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**13. ABSTRACT (Maximum 200 Words)**

Homeobox genes control anterior-posterior body axis patterning during development. Although expressed primarily in developing embryos, a growing body of evidence shows that homeobox gene re-expression in adult tissues is associated with tumorigenesis. Earlier work has shown that introduction of HOXB7, one of the members of this gene family, into non-expressing breast cancer cells induced expression of a number of pro-angiogenic factors and formed tumors upon xenograft into nude mice. Since a growing body of evidence showed that HOXB7 may be involved in ovarian, skin and breast cancer, we wanted to investigate its expression levels in breast carcinomas and evaluate its oncogenic potential in this study.

To characterize the role of HOXB7 in breast cancer, a number of HOXB7-interacting proteins in breast cancer cells were identified and binding to HOXB7 was confirmed *in vivo*. Interestingly, all of the HOXB7-associated proteins identified have well characterized roles in the non-homologous end joining (NHEJ) pathway for DNA double strand break (DSB) repair as well as a larger role in maintenance of genomic integrity. Cell survival experiments demonstrated that breast cancer cells stably transfected with HOXB7 survive better after induction of DNA DSB with fewer chromosomal abnormalities following exposure to gamma radiation. In addition, transduction of HOXB7 into the normal immortalized mammary epithelial cell line, MCF10A, induced anchorage independent growth that was dependent of PARP activity. These data have led us to propose that HOXB7 may serve a role in breast cancer tumorigenesis in which PARP shares a common pathway.

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## Introduction

Like all HOX genes, HOXB7 has a role in vertebrate patterning during development (reviewed in 1, 2). In addition, several lines of evidence point to the importance of HOXB7 in myeloid hematopoiesis (3), with expression found in many hematopoietic cell lineages (4-8). Nevertheless, there is increasing evidence of a role for HOXB7 in breast cancer. When transduced into breast cancer cells, HOXB7 expression has been shown to promote the growth of xenografts of SKBR3 breast cancer cells in nude mice, with concomitant upregulation of both mitogens and pro-angiogenic factors (9-11). In addition, Hyman et al recently showed that HOXB7 overexpression in human breast cancer may be attributed to gene amplification and is correlated with poor patient prognosis (12). Despite increasing evidence of the involvement of HOXB7 in cancers of the breast, ovary (13) and skin (9), the relevance of this gene to breast cancer, and its functional role in tumorigenesis is poorly understood.

The study of HOX protein-protein interactions is of great importance in understanding their distinct functions. Since all 39 known HOX proteins have been shown to bind to similar DNA sequences, the question of how the specificity of HOX gene function is determined has remained unclear. One level of specificity could be afforded by distinct protein-protein interactions which could mediate both specific DNA binding and transcriptional target activation. For example, cooperative interaction between HOXB7 and PBX1 aids in high affinity and site-specific DNA binding (14-15). Despite searches for HOX binding partners, few have been found to date (reviewed in 16). In addition to PBX1, HOXB7 has only been found to bind to two other proteins *in vitro* and *in vivo*. These include the CREB-binding protein (CBP) (17) and the NF- $\kappa$ B inhibitor, I $\kappa$ B- $\alpha$  (18). Both these proteins enhanced transcriptional activation by HOXB7. In order to further understand the role of HOXB7 in breast cancer and the mechanism whereby HOXB7 overexpression may contribute to breast cell transformation we sought to identify HOXB7 binding proteins in breast cancer cells. Interestingly, the most abundant HOXB7 interacting proteins all fell into a group of genomic caretakers, including members of the nonhomologous end joining pathway (NHEJ) of DNA DSB repair, and poly-ADP ribose polymerase (PARP), which have established roles in the maintenance of genomic integrity.

Maintenance of genomic integrity relies on both faithful DNA repair mechanisms as well as eradication of cells carrying cancer-causing changes or mutations. DNA DSB repair in mammalian cells is accomplished by two major pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ) (see 19-21 for review). Recent evidence indicates that the more error-prone NHEJ pathway (22-23) is dominant, especially in G1 through S phase. NHEJ relies on recruitment of the DNA-dependent protein kinase (DNA-PK) to sites of DNA damage by Ku80 (also known as Ku86) together with its heterodimeric binding partner, Ku70. Additional enzymes including XRCC4 and ligase IV are required for sealing the adjacent ends of the DSB (19-21). The DNA-PK also plays a major role in immunoglobulin VDJ recombination and as such, Ku 70 and 80 have been shown to bind to numerous nuclear proteins both as a complex and independently (24-29). While acting as a DS DNA end binding protein, Ku 80 also associates with telomeres (30-32) and telomere binding proteins where it is thought to prevent inappropriate

recognition of these DS ends by the NHEJ machinery. Here it is postulated to restrict access of telomerase to the DNA ends since Ku 80 knockout mice possess elongated telomeres (33-36).

Other proteins involved in maintenance of genomic integrity belong to the poly-ADP-ribose polymerase (PARP) family of enzymes. This growing family consists of 4 members (as well as telomere-associated relatives) that catalyze the transfer of polymers of ADP-ribose from an NAD substrate onto protein targets, including histones, transcription factors and PARP itself (37-39). PARP has been shown to play a major role in maintenance of genomic integrity both by its involvement in DNA repair (40-42), regulation of apoptotic signals (43-45) and maintenance of telomeres (46-47). Although present at a basal level in most cells, the catalytic activity of PARP is stimulated by DNA damage or other forms of genotoxic stress. In response to DNA damage, transcription factors that become poly-ADP-ribosylated carry a high net negative charge. This is thought to cause electrostatic repulsion between protein and DNA thereby abolishing DNA binding, which allows the necessary DNA repair to take place before initiation of further rounds of transcription. PARP activity is necessary for yet another mechanism for maintenance of genomic integrity, that is by a caspase-independent apoptotic pathway, by acting through its target AIF (43). Although there is some controversy regarding the telomeric maintenance of cells derived from PARP knockout mice (48-49), PARP appears to be required for maintenance of telomeric ends. A subpopulation of PARP exists in complex with centrosomes where its enzymatic activity is required to ensure faithful coupling of the centrosome duplication and cell cycles (50-51). This function of PARP is important for maintaining correct chromosome number and orderly segregation.

In this study we have found that HOXB7 is indeed overexpressed in breast cancer, and its interactions with PARP as well as members of the DNA-PK holoenzyme led us to examine whether HOXB7 could transform normal mammary epithelial cells, whether PARP activity would be required for this function, and whether HOXB7-expressing breast cancer cells had different DNA DSB repair profiles. Our results show that introduction of HOXB7 into normal mammary epithelial cells resulted in anchorage-independent growth on soft agar, and that its interaction and subsequent post-translational modification by PARP is required for transformation *in vitro*. In addition, we have found that HOXB7 was phosphorylated by DNA-PK *in vitro*, which blocked its DNA binding and that breast cancer cells engineered to overexpress this gene had enhanced DNA DSB repair *in vitro* and *in vivo*. Taken together, it appears that breast cancer cells which overexpress HOXB7 acquire a survival advantage which helps to sustain their oncogenic program, and that transformation of mammary epithelial cells by HOXB7 is dependent on PARP activity.

## 2004 Annual Summary

### Body

**Task 1 of the statement of work** investigates the transforming potential of HOXB7 *in vitro* by soft agar growth assay. While the majority of this work, including establishment of stably-transfected SKBR3 breast cancer and MCF10A immortalized normal mammary epithelial cell lines were completed in year one, MCF10A stable transfectants expressing mutant HOXB7 constructs were generated. HOXB7 expression plasmids containing mutations or deletions in the DNA binding domain (homeobox) (FB7Δh3pCDNA3), the glutamate-rich carboxy-terminal domain (FB7ΔGLUpCDNA3) and the PBX1-binding residues (FB7WM129, 130FI pCDNA3) were stably transfected into MCF10A cells to assess whether HOXB7 transformation capabilities were homeodomain, PBX1, or poly-ADP-ribosylation independent. These cells, together with the wild type transfectants positive and vector transfected negative controls, respectively, were plated on soft agar to measure anchorage-independent growth. Only the MCF10A-HOXB7 wild type transfectants were able to grow on soft agar.

To determine if modification by PARP (see year one, identification of HOXB7 interacting proteins, and below) is required for this function (as the glutamic-acid mutant did not grow on soft agar despite the presence of intact homeobox and PBX1-binding domains), the soft agar colony formation assay was repeated in the presence of 2 well-characterized PARP inhibitors: 3-Aminobenzamide (3-AB) and 1,5-dihydroxyquinolindione (DIQ) or vehicle (aminobenzoic acid). As a control, ras/neu transformed MCF10A cells which have a proven ability to grow on soft agar, were plated in kind. After culturing in the presence of the PARP inhibitors, the ras/neu transformed MCF10A cells showed no effect, while the HOXB7-transfected MCF10A failed to grow. This was not due to blocking protein-protein interactions (HOXB7 still co-immunoprecipitated with PARP in the presence of these drugs) but due to blocking enzymatic functions of PARP required for post-translational modification of HOXB7 as well as other proteins.

**Task 2 of the statement of work** aims to identify HOXB7 target genes and interacting proteins. While a microarray analysis of HOXB7 is currently in progress, identification of interacting proteins has proceeded following confirmation of the interaction of HOXB7 with Ku 70/Ku 80, DNA-PKcs and PARP. The majority of this work was completed in year one. Nevertheless, as these proteins were all DNA-binding proteins, we wanted to confirm that DNA as a common binding factor was not the sole determinant for the observed interaction between HOXB7 and DNA-PK and PARP. To ensure that DNA was not mediating the interaction, 4 experiments were performed:

1. GST-Pulldown assay in the presence of DNaseI
2. Co-immunoprecipitation in the presence of DNaseI
3. DEAE-Sepharose chromatographic fractionation prior to co-immunoprecipitation
4. Examination of nonspecific complex formation with other unrelated DNA binding proteins

First, bead-bound GST-HOXB7 was treated with DNaseI prior to mixing with DNaseI-treated SKBR3 cell lysates. 2000 units of enzyme was used per 1 mg of cell lysate and the incubation proceeded for 30 minutes at 30 degrees. Interacting proteins were eluted with 50 mM glutathione, separated by SDS-PAGE and a comparison of binding protein abundance was performed by silver staining. In this assay, no difference was found between binding protein abundance with or without DNA (DNaseI treatment).

Next, Flag-tagged HOXB7 was transiently transfected into SKBR3 cells. Half the cell extracts were treated with DNaseI as above and immunoprecipitation was performed on FLAG-agarose. HOXB7 and its interacting proteins were eluted with excess FLAG peptide and separated by SDS-PAGE. When comparing protein abundance by immunoblot using antibodies to Ku 70, Ku 80, PARP and DNA-PK, no differences were observed.

As an added control, Flag-HOXB7 expressing cell extracts were fractionated on the weak anionic exchange resin, DEAE-Sepharose, to bind cellular DNA. Following elution in 0.2 M NaCl, cell extracts were subjected to immunoprecipitation on FLAG-Agarose as above. Binding protein abundance in immune complexes after DEAE-Sepharose fractionation was compared to that of untreated lysates by western blot with appropriate antibodies, as above. In this assay, it appeared that while the presence of DNA stimulated the interaction between HOXB7 and Ku 70, Ku 80 and PARP, co-immunoprecipitation of these proteins was still observed in the absence of DNA.

Finally, western blots of immunoprecipitated Flag-HOXB7 were performed using antibodies to unrelated DNA

binding proteins including pRb, BRCA1, E2F1, Rad52 and Sp1. E2F1, BRCA1 and Sp1 did not appear to bind to HOXB7.

We therefore conclude that HOXB7 does not bind nonspecifically to any DNA binding proteins, that the interaction between HOXB7 and Ku, PARP and DNA-PKcs is specific and that DNA stimulates the interaction but is not required for binding to occur.

Work studying the regions of HOXB7 protein which may be involved in the interaction with PARP and Ku were initiated in year one of the award. In those studies, it appeared that HOXB7 may utilize its homeodomain for interaction with PARP, as mutants in this region exhibited reduced PARP binding in co-immunoprecipitation experiments. To examine this in more detail, sequential carboxy-terminal deletion constructs of HOXB7 were made and used to produce <sup>35</sup>S-labeled proteins in rabbit reticulocyte lysates. These proteins were mixed with those of full-length PARP and subjected to immunoprecipitation with PARP antibodies. Only HOXB7 constructs containing an intact homeodomain were able to complex with PARP, indicating the requirement for the homeodomain in mediating the interaction.

#### *Functional interaction of HOXB7 with DNA-PK and PARP*

Studies focusing on the functional interaction of HOXB7 with Ku/DNA-PK and PARP were begun in year one. First, since Ku/DNA-PK are major components of the nonhomologous end joining (NHEJ) repair machinery which is the major pathway for DNA double-strand break (DSB) repair in mammalian cells throughout all phases of the cell cycle except for G2/M, we wanted to examine whether HOXB7 expression would have any effect on DNA DSB repair. To do this, in year one, plasmid end-joining assays were employed to show a stimulation of end-joining activity *in vitro* in cell extracts overexpressing HOXB7. We also examined cell survival characteristics of SKBR3 cells stably transfected with HOXB7-YFP. These cells were exposed to increasing doses of gamma irradiation to induce DNA DSB. Compared to either parental or vector-transfected control cells, the cells expressing HOXB7 had enhanced survival rates and less residual chromosomal damage following irradiation. Finally, the MCF10A cells stably expressing HOXB7 were also assessed for their survival after exposure to radiation by clonogenic survival assay. Results show that even the normal immortalized mammary epithelial cell line had enhanced cell survival by this assay when overexpressing HOXB7 but not mutant forms lacking the homeodomain or glutamate carboxy terminal domain.

In addition, while we have analyzed the phosphorylation of HOXB7 by DNA-PK *in vitro* in year one, we have not yet been able to show that HOXB7 is phosphorylated *in vivo*. To study HOXB7 phosphorylation in living cells, we tried a number of approaches. First, SKBR3 cells transfected with Flag-HOXB7 were labeled with <sup>32</sup>P-orthophosphate for 3 hours prior to immunoprecipitation. HOXB7 thus precipitated did not incorporate the radiolabel when visualized by autoradiography. Next, proteins from Flag-HOXB7 transfected cells were separated by electrophoresis on low percentage gels to determine if a shift could be seen in molecular weight. This was not observed. However, when immunoprecipitated from these cells, the HOXB7 band did appear as doublets on FLAG-western blots. To determine if one of these bands was a phosphorylated form, immunoprecipitates of Flag-HOXB7 were treated with lambda phosphatase prior to gel electrophoresis. Results are expected soon. It is possible that failure to observe <sup>32</sup>P incorporation in immunoprecipitates of HOXB7 results from low level or stoichiometry of incorporated phosphate and the high level of background in the immunoprecipitates. It is also possible that HOXB7 phosphorylation *in vivo* is a transient event in the cell, regulated by a cell cycle or DNA damage-dependent signal. In this case, identification of HOXB7 residues modified by DNA-PK and generation of phospho-specific antibodies will aid in determining the phosphorylation status of HOXB7 in living cells.

Studies identifying HOXB7 as a substrate for PARP *in vivo* were initiated in year one of the award. In this work, cells expressing Flag-HOXB7 or mutant forms were permeabilized in the presence of <sup>32</sup>P-NAD prior to immunoprecipitation of HOXB7. HOXB7 bands on the resulting autoradiographed gels were apparent, but only in wild type HOXB7, not in proteins lacking the glutamic acid tail, indicating the carboxyl terminal glutamate rich region is the site of poly-ADP-ribosylation. We have also developed an *in vitro* method for modifying HOXB7 by poly-ADP-ribosylation. To do this, purified PARP enzyme obtained from a commercial source (Trevigen, Inc.,

Gaithersburg, MD) was mixed with bead-bound GST-HOXB7 in the presence of  $^{32}\text{P}$ -NAD. The beads were then washed extensively prior to elution of the GST-HOXB7. After gel electrophoresis and autoradiography, GST-

HOXB7 but not GST alone appeared to incorporate the label. We also performed an *in vitro* phosphorylation assay to phosphorylate GST-HOXB7 using purified DNA-PK (Promega) described in year one, thus obtaining sources of purified GST-HOXB7 modified by phosphorylation or poly-ADP-ribosylation. These proteins were then used in EMSA (gel shift or DNA-binding) assays to determine if modification by phosphorylation or poly-ADP-ribosylation would interfere with DNA binding as has been shown for poly-ADP-ribosylated p53 (52-56) and phosphorylated forms of other HOX proteins (3, 57-59). When mixed with DNA probes containing 2 HOX binding sites, only the unmodified GST-HOXB7 could form a protein-DNA complex. Thus we concluded that both phosphorylation and poly-ADP-ribosylation of HOXB7 blocks its DNA binding functions.

**Task 3 of the statement of work** is to develop HOXB7 as a detection marker for diagnosis. Initial attempts to detect either circulating antibodies to HOXB7 or HOXB7 protein in patient serum failed as discussed in the annual report of year one. It is possible that levels were too low to detect by ELISA methods employed. Nevertheless, we are characterizing a number of HOXB7 antibodies being developed by a commercial source (Zymed, Inc., San Francisco, CA) which we hope to use to screen breast cancer tissue arrays for presence of HOXB7 in tissue samples. To determine if HOXB7 mRNA levels were upregulated in breast cancer patients, quantitative, real-time PCR was employed. By comparison to levels of HOXB7 in normal samples (12 mammoplasty, 8 normal enriched epithelial organoid), mRNA for this gene was overexpressed an average of 4-fold in 39 of 41 mammary carcinomas, indicating that it may be of use as a diagnostic marker of the disease.

### Key Research Accomplishments

#### Task 1: Investigate the transforming potential of HOXB7 *in vitro* (months 1-12)

- Stably expressed HOXB7 mutant constructs in MCF10A normal mammary epithelial cells
- Showed that only the wild type HOXB7-expressing cell line, but none of the mutants (above) formed colonies in soft agar assays.
- Showed requirement for PARP activity specific to HOXB7-dependent transformation *in vitro*

#### Task 2: Identify HOXB7 target genes and interacting proteins (months 13-24)

- Prepared RNA for oligonucleotide microarrays.
- Further characterized the interaction between HOXB7 and Ku/DNA-PK and PARP *in vivo* in co-immunoprecipitation experiments
- Discovered that two previously uncharacterized post-translational modifications of HOXB7 block its DNA-binding functions
- Identified effects of HOXB7 on cell survival of MCF10A normal epithelial cells following exposure to radiation
- Attenuated HOXB7-mediated transformation by PARP inhibition

#### Task 3: Develop HOXB7 as a detection marker for diagnosis (months 1-36)

- Determined HOXB7 mRNA levels in 41 mammary carcinoma samples compared to normal controls.
- Characterized HOXB7 antibodies from a commercial source (ongoing).

#### List of reportable outcomes

1. Workshop presentation, March 2004  
The Role of HOXB7 Overexpression in Breast Cancer  
Homeobox Genes and Tissue Remodeling: Focus on Mammary Gland Workshop  
National Cancer Institute, Bethesda, MD
2. Zhu, T., E. Rubin, X. Wu, H. Chen, P.E. Lobie and S. Sukumar. 2004. HOXB7 overexpression enhances the tumorigenesis of human mammary epithelial cells via multiple receptor tyrosine kinases. Proceedings of the American Association of Cancer Research, 45, Abstract 29.
3. Wu, X., E. Rubin, T. Zhu, B. Parker and S. Sukumar. 2004. Induction of epithelial-mesenchymal transition of MCF-10A cells with high expression of HOXB7. Proceedings of the American Association of Cancer Research, 45, Abstract 2402.
4. Rubin, E., T. Zhu, X. Wu, H. Chen, G. Sharma, S. Dhar, T. K. Pandita and S. Sukumar. Physical and Functional Interaction of HOXB7 with PARP and Ku Proteins in Breast Cancer. *Manuscript in preparation.*

## Conclusions

This study examined the role of HOXB7 in breast cancer from a biochemical and cellular viewpoint. We have found that HOXB7 is a substrate for DNA-PK and PARP, two enzymes with which it interacts, and have determined that post-translational modification by these enzymes blocks DNA binding functions of HOXB7. This is the first report of poly-ADP-ribosylation of a homeobox protein and its functional consequences. Since HOXB7 was a substrate of PARP, and mammary cells overexpressing this protein exhibited anchorage-independent growth, we examined the relationship between PARP and HOXB7 in soft agar assays. Our results indicate that while chemical inhibition of PARP had no effect of *ras/neu* transformed MCF10A cells, soft agar colony formation of MCF10A/HOXB7 was dramatically reduced by over 75%. While the role of PARP in tumorigenesis is not clear-cut and requires more study, it appears from our studies that HOXB7 and PARP function in the same pathway in breast cancer.

We have also functionally analyzed different regions of HOXB7 with respect to interaction with PARP and anchorage independent growth. The abundance of PARP protein in HOXB7 immune complexes was reduced when HOXB7 expression constructs lacking helix 3 of its homeodomain were used to transfect cells. This led us to examine whether the homeobox of HOXB7 was mediating the interaction with PARP. Sequential carboxy-terminal deletions of HOXB7 were constructed and used to determine that the homeodomain of HOXB7 was indeed required for PARP binding. Soft agar colony formation assays also revealed a requirement for the homeodomain, poly-ADP-ribosylation and PBX1-interacting regions of HOXB7. Cell survival assays of MCF10A cells stably expressing HOXB7 and its mutant constructs also indicated a need for these regions for enhanced survival following induction of DNA DSB by gamma irradiation. Taken together, our results suggest that protein-protein interactions and post-translational modification of HOXB7 are crucial for modulating its biological activities and that mammary cells overexpressing this protein have oncogenic potential and survival advantages over non-expressing cells. As more than 95% of mammary carcinomas in our study exhibited upregulated levels of HOXB7 mRNA, it is important to understand the role of HOXB7 and its utility as a diagnostic marker for breast cancer.

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