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Introduction:

Although traditionally most laboratories have studied the tumor itself, or tumor cell lines, to determine the cause of the malignant phenotype, we are studying the histologically "normal" epithelial tissue outside the tumor, or Non-Tumor Adjacent (NTA) tissue. We hypothesize that this tissue is pre-malignant from the standpoint of deficient DNA repair capacity, although it does not have as many of the obvious karyotypic abnormalities associated with the tumor itself (1-2).

We have developed a novel primary Human Mammary Epithelial Culture system (3) for normal breast tissue that provides an epithelial architecture more consistent with in vivo architecture than older traditional systems. These normal cultures are long-lived as primary cultures and grow and differentiate from 3-dimensional "mammospheres", to luminal branching ducts and lobules in vitro. Tumor cells under the same culture conditions do not form epithelial architecture. In order to study the loss of DNA repair as an etiological factor in breast cancer etiology we have generated from these robust primary cultures, over 20 matched pairs of cell lines from human breast tumors and matching, isogenic non-tumor adjacent tissue without the use of immortalizing agents. In addition we have generated 24 cell lines from non-diseased breast reduction tissue. These cell lines show cytogenetic profiles that are consistent with the tissue of origin. Establishment of the primary cultures and the cell lines represent 90% success from normal breast and 80% from tumor. The published rate of success from tumors is 15% (4) showing that our method is very reliable.

Rationale/Purpose: We have found that 75% of our NTA primary cultures (n= 42) and cell lines manifest the same deficiency of Nucleotide Excision Repair (NER) as the matching tumor culture (**Fig. 1**). This subpopulation of breast cancers indicates that the loss of NER preceded the final mutations that led to full blown transformation. This hypothesis is in stark contrast to the hypothesis that cancer arises in a vacuum and the NTA tissue represents relatively normal tissue.

Objectives: We will use the isogenic pairs of cell lines we have established to determine whether loss of NER in the pairs is: 1. due to reduced expression of the same genes in tumor vs NTA; 2. due to the loss of expression from an epigenetic phenomenon; or 3. due to the loss of expression from a genetic phenomenon, i.e. actual mutation of these genes. If mutation is found to be the reason for the reduced expression, we will confirm this using Comparative Genomic Hybridization (CGH) at the chromosomal loci of interest and allelic loss of heterozygosity (LOH).

Methods: We have been using RNase protection analysis with a multiplex Pharmingen kit that includes 20 of the NER genes to confirm reduced expression of the same genes in NTA vs tumor cell lines. We have begun to treat these cell lines with azacytidine to attempt to restore NER gene expression in the deficient cell lines. We will also, in parallel use allele specific LOH PCR to determine whether mutations exist in the candidate genes identified and confirmed by RNase protection. At a point in the future, we will confirm that the protein expression of these genes mirrors the RNA expression using Western analysis.

Body (based upon the statement of work):

1. Identify 3 matched sets of isogenic (NTA and tumor) cell lines with reduced NER (these lines already exist in the lab). Work is ongoing with 4 matching sets of cell lines

Loss of NER is Intrinsic in Sporadic Breast Cancer

Tissue samples from 43 early stage invasive breast ductal carcinomas, histopathologically confirmed to be free of adjacent normal breast epithelium, were successfully cultured for functional

NER (UDS) analysis (**Fig. 1**). None of these patients reported a significant family history of breast or related cancers. The patients ranged in age from 35 to 82 years, including both premenopausal and postmenopausal onsets. The tumors ranged in size from 1.2 to 5.0 cm and included examples of nuclear grades 1-3. Estrogen (ER) and progesterone receptor (PR) status were categorized into three groups for each: positive, negative and over-expressed. None of these patients received pre-surgical chemo- or radiotherapy.

As shown in **Fig. 1A**, the NER capacity of the stage I tumor samples were significantly lower than those of normal breast epithelium, averaging only 47% of normal activity ($P = 0.0002$). Half of all tumor samples had NER capacities lower than the lowest normal epithelial sample; this proportion rose to 75% when compared to an age-matched subset of the breast reduction population. The stage II tumors averaged 64% of breast reduction NER capacity and 18 (72%) had less than 70% of normal breast reduction NER capacity.

The highest NER capacity observed in our patients with confirmed lymph node status was 0.70 of normal; one patient with unconfirmed lymph node status was higher than this (0.83). If 0.70 X the average of the NER capacity of the breast reduction samples is established as a cut-off, the sensitivity of detecting tumors based on reduced NER levels alone is 92%, the specificity is 77%, and the odds ratio is 37.4 (95% CI, 17.1-82.0).

Analysis of the histologically **normal non-tumor adjacent (NTA) tissue** located outside the margin of each stage I tumor is shown in **Fig. 1B** and **C**. This tissue was derived from the ipsilateral breast and showed two patterns of NER capacity. In **Fig. 1B**, representing 25% of the patients tested, the NTA tissue manifested NER capacity consistent with that of normal, non-diseased breast. In **Fig. 1C**, the NTA tissue manifested the same magnitude of NER deficiency as that shown by the matching isogenic tumor, despite the fact that the tissue was confirmed to be histologically normal by a pathologist before being processed for primary culture. The NTA tissues in **Fig. 1C** represent 75% of the patients tested. The simplest interpretation of this data is that 75% of breast cancer patients have a constitutive deficiency in NER capacity. However, our preliminary data on the NER of peripheral blood lymphocytes of breast cancer patients compared with normal controls shows that there is no significant difference between the overall genomic repair in these two groups (N = 20, data not shown). **We therefore believe that some or all of our NTA samples represent pre-neoplastic stages of the tumor itself, through a “field effect” as demonstrated by Deng *et al.* (5), that renders them inappropriate as controls for our studies. It is for this reason that we have created several matching sets of cell lines from contralateral breast and breast tumor for molecular analysis to be used in this and other studies. We chose NTA lines that showed matching low NER capacity with the isogenic tumor which represents 75% of the NTA cell lines.** One unmatched contralateral breast tissue from a woman who previously had breast cancer can be seen in the filled dot in **Fig. 1A**, and this explant showed normal repair capacity.

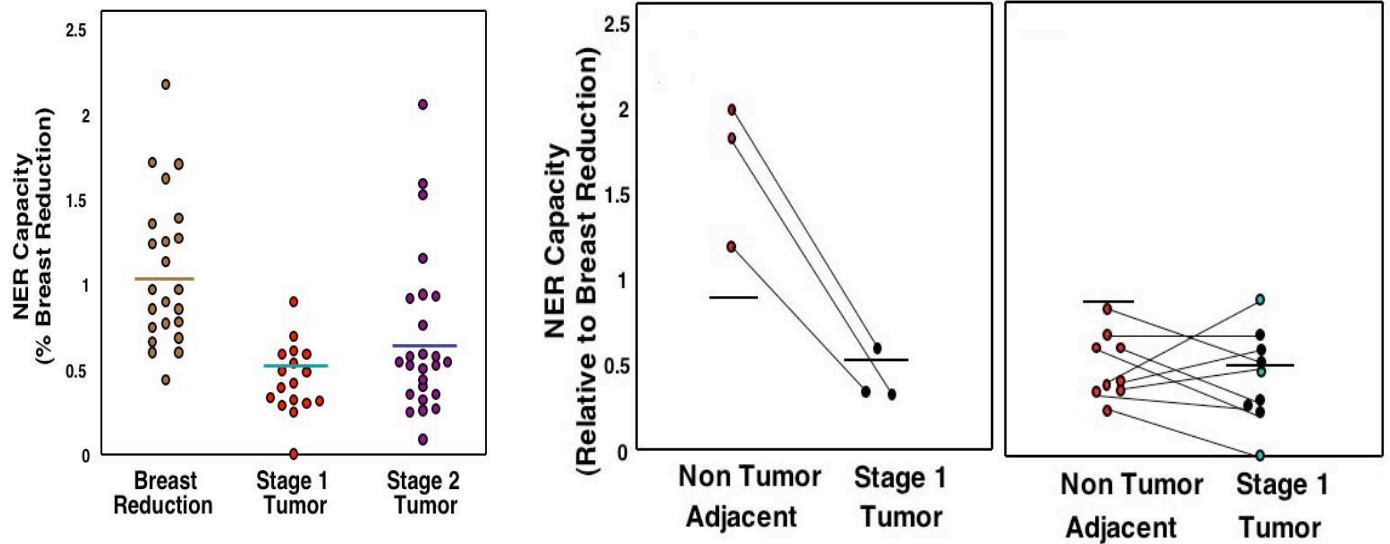


Fig. 1A. NER capacity of breast reductions vs. stage I and II tumors. Comparison of NER capacities of primary explant cultures established from normal breast reduction tissue and stage I and II ductal carcinoma.

B and C. The matching histologically normal tissues located outside the stage I breast tumors shown in **Fig. 1A** are shown in B and C in comparison with the matching tumor. Two types of non-tumor adjacent tissues were found: those with normal NER (panel **B**) and those with repair capacity as low as the matching tumor (panel **C**).

Characterization of Cell Lines Derived from Primary Cultures

Because of the robust nature of our primary cultures we can also derive explants (defined as passages ≤ 13) and cell lines (explants at passages > 13) and have over 100 extended explants to date (about 30% confirmed cultured into cell lines thus far). These cell lines retain the NER capacity that has been seen in the primary cultures. These explants and cell lines were not treated with any exogenous transforming or immortalizing agent and therefore represent a unique opportunity to study the molecular mechanism of the loss of NER in samples that still have relevance to early stage breast cancer. We have shown that the normal cell cultures retain a normal human karyotype (data not shown) whereas two representative cell lines derived from early stage malignant breast tissues have abnormal karyotypes.

We have 12 pre-existing matching isogenic sets of cell lines from adjacent normal tissue and tumor tissue or contralateral tissue and tumor tissue (ipsilateral). **Three matching sets of early stage cell lines have been analyzed and another set will be analyzed in the next year.** In addition 3 non-diseased breast reduction cell lines have also been analyzed for comparison. **The data shown in this report show the tumor cell lines, although the adjacent normal or contralateral counterparts show similar trends in repair capacity and gene expression.**

Evaluation of NER Gene Expression in Cell Lines Generated from Repair-deficient Primary Cultures

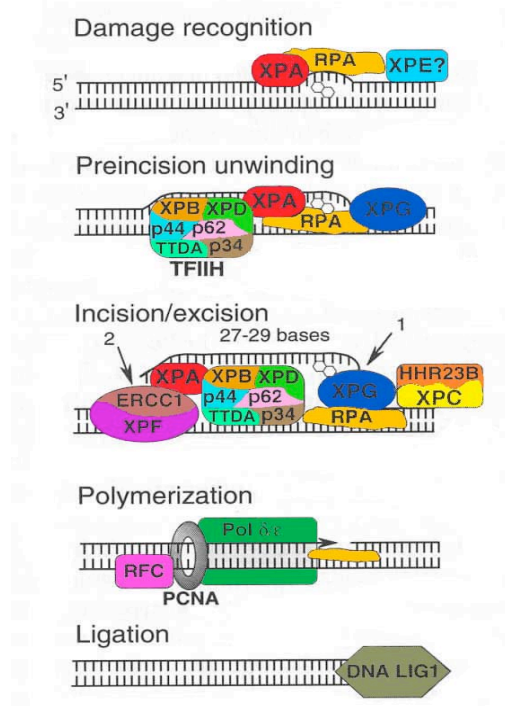
In our original studies we hypothesized that the NER pathway, requiring the products of 25-30 genes for function, represented a large target for mutational inactivation during the etiology of breast cancer. Thus, we were prepared to observe specific down-regulation or loss of a single unique NER gene in each tumor line analyzed. Instead, however, in the first set of three normal

and three tumor-derived cell lines analyzed, we have observed a concurrent down regulation of several genes in the NER pathway (**Figs. 3A, B, 4A**). Seven NER genes are significantly lower in mRNA expression in three tumor lines when compared to three unrelated normal breast epithelial samples: CSB ($P = 0.003$), XPA ($P = 0.004$), Cych ($P = 0.005$), TFIIHp34 ($P = 0.011$), Cdk7 ($P = 0.015$), TFIIHp52 ($P = 0.018$) and XPB ($P = 0.019$).

During human NER, damage is recognized, unwinding occurs at the damaged site, two incisions are made flanking the DNA lesions and residues are replaced by repair synthesis (**Fig. 2**). The minimal set of proteins required for repair of most lesions is RPA, XPA, XPC-hHR23B, XPG and ERCC1-XPF. An additional factor, the TFIIH complex (and one of the most complex to study), has a 6 subunit core comprised of XPB, XPD, p44, p34, p52, p62) and a 3-subunit kinase (CAK). TFIIH has roles both in basal transcription initiation and in DNA repair and several inherited human disorders involve mutations in TFIIH subunits. TFIIH from cells with XPB or XPD mutations was defective in supporting repair (6). Deletion of the C-terminal region of p52 results in a dramatic reduction of TFIIH NER and transcriptional activities. Intact p52 is needed to anchor the XPB helicase within TFIIH providing an explanation for the transcriptional and NER defects with the mutant p52 (7). Cyclin H is part of the cdk activating complex (CAK) and provides a link between basal transcription and the cell cycle. Cdk7 phosphorylates the carboxy terminal domain of RNA polymerase II.

In contrast, other NER genes, such as XPC, hHR23B and DDB2, show no significant changes in expression between FF, breast reduction and stage I tumors (**Fig. 4B**). XPC, with its helper hHR23B, and DDB2 are the primary genes identified in the NER pathway that bind directly to sites of DNA helical distortions (caused by DNA damage lesions). Lesion recognition and binding potency is dependent upon the amount of resulting helical distortion. The XPC-hHR23B complex is bound tightly together and binds directly to the lesion. Cells from XP-C patients have relatively low NER capacity and repair is confined to the actively transcribed strand. In comparison, XP-E patients (DDB2) have relatively high repair. The p127 and p48 subunits are involved in damaged DNA binding activity. This complex is required for the repair of moderately helix distorting lesions. Recruitment of XPC to the site of lesions is hampered in the absence of DDB, but not abolished. This may be because of the putative interaction between DDB and p300, the histone acetylase, since histone acetylation is known to enhance NER. DDB2 and XPC are the only genes from the pathway that have been shown to be transcriptionally regulated in a p53-dependent manner (8-9).

Fig. 2. Schematic of NER. The genes involved in damage recognition are XPA and XPE as well as Replication Protein A (RPA). RPA is a multifunctional ssDNA binding protein made up of 70, 34 and 14 kDa subunits. Incision then takes place via endonucleases XPF and XPG as well as the basal transcription factor TFIIH, which contains repair helicases XPD (ERCC2) and XPB (ERCC1). The incision step provides the closest linkage between replication coupled NER and transcription. After incision, excision of the damaged DNA strand occurs and repair synthesis of a new strand occurs. Ligation via DNA ligase then seals the nicks (13).



In addition to using RNase protection analysis, we have begun to characterize these cell lines by expression microarray, and very early results (N = 1, for both normal, stage I tumor cells and matching low repair Non tumor adjacent cells) are consistent with our RPA observations (**data not shown**).

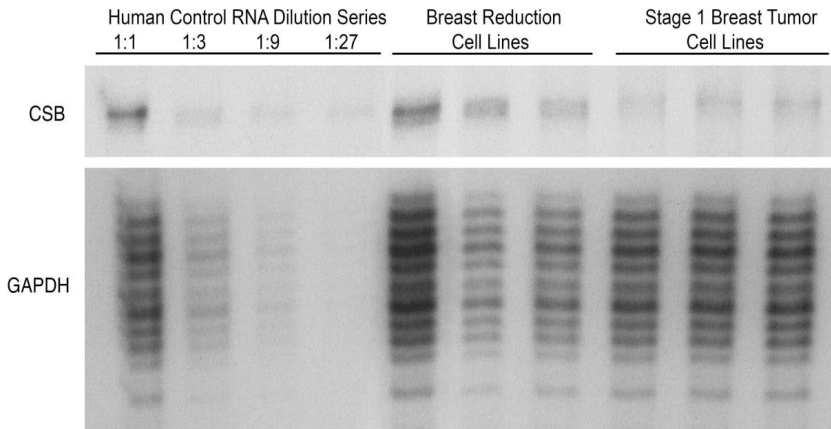
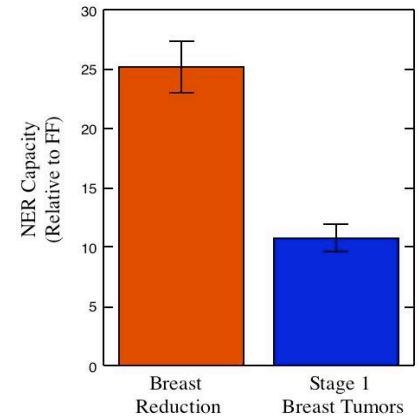
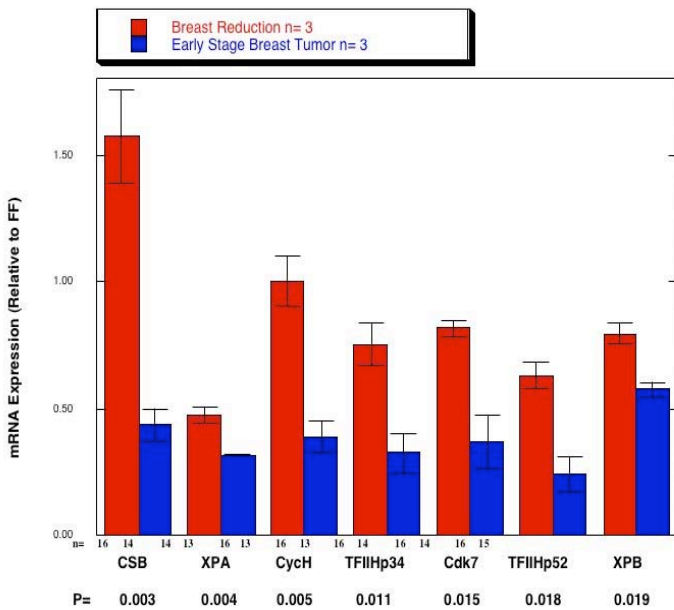


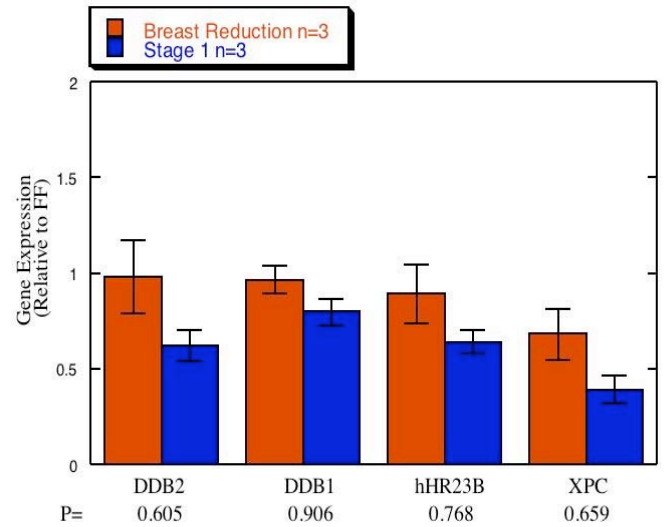
Fig. 3A. Representative RNase protection gel showing hNER2 kit hybridized to a dilution series of human control RNA and 2 ug total RNA derived from 3 breast reduction cell lines, and 3 stage I cell lines. Gene expression data for CSB and GAPDH are shown.



3B. Significant loss of functional NER is seen in primary explant cultures from 17 stage I tumors vs. those derived from 23 breast reduction mammoplasties.



4A. RNase protection data showing the significant loss of mRNA expression in 7 of our candidate genes between breast reduction and (normal) (red) and tumor cell lines (blue). *P* values are shown below the columns. Autoradiograms were normalized for loading using GAPDH and expressed relative to normal FF in each experiment. Total RNA was generated **from cell lines** originally derived from primary cultures. Three breast reduction cell lines were utilized and 3 different stage I tumor cell lines were utilized. Each bar represents at least 3 independent experiments. Non tumor adjacent cell lines showed similar results as the matching tumors.



4B. RNase protection data showing a group of genes with no significant changes in expression between breast reduction (normal) (red) and tumor cell lines (blue). Autoradiograms were normalized for loading using GAPDH an expressed as % of expression from normal FF. Total RNA was generated from cell lines that were passaged from primary cultures. Non tumor adjacent cell lines showed similar results as the matching tumors.

Restoration of DNA Repair is Part of Tumor Progression

In contrast with our findings in early stage tumors, we show that late stage tumor explants show an apparent recovery of NER activity relative to early stage tumor explants. Thus, NER activity is regulated during **breast oncogenesis and tumor progression in opposite ways**, suggesting that the changes may occur by a **reversible mechanism**. The NER capacities of tumor explants from tumors of stages I, II, III and IV are shown in **Fig. 5A**. All tumors cultured for this work were chemotherapy and radiation therapy naive (including stage III tumors, which are no longer available in the chemotherapy naive state). All tumor stages show some individual variation, which is to be expected in the human population, although we will probably not be able to increase the statistical power of the stage III results with more specimens.

The Same Candidate Genes are Restored in Expression in Later Stage Tumors as are Lost in Stage I Tumors

RPA data on several selected cell lines from advanced stage tumors are shown in **Fig. 5B**. There is a clear association between increasing gene expression and with increasing tumor stage (tumor progression) for CSB, XPB and TFIIHp52, TFIIHp34, Cych and Cdk7 (all $P < 0.001$) (**Fig. 5B**).

The only explanation for an initial loss of function in multiple genes (7), followed by an gain of function and expression is an epigenetic phenomenon which is by definition reversible. We have therefore pursued the possibility that methylation is the mechanism for this epigenetic phenomenon.

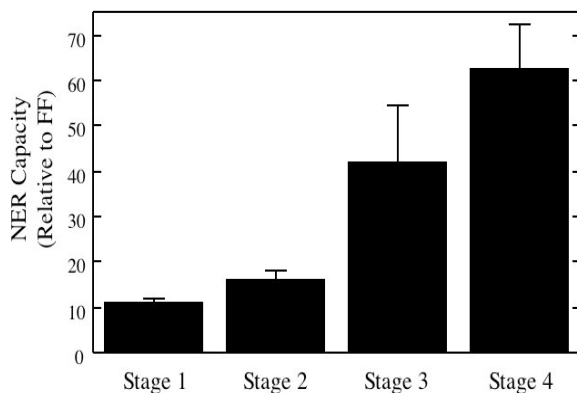
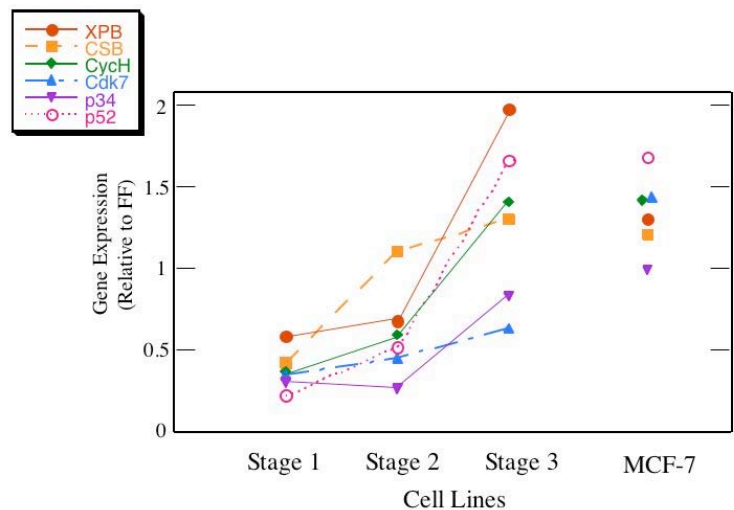


Fig. 5A. Functional UDS assay data showing the stage specific increase in NER capacity in primary explants of breast tumors. These differences are maintained in our selected cell lines. Stage I and II (N = 41) are significantly different from stage III tumor explants ($P = 0.0004$)



B. RNase protection data showing the increase in expression with tumor stage of our candidate genes. Autoradiograms were normalized for loading using GAPDH an expressed as % of expression from normal FF. Total RNA was generated from cell lines derived from primary cultures. Gain of expression and function after initial loss can only be explained by an epigenetic phenomenon.

2. Treat the lines with azacytidine and determine the range sensitivity

The appropriate dosage of azacytidine was determined (between 1 and 5 μM) and both of these doses are being used in subsequent analyses. In addition we performed analyses of the 5' promoter regions of the NER genes.

5-aza-2'-deoxycytidine is an inhibitor of maintenance methylation following DNA replication. Besides being unmethylatable itself, incorporation of this base analog causes the methylase to pause at the site of incorporation, delaying or preventing processive downstream methylation. AzaC must therefore be present during S-phase to affect DNA methylation and gene expression. Optimization experiments have demonstrated that optimal survival and induction of gene expression take place after only one or two cycles of base incorporation, peaking at 24-48 hours exposure. Optimal gene induction is not observed unless an expression time is allowed for transcription, translation and any other required processes, such as modification and localization, necessary for protein function to occur.

Preliminary CpG Analysis of Promoter/Exon Regions of NER Genes

We have used both Genomatix and MacVector software to analyze the promoter regions of the 20 NER genes for C/G content and putative transcription factor binding sites. Both of these companies used software that is linked with human genome databases. Genomatix uses 3 independent types of evidence (one experimentally based on 5' capping) to identify promoter regions. This analysis was done to determine 1) whether the CG profile of these genes could be consistent with DNA methylation as a possible mechanism of epigenetic gene inactivation, and 2) whether any common transcription factor elements could explain the correlations we are seeing in both mRNA expression and functional NER.

Using MacVector software we have found several patterns of CG content. In the top panel of **Fig. 6**, one can see the tissue specific gene α_1 -antitrypsin. It has a maximum of 4-5 in terms of the units of frequency of C/G content, and this content is distributed evenly along the gene including the promoter region (upstream of the blue vertical line). This is a well-characterized gene that is very strictly regulated by transcription factors in the liver. The GAPDH housekeeping gene is also rather low for GC content again reaching a maximum of 5 frequency units. It has more intensity in the region of the 5'-most exons. In contrast to these patterns, we show 4 of our candidate genes in the NER pathway show much higher frequencies of GC content ranging from 15 to 40 units, and they all show a sharp spike over the promoter region and first exons. Although this is not proof that methylation may be a controlling factor in the expression of these genes, it certainly indicates that it is possible mechanism of regulation.

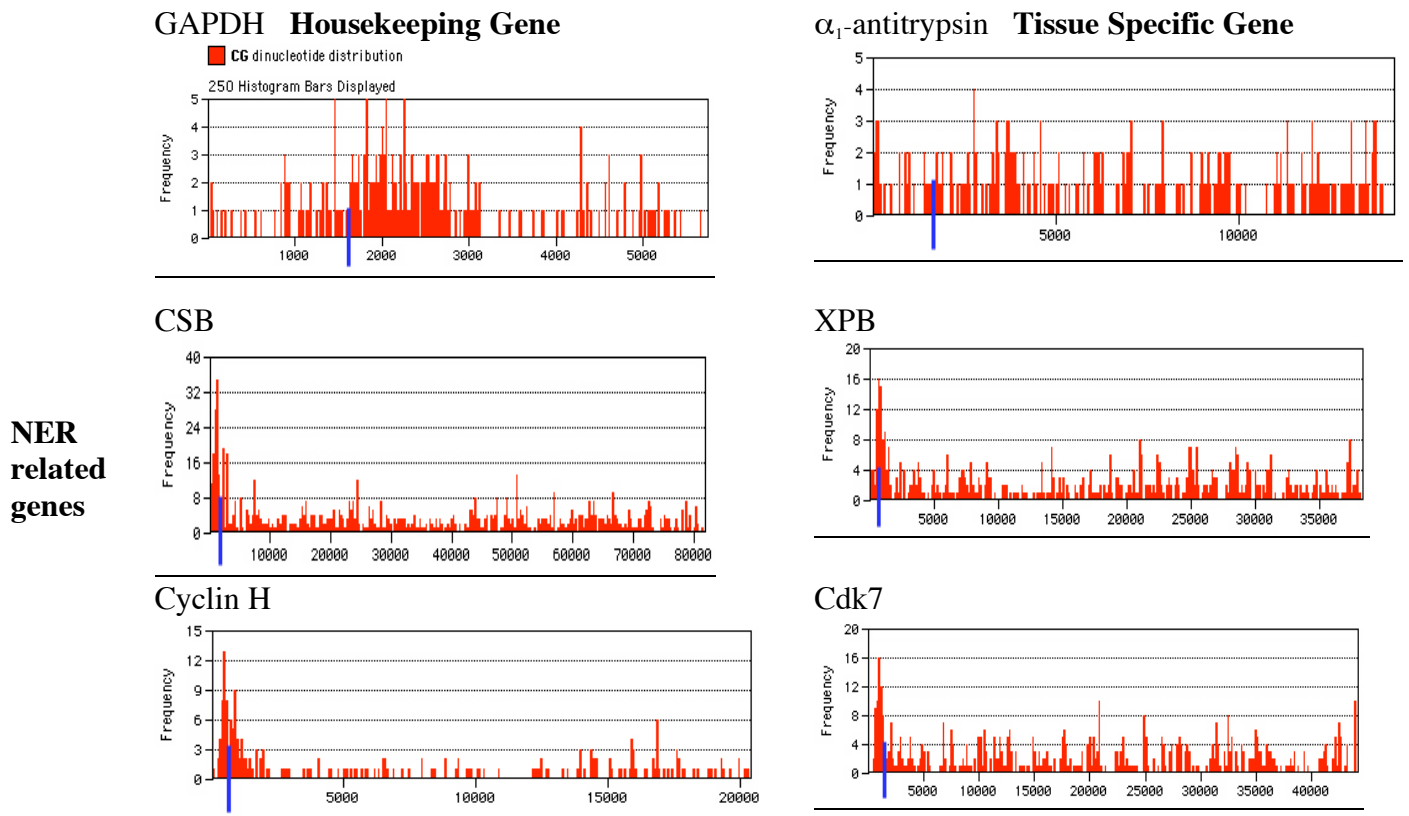
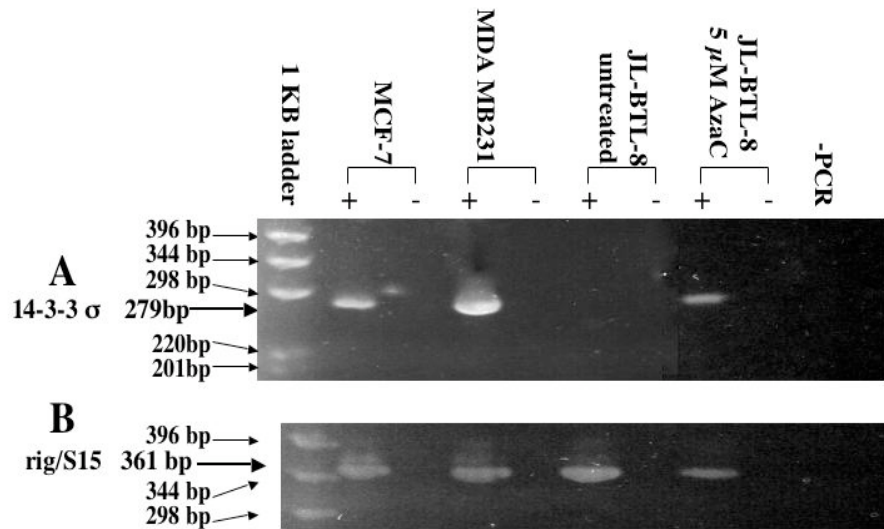


Fig. 6. Summary of three different C/G patterns we have identified in our C/G analysis. The vertical blue lines represents the transcriptional start site so this view of the gene shows the overall C/G content. Both the GAPDH housekeeping gene and the liver-specific A1-antitrypsin gene have rather low frequencies of C/G sites, with the maximum on the Y-axis of 5. In contrast the 4 NER related candidate genes shown show a peak of C/G rich sites in the promoter/exon 1 region and a rather equal distribution over the rest of the genomic sequences. The CSB gene has a maximum on the Y-axis of 40 and XPB shows a maximum of 26.

Reactivation of 14-3-3 sigma

Interestingly, the 14-3-3 sigma gene (originally identified as HME1), another gene involved in DNA repair, has been shown to be epigenetically reduced in expression in all breast tumors (10), suggesting that our results with the NER pathway may be part of a larger effect on DNA repair during breast oncogenesis. It has also previously been shown that expression of the BER (base excision repair) glycosylases is naturally low in the breast (11-12), and that microsatellite instability can occur during breast carcinogenesis due to gene methylation (13). **We have now utilized this gene as a validation of de-methylation to indicate that we can reactivate genes that have been methylated in our stage I tumor cell lines and where tested in the NTAL lines.** 14-3-3 sigma has been shown to be methylated in 94% of human breast tumors. A representative cell line JL BTL-8, which was derived from a stage I breast tumor, did not express this gene (**Fig.7**) until after treatment of 5 uM azacytidine for a period of 10 days. In order to show that RNA existed in all lanes shown, a control set of primers was utilized for the rig/S15 gene which proves that reverse transcribed DNA existed in all lanes (both positive and negative for sigma). Interestingly the 14-3-3 sigma gene is one of the few eukaryotic genes that lacks introns. Because of this, we were forced to use a DNase treatment to remove any contaminating genomic DNA (since primer design could not eliminate this issue). All samples are shown with and without the presence of reverse transcriptase to prove that there was no contaminating genomic DNA.

Figure 7A. Reactivation of the 14-3-3 sigma gene in the JL BTL-8 tumor cell line. Results of RT-PCR analyses of 14-3-3 sigma in the stage 1 cell line JL-BTL-8 after treatment with 5µM 5-aza-2'deoxyctidine (AzaC). The MCF-7 and MDA-MB231 cell lines are included as positive controls for the presence of 14-3-3 sigma.



7B. The presence of cDNA (and RNA) was confirmed by the use of control primers targeting the rig/S15 gene. The same cDNA samples were subjected to PCR using these primers and the same conditions as used for the 14-3-3 sigma PCR.

-PCR: water template
+/-: presence or absence of MMLV-RT enzyme in the RT reaction

3. Perform RNase protection to determine if candidate NER gene expression is restored
This work is in progress now.

4. If this drug will not work on its own add additional related drugs

The azacytidine is working on it's own and additional drugs will not be needed.

5. If no restoration is found, work on the LOH will be performed

Restoration in tumor and in NTA cell lines seems to be restored so that LOH should not be necessary except as an unequivocal negative.

6. Work will begin using PCR primers already designed for the 7 candidate genes

Westerns are currently being run as opposed to PCR (Fig. 8).

Protein Expression Levels- CSB

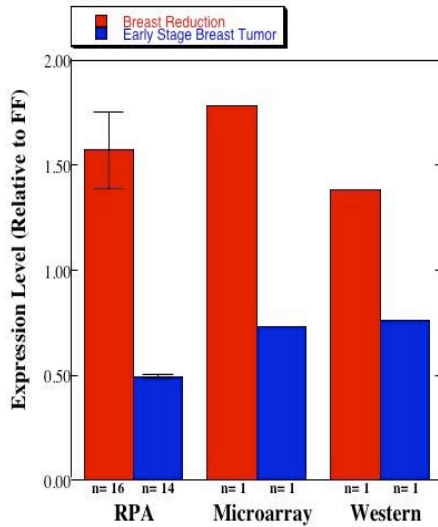


Fig. 8. A comparison of RNase protection data (RPA), microarray data and western analysis for the gene CSB, one of our candidate genes. There is good agreement between the 3 types of analyses although repeated microarray and westerns will allow for the estimation of standard error. Only RPA has been repeated multiple times to date.

7. If the LOH results indicate mutation, sequencing of specific genes will be performed

To date Mutation has not been indicated, but rather epigenetic regulation of 7 genes has been indicated.

8. Concurrently, CGH will be performed by our collaborator and these data will be compared with the data our laboratory is producing

This has not been necessary based on our current findings.

9. Write the data up for publication and presentation at AACR and the ERA of hope meeting
See the Reportable outcomes below.

Key Research Accomplishments:

-We have determined that our low repair NTA and matching tumor cell lines have similar gene expression patterns, and the same candidate genes operating in them. 7 candidate genes have been identified (CSB, XPA, Cych, TFIIHp34, Cdk7, TFIIHp52 and XPB.

-We have determined based upon the loss of NER in stage I and II tumors and the gain in more advanced stage tumors, that an epigenetic phenomenon is working rather than one based on mutation.

-We have also determined in related work that BRCA1 heterozygous tissue is not reduced in NER capacity which rules out BRCA1 as an implicated gene (see publication). This was one possibility when we were considering a mutational mechanism for the loss of NER in the NTAL and the early stage tumors.

-We have developed an azacytidine treatment regimen that works on our cells in a variation of our original medium without significant toxicity (which was not trivial).

-We have re-activated the unrelated 14-3-3 sigma gene in our cells to prove that we can demethylate the promoter gene regions in our cells.

-We have determined that the most dramatic of the 7 candidate genes showing loss of expression in low repair cell lines, CSB is also reduced in protein levels.

Reportable Outcomes:

Papers:

Kelly, C.M. and **Latimer, J.J.** (2005) Unscheduled DNA synthesis: a functional assay for global genomic nucleotide excision repair. *Methods in Molecular Biology* **291**: 303-320.

Johnson, J.M., and **Latimer, J.J.** . (2005) Analysis of DNA repair using transfection-based host cell reactivation. *Methods in Molecular Biology* **291**: 321-335.

Latimer, J.J., Rubinstein, W.S., Johnson, J.M., Kanbour-Shakir, A., Vogel, V.G., and Grant, S.G. (2005) Haploinsufficiency for *BRCA1* is associated with normal levels of DNA nucleotide excision repair in breast tissue and blood lymphocytes. *BMC Medical Genetics* **6**(26) online.

Meeting Abstracts:

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Conclusions:

We have made significant progress in terms of ruling out a mutational mechanism versus an epigenetic mechanism for loss of NER in early stage tumor and in NTA cell lines. Seven candidate genes have been identified by RPA and microarray analyses. These genes are decreased in expression in early stage BC tumors and in 75% of matching NTA cell lines and increased in expression in later stage tumors. This reversible phenomenon can only be explained by an epigenetic phenomenon. We will conclude this study in the next year by determining whether our 7 candidate genes can be reactivated in NTA and tumor cell lines with azacytidine (implicating methylation) using RPA, microarray and protein analysis and also sequence these genes and their 5'ends to verify that mutation can be absolutely ruled out. If methylation is not found to be the mechanism, then histone acetylation will be addressed using the drug trichostatin. Most of the methods shown here were initially worked out using early stage tumor lines so that the non-tumor adjacent cell lines need further repetitions to show statistically significant data. This will also be performed in the coming year. Several matching contralateral lines will also be included for analysis this year.

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