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by In Vivo Crosslinking

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FOREWORD

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Introduction

This report provides a detailed description of my accomplishments for the second year of funding supported by the USAMRMC Breast Cancer Research Program. The subject of my research is to identify and clone target genes for the transcription factor Egr-1. The purpose is to gain an understanding of the profile of genetic targets for Egr-1 in normal breast cells which are absent from breast cancer cells. The long term goal is to build transcriptional profiles for Egr-1 and to identifying key transcriptional defects occurring in breast cancer cells. For this report, the scope of the research is to first confirm the expression of Egr-1 in normal, but not in breast cancer cells. Subsequently, Egr-1 must be crosslinked to its target sites *in vivo* through the action of a buffered formaldehyde solution, and the crosslinked Egr-1, together with its bound DNA, isolated for further characterization.

Body

The results highlighted from the first year documented the successful use of the *in vivo* crosslinking technique to recover DNA targets directly bound by a transcription factor, in this case Egr-1. Additionally, the application of multiplex PCR allowed the amplification of cDNA sequences permitting target gene identification.

In this report, I present data describing my progress in the complex task of identifying target genes through which key Egr-1 regulatory effects result in the maintenance of normal breast cell function.

After successfully multiplex amplification, to achieve completion of the second phase of the project it was critical to determine whether the protocol faithfully accomplished the amplification of genuine Egr-1 target genes. To address this, I proceeded to first isolate individual cDNA's to allow their characterization and sequence identification. During this process, I have sequenced a full length cDNA for which no homolog or ortholog is known, by genomic database searching. I have named this new gene TEX1 for Target of Egr-1 by (X) crosslinking. Therefore, to identify this new gene as a target for Egr-1 transcriptional activity, it was necessary to perform an in depth characterization of the gene. As shown in figure 1, a full length cDNA

was isolated and sequenced. While homology searches of DNA databases revealed no match to the cDNA, I found that the human genome sequencing project was able to map the gene to human chromosome 1. In order to characterize and confirm the regulation of this new gene by Egr-1, I performed both RT-PCR and gel shift experiments. As shown in figure 2, following transfection of Egr-1 into H4 cells (which do not express endogenous Egr-1), a dose dependent induction of the expression of TEX1 was noted. This result indicated that Egr-1 was indeed able to regulate the expression of TEX1 by activating its transcription.

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ATG GCG CCG CAG TGA CG AT GG CG CAG TTT CA GCT CCG CAG CT TCC GA
M A A D S D D G A V S A P A A S D
CGG TG GT CAG CAA AA GC A CA CAT CT GG GAG GAG CT AG T AG TCC AG G
G G V S K S T T S G E E L V V Q
TTCCCGT AG TG GATG TG CAAAGCAACA CTTC AAG GAG ATG TG GCC ATCCC
V P V V D V Q S N N F K E M W P S
TCCGT CT AG CCA TAA AG ACAG CT AA TT TCGT GG CT GT GG ACACGG AG CT GA
L L L A I K T A N T A V D T R
GTG GG CT TG GG GAC AGG AAG AT TT GCTG AAC CAG TG CATT GAGG AACG TT
S G L G D R K S L L N Q C I E E R
ACAAG GCG TG TG TCAT GCTG CAG GAC CCG TT CT AT CCTT TC CCG TG CC
Y K A V C H A A R T R S I L S L G
T CG CCTG CT CAA GCGG CAG CCA GACA AG GG TG AAC TT CCT ATCTG GCTC
L A C F K R Q P D K G E H S V L A
AAG TG TT CAAT CT CACT CT GCTG TG CATG GAGG AG TATG TCAT AG AACCAA
Q V F N L T L L C M E E V V I E P K
G TCTG TG CAG TCTCT GATACAGCAT GG CT CAACT CAA CAG CAGT AT GCC
S V Q F L I Q H G F N F N Q Q Y A
CAAG G CATCCCCTACCA TAA G GCA AT GACA A GG GT GAT GAG AG CCA GAG
Q G I E Y H K G N D K G D E S Q S
CCAG T CAG T ACG GAC CCT AT T CCT G G AG CT AA TCC G AG CCG CCG G CCCCT
Q S V R T L F L E L I R A R R E L
GGT GCTACAAT GG CCTT AT AG ACTT GG TG TT CCTG TAC CAG AAC TCTAT
V L H N G L I D L V F L I V N F Y
G CACA CTCCCTG AG AG TCTG GG AACCTT CACCGCTG ACCT GT GT GAG ATG
A H L P E S L G T F T A D L C E M
TTCCCG CAGG CATT ATG ACACCA AATATG CT GCTG AG TT TCAT GCCCGT T
F P A G I Y D T K Y A A E F H A
T CG TG GCCT CTA CT TAGA AT AT GCCT TCCG GAA AT TG AACG GG AAA ATG
F V A S Y L E V A F R A K S V C G N
G GAAG CAG CCG G CAG CT GG CA GCCACACCTT ACCCT GG AG TT CT GCAAC
G K Q R A A G S P H L T L E F C N
T AT CCTT CCA G CATG AG GG ACCATA TT GATT ACCG CT GCTG CCTG CCCC A
Y P S S M R D H I D Y R C C L P P
G CA ACCC CCG T CCT CA TCCACCA CAG CAT CT GT GACA CT TT CT CG GCTT AT
A T H R P H P T S I C D N F S A Y
G GCT GG T G CCCCCT GG G A CCA CAG TG T CCT CAG T CT CAG AT AT TG ACCTT
G W C P L G P Q C P Q S H D I D L
ATCAT T GAC ACTG AT G AG GCT GCGG CAG AG G ACAAG CG GCG ACGG CGA CG
I I D T D E A A A E D K R R R R R
ACG TAGG GAAAA CG GAAG AG GG CT TT AT TG AACCTA CCGG GG ACACAG AC
R R E K R K R A L L N L P G T Q C
CTCTG G GG AAG CT AAG GATG G TCCT CCAAG AAG CAGG TCT GT GG G GATA
S G E A K D G P P K K Q V C G D
G CATCAAG CCT GAAG AAA CCG AG CAG GAGG T GG CT G CCGAT GAAACTAGG
S I K P E E T E G E V A A D E T R
AACCT GCCT CACT CCAAGCAA GG CAACAA AA AT GA CT TAGA GATG GG GATT
N L P H S K Q G N K N D L E M G I
AAG GCAG CAAG GCCT GAAA TAGCTG AT AG AG CT ACCT CAGA AG TG CCGAG
K A A R P E I A E R A T S E V P G
G AG CCAAGCCAGT CTA ACCCAG TG CCGT GG GG TG GATT GCACCG GG CTG
S Q A S P N P V P G G L H R A
G TT TT GATG CCTT ATG ACAG GT TATG TG AT GG CCTA TG TG GAAG TG AG CCA
G F D A F M T G Y V M A Y V E V S Q
GG GACCG CAACCTG CAGCT CT GG ACCCT GG CTCCCT GAAT G CCA CAAT AA
G P Q P C S S G P W L P E C H N K
GGT AT ATT TG AGT GG CAAAG CTG TACCCCT CACAG TGG CCAAG AG CCA GT T
V Y L S G K A V P L T V A K S Q F
CTCT CG TT CCT CCA AAG CCA CA ATCAG AAG AT G AAG CTC ACT TGG GCG G
S R S S K A H N Q K M K L T W G S
TAG CT GA
S stop

```

Figure 1. cDNA and protein sequence of TEX1; a new Egr-1 target gene.

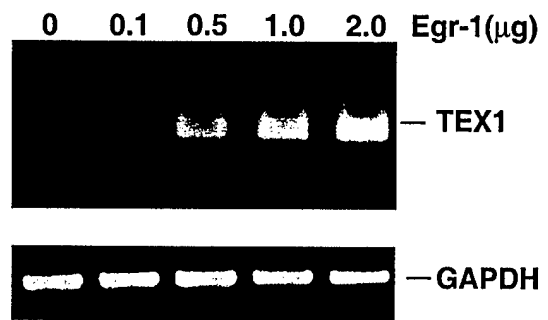


Figure 2. RT-PCR showing activation of TEX1 expression following Egr-1 transfection

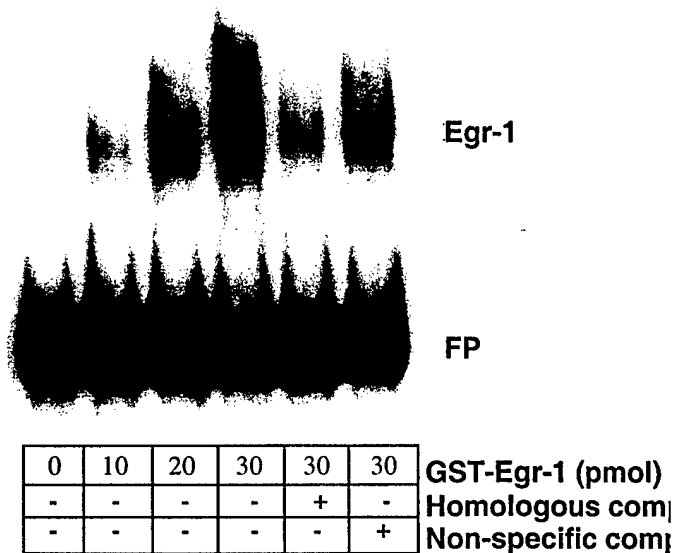


Figure 3. Gel shift analysis showing specific binding of Egr-1 to the TEX1 5' non-coding sequence.

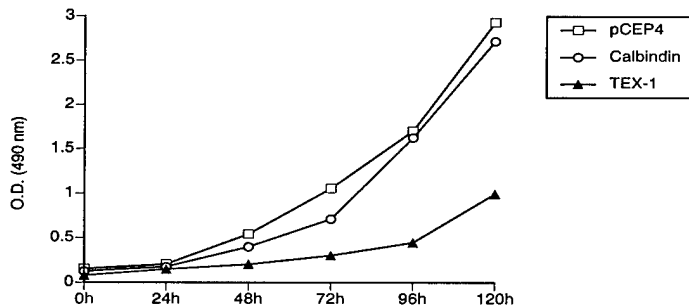


Figure 4. TEX1 inhibits cell growth. Growth curves for empty vector (pCEP4), an unrelated protein (calbindin), or cells expressing TEX1 (TEX1), are shown.

Further confirmation that TEX1 represents a direct target of Egr-1 was shown using the gel shift assay. As seen in figure 3, recombinant Egr-1 displayed direct and specific binding to the 5' non-coding portion of the TEX1 gene. Further characterization of TEX1 has included studies examining the biological activity of this newly found protein. I have found that following transfection of a TEX1 expression vector, cell growth is dramatically inhibited (figure 4). This result is currently being further characterized to identify the precise mechanism by which growth inhibition is achieved. Clearly, the cloning of this new gene has potential value to contribute a greater understanding of how the loss of Egr-1 in breast cancer cells contributes to their uncontrolled growth. Together, these results confirm not only the identification of an Egr-1 target gene, but have provided evidence that the crosslinking technology is capable of new gene identification. Having demonstrated the validity of the technology I am continuing with my planned statement of work by screening the captured and amplified cDNAs. To accomplish this task in the most efficient manner, I have applied the technology of DNA microarray screening to allow simultaneous identification of the entire profile of Egr-1 targets. A facility here at the Burnham Institute is in the final stages of set up, and I have been allowed to test the in house produced microarrays for my project. To date my profile of multiplex target have been analyzed three times, and I am in the process of repeating the entire experimental protocol to ensure reproducibility. This is necessary given the variation from array to array that currently exists. I anticipate that the results from the repeat experiments will be concluded shortly, following expansion

of the Institute's facility. This will lead to the compilation of consensus Egr-1 targets in breast cells and be followed by their analysis as detailed in the third phase of this project.

Appendix

1) Key research accomplishments:

- * Use of multiplex PCR amplification to identify a new gene target for Egr-1, named TEX1.
- * Characterization of TEX1 as a genuine direct target for Egr-1, validating the technology.
- * Identification of TEX1 as an inhibitor of cell growth.
- * Application of DNA microarray screening to profile captured Egr-1 targets simultaneously.

2) Reportable outcomes:

Manuscript: I. de Belle, J.-X. Wu, S. Sperandio, D. Mercola and E.D. Adamson (2001). In vivo cloning and characterization of a new growth suppressor protein TEX1: a direct target gene of Egr-1 (manuscript in preparation).

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