Targeting the HOX / PBX dimer in breast cancer

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Abstract

Introduction: The *HOX* genes are a family of closely related transcription factors that help to define the identity of cells and tissues during embryonic development and which are also frequently deregulated in a number of malignancies, including breast cancer. Whilst relatively little is known about the roles that individual *HOX* genes play in cancer, it is however clear that these roles can be both contradictory, with some members acting as oncogenes and some as tumor suppressors, and also redundant, with several genes essentially having the same function.

Methods: Here we have attempted to address this complexity by using the HXR9 peptide to target the interaction between HOX proteins and PBX, a second transcription factor that serves as a common co-factor for many HOX proteins.

Results: We show that HXR9 causes apoptosis in a number of breast cancer-derived cell lines and that sensitivity to HXR9 is directly related to the averaged expression of *HOX* genes *HOXB1* through to *HOXB9*, providing a potential biomarker to predict the sensitivity of breast tumors to HXR9 or its derivatives.

Conclusions: Measuring the expression of *HOX* genes *HOXB1* to *HOXB9* in primary tumors revealed that a subset of tumors show highly elevated expression indicating that these might be potentially very sensitive to killing by HXR9. Furthermore, we show that whilst HXR9 blocks the oncogenic activity of *HOX* genes, it does not affect the known tumor-suppressor properties of a subset of *HOX* genes in breast cancer.

Key words: breast cancer; SKBR3; MCF7; HXR9; HOX; PBX

Introduction

The *HOX* genes are a family of homeodomain-containing transcription factors that were first identified as determinates of cell and tissue identity in early development, although they are now also known to function in adult stem cell renewal and differentiation [1-3]. A series of duplication events is thought to have given rise to the four separate clusters of *HOX* genes found in vertebrates, with each cluster consisting of a group of closely linked members that often share enhancer regions. These clusters are named A, B, C and D, and together they contain the 39 *HOX* genes found in mammals[4]. Each gene within a cluster is labeled with a number according to their relative position in the chromosome, so for example *HOXB1* is the 3' most member of the B cluster, and *HOXB13* is the 5' most member[5]. The linkage of genes within each cluster is closely reflected in both their temporal and spatial order of expression in the embryo, with the 3' genes being expressed more anteriorly and earlier than their 5' neighbors. The relative position within the cluster is also reflected in the co-factor interactions, DNA binding specificity and regulation of each member[4].

In addition to a role in development, and subsequently in stem cell differentiation, the *HOX* genes are also frequently deregulated in a number of cancers including melanoma, mesothelioma, and lung, kidney, prostate, ovarian and breast cancer[6]. Their function in oncogenesis is still unclear; however it is apparent that the great complexity of *HOX* function in development is also reflected in oncogenesis, with some *HOX* genes functioning as tumor suppressors and others as oncogenes. The best known examples of

both have been identified in breast cancer, where *HOXA5* is known to function as a tumor suppressor[7], at least in part through activating the transcription of the key tumor suppressor gene *TP53*[8]. Conversely, members of the closely related *HOXB* genes including *HOXB5* and *HOXB7* are oncogenic through mechanisms which include an up regulation of *FGF2*[9], and the promotion of epithelial to mesenchymal transition[10]. *HOXB7* has also been shown to confer tamoxifen resistance through activation of the EGFR pathway[11]. Exactly how such similar transcription factors can have opposing functions is also unclear, although it may be related to differential co-factor binding and consequently differential regulation of target genes. Known co-factors include members of the *PBX*, *MEIS* and *PREP* families of transcription factors all of which can influence the binding selectivity of HOX proteins and their action as either a suppressor or activator of transcription[3]. As an additional complexity, HOX proteins can also regulate transcription through binding to DNA as monomers[12].

Whilst different *HOX* genes can have individual, specific functions in embryonic development, there is generally a high level of functional redundancy, especially with regards to fundamental and highly conserved patterning events such as anterior-posterior pattering of the spine and hindbrain[13, 14]. This is also true in cancer, where a similar oncogenic function is common to a number of HOX genes, especially *HOXB1* through to *HOXB9*[14, 15].

This mixture of opposing functionality and functional redundancy, combined with the lack of ligand binding sites, makes targeting *HOX* genes in cancer potentially very

difficult. One approach however is to target multiple groups of *HOX* genes in a way that also singles out specific *HOX* functions, something that could potentially be achieved by disrupting the binding of HOX proteins to specific co-factors. To date this has only been possible for the PBX co-factor that can bind to HOX proteins numbered 1 to 9[15-19]. PBX increases the nuclear translocation of HOX proteins and also influences the selection of DNA binding sites[20, 21]. Its interaction with HOX is mediated by a highly conserved hexapeptide region on HOX proteins[20-23] and previous studies have shown that a synthetic peptide consisting of these amino acids and a short polyarginine sequence, known as HXR9, is capable of blocking the interaction between HOX and PBX proteins both *in vitro* and *in vivo*. HXR9 causes apoptosis in a number of cancers including melanoma[15], myeloma[18], and kidney[16], non-small cell lung[17], and ovarian cancer[19]. Here we show that HXR9 also causes apoptosis in cell lines derived from different breast cancers, and that HXR9 specifically blocks the oncogenic function of *HOX* genes without blocking the known tumor-suppressor role of *HOXA5*. Importantly, we also show that there is an extremely high correlation between the average expression of *HOX* genes *HOXB1* through *HOXB9* and the sensitivity of cell killing by HXR9.

Materials and Methods

Cell lines and culture

The cell lines used in this study are listed in Supplementary Table 1. They were obtained from the ATCC through LGC Standards Ltd (UK), and were cultured according to the instructions on the LGC Standards website.

Synthesis of HXR9 and CXR9 peptides

HXR9 is an 18 amino acid peptide consisting of the previously identified hexapeptide sequence that can bind to PBX and nine C-terminal arginine residues (R9) that facilitate cell entry[15]. The N-terminal and C-terminal amino bonds are in the D-isomer conformation, which has previously been shown to extend the half life of the peptide to 12 hours in human serum[15]. CXR9 is a control peptide that lacks a functional hexapeptide sequence but which includes the R9 sequence. All peptides were synthesized using conventional column based chemistry and purified to at least 80% (Biosynthesis Inc, USA).

Imaging of cell cultures

Cells were plated in 6-well plates using 2 ml of medium and allowed to recover for at least 24 hours. When approximately 60% confluent, cells were treated with the active peptide HXR9 (60 μM) or the control peptide CXR9 (60 μM) for 3 hours. For phase contrast micrographs, the cells were washed twice with cold PBS and visualized using a

Nikon Eclipse TS100 inverted microscope and images recorded using a Nikon camera and capture software (Jencons).

Analysis of cell death and apoptosis

Cells were treated with HXR9 or CXR9 as described above. Assessment of cell viability was done using the MTS assay (Promega) according to the manufacturer's instructions. Cells were harvested by incubating in trypsin-EDTA (Sigma) at 37ºC until detached and dissociated. Apoptotic cells were identified using flow cytometry (Beckman Coulter Epics XL Flow) and the Annexin V-PE apoptosis detection kit (BD Pharmingen) as described by the manufacturer's protocol. Caspase-3 activity was measured using the EnzCheck Caspase-3 Assay Kit (Molecular Probes), using the protocol defined by the manufacturer.

RNA purification and reverse transcription

Total RNA was isolated from cells using the RNeasy Plus Mini Kit (Qiagen) by following the manufacturer's protocol. The RNA was denatured by heating to 65ºC for 5 minutes. cDNA was synthesized from RNA using the Cloned AMV First Strand Synthesis Kit (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR

Quantitative PCR was done using the Stratagene MX3005P real-time PCR machine and the Brilliant SYBR Green QPCR Master Mix (Stratagene). Oligonucleotide primers were

designed to facilitate the unique amplification of *β-actin*, *c-Fos*, *TP53* and each *HOX* gene.

Transcriptional profiling

Total RNA was extracted from SKBR3 cells treated with CXR9 or HXR9 (60 μM) for three hours, and was used as a template to generate Cy3-labelled cRNA using the Low RNA Input Linear Amplification Kit (Agilent). Each Cy3-cRNA was used as a probe on the Whole Human Genome Microarray (4x44K) slide. This microarray consists of 60-mer oligonucleotides with sequences representing more than 41,000 human genes. The microarray slides were scanned and data were extracted using the Agilent Feature Extraction Software (version 9.5.3). Data was analyzed using GeneSpring GX software. The full data set and further experimental details have been deposited in the Array Express repository; accession number E-MEXP-3634.

Mice and in vivo trial

All animal experiments were conducted in accordance with the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the Welfare of Animals in Experimental Neoplasia[24] and were approved by the University of Surrey Research Ethics Committee. The mice were kept in positive pressure isolators in 12 hour light / dark cycles and food and water were available *ad libitum*.

Athymic nude mice were inoculated subcutaneously with a suspension of 2.5 x 10^6 MDA-MB231 cells in culture media (100 μl). Once tumors reached volumes of approximately 100 mm^3, mice received an initial dose of 100 mg/Kg CXR9 or HXR9

intratumorly, with subsequent dosing when or if the tumor reoccurred. Each treatment group contained 10 mice. The mice were monitored carefully for signs of distress, including behavioral changes and weight loss.

Patient samples

Patient breast tissue samples and clinico-pathological data were obtained from the Guy's and St Thomas' (GST) Breast Tissue and Data Bank, London, UK (REC ref: 07/H0804/131).This collection has approval from the GST Research Ethics Committee (ref: 07/H0804/131) and adheres to the Helsinki Declaration. Patients had given consent for the inclusion of their tissue in this bank; individual permission from each patient was not subsequently required for the particular study described here. Frozen sections were cut and using a stained guide slide, malignant cells were dissected from the surrounding tissue. RNA was extracted using the AllPrep DNA/RNA Micro kit (Qiagen). The manufacturer instructions were followed with the exception of an enhanced homogenization step.

Results

HOX *gene expression in breast cancer cell lines and normal breast tissue*

In order to assess the expression of *HOX* genes in breast cancer and in normal breast tissue five breast cancer-derived cell lines were used, SKBR3, MB231, MCF7, ZR75.1 and UACC, together with MCF10a, derived from a non-malignant mammary epithelium (detailed in Supplementary Table 1). RNA was extracted from cultures of each of these, together with normal breast tissue. *HOX* expression was determined by semi-quantitative PCR, and calculated as a ratio with the expression of the house keeping gene GAPDH (Fig 1). This reveals a significant, but variable degree of *HOX* deregulation between breast cancers derived cell lines and normal breast tissue, with normal breast tissue and the non-malignant cell line MCF10a generally having lower expression than the cancer derived cell lines.

HXR9 is toxic to breast cancer cells

Previous studies have indicated that HXR9 is taken up by and is selectively cytotoxic to cancer cells. A fluorescently labeled derivative of HXR9 was incubated with MDA-MB231 cells and localized to both the cytoplasm and the nucleus (Fig 2a). In order to determine the IC50 for cell killing by HXR9 we used an MTS assay at varying concentrations of the peptide. This indicated that the IC50 for cell killing for SKBR3, MDA-MB231, MCF7, ZR75.1, UACC and MCF10a were 16μM, 23μM, 33μM, 42μM, 48μM and 51μM, respectively. Thus MCF10a and the breast cancer derived cell lines UACC, ZR75.1 and MCF7 are relatively insensitive to HXR9 whilst MDA-MB231 and

SKBR3 are significantly more sensitive ($p<0.01$; Fig 2b). As a control a second peptide was used, CXR9, which has an identical polyarginine cell penetrating sequence to HXR9 but which lacks an active hexapeptide sequence. None of the cell lines were sensitive to CXR9 (i.e. IC50 > 100μM).

Treatment with HXR9 increases the expression of cFos *but not* TP53

Previous studies have shown that *HOX* genes can prevent apoptosis, at least in part, by blocking the expression of *cFos*, and that HXR9 can induce apoptosis by mediating a rapid increase in the number of *cFos* transcripts[15]. SKBR3 and M cells similarly show a very large increase in *cFos* expression (553 fold) two hours after HXR9 treatment (Fig 3). Conversely, some HOX genes have been identified as potential tumor suppressors, most notably *HOXA5* in breast cancer through its regulation of *TP53*[8]. The detailed mechanism for this regulation is yet to be elucidated, but if it requires a HOXA5 / PBX dimer then it might be expected that HXR9 could reduce *TP53* expression and therefore be pro-oncogenic in this respect. In order to determine whether this is the case we assayed *TP53* expression in SKBR3 and MDA-MB231 cells treated with HXR9 by semi-quantitative PCR. This revealed that there is no significant change in *TP53* expression in response to HXR9 treatment in either cell line (Fig 3).

HXR9 primarily causes transcriptional activation

In order to identify additional target genes that are regulated by a HOX/PBX dimer, and are thus differentially expressed upon treatment with HXR9, we used whole genome based microarray to study the transcriptome of HXR9 treated SKBR3 cells. This revealed

that the majority of HXR9 target genes identified are upregulated by HXR9 treatment (Fig S1), indicating that the HOX / PBX dimers act predominantly to repress transcription in these cells. The promoter regions of the 20 genes that are most strongly upregulated by HXR9 were analyzed for the presence of the HOX / PBX binding consensus[25]. Of these 20 genes 18 had at least one potential site, and many of the promoter regions had multiple and often overlapping consensus binding sites (Fig S2), including *ATF3, NR4A3, ZFP36* and *PPP1R15A*, that have tumor suppressor functions (Supplementary Table 2). Furthermore, amongst those genes that were upregulated by ten fold or more, 22 have known tumor suppressor function (Supplementary Table 2).

HXR9 induces apoptosis

The mechanism of cell death was studied in the most sensitive of six cell lines, SKBR3. Previous studies have shown that HXR9 can induce apoptosis through, at least in part, a rapid increase in expression of *cFos*[15]. SKBR3 and MDA-MB231 cells similarly show a very large increases in *cFos* expression (553 and 19.3 fold, respectively) two hours after HXR9 treatment (Fig 3). In order to establish whether HXR9 induces apoptosis, HXR9 treated cells were analyzed by FACS after staining with Annexin-7AAD and propidium iodide. The former allows the detection of changes in the cell membrane that are characteristic of apoptosis, whilst the later is used to evaluate membrane integrity (Fig 4a). This shows that HXR9 treated SKBR3 undergo cell death predominantly through apoptosis, with the majority of cells being in an early stage of apoptosis two hours after HXR9 treatment (Fig 4b). There is also a corresponding increase in Caspase 3 activity

over two hours with the same concentration of HXR9 (Fig 4c). Similar results were obtained for the MDA-MB231 cell line (not shown).

Sensitivity to HXR9 correlates with HOX expression

As HXR9 targets the HOX / PBX interaction, we calculated the average expression of all *HOX* genes numbered between 1 and 9 (i.e. the 3' most 27 members of the *HOX* family that bind PBX), for all of the cell lines and for normal breast tissue. When ranked against sensitivity to HXR9 there is an apparent positive relationship between mean *HOX* expression and IC50 with the exception of MCF7 (Fig 5a). However, when cell line sensitivity is ranked against mean expression of only the *HOX* genes *HOXB1* through to *HOXB9* the ranked order is complete (Fig 5b). When mean expression of *HOXB1* through *HOXB9* is plotted against the IC50 for HXR9 toxicity a linear relationship is apparent, the line of regression for this putative relationship has an r^2 value of 0.9778 ($p=0.0002$), (Fig 5c).

HOX genes HOXB1 through to HOXB9 are highly expressed in a subset of primary breast tumors

In order to explore the possible relevance of these findings to primary tumors we measured the transcripts of *HOX* genes *HOXB1* through to *HOXB9* in 78 primary tumor samples obtained from the GST Breast Tissue and Data Bank, the associated pathological characteristics of which are listed in Supplementary Table 3. This revealed that whilst the majority of tumors expressed these genes to a relatively low level, a subset (7.4%) showed a highly elevated level of expression that could indicate that these cells would be

sensitive to killing by HXR9 (i.e. with an IC50 <1μM, Fig 5d). High levels for *HOXB1* through to *HOXB9* expression did not correlate with expression of the estrogen receptor (ER), progesterone receptor (PR), or HER2, nor with survival, tumor grade, or spread to axillary lymph nodes (Fig S3). There was however a positive association between low *HOXB1* through to *HOXB9* expression and mucoid or lobular histology (Fig S3).

HXR9 retards tumor growth in vivo

In order to assess the efficacy of HXR9 *in vivo* we established a xenograft model of MDA-MB231. Although these cells are less sensitive to killing by HXR9 than SKBR3, it is more widely used as a xenograft model (605 references in PubMed compared to 35 for SKBR3), and represents a form of breast cancer with a poor prognosis, having been derived from a tumor negative for HER2, ER and PR[26]. Tumors were initiated by injection of cells into the flank, and treatment was started when the average tumor volume had reached 100 mm^3 with an initial dose of HXR9 of 20 mg/Kg intratumorally, followed by an additional dose if tumor growth reoccurred (with no more than one additional dose per animal). Tumor growth in HXR9 treated mice was retarded 7.5 fold at 13 days compared to the control group (Fig 6a), and the HXR9 treated animals survived for significantly longer (88% survival at 30 days as compared to 0% for the control group, p=0.004; Fig 6b) RNA was extracted from tumors at the end of 32 days in order to measure *cFos* expression by QPCR; *cFos* was found to be expressed at an 18-fold higher level in tumors from HXR9 treated mice than in tumors from untreated mice (Fig 6c). Histological analysis of CXR9 and HXR9 treated tumors revealed extensive cell death in the later (Fig 6d).

Discussion

In this study we have shown that *HOX* genes are generally deregulated in breast cancer, and mostly there is an increase in expression when compared to normal breast tissue or non-malignant MCF10a cells, although the exact pattern of expression varies between cell lines. The significance of the variation between cell lines is unclear, although the functional redundancy between *HOX* genes may mean that the net effect of *HOX* over expression is similar. There are however cases where cancer phenotype can be determined by specific *HOX* genes, for example in ovarian cancer *HOXA11* expression confers a mucinous (as opposed to serous) phenotype[27], and distinct patterns of *HOX* expression are associated with lymphoblastoid myeloma[18].

We also show that treating breast cancer cell lines with the HOX / PBX inhibitor HXR9 causes apoptosis in SKBR3 and is cytotoxic to all of the lines tested. This suggests, as it has done in studies on other cancers, that targeting the HOX / PBX dimer allows a specific subset of *HOX* functions to be modified, one of which includes the repression of apoptosis[15-19]. In the case of melanoma, apoptosis is induced in part through the induction of *cFos*[15], and a large increase in *cFos* expression is also observed in this study when SKBR3 cells are treated with HXR9. Selective targeting of anti-apoptotic functions is important as some *HOX* genes, notably *HOXA5* in breast cancer[8], have a tumor-suppressor role rather than an oncogenic role. This is mediated by an increase in *TP53* expression[8], however we show here that HXR9 does not change *TP53* expression in breast cancer cells, suggesting that the regulation of *TP53* by *HOXA5* does not depend

upon a HOXA5 / PBX dimer, but may instead be dependant on other co-factors, or may possibly not require a co-factor. The later possibility is supported by the observation that forced expression of *HOXA5* alone is sufficient to drive *TP53* transcription, and that the HOXA5 binding site identified in the *TP53* promoter does not appear to include a PBX binding consensus[8]. Further, whilst confirming a positive interaction between *HOXA5* and *TP53*, a more recent study also suggests that *TP53* is not transcriptionally regulated by *HOXA5*[28]. These findings support the conclusions of previous studies that indicate the HOX / PBX dimers are predominantly anti-apoptotic in the context of cancer[15-19].

An oncogenic role for HOX / PBX dimers is also supported by the transcriptional repression of genes with known tumor suppressor functions, all of which exhibit a correspondingly large increase in expression upon HXR9 treatment (Table 2). These include *EGR2*[29] and *ATF3*[30], both of which function in p53 mediated apoptosis, the GTPase encoding gene *RRAD* which is frequently lost in malignancy[31], and the EGFR / ERBB2 inhibitor *ERRFI1*[32]. These findings are of particular interest because they suggest potential synergistic interactions between HXR9 and other classes of drug.

Identifying patients that are likely to respond to a particular treatment is a now a key requirement in clinical trials, and the development of predictive biomarkers for this purpose is most effective at early drug development stages. We hypothesized that sensitivity to HXR9 in breast cancer lines should depend, at least to some extent, on *HOX* gene expression. Given the functional redundancy found in the PBX binding HOX proteins, we examined whether sensitivity might depend on the averaged level of *HOX*

gene expression. In fact, our results suggest that this is not the case. However, the averaged expression level of a subset of *HOX* genes, namely *HOXB1* through *HOXB9*, does correlate with sensitivity to HXR9 with a highly significant line of regression. High levels of *HOXB1* through *HOXB9* expression are associated with increased sensitivity to killing by HXR9, whilst low levels of expression are associated with insensitivity to HXR9. Here we show that both normal breast tissue and the non-malignant mammary line MCF10a have relatively low levels of expression, and the later is also relatively resistant to HXR9 mediated cell killing.

Conclusions

The significance of these finding requires further study, although we note that *HOXB* genes have in general been more frequently implicated as having a role in cancer, both through direct mechanistic studies[6] and through a number of clinical observations including the association of elevated *HOXB7* expression with a poor prognosis in HER2 positive breast cancer[33]. Our analysis of 78 primary breast tumors showed that whilst the majority had relatively low levels of *HOXB1* through to *HOXB9* expression, a subset had very highly elevated expression of these genes, indicating that they could potentially be extremely sensitive to HXR9. Determining the expression of these genes from biopsies could form the basis by which patients might be selected for treatment by HXR9, or its derivative.

All authors declare no financial or non-financial competing interests.

Author contributions:

RM -Project lead, coordinated research project and planned experiments, drafted manuscript. **AB-**Conducted in vitro assays. **KH-**Provided scientific criticism of project and manuscript, advised on statistics. **GS**-Conducted in vivo modeling. **CG**-Patient tissue procurement and analysis of results**, AM**-Provided scientific criticism of project and manuscript, advised on statistics. **HP-**Experimental and statistical design and critical analysis**. All authors read and approved the final manuscript.**

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Abbreviations

ER – Estrogen Receptor

UKCCCR - United Kingdom Co-ordinating Committee on Cancer Research

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

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Figure Legends

Figure 1. HOX gene expression in breast cancer derived cell lines and in normal breast tissue. The expression of each gene was determined by semi-quantitative PCR and is shown relative to the house keeping gene GAPDH (x10000). The values shown are the mean of three independent experiments and the error bars represent the SEM. NBT – normal breast tissue.

Figure 2. (a) **HXR9 enters the cytoplasm and nuclei of MDA-MB231 cells *in vitro***. MDA-MB231 cells were incubated with 22μM FITC labeled HXR9 (green) for two hours and then stained with DAPI (a fluorescent dye staining nuclei blue). Scale bar: 5μm (b) **IC50 values for HXR9 treatment**. The negative control peptide CXR9 was not toxic at any of the concentrations tested for any of the cell lines (i.e. the IC50 > 100μM). Error bars represent the SEM (n=3), the p values are shown where $p<0.01$ with respect to MCF10a.

Figure 3. Semi-quantitative PCR of *TP53* and *cFos* in HXR9 treated SKBR3 (a) and MDA-MB231 (b) cells. RNA was extracted from cells cultured in *vitro* and treated with 60μM peptide for two hours. Results are expressed as a ratio with the housekeeping gene GAPDH and are the mean of 3 independent experiments, error bars show the SEM. *** $p<0.001$ with respect to untreated cells

Figure 4. HXR9 induces apoptosis in SKRB3 cells. SKBR3 cells were treated with 60μM HXR9 or CXR9 for two hours and cells were assessed for apoptosis or necrosis through Annexin / Propidium iodide staining. (a) Example plots for each treatment. (b) The % of cells in early apoptosis (EA), late apoptosis (LA), necrosis (N), or which are still viable (V) is shown. (c) Caspase 3 activity in SKBR3 cells treated with 60μM HXR9 or CXR9 for two hours. Cells lysates were treated with a Caspase inhibitor (Ac-DEVD-CHO) to establish background signal ('-AcDEVD', '+AcDEVD', lysates untreated / treated with inhibitor). Error bars show the SEM. *$p<0.05$, **$p<0.01$ with respect to untreated cells

Figure 5. The IC50s for HXR9-treated breast cancer derived cell lines correlate with the mean expression of *HOX* genes *HOXB1* through to *HOXB9*. (a) The mean expression of all *HOX* genes or (b) of *HOX* genes *HOXB1* to *HOXB9* were calculated and are shown for each cell line. The cell lines are arranged in order of decreasing sensitivity to HXR9 and the IC50 for each cell line is shown below the chart. Data are the mean of three independent experiments, error bars show the SEM. * $p<0.0073$ (applying Bonferonni's correction for multiple hypothesis testing), as compared to SKBR3. P values are not shown where $p>0.1$. NBT – normal breast tissue. (c) Linear regression of cell line IC50s and mean *HOX* expression. The IC50 values for cell killing by HXR9 were plotted against the mean expression level of *HOX* genes *HOXB1* through to *HOXB9* for each of the cell lines tested. A linear regression (solid line) gives an r^2 value of 0.9778. The probability for the null hypothesis that the slope of the line is actually zero was calculated as to be $p = 0.0002$. NBT – normal breast tissue. (d) The distribution of

mean *HOXB1* through to *HOXB9* expression in primary tumors. The expression of these genes in 78 primary tumors (red) were plotted according to their theoretical sensitivity to HXR9, as determined using the linear regression described above. The regression line shown in part (c) is included for reference.

Figure 6. HXR9 retards MDA-MB231 tumor growth *in vivo*. (a) Growth curve for MDA-MB231 tumors treated intratumorally with a single dose of HXR9 or CXR9 when the tumor volume reached 100mm^3. Error bars show the SEM. (b) Survival plot for HXR9 and CXR9 treated tumors shown in (a). (c) Expression of cFos in tumors treated with HXR9 or CXR9, shown as a ratio between cFos and GAPDH transcripts detected by QPCR. Error bars show the SEM. (d) Section through MDA-MB231 tumors in mice treated with CXR9 or HXR9. The CXR9 treated section shows highly undifferentiated tumor cells, whilst the HXR9 section shows the remains of dead tumor cells (T) surrounded by stroma (S). Scale bar: 20μm.

Supplementary figure 1. Changes in expression of HXR9 target genes in SKBR3 cells. The relative expression of target genes are shown as a normalized intensity value (Log10), blue lines represent targets that increase expression in response to HXR9 compared to untreated cells [Unt], whilst the red lines represent targets that are repressed by HXR9.

Supplementary figure 2. Distribution of HOX/PBX consensus sites in the promoter regions of the 20 most responsive HXR9 target genes. Possible HOX / PBX consensus

binding sites are shown shaded according to the number of nucleotides that match the consensus (out of a maximum of 10). *Multiple consensus sites that overlap each other.

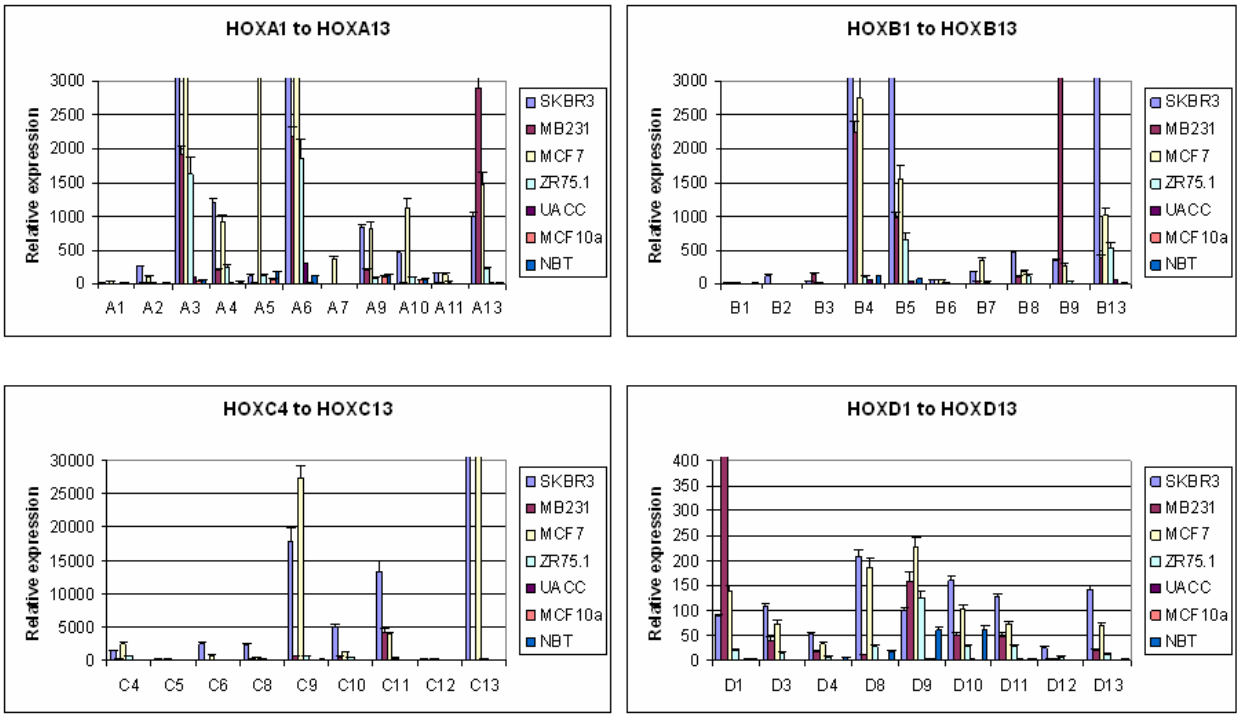
Supplementary figure 3. The mean expression values of *HOX* genes *HOXB1* through to *HOXB9* in primary tumors grouped according to (a) histopathological type, (b) estrogen receptor (ER) status, (c) spread to nodes as determined by pathology, (d) 5 year survival, (e) grade (G2, grade 2; G3, grade 3), (e) whether tumors were from the left or right breast, (g) progesterone receptor status (PR), and HER2 status. Error bars show the SEM.

Supplementary table 1. Summary of breast cancer derived cell lines used in this study.

Supplementary table 2. Genes with potential tumor suppressor functions that are upregulated by HXR9.

Supplementary table 3. Summary of pathological data for the primary tumors analyzed in this study.

Figure 1


HOXA1 to HOXA13
Relative expression
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2500
2000
1500
1000
500
0
A1 A2 A3 A4 A5 A6 A7 A9 A10 A11 A13
SKBR3
MB231
MCF7
ZR75.1
UACC
MCF10a
NBT
HOXB1 to HOXB13
Relative expression
3000
2500
2000
1500
1000
500
0
B1 B2 B3 B4 B5 B6 B7 B8 B9 B13
SKBR3
MB231
MCF7
ZR75.1
UACC
MCF10a
NBT
HOXC4 to HOXC13
Relative expression
30000
25000
20000
15000
10000
5000
0
C4 C5 C6 C8 C9 C10 C11 C12 C13
SKBR3
MB231
MCF7
ZR75.1
UACC
MCF10a
NBT
HOXD1 to HOXD13
Relative expression
400
350
300
250
200
150
100
50
0
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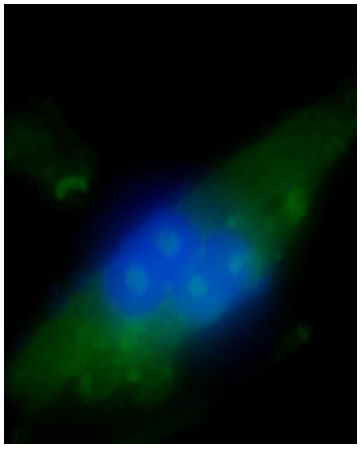
SKBR3
MB231
MCF7
ZR75.1
UACC
MCF10a
NBT

Figure 2



b

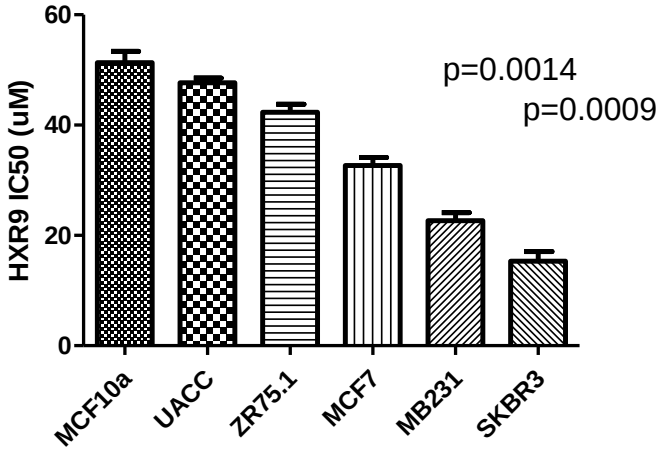
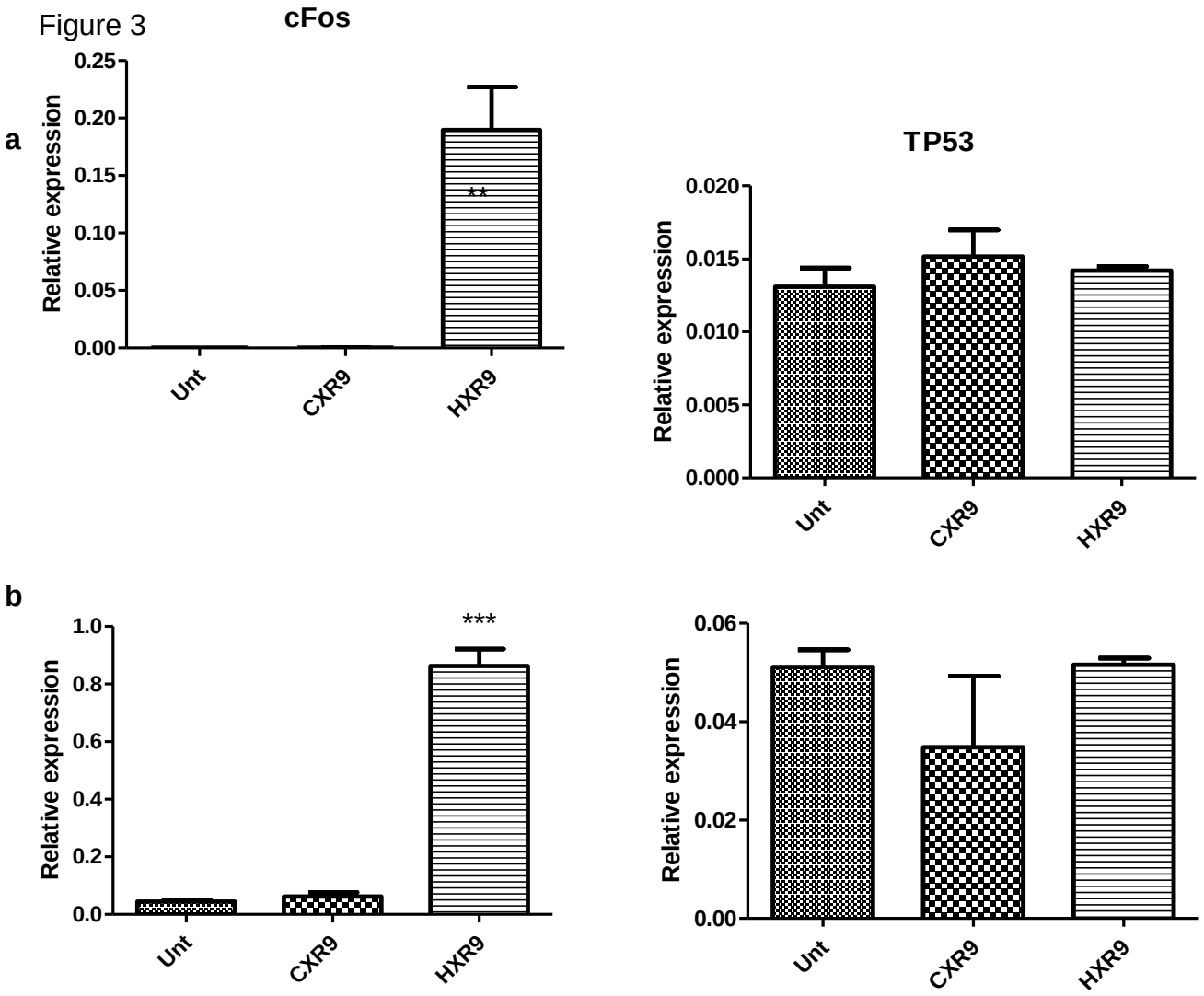

60
40
20
0
HXR9 IC50 (uM)
p=0.0014
p=0.0009
MCF10a
UACC
ZR75.1
MCF7
MB231
SKBR3

Figure 3


a
cFos
Relative expression
0.25
0.20
0.15
0.10
0.05
0.00
**
Unt
CXR9
HXR9
TP53
Relative expression
0.020
0.015
0.010
0.005
0.000
Unt
CXR9
HXR9
b
Relative expression
1.0
0.8
0.6
0.4
0.2
0.0
***
Unt
CXR9
HXR9
Relative expression
0.06
0.04
0.02
0.00
Unt
CXR9
HXR9