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13. ABSTRACT (Maximum 200 Words) Two important transcriptional targets of p53 tumor suppressor protein are genes encoding the Proliferating Cell Nuclear Antigen (PCNA) and p21/WAF1/Cip1. PCNA is a necessary component of DNA replication and DNA repair machinery. p21/WAF1/Cip1 is a cyclin-dependent kinase (cdk) inhibitor which can interact with PCNA to modulate the balance of DNA repair versus replication. We hypothesize that correct ratio of PCNA and p21 proteins is crucial for normal regulation of DNA repair and cell cycle control, and hence, dysregulation of pcna and p21 transcription in response to genomic damage is an important aspect of breast cancer formation. To test this hypothesis in vivo, we are developing a mouse model where p53 signaling specifically to the pcna and p21 gene transcription is disrupted. Toward this goal, we are characterizing p53 interaction sites on mouse p21 and pcna gene promoters (from a series of BAC clones isolated with the help of Roswell Park Cancer Institute Microarray and Genomics Facility). This mouse model will enable testing the relevance of specific transcriptional signaling by p53 to mammary oncogenesis, identification of new therapeutic targets, and analyzing the role of specific p53 transcriptional targets in modulating responses to chemotherapeutic drugs and radiation therapy.					
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INTROUCTION:

Elimination of functional p53 tumor suppressor protein through various mechanisms is the single most common event in human cancer, occurring in more than 50% of human cancers (1). p53 elicits its biological effects mainly by functioning as a transcriptional regulator of various target genes (2). Two such p53 targets important in cellular response to genomic damages are genes encoding the Proliferating Cell Nuclear Antigen (PCNA) and p21/WAF1/Cip1 (3). PCNA is a necessary component of DNA replication and DNA repair machinery. It forms a doughnut-like structure around DNA thereby forming a sliding platform for interaction of various proteins involved in DNA repair, DNA replication, and cell cycle control. p21/WAF1/Cip1 is a cyclin-dependent kinase (cdk) inhibitor which can interact with PCNA to modulate the balance of DNA repair versus replication.

In response to genomic damage, p21/WAF1/Cip1 is induced at the transcriptional level by p53. Results from several laboratories including ours have shown that PCNA is co-induced with p53 in response to genomic damage, and that p53 is a transcriptional activator of PCNA (4). However, the mechanisms by which p53 exert transcriptional regulation of PCNA and p21/WAF1/Cip1 genes in normal cells and in cells subjected to genomic damage remain unknown. We hypothesize that correct balance of PCNA and p21 amounts is crucial for normal regulation of DNA repair and cell cycle control, hence, dysregulation of PCNA and p21 transcription in response to genomic damage is an important aspect of breast cancer formation. To test this hypothesis in vivo, we are developing a mouse model where p53 signaling specifically to PCNA and p21 gene transcription is disrupted by knocking out the p53 interaction sites on the promoters of the genes. Since we will be disrupting p53 signaling to the PCNA or p21 transcription ONLY at a time, this model will facilitate analyzing the role of gene-specific transcriptional regulation by p53 in breast cancer. Such analysis has been impossible with the conventional p53 knockout (p53^{-/-}) mouse model where regulations of multiple p53 target genes besides PCNA are disrupted. This animal model will enable us to analyze in breast tissue the developmental and physiological consequences of uncontrolled PCNA and p21 expression when their transcription is divorced from the regulatory role of p53. Moreover, unlike conventional knockouts, our model will not be completely compromised for PCNA expression, thereby preventing the possibility of embryonic lethality. PCNA is expressed in cells, which are null for p53, and these cells can divide suggesting that they are capable of DNA replication. We hypothesize that is DNA repair that would mainly be affected if PCNA and/or p21 transcription is unlinked from p53 control, and hence, more relevant for tumorigenesis.

The novel mouse model proposed here will provide physiological in vivo experimental system to test the relevance of specific transcriptional signaling by p53 to mammary oncogenesis, which in turn, could be used for identifying specific signaling pathway targets in developing therapeutic strategies to combat breast cancer. This

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would also be a promising system to analyze the role of specific p53 transcriptional targets in modulating responses to chemotherapeutic drugs and radiation therapy.

BODY:

The first step toward generating the mouse model described above was to characterize the mouse pcna gene promoter with respect to transcriptional regulation by p53. Although the p53-binding site on the human pcna gene is well documented (including work from our laboratory), there are no reports available on p53 sites on the mouse pcna gene promoter. Also, relatively short region of the mouse gene promoter has been reported thus far. Therefore, our first attempt was to obtain long stretches of mouse pcna promoter. We wanted to obtain the gene from the same strain of mouse that is the source of embryonic stem cells (ES cells) to be used for making the chimeric mouse. Introduction of the altered pcna and p21 genes into ES cells, generation of chimeric mouse, and finally mice homozygous for the pcna and p21 mutant genes will be accomplished with the help of Gene Targeting and Transgenic Facility at the Rosewel Park Cancer Institute, Buffalo, New York. The ES cells used at this facility was derived from 129SVJ strain of mouse. Therefore, we decided to screen a bacterial artificial chromosome BAC) library (RPCI-22) of genomic DNA from 129SVJ mouse strain for genomic clones of pcna and p21 genes.

Screening of BAC library.

The screening of BAC library was performed with the help of the DNA Microarray Facility at the Rosewel Park Cancer Institute. Sequence of mouse p21pcna genes was obtained from Celera mouse database. DNA sequences identified from the database and the sequence of the 40 bp overgo probe for screening the RPCI22 IBAC library are shown below:

>p21_5_UTR (120-200 specific)

aagaccagagggagcctgaagactgtgatgggtagttccatagtgacccgggtccttctgtgttcagccacagcgac
c

Complete overgo sequence for P21_5_UTR2 :
AGCCTGAAGACTGTGATGGGGTAGTTTCCATAGTGACCCG

>pcna_5_UTR (highly specific)

taggggtgttaaaataggtggcctcttattactcgatatattgcagcgtatttcttacgtagggaaaacgctccgtagtgtta
aaatactctccagcttaaggcaggcgcgcgcacagctcgatttgctgtgacttccacttccgtggcgcggaacttcc
taaggatggaaactgcagcctaaactcccacaaacttggcggtgacgacagcctacgcgaaccccgtagtcccctc
gcctccaggctcctacccgcagccccgccttgcatacgcggtggggcgggccttgcctaaaccagggtagcattgg
tccttgaggagaggtgggtggatcagcgctgtggcgatgacctcgcgagggaaaaggcgcgcgcttaggaagccg
cg

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Complete overgo sequence for PCNA_5_UTR :
CGCGGAAACTTCCTAAGGATGGAACTGCAGCCTAACTC

Initial screen of the BAC library was performed by Southern hybridization using the overgos specific for p21 and pcna as probes. 18 positive clones were obtained. To confirm the authenticity of the clones, all the 18 clones were re-screened by PCR with oligonucleotides specific for p21 and pcna genes. From the PCR screen, 14 clones were confirmed positive of which 11 were p21 clones and 3 were pcna clones.

Identification of p53-binding sites on pcna gene promoter.

For disrupting the p53 signaling specifically to the pcna and to the p21 gene trnscription, it is essential to know what are the p53-responsive sites on the proter regions of these genes. As discussed earlier, p53-responsive elements are reasonably well characterized in the case of p21 gene, whereas no such information exists for the mouse pcna gene. As a first step toward unraveling the p53 -responsive elements on the pcna gene promoter, we searched pcna promoter region (3496 bp upstream of the transcription initiation site) for consensus p53-binding site. The results we obtained in a search using the MacVector software are as follows:

Sequence Range: 1 to 3495

```

      10      20      30      40      50      60
CTTGCTCTCCCTGTGGTCCTGAGTTCCAATCCTAACAACCACATGGTGGCTCACAACCAC
GAACGAGAGGGACACCAGGACTCAAGTTAGGATTGTTGGTGTACCACCGAGTGTGGTG

      70      80      90     100     110     120
CCAAAATGAGATCTGATGCCCTCTTCTGGGTGACAGCTACAGTGTACTTATGTATAATAA
GGTTTTACTCTAGACTACGGGAGAAGACCACATGTCGATGTCACATGAATACATATTATT

      130     140     150     160     170     180
TAAGTACATCTTTAAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAT
ATTCATGTAGAAATTTTATTATTATTATTATTATTATTATTATTATTATTATTATTATTATA

      190     200     210     220     230     240
CTCCAGGAGGCTGAGGCATAGCTCAAAGGCGGGTTTCTGTTTAGCTTTTCTGAAGTCCTG
GAGGTCCTCCGACTCCGTATCGAGTTTCCGCCCAAAGACAAATCGAAAAGACTTCAGGAC

      250     260     270     280     290     300
ACATCAGTTCCCAGCATTAAGAAAAAGTCTCTAAAATAATTACATTCTAGGCATTAAAG
TGTAGTCAAGGGTCGTAATTCTTTTTCAGAGATTTTATTAATGTAAAGATCCGTAATTC

      310     320     330     340     350     360
AAAATATTATATAAGCCCCAGCACCGAGACAGAGCAACAGAAGAAACATAGTGTGTGTA
TTTTATAATATATTCGGGGGTCGTGGCTCTGTCTCGTTGTCTTCTTTGTATCACAACAAT

      >p53
      |
      370 | 380     390     400     410     420
GTGTTGTTAGCAAAGCAAGACCACAAGTCAGAAAGAACACATTCTATGGTTTGCAAAGGA
CACAACAATCGTTTCGTTCTGGTGTTCAGTCTTCTTGTGTAAGATACCAAACGTTTCCT

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430 440 450 460 470 480
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 TTA CTCTAAAATTCTTTGAGTTCTTTTCTTTTAAAGAGTGTATATACATCGACATACTA

 490 500 510 520 530 540
 GGGTTTTTATTTTGTGCTGTGTTGTTGGACACACAGTAACACTAAGCTGCAAGAC
 CCCAAAAATAAAACAAACACGACACAACAACCTGTGTGTCATTGTGATTGACGTTCTG

 550 560 570 580 590 600
 CAGTCCTACATTATCTCTTGCTGTGTGAAGGAATAATTCAGGCTGTCAAGACAGCTTAGC
 GTCAGGATGTAATAGAGAACGACACACTTCCTTATTAAGTCCGACAGTTCTGTGCAATCG

 610 620 630 640 650 660
 AGGTAAAGGGACTTAACTTACCACTAAGCCTGGCAACCTGAGTTCTATCCTGGGATCCAG
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 670 680 690 700 710 720
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 <p53
 |
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 790 800 810 820 830 840
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 >p53 <p53
 | |
 850 860 870 880 890 900
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 910 920 930 940 950 960
 CTTAACAAGCAGAGTGAATACATTAAAAACAGCAGCAGCAGCTCAGTGGGAAACT
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 970 980 990 1000 1010 1020
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 |
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 1090 1100 1110 1120 1130 1140
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<p53

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1150 1160 1170 1180 1190 1200
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1210 1220 1230 1240 1250 1260
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 GCTTGAGTCTTTAAGTGGACGGAGACGGAGGGTTCACGACCCTAATTTCCGCACCTGGTG

1270 1280 1290 1300 1310 1320
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1330 1340 1350 1360 1370 1380
 GTGGGGGCTGAAAGTATAGTAGTGGGGTTTTCTGGGTTTTGTTTTTAATTTTAAAAAT
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1390 1400 1410 1420 1430 1440
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1450 1460 1470 1480 1490 1500
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<p53

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1570 1580 1590 1600 1610 1620
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1630 1640 1650 1660 1670 1680
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1690 1700 1710 1720 1730 1740
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<p53

|

<p53

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1810 1820 1830 1840 1850 1860
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1870 1880 1890 1900 1910 1920

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TCACTTGTACATCCCTTTGGCTTGTGAACGGTAGTCTCACCAGCTCTCATAAGATCTGG

1930 1940 1950 1960 1970 1980
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1990 2000 2010 2020 2030 2040
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2050 2060 2070 2080 2090 2100
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2170 2180 2190 2200 2210 2220
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2230 2240 2250 2260 2270 2280
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2350 2360 2370 2380 2390 2400
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2410 2420 2430 2440 2450 2460
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2470 2480 2490 2500 2510 2520
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2590 2600 2610 2620 2630 2640
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2650 2660 2670 2680 2690 2700
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2890 2900 2910 2920 2930 2940
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2950 2960 2970 2980 2990 3000
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3070 3080 3090 3100 3110 3120
 GAAAAGTGATATGAAATGGGGGGGGGTAGGGGTGTTAAATATGGTGGCCTCTTTATTA
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3130 3140 3150 3160 3170 3180
 CTCGATATTTTGCAGCGTATTTCTTACGTTAGGGAAAACGCTCCGTAGTGTTTAAATAC
 GAGCTATAAAACGTCGCATAAAGAATGCAATCCCTTTTGCAGGCATCACAAATTTTATG

3190 3200 3210 3220 3230 3240
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 AGAGGTGCAAGTTCCGTCCGGCGGCGCGTGTGAGCTAAACGGACACTGAAGGTGAAGGC

3250 3260 3270 3280 3290 3300
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3310 3320 3330 3340 3350 3360
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3430 3440 3450 3460 3470 3480

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AGGAGAGGTGGGTGGATCAGCGCTGTGGCGTCATGACCTCGCGCAGGGAAAAGGCGCGCG
TCCTCTCCACCCACCTAGTCGCGACACCGCAGTACTGGAGCGCGTCCCTTTTCCGCGCGC

3490

CCTAGGAAGCCGCGG
GGATCCTTCGGCGCC

Further sequence examination in terms of conservation of crucial residues suggested that sites at 727, 1052, and at 1555 are the most likely functional sites.

It is essential to ensure the functional relevance of the binding sites identified based on sequence homology. To address this issue, we are planning to verify which one of these sites is transcriptionally p53-responsive in a transfection assay. Toward this goal, we are in the process of cloning the > 3 kb pcna promoter fragment into pGL3 Luciferase assay vector that has been extensively used in our laboratory for transcription assays in vivo.

Once we ascertain the functional authenticity of p53 sites on the mouse pcna and p21 gene promoters in a highly sensitive luciferase reporter-based transcription assay, we will also construct mutant versions of the promoter where the p53-binding site(s) are functionally debilitated by mutagenizing critical residues. We will clone a large region (~9kb) of pcna gene promoter encompassing the mutant p53-binding sites into a ES cell targetting vector. The ES cells carrying these mutant pcna gene will be injected into blastocysts to create chimeric mouse followed by breeding to generate mice which are homozygous for the engineered mutation. To direct conditional p53 signaling disruption to the pcna gene in mammary tissue, we will use the Cre/lox-recombinase system (5). Using a similar strategy, we will also generate mice that are homozygous for mutant p21 gene.

KEY RESEARCH ACCOMPLISHMENTS:

- Isolated mouse genomic clones of pcna and p21 genes.
- Identified putative p53-interaction sites on the pcna gene promoter.

REPORTABLE OUTCOMES:

Too early to be submitted as a manuscript for publication.
An abstract has been submitted to the upcoming third Era of Hope meeting to be held in Florida, September 25-28, 2002.

LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

Hui Yang, M.D., Ph.D.
Ying Yang, M.S.

PROPRIETARY/UNPUBLISHED

CONCLUSIONS:

Once we succeed in generating the mouse carrying mutations that would specifically disrupt p53 signaling to the transcriptional regulatory machinery of pcna and p21 genes, such mice will be an important model system to analyze mechanisms of mammary oncogenesis.

"So What?"

Information gained could be used for identifying specific signaling pathway targets in developing therapeutic strategies to combat breast cancer. This would also be a promising system to analyze the role of specific p53 transcriptional targets in modulating responses to breast cancer chemotherapeutic drugs and radiation therapy.

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DEPARTMENT OF THE ARMY
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REPLY TO
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11 Mar 03

MEMORANDUM FOR Administrator, Defense Technical Information
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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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