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## HOX genes: Major actors in resistance to selective endocrine response modifiers

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### Abstract

Long term treatment with therapies aimed at blocking the estrogen- (ER) or androgen receptor (AR) action often leads to the development of resistance to selective modulators of the estrogen receptor (SERMs) in ER $\alpha$ -positive breast cancer, or of the androgen receptor (SARMs) in AR-positive prostate cancer. Many underlying molecular events that confer resistance are known, but a unifying theme is yet to be revealed. Receptor tyrosine kinases (RTKs) such as EGFR, ERBB2 and IGF1R are major mediators that can directly alter cellular response to the SERM, tamoxifen, but the mechanisms underlying increased expression of RTKs are not clear. A number of HOX genes and microRNAs and non-coding RNAs residing in the HOX cluster, have been identified as important independent predictors of endocrine resistant breast cancer. Recently, convincing evidence has accumulated that several members belonging to the four different HOX clusters contribute to endocrine therapy resistant breast cancer, but the mechanisms remain obscure. In this article, we have reviewed recent progress in understanding of the functioning of HOX genes and regulation of their expression by hormones. We also discuss, in particular, the contributions of several members of the HOX gene family to endocrine resistant breast cancer.

### 1. Introduction

Breast cancer is the second most common cancer among women in the United States, affecting one out of eight females during their lifetime. In 2015, an estimated 231,840 new cases of invasive breast cancer were diagnosed, and during this time, almost 40,290 died from this disease [1]. At least 70% of breast cancers are identified as ER-positive in that their growth is dependent on the continued presence of estrogen. Despite the relative safety and significant therapeutic effects of endocrine therapy such as tamoxifen and aromatase

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inhibitors, about thirty percent of breast tumors develop resistance and eventually recur as distant metastasis. For decades, extensive studies have identified multiple mechanisms including activation of EGFR, HER2, ER $\alpha$  and IGF1R signaling pathways by which breast cancers mediate endocrine resistance [2]. However, which critical pathway should be targeted in order to overcome endocrine resistance in breast cancer is not clear.

The HOX proteins contain a homeodomain that is a highly conserved 61 amino acid helix-turn-helix motif. The homeodomain recognizes and binds to the consensus core DNA sequence 5'-TAAT-3' in the proximal promoter or enhancer regions of its target genes, along with cofactors such PBX1, PBX2, MEIS1, PBC and TALE, to promote transcription [3–5]. First found in the fruit fly, *Drosophila melanogaster*, in two clusters consisting of the bithorax and antennapedia complexes, HOX genes are intimately involved in spatial patterning of the body during development [6]. In human, there are 39 HOX genes distributed in 4 paralogous clusters named A, B, C, and D which are located on chromosomes 7p15, 17q21.2, 12q13, and 2q31 respectively, and are named according to their 3' to 5' order of alignment. The number of HOX genes in each cluster is not uniform and varies from 9 to 11 genes in mammals [7].

HOX genes are transcription factors involved in the specification of segmental pathways during embryonic development [8]. Belonging to one of the largest superfamily of homeotic genes, members of the HOX gene family are primarily responsible for maintaining cell pluripotency, determining cell fate and promoting differentiation in multicellular organisms [9, 10]. Continued expression of some of the members of the HOX clusters into adulthood suggests a functional requirement of HOX proteins in maintaining cellular homeostasis [11]. Aberrant changes in HOX clusters including DNA hypermethylation and altered gene expression are frequently associated with cancer development [12]. In cancer, HOX genes have been reported to regulate cell cycle and apoptosis [13]. A number of strong pieces of evidence suggest that HOX genes play a critical role in the development of resistance against therapy in cancers. In this review, we will discuss studies on the HOX clusters including HOX genes, long non-coding RNA and microRNAs along with their functional characterization, clinical implications of their dysregulation, and regulation of gene expression in endocrine resistant breast cancer.

## 2. Germline mutations in HOX genes

Germline mutations in HOX genes manifest themselves as abnormal human phenotypes at birth or during sexual development (Table 1) [14–31]. Germline missense mutations are found in HOXB13 (G84E) in men with early-onset or with a positive history of prostate cancer (up to 3.1%) and is linked less frequently with familial breast cancer (0.7%) [25]. In sporadic prostate [32] and breast cancer [33], HOXB13 overexpression predicts poor prognosis [34]. In breast cancer cells, HOXB13 expression is regulated by estrogen, and its overexpression is an important marker of tamoxifen-resistant ER+ breast cancer [35]. In prostate cancer, HOXB13 interacts directly with AR, thus regulating AR-target gene expression [36]. These results imply that germ line mutations of HOXB13 are also likely associated with endocrine regulation. In addition, HOX genes play an important role in homeostasis in adult tissues. Several HOX genes are responsible for endometrial receptivity,

pregnancy and hematopoiesis [37]. As transcription factors, HOX genes function to regulate crucial processes in embryonic development as well as cell differentiation, proliferation and motility in adult [10].

### 3. Regulation of HOX gene expression

#### 3.1. Retinoic acid

Retinoic acid (RA) plays a critical role in embryogenesis. When RA binds to RA receptors (RARs), a member of the ligand activated nuclear receptor family, the RAR homo-, or heterodimeric complex with rexinoid receptors (RXR), is recruited to the proximal promoter regions at HOXA1, HOXB1 and HOXD4 genes to enhance their transcription [38–42]. The RA signaling pathways are involved in various types of cancer and thus the RARs and their downstream genes are potential therapeutic targets due to their functions in differentiation, proliferation, apoptosis, and oxidation [43]. In 2007, Chen et al. reported that RA can induce HOXA5 expression in RAR $\beta$ -positive breast cancer cells [44]. RAR $\beta$  binds directly to the RA response element (RARE) present in the 3' region of *HOXA5* gene. In addition, we observed a decrease in both HOXA5 and RAR $\beta$  expression during neoplastic transformation and progression. Knockdown of HOXA5 partially abrogates retinoid-induced apoptosis and promotes cell survival upon RA treatment, suggesting that HOXA5 acts directly downstream of RAR $\beta$  and may contribute to its differentiation functions [44].

#### 3.2. Vitamin D, Testosterone, and Progesterone

Vitamin D and its receptor, VDR (Vitamin D receptor) regulate HOXA10 and HOXA11 expression in both hematopoietic and endometrial cells [45]. Also, testosterone binds to the androgen receptor causing a decrease in HOXA10 and HOXA11 mRNA expression in an endometrial adenocarcinoma cell line [46]. The progesterone receptor (PR), by binding to its cognate ligand, progesterone, co-ordinates morphogenesis of the mammary gland. Progesterone upregulates HOXA10 and HOXA11 expression in myocytes, in endometrium and in primary endometrial stromal cells [47–49]. In breast cells, on the other hand, HOXA5 directly activates PR gene expression and function through binding to a single HOX-binding site in its promoter region; this is not the case for HOXB4, HOXB5, or HOXB7 [50].

#### 3.3. Estrogen

Estrogens are required for embryonic development of the female reproductive tract as well as functional differentiation of the adult endometrium. Several papers have shown that the HOXA genes are regulated by estrogen. In the endometrium, HOXA10 expression is significantly increased in a menstrual cycle stage-dependent manner. HOXA10 mRNA levels is not altered upon pretreatment with cycloheximide, which supported the notion that estrogen bound ER (estrogen receptor) binds to ERE (ER binding element) at the HOXA10 promoter to directly enhance HOXA10 transcription [51, 52]. In addition, the level of HOXA10 expression is dramatically increased in MCF-7 cells treated with estradiol and tamoxifen as determined by semi-quantitative RT-PCR and northern blot analysis [53]. In endometrial cells, HOXA10 expression is regulated by estrogen and tamoxifen [54]. HOXA1 is also an estrogen-responsive gene [55]. The authors show that ACK1, a ubiquitously expressed non-receptor tyrosine kinase, interacts with ER and the ER co-

activator, KDM3A, a H3K9 demethylase, by phosphorylation in presence of tamoxifen. The complex of activated ACK1, ER and KDM3A binds to the HOXA1 promoter regions directly and regulates HOXA1 transcriptional activity. These results suggest that the ACK1-KDM3A-HOXA1 signaling axis might be a new target in acquired tamoxifen-resistant breast cancer [55]. Another HOXA protein, HOXA7, influences ER expression in MCF-7 cells. Knockdown of HOXA7 decreases cell proliferation and ER downregulation [56]. In ER+ MCF-7 cells, in contrast to ER-negative cells, the expression level of HOXB7 and HOXB13 shows a dramatic decrease upon treatment of the cells with estrogen. However, this effect is abrogated when MCF-7 cells are treated with tamoxifen [57, 58]. HOXC10 is upregulated by estrogen, through interaction with several ERE elements in its promoter region, but is downregulated by promoter methylation when the breast cancer cells acquire aromatase inhibitor resistance [59]. In osteoblast-like cell lines, the expression of HOXD11 is increased by exemestane, an aromatase inhibitor (AI) [60]. Since the expression of many HOX genes is altered by estrogen and SERMs, HOX genes have been implicated as significant players in endocrine-resistant breast cancer.

## 4. HOX genes in endocrine resistant breast cancer

### 4.1 HOX genes

It is well established that, at diagnosis, overexpression of HOXB13 is a strong marker of tamoxifen resistant breast cancer. By analysis of gene expression profiles in ER+ primary breast cancer in patients treated with adjuvant tamoxifen monotherapy, the ratio of HOXB13 to interleukin-17B receptor (IL17BR) was identified as an important independent predictor of outcome [35]. A high ratio of HOXB13/IL17BR (H:I) is associated with increased relapse, tumor aggressiveness, tamoxifen treatment failure, and death in breast cancer patients [61]. Breast cancers in tamoxifen resistant patients is also found to exhibit high HOXB13 expression, where HOXB13 overexpression is shown to be repressed by estrogen and this repression is lifted by tamoxifen. Refining this signature further, they developed the Breast Cancer Index or BCI by combining the H:I ratio with molecular grade index or MGI. MGI utilizes five other tamoxifen resistance biomarkers [62], shown to predict long-term recurrence risk after 5 years to not only tamoxifen, but also to aromatase inhibitor treatment, making this biomarker relevant to the clinical management of breast cancer [63]. For nearly a decade, HOXB13 has been recognized as an important biomarker of tamoxifen resistance, but its mode of action remained unclear. Our recent studies provides some insights into the mechanism whereby HOXB13 mediates tamoxifen resistance and metastasis. We showed that HOXB13 confers tamoxifen resistance via repression of ER expression, as well as induction of IL6 expression [34]. Using anti-IL6-antibodies [34] or Tocilizumab (an anti-IL6R antibody) (our unpublished data) combined with tamoxifen, we found that the inhibition of IL-6 signaling pathway reduces tumor growth of high HOXB13 expressing breast cancer cells. Thus, the IL-6 signaling pathway may be a novel therapeutic target in HOXB13-overexpressing tamoxifen resistant breast cancer.

Among the HOX genes, HOXB7 has emerged as a new master regulator of tamoxifen resistance. In preclinical models, HOXB7 expression promotes the growth of ER+ breast tumors. Interestingly, even in the absence of exogenous estrogen supplementation, HOXB7

overexpressing tumors grow rapidly in athymic nude mice. We showed that HOXB7 overexpression renders breast cancer cells resistant to tamoxifen through activation of several receptor tyrosine kinase pathways, including the EGFR pathway [58]. Recently, we investigated other pathways that may be involved, and the interplay between them. HOXB7 enhances ER target gene expression via the ER–HOXB7 complex directly bound to the promoter regions of key ER target genes such as *CA12*, *MYC* and *HER2*. What is the consequence of overexpression of HER2 in tamoxifen-resistant breast cancer? Studying the upstream regulation of HOXB7, we discovered the HOXB7-regulatory function of miR-196a, which, in turn, is transcriptionally regulated by c-MYC. High c-MYC, stabilized by activated HER2, downregulates miR-196a, which results in upregulation of HOXB7 in tamoxifen-resistant breast cancer cells. The identification of the cooperative role of HOXB7 and ER in controlling the expression of a myriad of genes, including HER2, the phosphorylation mediated activation of c-MYC by HER2 leading to downregulation of miR-196a, and its effects on HOXB7 expression revealed the existence of the HER2-MYC-miR-196a axis and its function. With the uncovering of this axis also came the identification of multiple, yet unrecognized ER/HOXB7 targets that are capable of attenuating tamoxifen resistance in breast cancer [64].

The role for epigenetic silencing of HOX genes was long suspected due to the frequent presence of dense CpG islands in their promoter regions. Using a genome-wide methylation screen it was reported that HOXC10 expression is repressed by promoter hypermethylation in two independent long-term estrogen-deprived cell lines, C4-12 and LTED [65]. Downregulation of HOXC10 by estrogen supplementation enhances breast tumor growth. On the contrary, upregulation of HOXC10 expression by aromatase inhibitors that suppressed estrogen production initiates apoptosis and subsequently reduces tumor growth. Thus, epigenetic silencing of HOXC10 in tumor cells results in continued growth and eventually to acquired aromatase inhibitor resistance [65]. In addition, it was demonstrated that HOXC11 is involved in endocrine resistance by interacting with SRC-1 as ER coactivator [66]. While the frequent presence of CpG island and promoter methylation may be one mechanism of epigenetic control of expression of HOX genes, multiple other factors play a role in the regulation of their expression (Figure 1).

#### 4.2. HOX cluster non-coding RNA (ncRNA)

The HOX gene clusters harbor five different microRNAs: miR-10a, miR-10b, miR-196a1, miR-196a2 and miR-196b. The miR-10a gene is located between HOXB4 and HOXB5 [67]. Using a global microRNA screen (1,105 human miRNAs) of primary tumors of patients with ER-positive breast cancer treated with tamoxifen, who were either recurrence-free or had suffered a recurrence, miR-10a was found as one of top 20 deregulated miRNAs. It is also a major predictor of ER-positive tumor relapse in early postmenopausal breast cancer patients who are treated with tamoxifen [68]. In addition, miR-10b gene, located upstream of HOXD4, is a well-known enhancer of breast cancer invasion and metastasis [69]. Recently it was shown that in MCF-7 and T47D cells, overexpression of miR-10b renders cells tamoxifen resistant by reduction of HDAC4 expression, while depletion of miR-10b enhances sensitivity to tamoxifen in MCF-7 tamoxifen-resistant cells [70]. Thus, the

miR-10b-HDAC4 axis is a potential therapeutic target in tamoxifen resistant breast cancer [70].

The HOXB7 downregulator, miR-196a, is located upstream of HOXB7, and is repressed by MYC protein, which is stabilized by phosphorylation by the highly activated HER2 signaling pathway in tamoxifen resistant MCF-7 cells. Predictably, overexpression of miR-196a sensitizes MCF-7-TMR cells to tamoxifen by downregulation of HOXB7. In order to overcome tamoxifen resistance induced by miR-196a, both HER2 and MYC signaling pathway could be potentially exploited through novel targeted therapeutic approaches [64].

The long non-coding RNA, HOTAIR, which is located upstream of HOXC11, is well known to promote breast tumorigenesis and metastasis by silencing, in trans, the expression of genes in the HOXD cluster. Recently, it was reported that HOTAIR is overexpressed in tamoxifen-resistant breast cancers [71]. In addition, the expression HOTAIR is directly inhibited by estrogen. The interaction between HOTAIR and ER promotes ER target gene transcription in estrogen-depleted conditions. Thus, HOTAIR also promotes growth and tamoxifen resistance in breast cancer cells. HOTAIR could serve as an additional potential therapeutic target and a crucial biomarker (Figure 1) [71].

## 5. Future clinical directions

Over the past few decades, many HOX genes and noncoding RNAs in the HOX clusters have been found to be deregulated in different tumor types and to actively drive diverse hallmarks of cancer. Despite their obvious clinical significance, it has been difficult to therapeutically target them with high specificity because of their extreme shared homology. Recent advances in gene therapy and computational biology might help in overcoming this limitation: by using siRNAs/shRNAs or RNAs, stable, selective and efficient knockdown or overexpression of genes can be achieved. Brock et al. [72] took it one step further. Gene network inference allowed them to identify HOXA1 to be causally involved in cancer development. They then designed HOXA1 siRNA nanoparticles and injected the particles intraductally through the nipple of transgenic mice with early-stage breast disease. This led to a decrease of proliferation and mammary tumor incidence. This approach, taking advantage of novel therapeutics and a unique delivery modality offers a novel noninvasive strategy to identify and specifically target oncogenes such as the HOX genes [72].

Other RNA targeting tools (like ribozymes, aptamers, ASOs and microRNAs) might be as effective and even have unique features that provide them advantages over siRNAs, especially for targeting the HOX lncRNAs. For example, at least 30 of the 39 HOX gene 3'UTR harbor conserved matches to miRNAs, many of these have been validated experimentally, and thus represent new opportunities for clinical intervention. Interestingly, the interaction of microRNAs with lncRNAs- for example, miR-141 with the oncogenic lncRNA, HOTAIR- may serve not only to decrease transcript levels but also to interfere with binding to their factors, and thus blunt their function [73].

Recently, the high levels of expression of the lncRNA H19 in cancer cells was exploited by BioCancell Therapeutics, who have developed a plasmid (BC-819) composed of the H19 promoter driving the expression of diphtheria toxin. BC-819 is being tested in phase I/II clinical trials to target invasive bladder, ovarian, lung and pancreatic cancers [74]. Given that HOTAIR is highly expressed in many aggressive and chemo-resistant cancers, using such a gene therapy strategy might provide promising clinical benefits.

Pharmaceutically, though targeting individual HOX proteins may still be not feasible, designing drugs that interfere with their interaction with downstream factors is a promising approach to inhibit their function. HXR9 is a successful example: HXR9, a small, cell-permeable peptide, acts by antagonizing HOX/PBX interactions (which influence HOX DNA binding specificity). HXR9 inhibits growth of prostate, melanoma, breast and ovarian cancer in cell culture and in preclinical models [75]. With further discoveries of novel HOX partners and the importance of these interactions in oncogenesis and drug resistance, and with advances in computational structural biology, it might be possible to design new drugs in future that specifically interfere with HOX functions.

Finally, the expression, methylation, translocation or mutation of many of the HOX genes and ncRNAs is increasingly found to function as novel biomarkers for diagnosis, prognosis, metastasis, and predictors of responsiveness to therapy. So far, only HOXB13 testing has entered clinical practice where high HOXB13/IL-17BR expression ratio is associated with increased relapse and death in ER-positive breast cancer patients treated with tamoxifen [76]. Strong enrichment of HOX genes is present within the 100 methylated markers that correlate with recurrence markers in breast cancer [77]. In fact, methylated HOXB4 in circulating tumor DNA is one among the 10-gene panel of biomarkers that can detect more than 90% of cases with metastatic breast cancer, help monitor response to treatment, and possibly prognosticate long term outcome [78]. The potential of HOX genes and lncRNAs as markers in human fluids is still unexplored and may provide new specific and facile diagnostic biomarkers, and minimize unnecessary biopsies.

In summary, the exploration of the HOX clusters has taken an interesting twist in the last few decades as we begin to unveil their complex interactions and discover their roles in diverse cellular processes, far beyond just the control of embryonic development. The investigation of HOX genes for understanding, discovering and treating endocrine resistant breast cancer might still be at its early stages of development, but is full of future promise.

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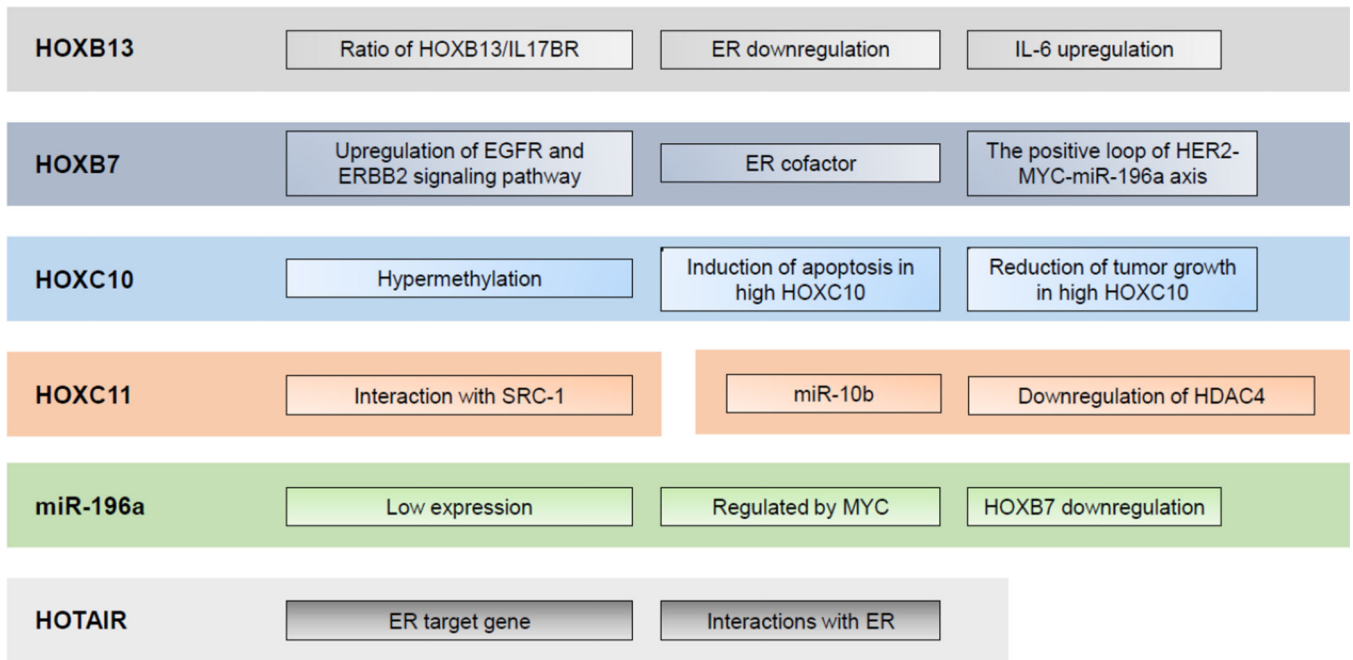
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### Highlights

- HOX genes are often overexpressed in breast cancer.
- Hormones regulate expression of many HOX genes.
- HOX genes and miRNAs/ncRNA in the HOX cluster can contribute to endocrine resistance.
- Targeting specific HOX gene can overcome endocrine-resistance in breast cancer.



**Figure 1.** Overview of known HOX gene functions in endocrine resistant breast cancer.

**Table 1**

Disorders associated with mutations in HOX genes

HOX gene	Condition	References
HOXA1	Bosley–Salih–Alorainy syndrome	14
HOXA1	Athabaskan brainstem dysgenesis syndrome	15
HOXA2	Microtia, hearing impairment and cleft palate	16
HOXA11	Radioulnar synostosis with amegakaryocytic thrombocytopenia	17
HOXA13	Hand–foot–genital syndrome	18–20
HOXA13	Guttmacher syndrome	21
HOXB1	Hereditary congenital facial paresis-3	22
HOXB13	Prostate, Colon and Breast	23–25
HOXC13	Ectodermal dysplasia 9, hair/nail type	26
HOXD4	Lymphoid malignancy and skeletal malformations	27
HOXD10	Congenital vertical talus and Charcot–Marie–Tooth disease	28
HOXD13	Synpolydactyly type II	29–31
HOXD13	Brachydactyly types D and E	
HOXD13	Syndactyly type V	
HOXD13	Brachydactyly–syndactyly	

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