of infection on the two different 4F-tkneo/hygro clonal cell lines selected by previous screening, these cell lines were infected by the same U3Cre viral stock. Hygromycin resistant colonies resulting from the integration of the U3Cre provirus behind a constitutively active cellular promoter were counted. Results indicated that for these two target cell lines, clone #21 had a slightly higher infection efficiency. These hygromycin resistant cells were pooled and analyzed further. First, to test for neomycin sensitivity, these

cells were put under neomycin selection. After 12 days of selection with 1mg/ml G418, both clone 21 and 29 showed 100% neomycin sensitivity. Second, to confirm the existence of both functional GR and PR in the 4Ftkneo/hygro clone 21 cell line, a MMTV promoter-luciferase reporter and an internal control pCMVβ-gal were transiently transfected into the clone 21 cells by a DEAE/Dextran method (47). Forty-eight hours after transfection, cells were treated with dexamethasone (10^{-6}M, 10^{-7}M, 10^{-8}M) or R5020 (10^{-8}M) for 24 hours. Cells were harvested, and luciferase and β-gal assays were performed as described (13). Results shown in Fig. 3 demonstrated that both dexamethasone and R5020 treatments can lead to good induction of the MMTV promoter, indicating functional GR and PR were expressed in the 4Ftkneo/hygro clone 21 cells. Furthermore, this result demonstrated that the MMTV promoter can be induced at the concentration of the hormones that ensures receptor-specific binding ($\leq 10^{-7}$M for Dex and $\leq 10^{-8}$M for R5020). These concentrations for the hormones will be used for the promoter-trap selection.

The screening strategy I used is shown in Fig. 4. The 4Ftkneo/hygro cells were infected with U3Cre retrovirus at MOI of 0.5-1. The cells were placed under G418 selection. Those cells that harbor a retrovirus intergrated downstream of a constitutive active promoter will die due to the expression of Cre and the deletion of the neomycin resistance gene. The survivors were split into 2 groups, one treated with R5020 (10^{-8}M) , the other with dexamethasone (10^{-7}M). To identify promoters that can be induced by one hormone, the cells were switched to hygromycin selection, therefore cells represent a progestin inducible promoter were identified in group A and cells represent a glucocorticoid inducible promoter were identified in group B. To identify promoters that can be induced only by glucocorticoid but not progestin, the cells treated with R5020 were maintained in G418 media, therefore those cells represent a progestin-inducible promoter will die. Now the cells were treated with dexamethasone (10^{-7}M) and then switched to hygromycin selection to select those cells represent a promoter that is only inducible by glucocorticoid but not progestin (group C). Likewise, cells represent a promoter that is only inducible by progestin but not glucocorticoid were identified in group D. For each of the four groups, 30-60 cell lines were cloned and expanded. In the future studies, I will analyze the genes and promoters represented in each of these clonal cell lines. For those genes that is truely differentially regulated by progestins and glucocorticoids, I will study the mechanism of their differential regulation.

AIM TWO Implement Affymetrix oligonucleotide array analysis to identify genes differentially regulated by progestins and glucocorticoids in the human breast cancer cell line T47D/A1-2.

T47D/A1-2 cells were treated with vehicle, dexamethasone (10^{-7}M) or R5020 (10^{-8}M) for 2hrs or 6hrs. Total RNA was harvested from each sample using purescript RNA isolation kit (Gentra system). Total RNA was DNaseI (RNase-free) treated to remove trace amount of genomic DNA contamination and purified by phenol/chloroform extraction. 10μg of total RNA were used to synthesize double-strand cDNA. cRNA were produced using in vitro transcription with biotin-labeled nucleotides incorporated according to the procedure recommended by Affymetrix. cRNA were purified and used to hybridize to the HuGeneFL oligonucleotide chip array. After washing and staining, the chip array was scaned. In the future studies, the raw data obtained from the scan result of each array will be analyzed and genes that are differentially regulated by progestin and dexamethasone in T47D/A1-2 cells will be identified. The differential regulation will be confirmed by RT-PCR, Northern blot analysis and/or western blot analysis. The mechanisms of their differential regulation will be studied.

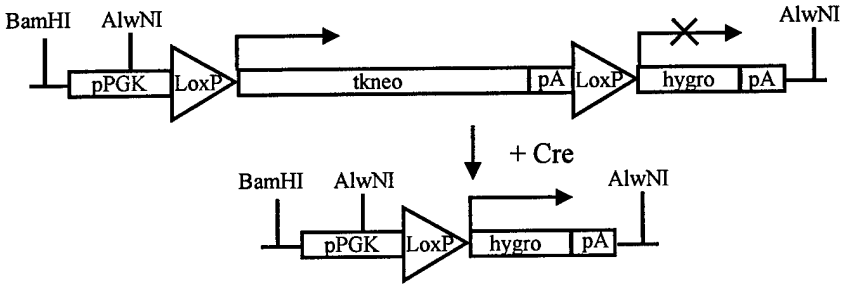

Figure 1 **Structures of the double-selection reporter construct before and after Cre-mediated recombination at loxP sites.** Gene sequences are labeled as following: pPGK, mouse phosphoglycerate kinase promoter; tkneo, herpes simplex virus 2 thymidine kinase (tk)-neomycin phosphotransferase (neo) fusion gene; hygro, hygromycin-B phosphotransferase; pA, a pair of polyadenylation signals from the growth hormone gene.

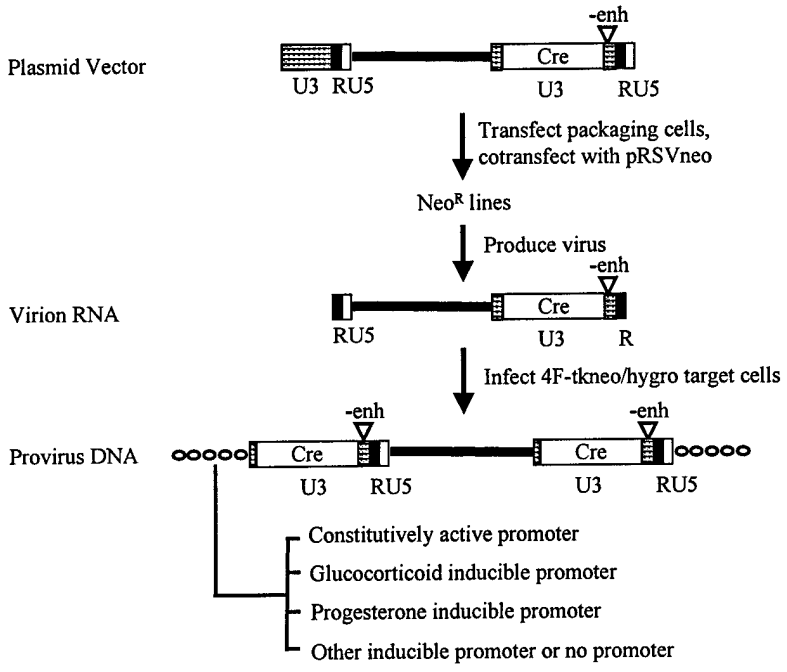

Figure 2 **Structure and replication of the U3Cre retroviral vector.** Gene sequences are labeled as following: Cre, modified bacterial phage P1 Cre recombinase; U3, R, and U5 are segments of the viral LTR. Infection of 4F-tkneo/hygro target cells generates provirus flanked by Cre sequences. Neo^R; neomycin-resistant.

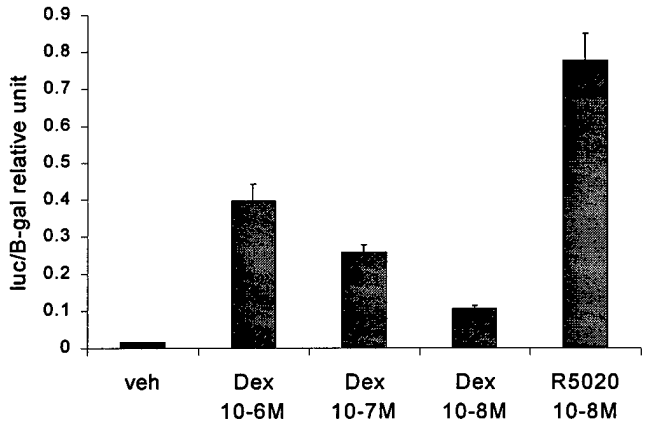

Figure 3 **Detection of functional GR and PR in 4Ftkneo/hygro #21 clonal cell line.** A MMTV-luciferase (pAHluc) reporter gene and an internal control pCMVβ-gal were transiently transfected into clone 21 cell line with a DEAE/Dextran method. 48 hours after transfection, cells were treated with dexamethasone or R5020 for 24 hours. Results are shown as luciferase activity normalized by β-gal activity. Results are shown as the average of triplet samples ± SD.

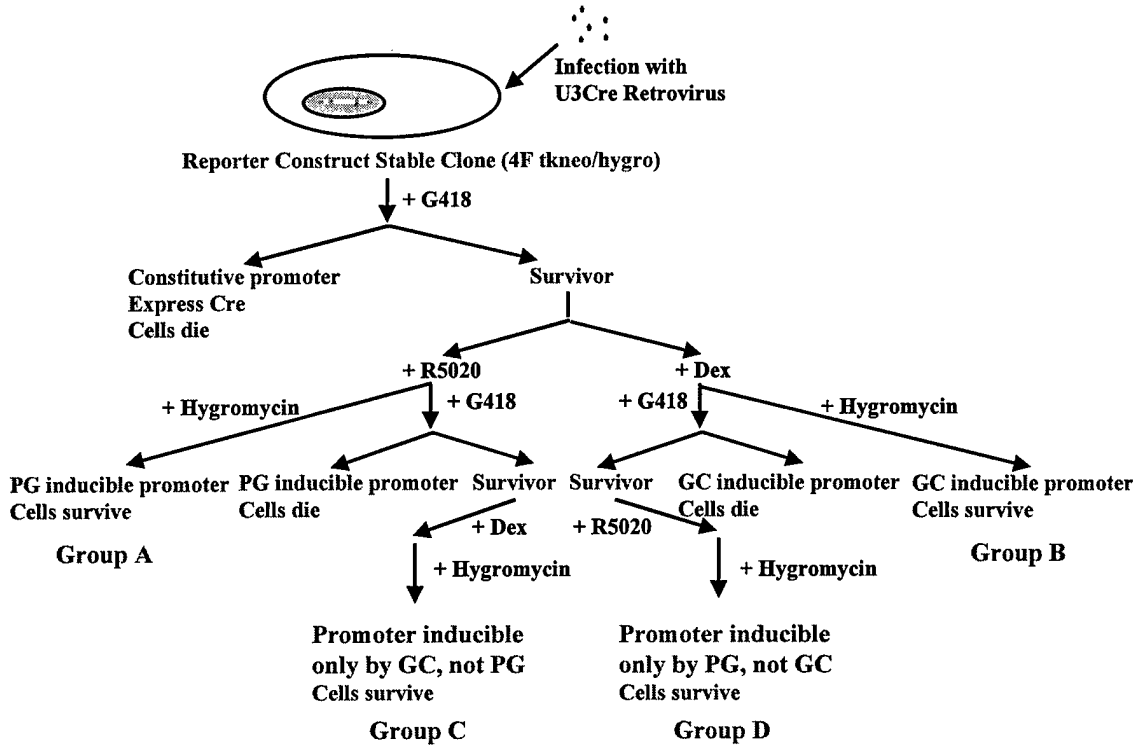

Figure 4 **Diagram of the screening strategy.** "Dex", dexamethasone; "PG", progestin; "GC", glucocorticoid.

Key Research Accomplishments

Aim One:

- A double-selective reporter plasmid was modified from the original construct.
- A stable cell line 4Ftkneo/hygro that can undergo drug-resistance phenotype switch after cre expression in the cells was established.
- A PA317 packaging cell line that can produce U3Cre retrovirus at high titer was established.
- Sequential selection procedures were performed and clonal cell lines representing potential hormone inducible and differentially inducible genes were cloned.

Aim Two:

- Total RNA was isolated from T47D/A1-2 cells treated with vehicle or hormone. cRNA were synthesized for the oligonucleotide array hybridization.
- Affymetrix HuGeneFL oligonucleotide arraies were hybridized to cRNA. Initial scan data were obtained.

Reportable Outcomes

Aim One:

- A stable cell line 4Ftkneo/hygro that can undergo drug-resistance phenotype switch after cre expression in the cell was established.
- A PA317 packaging cell line that can produce U3Cre retrovirus at high titer was established.

Aim Two:

- A database of genes that are regulated by progestins or glucocorticoids in T47D/A1-2 cells were generated based on the result of Affymetrix HuGeneFL oligonecleotide array analysis.

Conclusions

Several clonal cell lines that represent potential hormone differentially regulated genes were generated in the retroviral promoter-trapping system. Genes that are regulated and differentially regulated by progestins and glucocorticoids were identified in the oligonucleotide array analysis.

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Appendices N/A