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14. ABSTRACT

Homeobox genes control anterioposterior 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. 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. 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 had 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. HOXB7 was overexpressed in more than 50% of primary tumors. Expression in normal mammary epithelial cells conferred the ability to form colonies in agar, and tumors in nude mice. Thus it is possible that HOXB7 is a novel marker for identifying aggressive tumors.

15. SUBJECT TERMS
Breast cancer, HOX genes, DNA repair, marker

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Introduction

HOX genes encode transcription factors that are characterized by a highly conserved tri-helical homeodomain that binds to specific DNA sequences. A total of 39 HOX genes have been identified that are organized into four paralogous clusters, HOX-A to –D, on autosomal chromosomes (Garcia-Fernandez, 2005). The functions of homeodomain-containing proteins are diverse and include roles as both classical regulators of transcription and novel roles outside of transcriptional regulation. HOX genes are functionally important in anterioposterior patterning during embryogenesis, homeostasis in adult tissue, cell-cell interactions, and cell-ECM interactions (reviewed in Chen and Sukumar, 2003). Examples of novel roles for homeodomain-containing proteins include the role of human proline-rich homeodomain protein, PRH (known as Hex in studies on hematopoiesis), which interacts with eIF4E to inhibit its mRNA nuclear-cytoplasmic transport function (Topisirovic et al., 2003). Given that HOX proteins can bind to very similar sequences in vitro but exert diverse functions in vivo, a fundamental question is how each HOX protein achieves functional specificity. One hypothesis is that functional specificity is attained by physical interaction with various co-factors.

DNA double strand breaks (DSBs), caused by exposure to ionizing radiation (IR), certain chemicals or occurring during replication, V(D)J recombination, and meiosis, pose a major challenge to maintenance of genomic integrity. If they are left unrepaired, cell cycle arrest, apoptosis or mitotic cell death ensues, while faulty repair can lead to neoplastic transformation (Richardson et al., 2004; Scott and Pandita, 2006). NHEJ is the major mechanism for the repair of IR-induced DSB, and involves the DNA end-binding heterodimer, Ku70/Ku80; DNA-dependent protein kinase (DNA-PK); the XRCC gene product; and DNA ligase IV, (Karran, 2000). The Ku antigen binds to and recruits DNA-PK to sites of DNA strand breaks, where DNA-PK is activated to participate in DNA repair. HOXC4 and HOXD4, along with homeodomain-containing proteins Octamer transcription factor-1 and -2 and Dlx2, interact with the C-terminus of the Ku antigen causing their recruitment to broken DNA ends and phosphorylation by DNA-PK (Schild-Poulter et al., 2001). However, the functional significance of this interaction is not known.

Another protein that contributes to genomic stability is Poly (ADP) ribose polymerase (PARP). PARP catalyzes the transfer of polymers of ADP-ribose from NAD+ onto protein targets (Schreiber et al., 2006) and regulates both cell survival and cell-death programs. A recent study has shed some light on their involvement in double-strand break (DSB) repair mediated by NHEJ and by homologous recombination (HR). Hochegger et al (Hochegger et al., 2006) showed that PARP-1(-/-) mutant chicken cells have reduced levels of HR and are sensitive to various DSB-inducing genotoxic agents. Interestingly, this phenotype is strictly dependent on the presence of Ku70. PARP-1/KU70 double mutants are proficient in the execution of HR and display an elevated resistance to DSB-inducing drugs. These results suggest PARP might function by minimizing the suppressive effects of Ku and the NHEJ pathway on HR.

In this study we found that HOXB7 has the ability to confer both a transformed phenotype and resistance to IR to cultures of human mammary epithelial cells, MCF10A. A search for protein interaction partners of HOXB7 that might contribute to this transformation led to the identification of the DNA repair proteins, Ku70, Ku 80, and the catalytic subunit of DNA-PK (DNA-PK_{cs}) and PARP. This, among other functions, suggests a role for HOXB7 in DNA repair through NHEJ. We present evidence to indicate that interaction between HOXB7 and the Ku antigens is functionally significant since HOXB7 expression enhances NHEJ, DNA-PK activity and DNA damage repair in mammalian cells.

Our work in the last 3 years on HOXB7 and its interaction with DNA doubles strand repair proteins DNAPKcs, Ku-70 and Ku-80 and the PARP. In the last year we refined these studies by showing that this interaction is not only prevalent in cells transfected with HOXB7 but also in breast cancer cells with endogenous high level expression of HOXB7, and that this overexpression renders these cells better able to perform double strand break repair.

Body

Task 1 of the statement of work investigates the transforming potential of HOXB7 *in vitro* by soft agar growth assay. Completion of this task has been reported in 2004.

Task 2 of the statement of work aims to identify HOXB7 target genes and interacting proteins. This task has been completed. The results are summarized below.

Task 3 of the statement of work is to develop HOXB7 as a detection marker for diagnosis.

Work completed-

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

- Stably expressed HOXB7 mutant constructs in MCF10A normal mammary epithelial cells
- Showed growth enhancement of MCF10A by HOXB7 in low factor medium
- 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 12-36) **Completed**

- 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
- **Months 24-36.** Repeated all the above experiments in cells with endogenous overexpression of HOX B7
- Showed that- MDAMB 468 cell extracts perform DSB efficiently, an effect that is abrogated by transfecting the cells with an antisense construct to HOXB7
- MDAMB468 and MDAMB 435 breast cancer cells have a greater ability to resist the deleterious effects of gamma radiation. siRNA to HOXB7 abrogates this protection as shown by colony formation assays
- Months 36-42
- Established that DNAPK activity is enhanced in HOXB7 expressing cells. Found that homeodomain of HOXB7 is indispensable for its interaction with Ku70/Ku80 and for performing its DNA repair function of NHEJ.
- Performed definitive pulse field gel electrophoresis to establish that HOXB7 enhances NHEJ.
- Performed si RNA experiments and experiments that included delta H3 deletion of HOXB7 as controls to prove that HOXB7 interacts with components of NHEJ and that this interaction is essential for the higher NHEK observed in these cells.
-

Task 3: Develop HOXB7 as a detection marker for diagnosis (months 1-42)

- Determined HOXB7 mRNA levels in 41 mammary carcinoma samples compared to normal controls.
- Characterized HOXB7 antibodies from a commercial source.
- Characterized HOXB7 antibodies for immunohistochemistry without success.
- Showed by Q-RT-PCR that breast cancer metastasis also overexpress HOXB7.

- By Q-PCR showed that overexpression is likely due to gene amplification of HOXB7
- Array analysis of a group of 89 tumors with followup after Tamoxifen monotherapy showed that HOXB7 is overexpressed, but only borderline significance p=0.6 was achieved.
- FISH analysis of a 500 patient cohort with 10 years of followup after Tamoxifen monotherapy showed that HOXB7 amplification occurs only in 10% of Cau breast cancers and does not correlate significantly with recurrence.

Key Research Accomplishments. We have shown that-

1. *HOXB7 elicits a transformed phenotype in MCF10A cells.*

Overexpression of HOXB7 enabled nontumorigenic breast cancer cells, SKBR3, to form well-vascularized tumors in immunodeficient mice (Care et al., 2001). To investigate whether HOXB7 expression transforms normal breast epithelial cells, a FLAG-tagged (Fl) HOXB7 expression plasmid was transfected into immortalized, human mammary epithelial cells (HMEC), MCF-10A, and pooled G418-selected colonies stably expressing HOXB7 were tested for alterations in growth properties compared to the vector-transfected cells (Figure 1A, B). MCF10A cells require a highly growth factor-supplemented medium for optimal growth (Heppner et al., 2000). In low growth nutrient RPMI medium with 10% or 1% serum supplementation, unlike vector-control cells, MCF10A-Fl-HOXB7 cells displayed a continued ability to proliferate (Figure 1A). Grown in Matrigel in complete medium, in contrast to the minute colonies formed by MCF-10A-Vec transfected cells (Figure 1C-1), MCF10A-Fl-HOXB7 cells formed 200 to 300-cells, anchorage-independent colonies (Figure 1C-2) within three weeks. Grown on Matrigel coated plates, MCF10A cells formed discrete acini-like structures with a hollow lumen (Fig. 1C-3), while MCF10A-Fl-HOXB7 cells displayed large irregular solid colonies with cells pushing haphazardly into the surrounding extracellular matrix (Figure 1C-4). Taken together, the results indicate that the MCF10A-Fl-HOXB7 cells exhibit a transformed phenotype like that of MCF10A cells expressing RAS or HER2/neu oncogenes (Heppner et al., 2000; Shekhar et al., 2000).
Some activated oncogenes render cells resistant to radiation while others enhance their susceptibility to IR. The molecular basis of sensitivity to IR is a complex product of cellular responses; loss of cell cycle checkpoints may result in increased sensitivity, particularly if the checkpoint controls G2 transitions. To

determine the effects of overexpressed HOXB7 on the response to IR exposure, several tests were performed. Clonogenic survival assays were performed using stably transfected MCF10A cells (Figure 1B). Upon exposure to low-dose gamma radiation, MCF10A-Fl-HOXB7 cells had about a 2-fold enhanced survival advantage over the vector-transfected and parental MCF10A cells (Figure 2 and B).

We also examined G1 (Figure 2C) and G2-type (Figure 2D) chromosomal aberrations in metaphase spreads from subcultured SKBR3 cells at various time points post-irradiation (Dhar et al., 2000). Cells in plateau phase were irradiated with 3 Gy, subcultured and examined for G1-type aberrations at metaphase. The frequency of aberrations was calculated in parental SKBR3, SKBR3-HOXB7-YFP, and SKBR3-vec cells and was significantly lower in SKBR3-HOXB7-YFP cells than in the other two groups (Student's t test , $P<0.05$). For G2-type aberrations, cells in exponential phase growth were irradiated with 1 Gy gamma radiation. Metaphases were examined for chromatid breaks and gaps. Fifty metaphases were scored for each post-irradiation time point. Results for SKBR3-HOXB7-YFP expressing cells were compared to those of vector-transfected (SKBR3-YFP) and parental (SKBR3) controls. SKBR3-HOXB7-YFP cells showed a significant decrease in G2 type chromosome aberrations as compared to parental control cells (Student's t test: $P<0.035$). These cells possessed an intact G2/M checkpoint (data not shown), though their elevated mitotic index (Figure 2E) appears to indicate enhanced recovery and repair of DNA damage. The nature of the protection against radiation conferred by HOXB7 in these assays suggests that HOXB7 may affect DNA repair kinetics through the NHEJ pathway.

To explore this further, we tested the DNA repair activities of HOXB7-containing nuclear extracts *in vitro* by plasmid end-joining assays (Sharma et al., 2003). This analysis revealed that expression of HOXB7-YFP in SKBR3 cells stimulated the end-joining activity almost 2.5-fold (Figure 2F). These results were verified by

knockdown of endogenous HOXB7 expression in breast cancer cells, MDA-MB-468, using antisense constructs. Transient transfection of HOXB7-antisense plasmids into MDA-MB-468 cells could suppress the expression of HOXB7 (>75%), and reduce NHEJ activity by about 1.6-fold (data not shown). These results suggest a role for HOXB7 in stimulating DNA repair, and raise the possibility that it occurs through NHEJ.

Figure 1

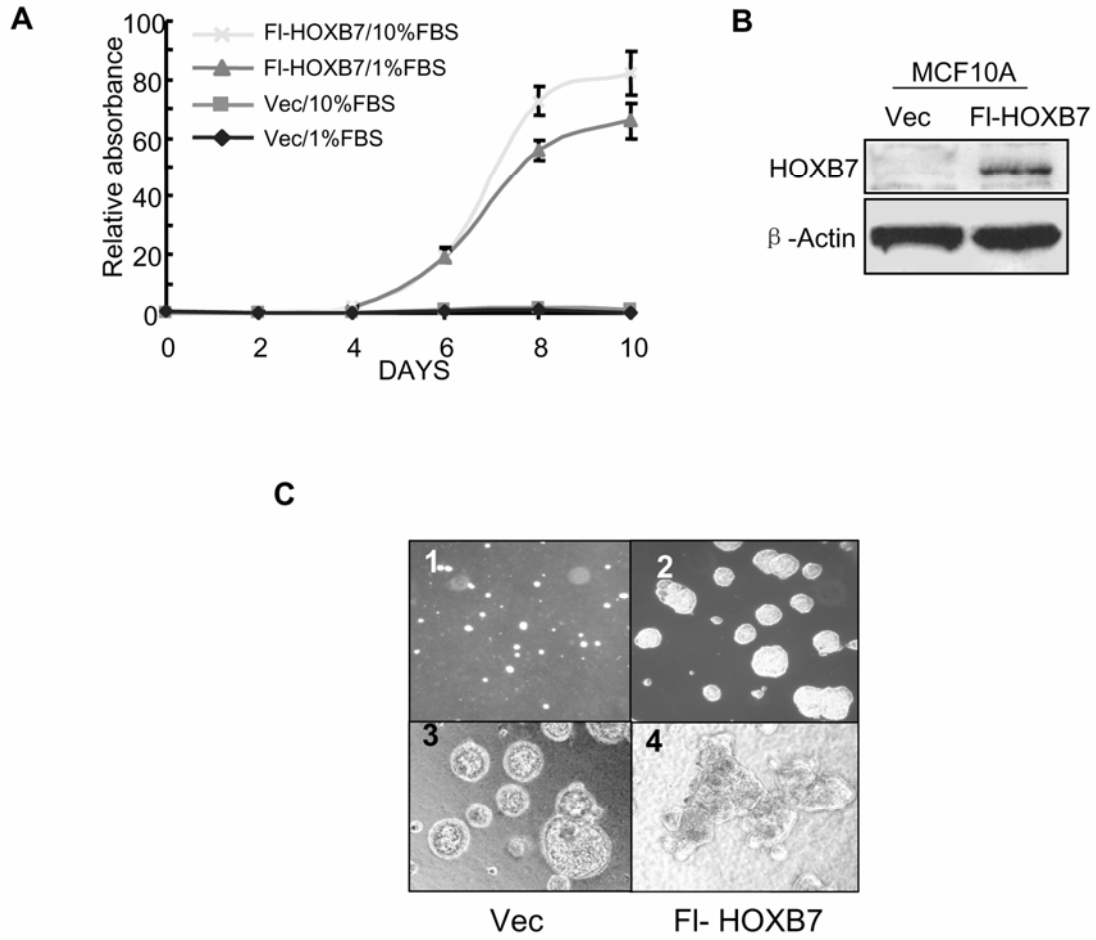


Figure 1: Overexpression of HOXB7 can confer a transformed phenotype upon MCF10A.

A. MCF10A cells stably expressing HOXB7 or vector control cells were grown in RPMI medium supplemented with 1% or 10% fetal bovine serum, and the cells were stained with crystal violet. Calorimetric measurements were done for samples in triplicate and the experiment was repeated 3 times. **B.** Western analysis using HOXB7 antibodies confirmed its expression in the stably transfected MCF10A-Fl-HOXB7 cells. Beta-actin levels served as loading controls. **C-1.** MCF10A-Vec cells remaining as 2-10 cell clusters grown in Matrigel and **C-2**, MCF10A-Fl-HOXB7 cells forming large, anchorage-independent colonies when grown in Matrigel, **C-3**, MCF10-vec cells forming acinar structures and **C-4**, MCF10A-Fl-HOXB7 forming irregular, solid colonies when grown on the surface of Matrigel. Phase contrast, magnification: 20X.

Figure 2

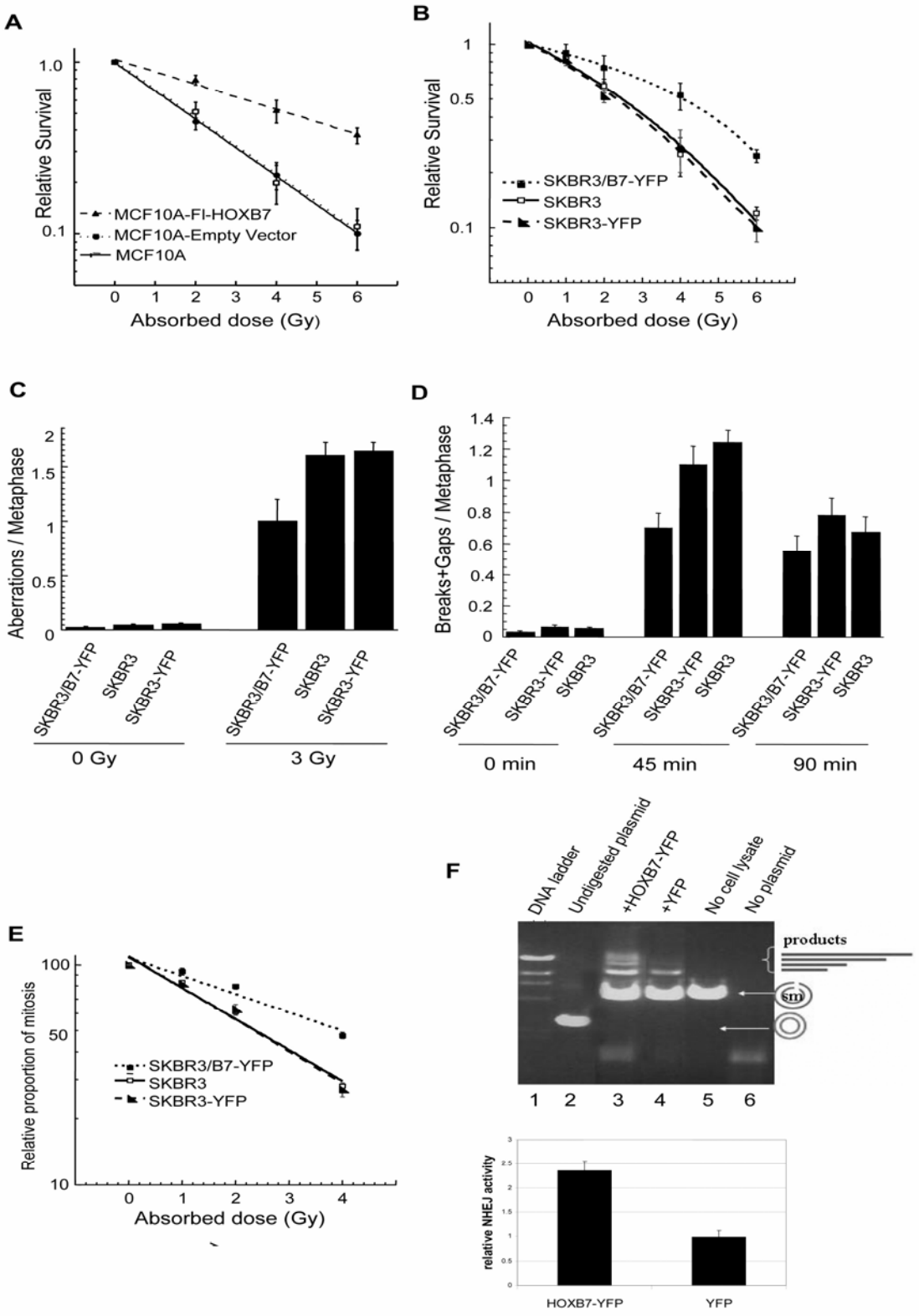

A
Relative Survival
1.0
0.1
MCF10A-FI-HOXB7
MCF10A-Empty Vector
MCF10A
0 1 2 3 4 5 6
Absorbed dose (Gy)
B
Relative Survival
1
0.5
0.1
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
0 1 2 3 4 5 6
Absorbed dose (Gy)
C
Aberrations / Metaphase
2
1.5
1
0.5
0
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
0 Gy
3 Gy
D
Breaks+Gaps / Metaphase
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SKBR3/B7-YFP
SKBR3-YFP
SKBR3
SKBR3/B7-YFP
SKBR3-YFP
SKBR3
SKBR3/B7-YFP
SKBR3-YFP
SKBR3
0 min
45 min
90 min
E
Relative proportion of mitosis
100
50
10
SKBR3/B7-YFP
SKBR3
SKBR3-YFP
0 1 2 3 4
Absorbed dose (Gy)
F
DNA ladder
Undigested plasmid
+HOXB7-YFP
+YFP
No cell lysate
No plasmid
products
sm
1 2 3 4 5 6
relative NHEJ activity
3
2.5
2
1.5
1
0.5
0
HOXB7-YFP
YFP

Figure 2: A-B. Clonogenic survival assays. Percent survival of **A.** MCF10A parental cells and MCF10A-Fl-HOXB7 or MCF10A-vec, or **B.** SKBR3 parental cells, SKBR3-HOXB7-YFP, SKBR3-YFP cells irradiated at the indicated doses was calculated and compared to mock-irradiated (0 Gy) controls.

C. G1-type chromosomal aberrations after radiation treatment. The frequency of aberrations following irradiation with 3 Gy was calculated in parental SKBR3, SKBR3-HOXB7-YFP, and SKBR3-vec cells.

D. G2-type chromosomal aberrations after radiation treatment. Number of chromatid breaks and gaps in metaphases were scored for SKBR3-HOXB7-YFP cells and compared to those of SKBR3-YFP and parental SKBR3 controls.

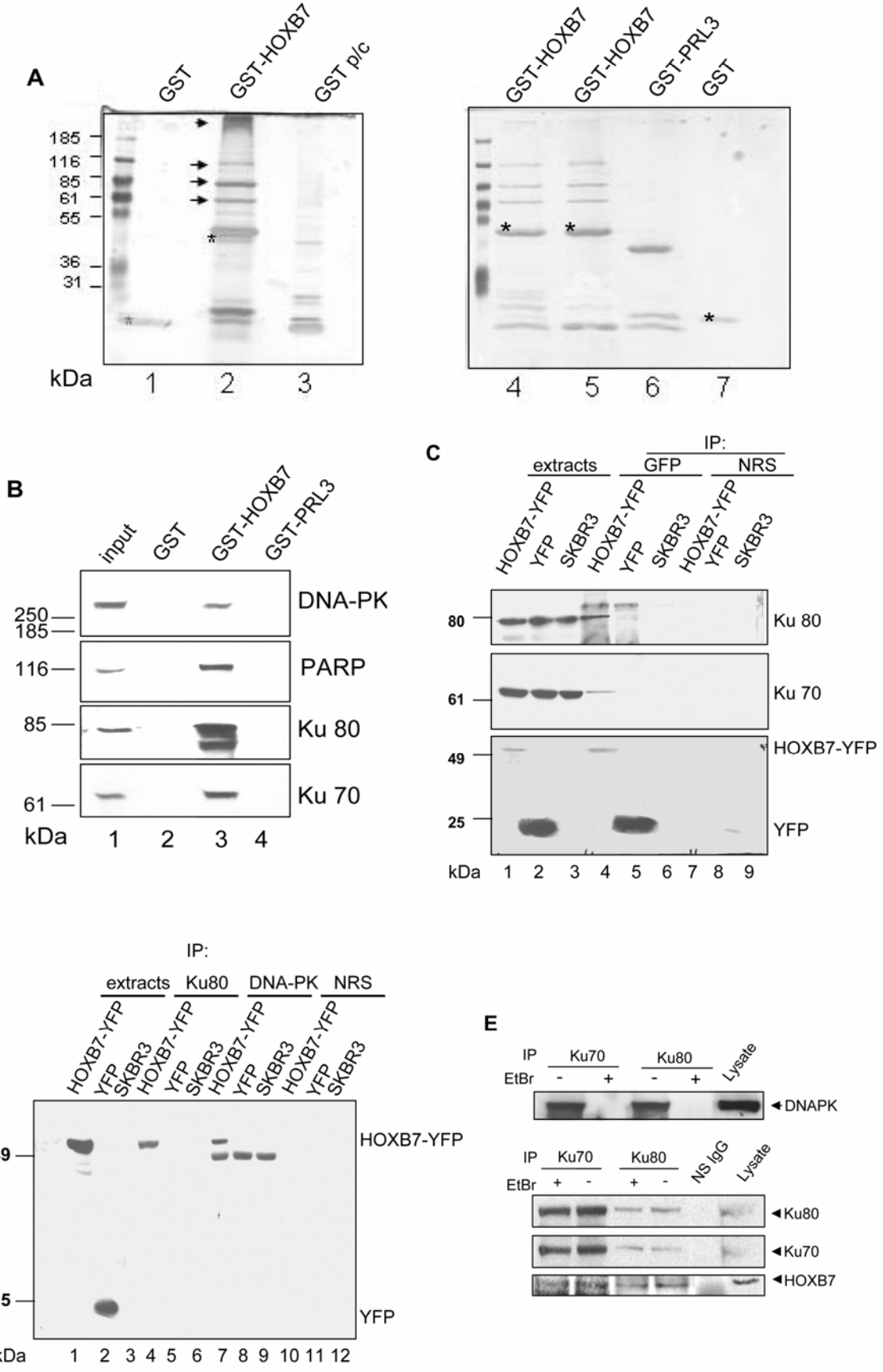
E. Mitotic index after radiation treatment. Parental SKBR3, SKBR3-YFP and SKBR3-HOXB7-YFP cells in exponential phase were irradiated with increasing doses of gamma radiation and then examined for the frequency of mitotic cells. The mean represents the value from three independent experiments. For each experiment, 200 metaphases were scored.

F. HOXB7 stimulates DNA repair *in vitro* and *in vivo*. Plasmid end-joining assays were performed. Nuclear extracts of SKBR3 cells expressing HOXB7-YFP or YFP alone were mixed with 0.25 ug of blunt-digested pCDNA3.0 in a plasmid end-joining reaction. Products were resolved on 0.7% EtBr stained agarose gels Lane 1: DNA ladder, lane 2: undigested pCDNA3.0, lane 3: digested plasmid plus SKBR3-HOXB7-YFP nuclear extract, lane 4: digested plasmid plus SKBR3-YFP nuclear extract; lane 5: digested plasmid plus extraction buffer; lane 6: SKBR3-HOXB7-YFP nuclear extract without plasmid. Band intensities were quantitated on Eagle Eye software and data from 2 separate analyses are shown as values +/- standard deviations.

3. HOXB7 interacts with DNA repair proteins

To investigate whether HOXB7 plays a role in NHEJ, we attempted to identify proteins interacting with HOXB7 in breast cells. Cell extracts of SKBR3 (Figure 3A, lanes 2 and 4) and MCF10A (Figure 3A, lane 5) were fractionated by affinity chromatography on GST-HOXB7-Sepharose. Analysis of the proteins in column eluates by silver and Coomassie staining after SDS-PAGE revealed the presence of 4 polypeptides of approximate sizes of 70, 85, 110, and >250 kD which did not bind to the GST (lanes 1 and 7), or to the unrelated GST-fusion protein, GST-PRL3 (lane 6). Similar results were obtained with extracts of HMECs, MCF-12A, and breast cancer cells, MDA-MB-231 (data not shown). To identify the eluted proteins, several methods were used. Direct sequencing from PVDF membranes yielded results for the 85 kD band, which identified Ku80 from an N-terminal 17-amino acid sequence (VRSGNKAAVVLCMDVGF). For the 110 and 70 kD protein bands, peptide mass fingerprints were obtained by MALDI-TOF and compared against those in public databases. Both ProFound and MS-FIT public database searches for the peptide mass maps obtained from the 110 and 70 kD protein bands identified PARP and Ku70, respectively. Protein identities were confirmed by immunoblotting with antibodies against PARP, Ku80 and Ku70 (Figure 3B). Since Ku70/80 are known binding subunits of the DNA-dependent protein kinase (DNA-PK), the high molecular weight band appearing at the top margin of the gel (>250 kD) was predicted and confirmed as the catalytic subunit of DNA-PK (DNA-PK_{cs}) by immunoblot analysis (Figure 3B). The finding of several components of the DNA-PK complex suggested that DNA repair observed in the experiments described above are most likely mediated by NHEJ. We therefore focused our efforts on understanding the interaction of HOXB7 with components of the NHEJ complex. Next, to test these interactions in intact cells, the associations between HOXB7 and Ku70, Ku80 and DNA-PK_{cs} were analyzed *in vivo* by co-immunoprecipitation. A HOXB7-YFP fusion protein was stably introduced into the HOXB7-null breast cancer cell line, SKBR3. Fluorescence microscopy confirmed that HOXB7-YFP localized solely to the nucleus (data not shown). Immunoprecipitation (IP) with GFP antibodies (which also recognize the YFP variant) showed that Ku70 and Ku80 associated with HOXB7 *in vivo* (Figure 3C, lane 4). Complementary IP using Ku80 (Figure 3D, lanes 4-6) or DNA-PK_{cs} (Figure 3D, lanes 7-9) antibodies confirmed the presence of HOXB7-YFP in their complexes (lanes 4 and 7following transient transfection of this construct into SKBR3 cells. Identical results were obtained when Fl-HOXB7 (Fl-HOXB7pcDNA3) was transiently expressed in SKBR3 cells (data not shown). Complex formation was not affected by DNA damage by UV or IR (data not shown). The interaction between HOXB7 and Ku70/Ku80 was also confirmed by co-IP analysis using protein extracts of breast cancer cells, MDA-MB-435, which express high levels of endogenous HOXB7 (data not shown).

Figure 3


A
GST
GST-HOXB7
GST p/c
185
116
85
61
55
36
31
kDa
1
2
3
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GST-HOXB7
GST-PRL3
GST
4
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GST-PRL3
250
185
116
85
61
DNA-PK
PARP
Ku 80
Ku 70
kDa
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NRS
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IP
Ku70
Ku80
EtBr
Lysate
DNAPK
NS IgG
Ku80
Ku70
HOXB7

)

Figure 3: Identification and analysis of HOXB7 interacting proteins.

A. Affinity chromatography. GST-HOXB7 interacting proteins from SKBR3 cells (lanes 1-3, silver stained gel, lane 4, Coomassie-stained PVDF membrane) or MCF10A cells (lanes 5-7, Coomassie-stained PVDF membrane). Lanes 1 and 7, proteins bound to GST alone (GST); Lane 3, proteins bound to GST alone during the preclearing (GST p/c) step; Lane 6, proteins bound to an unrelated control (GST-PRL3). Positions of GST (lane 1 and 7) and GST-HOXB7 (lanes 2, 4, 5) are indicated by asterisks.

B. **Immunoblot confirmation of HOXB7-interacting proteins.** Proteins which bound to GST-HOXB7 (lane 3) or control matrices (lanes 2 and 4) were eluted, separated by SDS-PAGE and transferred to nitrocellulose, and immunoblotted with antibodies to the catalytic subunit of DNA-PK (DNA-PK_{cs}), PARP, Ku 86 and Ku 70. SKBR3 cell extracts (100 ug of total extract, 2% of input) (lane 1) served as a positive control for proteins detected by immunoblot.

C. Co-immunoprecipitation of PARP, Ku 80 and Ku 70 with HOXB7-YFP in SKBR3 cells. SKBR3 cells were stably transfected with HOXB7-YFP (lanes 1, 4 and 7) or YFP alone as a vector control (lanes 2, 5 and 8) prior to immunoprecipitation with GFP antibodies and subsequent western blot of precipitated proteins. Parental SKBR3 cells, which lack detectable HOXB7, were used as controls as well (lanes 3, 6 and 9). Lanes 1-3 show protein levels in 100 ug of total cell extracts (5% of input), while lanes 4-6 are proteins that precipitated with HOXB7-YFP or controls that did not express HOXB7 (SKBR3-YFP and parental cells). Normal rabbit serum (NRS) was used to control for specificity (lanes 7-9).

D. Co-immunoprecipitation of HOXB7-YFP with DNA-PK_{cs} and Ku 80 in SKBR3 cells. Complementary immunoprecipitations to those shown in Figure 3C were performed using SKBR3 cells transiently transfected with HOXB7-YFP (lanes 1, 4 and 7), YFP alone (lane 2, 5 and 8) or SKBR3 parental cells, which do not express HOXB7 (lanes 3, 6 and 9). Normal rabbit serum (NRS) (lanes 10-12) was used as a control.

E. DNA is not required for the interaction between HOXB7 and the DNA-PK complex. Extracts of MCF-7 cells were treated with EtBr prior to co-immunoprecipitation with antibodies to Ku70 and Ku80, or p53 as a nonspecific IgG (NS IgG). Subsequent immunoblotting was performed with antibodies indicated on the right. Top panel: Effective blocking of the interaction between Ku70/80 and DNA-PK by using EtBr depletion of DNA (positive control). Bottom panel: No effect of DNA depletion on the interactions between HOXB7 and Ku70 and Ku80.

The common DNA-binding properties of these proteins raised the possibility that the interactions observed above are mediated through DNA rather than through direct protein-protein interaction. We tested this possibility by two methods. First, DNase I had no effect on binding of HOXB7 to Ku70/80 in HOXB7-transfected SKBR3 cells (data not shown). This finding was also verified more stringently using extracts of MCF-7 cells which express endogenous HOXB7. As previously shown (Yavuzer et al., 1998), treatment with an intercalating agent, ethidium bromide, (EtBr) effectively blocked the interaction between Ku70/80 and DNA-PK, since this reaction is completely dependent on the presence of DNA (Figure 3E, top panel). In contrast, depletion of DNA in the extracts using EtBr did not reduce the interactions between endogenous HOXB7 and Ku70 or Ku80 (Figure 3E, bottom panel). We also found no evidence for interaction of HOXB7 with two other DNA–binding proteins, i.e., BRCA-1 and E2F1 (data not shown). These results suggest that the interaction between HOXB7 and Ku70 and Ku80 are, in all likelihood, specific and not mediated by DNA.

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Figure 4

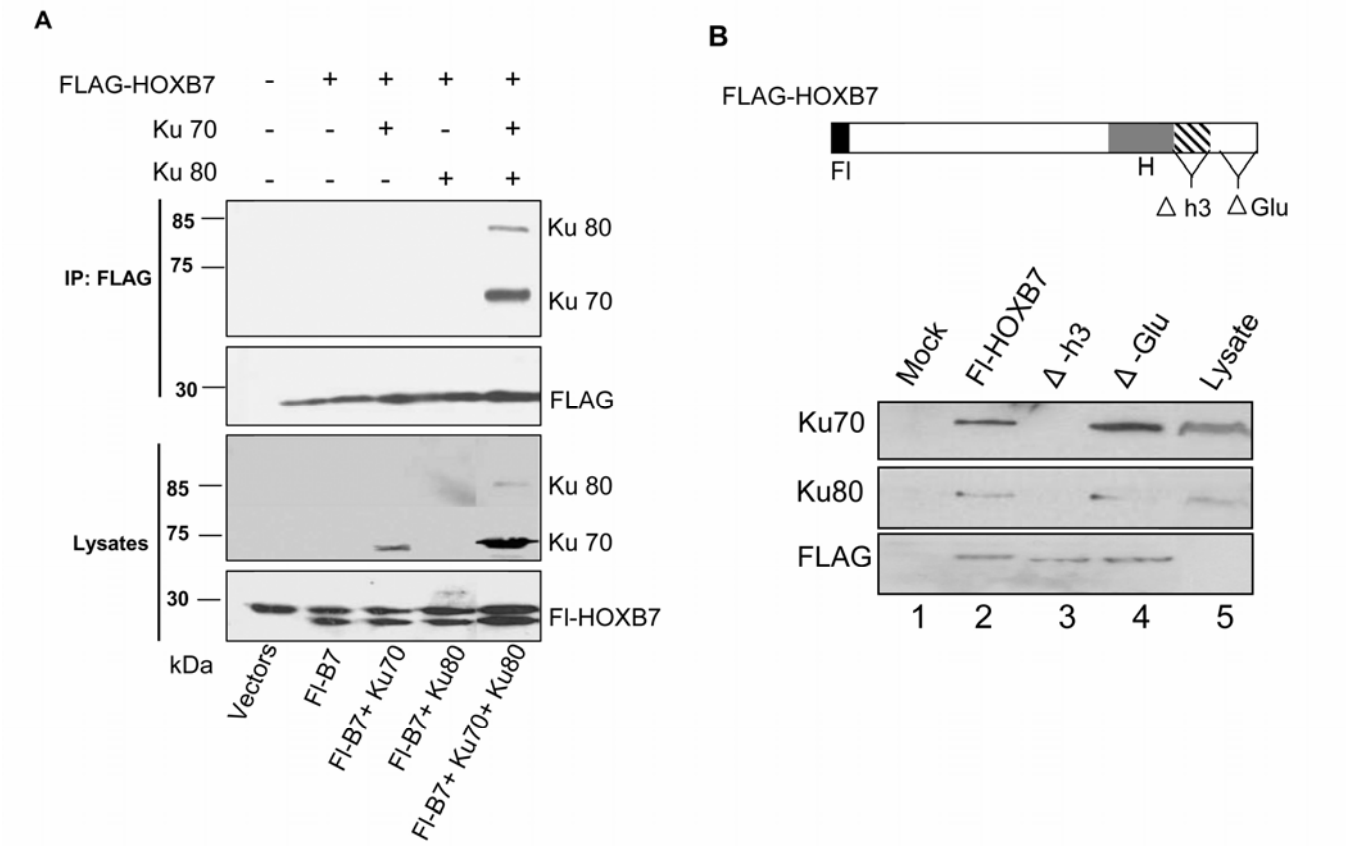


Figure 4: Analysis of HOXB7 complexes.

A. Ku70/80 heterodimer formation is a prerequisite for HOXB7 binding. Fl-HOXB7 was transiently expressed in the CHO cells alone or with Ku70 and/or Ku80, co-immunoprecipitated with FLAG-antibodies, and immunoblotted with the antibodies indicated on the right. 100 ug of total protein lysates (5% of input) are shown.

B. Defining the region of HOXB7 that interacts with Ku70/80 proteins:

Top panel: Schematic representation of FLAG-tagged HOXB7 (FLAG-HOXB7), and deletion constructs. Locations of the FLAG tag (Fl), homeodomain (H) and deletions of the third helix (Δh3) of the homeodomain, and of the glutamic acid-rich tail (ΔGlu) are shown.

Since complexes formed by interactions between Ku70, Ku80 and DNA-PK_{cs} are well- established, we investigated the nature of these complexes with HOXB7 and the order of their formation. Experiments introducing Fl-HOXB7 into CHO cells, which have undetectable Ku70 and Ku80 protein expression (Figure 4A), showed that expression of both Ku70 and Ku80 is required for association of either protein with HOXB7. These results suggest that Ku70/Ku80 heterodimer formation is a prerequisite for HOXB7 binding. In order to define the region of HOXB7 that interacts with Ku70/80 proteins, full length Fl-HOXB7 and HOXB7 with deletions of helix 3 of the homeodomain (HOXB7- Δh3) or of the glutamic acid tail (HOXB7-ΔGlu) (Figure 4B, top panel) (Yaron et al., 2001) were transfected into SKBR3 cells, and cell lysates were subjected to co-IP with FLAG antibody (Figure 4B, bottom panel). The results showed that deletion of helix 3 from the homeodomain in HOXB7 (lane 3) completely abolished the interaction between HOXB7 and Ku70/80 proteins. In contrast, removal of the glutamic acid tail from HOXB7 (lane 4) did not affect the interaction. These results show that the integrity of the homeodomain is essential for the interaction between HOXB7 and Ku70/Ku80.

Figure 5

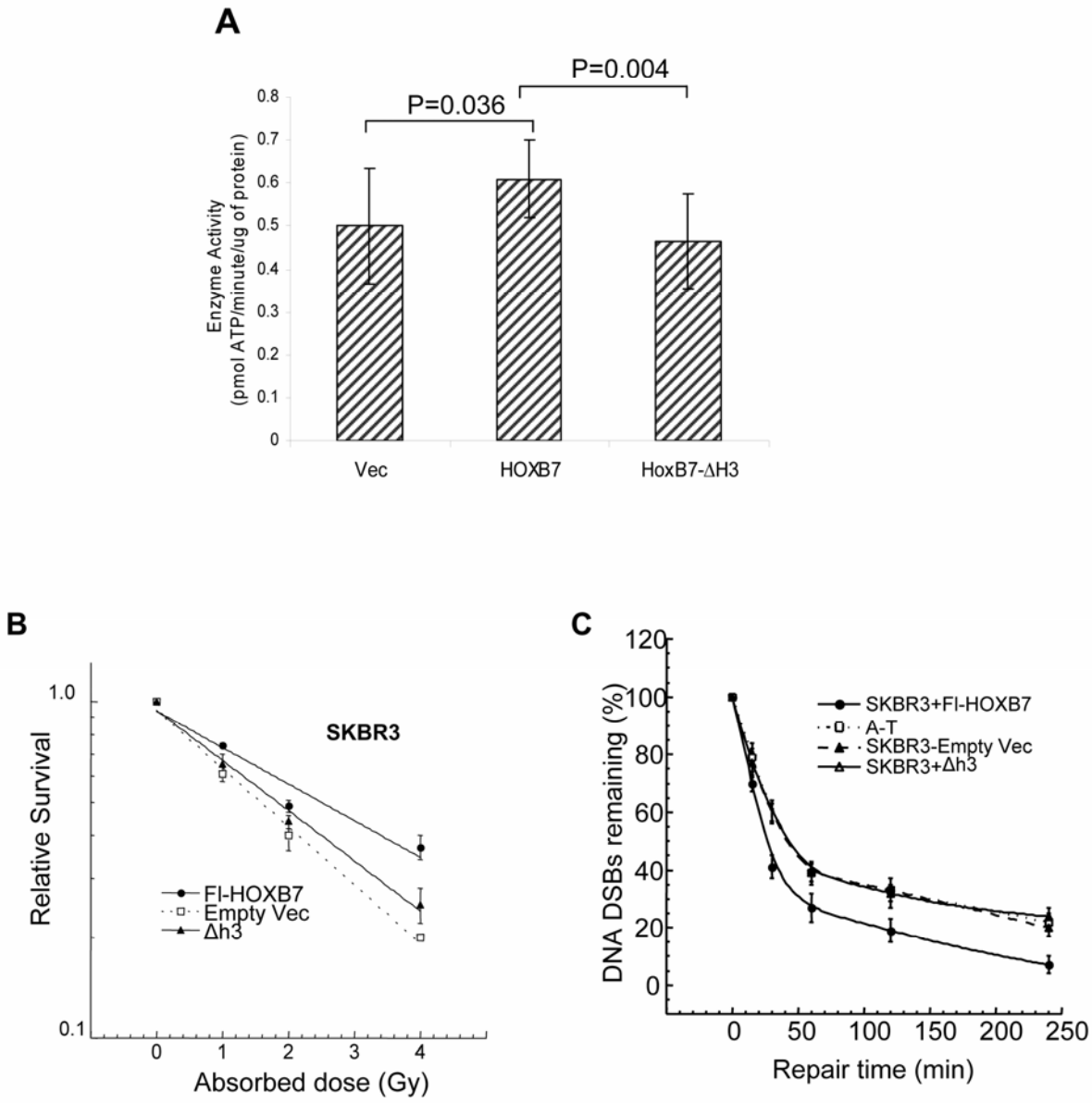

A
P=0.004
P=0.036
Enzyme Activity
(pmol ATP/minute/ug of protein)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Vec
HOXB7
HoxB7-ΔH3
B
SKBR3
Relative Survival
1.0
0.1
Fl-HOXB7
Empty Vec
Δh3
0
1
2
3
4
Absorbed dose (Gy)
C
DNA DSBs remaining (%)
120
100
80
60
40
20
0
SKBR3+Fl-HOXB7
A-T
SKBR3-Empty Vec
SKBR3+Δh3
0
50
100
150
200
250
Repair time (min)

Figure 5: HOXB7 stimulates DNA-PK activity and helix-3 is indispensable for HOXB7-mediated enhancement of cell survival and NHEJ

A. DNA-PK activity is enhanced in HOXB7-expressing cells. DNA-PK activity was measured in DNA-depleted whole cell extracts prepared from cells transiently transfected with either the empty vector or with plasmids expressing Fl-HOXB7 or HOXB7-Δh3. Data are the mean of values from duplicate samples from three experiments expressed as mean with +/- standard deviation.

B. Clonogenic survival assays. Survival of SKBR3 cells transiently transfected with Fl-HOXB7, HOXB7-Δh3, or the empty vector, after irradiation, was compared to mock-irradiated (0 Gy) controls.

C. DNA DSB repair. Cells were irradiated with 50 Gy and lysed at different periods after irradiation. Control cells are repair-deficient ataxia telangiectasia (AT) cells, GM5823. Unrepaired DNA breaks were measured under nondenaturing conditions. The data represent the means of three independent experiments.

4. Expression of HOXB7 stimulates DNA-PK activity and enhances NHEJ:

Because Ku70/80 is the DNA-binding subunit of DNA-PK, it is plausible that the observed interaction between Ku70/80 and HOXB7 may affect the catalytic activity of DNA-PK, and therefore NHEJ. To investigate the effect of HOXB7 expression on DNA-PK activity, we carried out transient transfections of MDA-MB-435 cells (Supplementary Figure 2). As shown in Figure 5A, expression of HOXB7 resulted in an increase in DNA-PK activity (P=0.036). Expression of HOXB7 lacking helix 3 of the homeodomain eliminated this effect, consistent with the finding (Figure 4) that interaction between HOXB7 and Ku70/80 proteins is abolished by deletion of helix 3 from the homeodomain in HOXB7.Since increased DNA-PK activity was abrogated by deletion of helix 3 of HOXB7 (Figure 5A) we further studied the effects of this deletion in clonogenic assays and in a DNA DSB repair assay. SKBR3 cells were transiently transfected with Fl-HOXB7, HOXB7-Δ h3, or empty vector (Supplementary Figure 3) and exposed to IR; survival of the cell clones was compared to mock-irradiated (0 Gy) cells. Unlike full-length HOXB7 protein, HOXB7- h3 was unable to efficiently protect cells from the effects of IR. The difference in cell survival post-irradiation between cells with full-length and those with mutant HOXB7 was significant (Student's t test, $P<0.05$) (Figure 5B). Thus, deletion of h3 domain of HOXB7 eliminated protection against IR afforded by the full length HOXB7 protein.

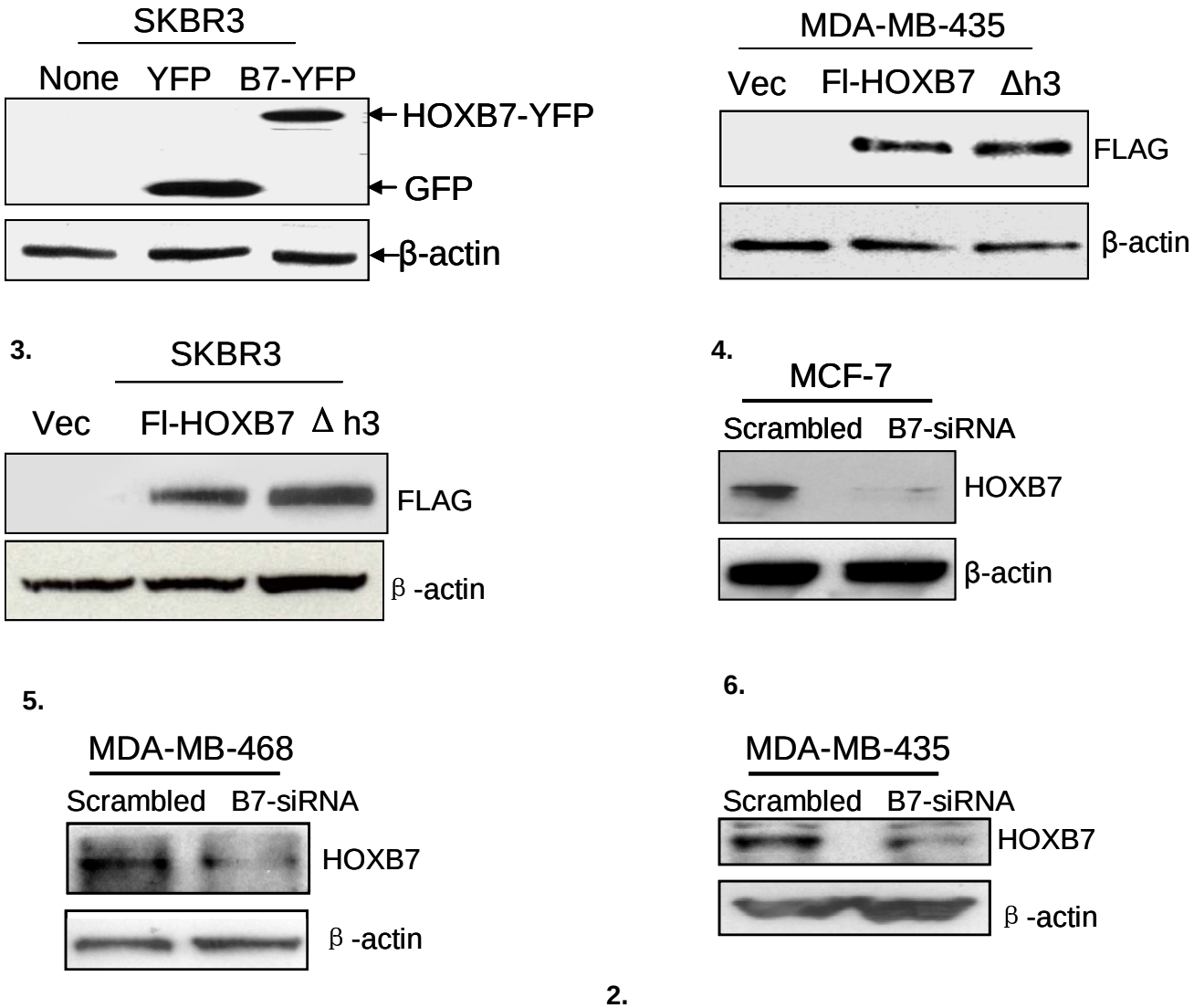
To determine if improved survival after radiation was a reflection of higher efficiency of repair of the DNA DSBs caused by the presence of HOXB7, SKBR3 cells transfected with Fl-HOXB7, HOXB7-Δ h3, or empty vector were used. An ataxia telangiectasia (AT) cell line, GM5823, was used as a known repair-deficient control. Cells were irradiated with 50 Gy and lysed at different intervals after irradiation. Unrepaired DNA breaks were resolved by pulsed field gel electrophoresis (PFGE) under nondenaturing conditions. SKBR3 cells were as inefficient at DSB repair as the AT cells. Cells overexpressing HOXB7 had the least amount of residual DNA DSBs. The effect of wild type HOXB7 on residual DNA damage in cells was significant (Student's t test, $P<0.05$). Deletion of the helix 3 abrogated the protective effect (Figure 5C). Collectively, these experiments provide evidence that HOXB7 plays an important role in DNA DSB repair. Further, the h3 domain of HOXB7 is indispensable for enhancement of DNA DSB repair through NHEJ.

5. Knockdown of endogenous HOXB7 can reduce efficiency of DNA repair

Our results provide strong support that HOXB7 associates with members of the DNA-PK holoenzyme. Initial findings had pointed to enhanced DNA repair capability in HOXB7 over-expressing cells (Figure 2 and 5). To further test the relevance of these findings and the contribution of HOXB7 to DNA repair, survival after IR exposure following suppression of HOXB7 expression using siRNA was investigated. In both MCF-7 (Supplementary Figure 4) and MDA-MB-468 cells (Supplementary Figure 5), HOXB7-specific siRNA reduced clonogenic survival significantly ($p<0.01$) (Figures 6A, B). Next, chromosomal aberrations were analyzed at metaphase after irradiation of MDA-MB-435 cells with or without reduced levels of HOXB7 (Supplementary Figure 6). All categories of asymmetric chromosome aberrations were scored. The frequency of chromosomal aberrations was higher in cells with reduced levels of HOXB7 indicating defective repair of chromosome

damage (Figure 6C). Cells with HOXB7 knockdown showed significant differences (P<0.01) in chromosomal aberration frequencies compared to control cells (Figure 6C). To further investigate the capacity of the G1-arrested cells to repair DSBs induced by IR, and to determine if this effect is mediated by HOXB7, we performed PFGE on DNA from gamma-irradiated MDA-MB-435 cells transfected with scrambled siRNA or with HOXB7-specific siRNA (Supplementary Figure 6). Indeed, the specific siRNA treatment significantly (p<0.04) increased the level of unrepaired DNA DSB (Figure 6D). Collectively, these data strongly suggest that HOXB7 can protect cells against DNA damage induced by IR exposure, possibly by conferring a higher efficiency of DNA DSB repair.

Supplementary figures 1-6



Supplementary figures 1-6: Western blot analysis of breast cancer cells transfected with the indicated plasmids to confirm expression using the antibodies shown on the right of the figure.

Figure 6

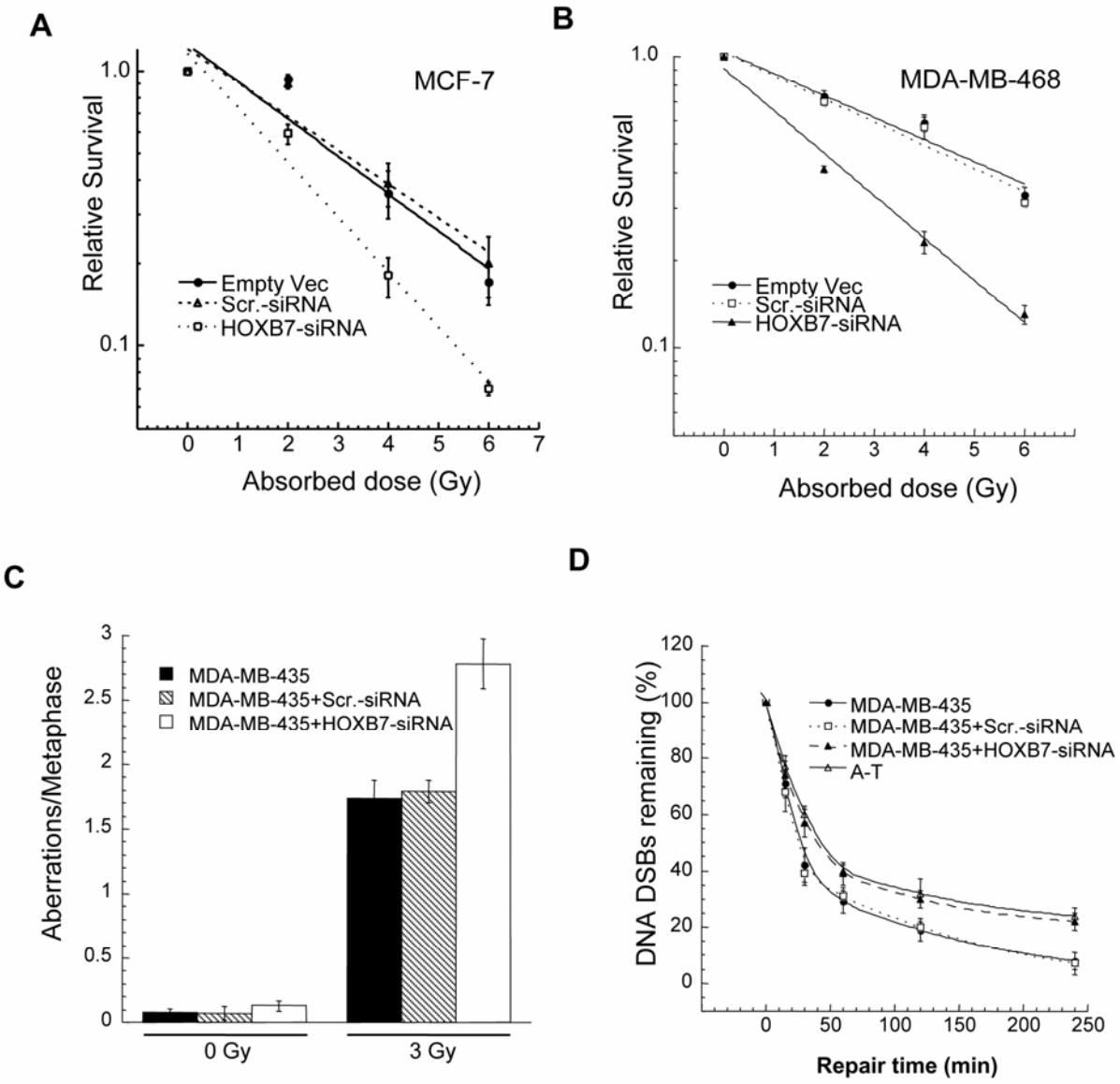


Figure 6. Knockdown of endogenous HOXB7 reduces DNA repair efficiency.
A, B: Clonogenicity after exposure to radiation of MCF-7 and MDA-MB-468 cells transfected with HOXB7-specific siRNA. Clonogenic survival assays of **A**. MCF-7 or **B.** MDA-MB-468 cells stably transfected with plasmids expressing either scrambled sequence siRNA (Scr.-siRNA) or HOXB7-specific siRNA (HOXB7-siRNA) were performed. **C. Analysis of chromosome damage and repair in MDA-MB-435 cells with or without reduced levels of HOXB7.** Cells with HOXB7 knockdown (HOXB7 siRNA) showed significant differences in chromosomal aberration frequencies per metaphase compared to control cells (Scr.-siRNA). **D. Knockdown of HOXB7 reduces level of DNA DSB repair in MDA-MB-435.** Cells with and without reduced levels of HOXB7 by transfection of HOXB7-specific (HOXB7-siRNA) or scrambled siRNA (Scr.-siRNA) along with parental cells were irradiated with 50 Gy and unrepaired DNA breaks were measured by PGFE. The data represent the mean of three independent experiments.

6. 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. With tnew antibodies, we were able to detect HOXB7 by Western analysis and by immunofluorescence but not by IHC. We have now generated antibodies in chicken since the antigenicity of the human peptide may not be high in rabbits or mice since HOX proteins are so well conserved through evolution. These are still undergoing characterization.

7. HOXB7 as a prognostic marker. HOXB7 mRNA levels were upregulated in breast cancer patients, as detemined by quantitative, real-time PCR . 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. We have since repeated this analysis with an additional set of 10 metastatic tissues from lymph nodes, ovary and liver. These tissues also displayed high levels of HOXB7.

8. A microarray analysis of purified epithelial cells revealed that compared to normal breast epithelial cells, HOXB7 was overexpressed 4-fold higher levels in primary metastatic breast carcinoma cells, and 12 –fold higher levels in bone metastasis.

9) Array CGH analysis of 146 breast cancers revealed that HOXB7 was amplified in breast cancer- 25% at log2ratio of >0.3 and 5% at >0.7. Independently, Hyman et al had published that HOXB7, as analyzed by FISH was systematically amplified in breast cancer cell lines, and in 10% of 363 primary breast cancers by FISH to a tissue microarray and was associated with poor prognosis (p=<0.001). Our efforts in the next few months will be focused on developing a FISH analysis to test amplification of HOXB7 of archival breast cancers.

Conclusions.

The project set out to study the oncogenic potential of HOXB7 and its possible utility as a cancer marker. Unexpected novel functions of HOXB7 were discovered. HOXB7 transformed breast epithelial cells, MCF10A, to grow in minimally supplemented medium, to form colonies in Matrigel, and display resistance to ionizing radiation (IR). Searching for protein partners of HOXB7 that might contribute to resistance to IR, we identified four HOXB7-binding proteins by GST pull down/affinity chromatography and confirmed their interactions by co-immunoprecipitation in vivo. Interestingly, all four HOXB7-binding proteins shared functions as genomic caretakers and included members of the DNA-PK holoenzyme (Ku70, Ku80, DNA-PK_{cs}) responsible for DNA double strand break (DSB) repair by the non-homologous end joining (NHEJ) pathway, and PARP. Exogenous and endogenous expression of HOXB7 enhanced NHEJ and DNA repair functions *in vitro* and *in vivo*, which were reversed by silencing HOXB7. This study provided definitive mechanistic evidence for involvement of any HOX protein in DNA DSB repair.

HOXB7 was shown to be oncogenic to HMECs and also conferred tumorigenic properties to non-tumorigenic cancer cells and metastatic properties to non-metastatic cancer cells. Thus far, we have not been able to show that sera of patients carry HOXB7 detectable by antibodies or that HOXb7 is predictable of recurrent disease.

Reportable outcomes

1. **A role for homeodomain protein, HOXB7, in DNA repair** Ethel Rubin[1], Xinyan Wu[1], Tao Zhu, Hexin Chen, Annaka Lorincz, Raj K. Pandita, Girdhar G. Sharma, Judith Gasson, Tej K. Pandita and Saraswati Sukumar *(submitted revision to EMBO, September, 2006)*

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.

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 Ku70/80 and anchorage independent growth. 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.

2. **HOXB7, a homoedomain protein is overexpressed in breast cancer and confers epithelial-mesenchymal transition.** Xinyan Wu, Hexin Chen, Belinda Parker, Ethel Rubin, Tao Zhu, Ji Shin Lee, Pedram Argani and Saraswati Sukumar.Cancer Res. In press, 2006
HOXB7 mRNA levels were upregulated in breast cancers as compared to normal tissues as shown by quantitative, real-time PCR. Cancer cells overexpressing HOXB7 show properties of greater invasion, metastasis, with activation of Rho/RAC pathway.
Metastatic tissues from lymph nodes, ovary and liver also displayed high levels of HOXB7. A microarray analysis of purified epithelial cells revealed that compared to normal breast epithelial cells, HOXB7 was overexpressed 4-fold higher levels in primary metastatic breast carcinoma cells, and 12 –fold higher levels in bone metastasis. Overexpression could be attributed to gene amplification. Array CGH analysis of 146 breast cancers revealed that HOXB7 was amplified in breast cancer- 25% at log2ratio of >0.3 and 5% at >0.7. HOXB7 expression correlated with epithelial-mesenchymal transition.

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