HOXA5 Acts Directly Downstream of Retinoic Acid Receptor β and Contributes to Retinoic Acid–Induced Apoptosis and Growth Inhibition

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Abstract

The promise of retinoids as chemopreventive agents in breast cancer is based on the differentiation and apoptosis induced upon their binding to the retinoic acid (RA) receptor β (RARβ). We have previously shown that HOXA5 induces apoptosis in breast cancer cells. In this study, we investigated whether RA/RARβ and HOXA5 actions intersect to induce apoptosis and differentiation in breast cancer cells. We found that HOXA5 expression can be induced by RA only in RARβ-positive breast cancer cells. We have, for the first time, identified the RA response element in *HOXA5,* which was found to be located in the 3′ end of the gene. Chromatin immunoprecipitation assays showed that RARβ binds directly to this region *in vivo*. Overexpression of *RARβ* strongly enhances RA responsiveness, and knocking down *RARβ* expression abolishes RA-mediated induction of HOXA5 expression in breast cancer cells. In addition, there is coordinated loss of both *HOXA5* and *RARβ* expression during neoplastic transformation and progression in the breast epithelial cell model, MCF10A. Knockdown of HOXA5 expression partially abrogates retinoid-induced apoptosis and promotes cell survival upon RA treatment. These results strongly suggest that HOXA5 acts directly downstream of *RARβ* and may contribute to retinoid-induced anticancer and chemopreventive effects. [Cancer Res 2007;67(17):8007–13]

Introduction

Retinoids, the natural and synthetic derivatives of vitamin A, exert profound effects on cell growth, differentiation, apoptosis, and morphogenesis (1). They are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of human cancers, including breast cancer (2–4). The effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid (RA) receptors (RAR) and retinoid X receptors (RXR; refs. 1, 5). 9-*Cis*-RA is a high-affinity natural ligand for both RARs and RXRs (6), whereas *all-trans*-RA is a high-affinity natural ligand only for the RARs (7). RARs and RXRs are each encoded by three distinct genes (*α, β, γ*), which function as ligand-activated transcription factors (1). RARs interact with RXRs, forming RXR-RAR heterodimers that bind to RA response elements (RARE) to control the expression of RA-responsive genes in the presence of retinoids (1). Some of the target genes are RARs themselves, in particular the *RARβ* gene, for which a RARE (βRARE) was identified in the promoter region (8).

Altered nuclear receptor activities are associated with carcinogenesis. In particular, loss of *RARβ* expression was found in a number of malignancies, including carcinomas of lung and breast, and squamous cell carcinomas of the head and neck (9–11). A growing body of literature has shown that the anticancer effect of RA is primarily mediated by RARβ, which is a potent tumor-suppressor protein (12, 13). For example, inhibition of *RARβ* expression in *RARβ*-positive cancer cells abolished the RA-induced apoptotic effect (14). Conversely, reexpression of *RARβ* in *RARβ*-negative cancer cells restored RA-induced growth inhibition, apoptosis, and decreased tumorigenicity (12, 15). In phase I/II clinical trials of breast cancer testing a combination of *all-trans*-RA and tamoxifen, RARβ expression was found to be consistently elevated in the breast tissue (16). However, how RARβ exerts its anticancer activity is still largely unknown.

HOX gene expression is regulated by RA in embryonic cells and tissues (17). In vertebrates, there are 39 *HOX* genes that are organized into four clusters (i.e., *HOXA, HOXB, HOXC*, and *HOXD*; ref. 18). In embryonic carcinoma cells, expression of the entire *HOXB* gene cluster can be induced by RA treatment (19). In contrast, in differentiated cells and tumor cells, very few *HOX* genes can be induced by RA (20). Despite efforts to identify the RAREs for *HOX* genes, only five RAREs have been identified for 5 of 39 *HOX* genes (*HOXA1, HOXB1, HOXA4, HOXB4*, and *HOXD4*; refs. 21–24). *HOXA5* expression has been shown to be regulated by RA in embryonic carcinoma cells and in developing mouse lung (25, 26). No RARE has yet been identified for *HOXA5*. How *HOXA5* expression is regulated by RA and the role of *HOX* gene in RA-mediated cellular function is poorly understood.

Recently, our laboratory has shown that HOXA5 expression is lost in >60% breast cancer cell lines and primary tumors (27, 28). Overexpression of HOXA5 in breast cancer cells induced apoptosis (27, 29). To determine if HOXA5 expression can be reactivated by RA, we treated a variety of breast cancer cells with RA and its analogues. We found that HOXA5 expression can be induced only in *RARβ*-positive breast cancer cells. Further, we identified the RARE to which RARβ binds directly. We also showed that induction of HOXA5 is important for RA-mediated apoptosis and cellular growth inhibition. This study suggested that HOXA5 acts directly downstream of *RARβ* and plays an important role in RA-mediated anticancer activity.

Materials and Methods

Reagents and cell culture. All of the retinoids (*all-trans*-RA, 9-*cis*-RA, 13-*cis*-RA, and 4-HPR) were purchased from Sigma-Aldrich. RARβ

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doi:10.1158/0008-5472.CAN-07-1405

antagonist LE135 was kindly provided by Dr. Hiroyuki Kagechika (Tokyo Medical and Dental University, Tokyo, Japan). Western blot analysis was done using standard procedure with antibodies to HOXA5 (Zymed) and RARβ (Santa Cruz Biotechnology).

All breast cancer cell lines except MCF10A were maintained in DMEM with 10% fetal bovine serum. MCF10A were maintained in DMEM/F12 containing 5% horse serum, 10 μg/mL human insulin, 0.5 μg/mL hydrocortisone, and 100 ng/mL cholera toxin. MCF10A, T1K.C12, and MCFCA.1h were obtained from Fred Miller and Robert Pauley (Karmanos Cancer Institute, Detroit, MI) and were cultured in MCF10A medium (30).

Plasmids and transient transfection. The pLc-P1-Luc was constructed by replacing the cytomegalovirus promoter sequence of the reporter plasmid pLc-Luc with *HOXA5* promoter sequence, which was amplified using PCR with forward primer 5′-GAGATCTAACCTCCTTCGGCTTTGCC-3′ and reverse primer 5′-CAAGCTTTGTGGCTCGCGGTCGTTTG-3′. The pLc-A5GF3-Luc was generated by inserting 6.5 kb of *HOXA5* downstream sequence into pLc-P1-Luc vector. To construct the deletion plasmid D1, pLc-A5GF3-Luc was digested with (*Kpn*I) and (*Eco*RI) to release a small fragment of the downstream sequence followed by filling with Klenow enzyme and self-ligation with T4 ligase. Similarly, pLc-A5GF3-Luc was cut with *Eco*RI/*Nde*I to construct D2, *Bsa*BI/*Nde*I to construct D3, *Kpn*I/*Afl*II to construct D4, and *Kpn*I/*Mlu*I to construct D5. The deletion sequences in F1 to F4 was PCR amplified with primers (F1 forward primer 5′-AGGTACCATGAAGACATCCAGCCTCAG-3′; F2 forward primer 5′-AGGTACCCATGGAGAGTGCAGAGCATT-3′; F3 forward primer 5′-AGGTACCAGTCTGTCATCGCCTAACAA-3′; F4 forward primer 5′-AGGTACCGGTGCTAACGAAGAGGTCAT-3′ and reverse primer 5′-ACATATGCAACCACTAGGGTTCACCTG-3′), and cloned into D1 at the *Kpn*I/*Mlu*I sites. The oligonucleotides containing one or three copies of either site I or II were synthesized and inserted into pGl3-Promoter vector to generate I-1, I-3, II-1, and II-3 plasmids. Similarly, II-3M, which contained three copies of mutated site II sequence, was constructed. The same mutations were introduced into F1 construct to generate M1 and M2 constructs, respectively.

Transient transfection experiments were done as described previously (31). At 24 h posttransfection, the cells were treated with 1 μmol/L of RA for another 24 h before harvesting for luciferase assay according to the manufacturer's instructions (Promega).

Establishment of *HOXA5*-short hairpin RNA stably transfected cell lines. The *HOXA5* short hairpin RNA (shRNA)-expressing plasmid was constructed by inserting the synthesized oligonucleotides (oligo 1a: 5′-GATCTCAGCGTCGGCCGCTTA-3′ and oligo 1b: 5′-AGCTTAAGCGGCCGACGCTGAGATC-3′; oligo 2a: 5′-AGCTTAGCGGCCGACGCTGAGATCCTTTTTG-3′ and oligo 2b: 5′-AATTCAAAAAGGATCTCAGCGTCGGCCGCTA-3′) into a modified vector pBS/U6 (32). The modified vector carries both a green fluorescent protein (*GFP*) gene and a neomycin gene sequence. MCF7 cells were transfected with *HOXA5* shRNA-expressing plasmid and empty vector, and selected with 500 μg/mL of G418 for 2 to 3 weeks. The GFP-expressing colonies were pooled and used in further experiments.

Quantitative real-time reverse transcription-PCR analysis. Real-time reverse transcription-PCR (RT-PCR) analysis was done as previously described (31). The primers used for the PCR are as follows: *HOXA5* forward: 5′-TCTCGTTGCCCTAATTCATCTTT-3′ and reverse primer: 5′-CATTCAGGACAAAGAGATGAACAGAA-3′; and *RARβ* forward primer: 5′-CTGGATTTGGTCCTCTGACT-3′ and reverse primer: 5′-CATGTGAGGCTTGCTGGGTC-3′. Native gel electrophoresis was used to characterize the final products. Data were first analyzed using the Sequence Detector Software SDS 2.0 (Applied Biosystems). Results were calculated and normalized relative to the glyceraldehyde-3-phosphate dehydrogenase control by using the Microsoft Excel program. All of the PCR assays were done in triplicate, and mean values are shown in figures.

Small interfering RNA transfection. *RARβ* small interfering RNA (siRNA) was purchased from Dharmacon, Inc. The breast cancer cell line, Hs578T, was transiently transfected with *RARβ* siRNA as described previously (29).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) analysis was done with the ChIP (Upstate Biotechnology) according to the manufacturer's instructions. Chromatin preparations were made from 2×10^6 MCF7 cells transiently transfected with vector or *RARβ*-expressing plasmids. The PCR primers included site II-specific forward primer 5′-AGAAGCAGGGCATCCTGAGA-3′and reverse primer 5′-CAACCACTAGGGTTCACCTG-3′, and the control forward primer 5′-TTGTGGCTCGCGGTCGTTTG-3′ and reverse primer 5′-CTCGCGGTCGTTTGTGCGTC-3′.

Apoptosis analysis. Cells (1×10^5) in six-well plates were treated with the desired concentration of RA and vehicle for 6 days. A total of 5×10^5 cells were used for cell cycle distribution analysis as described previously (29). The sub–G_1-G_0 population represents the apoptotic cells.

Clonogenic assay. Five hundred cells per well were seeded in six-well plates and allowed to attach overnight to the plastic substrate before the addition of the indicated concentration of retinoids or vehicle. The medium was replaced with retinoid-free medium for 2 to 3 weeks. As the colonies became visible, cells were fixed with methanol, stained with Giemsa (1:10 in distilled water), and counted.

Results

Induction of HOXA5 expression by RA in breast cancer cells. With a goal to reactivate the expression of HOXA5 and inhibit breast cancer cell growth, we treated a variety of breast cancer cells with different concentrations of RA. We found that HOXA5 expression was induced in three of eight breast cancer cell lines tested (Fig. 1*A*). To gain additional evidence to support the concept that RA/HOXA5 signaling pathway was specifically blocked in most breast cancer cells, we tested RA response using reporter plasmid containing RARE in the promoter. The results indicated that most of the breast cancer cell lines, except for MDAMB231 cells, responded to RA treatment (Supplementary Fig. S1). A few cell lines, such as ZR75.1 and MDAMB435, responded strongly to RA treatment but failed to express HOXA5 upon RA induction. Therefore, RA regulates the expression of its target genes through more than one mechanism in breast cancer cells.

RA regulates gene expression through binding to its receptors (RARs and RXRs). Because RXRα and RXRβ are stably expressed during mammary carcinogenesis and progression, and RXRγ is not detected in breast cells (33, 34), we examined the expression status of RARs in normal breast and breast cancer cell lines. RARα and RARγ were expressed in almost all of the 12 breast cancer cell lines examined, although the expression level varied from cell line to cell line. However, RARβ expression was detected in only four cell lines (MCF10A, MCF7, MDAMB435, and Hs578T cells; Fig. 1*B*). Interestingly, HOXA5 expression was induced by RA in three of these four *RARβ*-positive cell lines, suggesting that RARβ expression is important for HOXA5 induction.

Consistent with this finding, we found that *all-trans*-RA, which specifically binds to RARs, strongly induced HOXA5 expression. 9-*Cis*-RA and 13-*cis*-RA, which bind to both RARs and RXRs, moderately activated HOXA5 expression (7). 4-HPR, which selectively binds to RARγ, very weakly activated HOXA5 expression (35; Fig. 1*C*). Further, RA-induced HOXA5 expression was competitively inhibited by RARβ-specific antagonist, LE135 (Fig. 1*D*; Supplementary Fig. S2; refs. 36, 37).

RARE is located in the 3′-end of *HOXA5* gene. To elucidate the mechanism of the response of *HOXA5* to RA, we first generated a luciferase construct (pLc-P1-Luc) carrying the DNA sequence upstream of *HOXA5* coding region (Fig. 2*A*). Transient transfection assays showed that the upstream DNA sequence failed to confer RA responsiveness (Fig. 2*B*). A previous transgenic mouse study had shown that the downstream DNA sequence is also important for controlling spatiotemporal expression of murine *Hoxa5 in vivo* (38). To test whether the RA-responsive element is located in the 3′-end

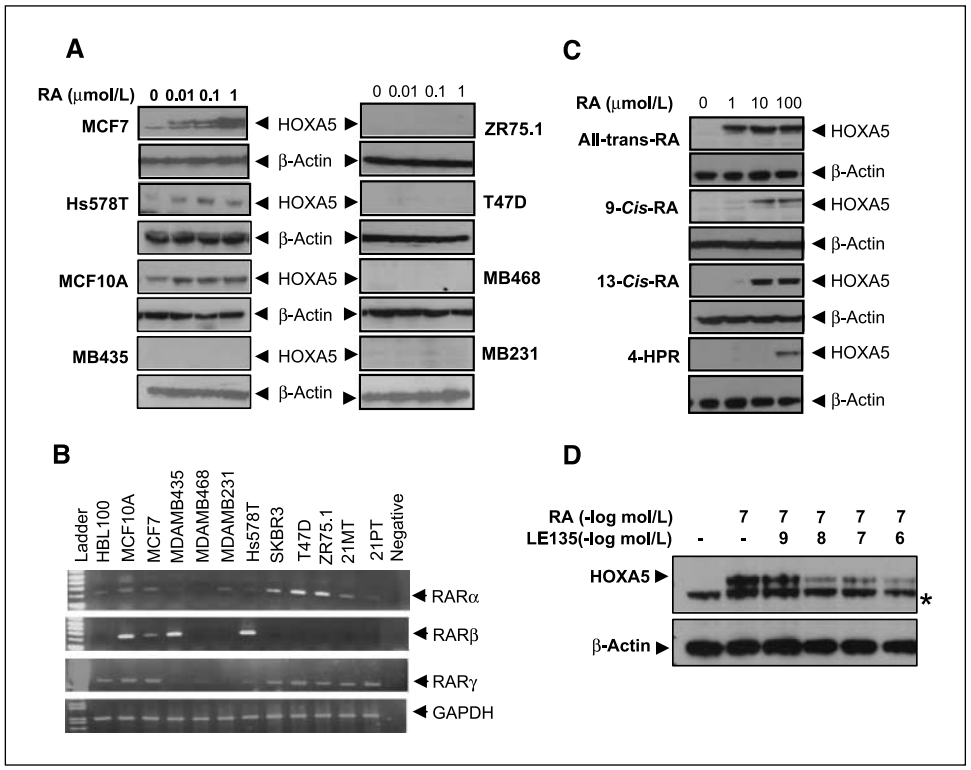

Figure 1. RA-induced HOXA5 expression in breast cancer cells. *A,* Western blot analysis of eight breast cancer cell lines treated with different concentrations of *all-trans*-RA (0, 0.01, 0.1, and 1 μmol/L) for 24 h. *B,* examination of expression of the three RARs in breast cancer cell lines using RT-PCR. *C,* induction of HOXA5 expression in MCF7 cells with RA analogues. *D,* inhibition of RA-induced expression of HOXA5 by RARβ antagonist LE135. MCF7 cells were treated with RA alone or in combination with LE135 for 24 h before harvesting for Western blot analysis. *, nonspecific band.

of *HOXA5* coding region, we added the 6.5 kb 3′-end DNA sequence to our construct (pLc-A5GF3-Luc; Fig. 2*A*). The new construct showed a strong response to RA treatment, indicating that the RARE is located in this 3′-end fragment (Fig. 2*B*).

To identify the RARE in this 6.5-kb fragment, we generated a series of deletion constructs. Transient transfection assays clearly showed that the ability of the reporter constructs to respond to RA depended on a 300-bp fragment located between the 5′ ends of D5 and F4 constructs (Fig. 2*C*). The sequence of this 300-bp fragment revealed two potential RAREs, which match or closely resemble the RA half-site consensus sequence AGGTCA. To analyze the role of these two potential binding sites in RA responsiveness, point mutations were introduced into each of these two sites. Mutation of site I had no dramatic effects on the responsiveness to RA, whereas the mutation of site II significantly blocked the increase in reporter activity in response to RA treatment (Fig. 2*C*). To confirm that the site II sequence is a functional RARE, one or three copies of the 13-bp site I and 15-bp site II nucleotides were synthesized, and each was inserted in front of a SV40 promoter (Fig. 2*D*). Transient transfection assay showed that the second binding site, but not the first, conferred responsiveness to RA. Further, mutation of this putative binding motif completely abolished its responsiveness to RA treatment (Fig. 2*D*).

RARβ directly binds to the RARE and mediates HOXA5 induction in breast cancer cells. To further confirm that RARβ expression is important for HOXA5 induction, we cotransfected the reporter construct (pLc-A5GF3-luc) with *RAR*- and *RXRα*-expressing vectors into CV-1 cells. CV-1 monkey kidney fibroblast cells are widely used for testing RAR isotype-specific activities, partly due to the low or absent endogenous level of expression of all of the RARs (7, 39, 40). Although expression of each of the RARs and RXRα restored *HOXA5* RARE function to varying levels in CV-1 cells, RARβ expression most strongly up-regulated the response of *HOXA5* RARE to RA treatment (Fig. 3*A*). Conversely, knocking down of *RARβ* expression in breast cancer cell line Hs578T cells using siRNA blunted the induction of HOXA5 expression by RA (Fig. 3*B*). Further, ChIP assays confirmed that both RARα and RARβ bound strongly to the site II–containing DNA region *in vivo*, whereas RARγ bound weakly to this region (Fig. 3*C*). These experiments provide strong support for the hypothesis that RARβ expression is required for HOXA5 induction in breast cancer cells.

Because the expression of both *RARβ* and *HOXA5* has been reported to be lost during breast cancer progression, we wanted to test whether there is a correlation between the expression patterns of these two genes. We examined the expression status of *HOXA5* and *RARβ* in a MCF10A model of breast cancer development and progression using real-time PCR (Fig. 3*D*). Each of these cell lines is derived from parental MCF10A, an immortalized normal breast epithelial cell line. Upon transplantation into immunodeficient mice, MCF10A cells do not grow; T1k.C12 grow slowly and develop morphologic structures similar to the hyperplasic lesions and carcinoma *in situ* of the human breast; whereas MCFCA.1h cells are malignant and develop tumors that infiltrate the surrounding tissues, thus recapitulating multiple steps of progression in breast cancer (30). MCF10A cells express both RARβ and HOXA5. During tumor progression in this model, the expression of both *HOXA5* and *RARβ* were coordinately lost, suggesting a correlation between the loss of HOXA5 and RARβ in a biologically relevant model system (Fig. 3*D*). Due to the relatively low-level expression of RARβ in each member of the MCF10A series of cells, the reduction in RARβ expression at the mRNA level does not seem to be so dramatic. However, previous studies, using the same model system, have shown that progression and malignant transformation of MCF10A cells is associated with loss of the induction of RARβ at both transcription and translation levels by RA (41).

HOXA5 induction contributes to RA-induced apoptosis and cellular growth inhibition. RARβ has been shown to mediate the growth-inhibitory effect of RA by promoting apoptosis in breast cancer cells (12, 15). In this study, we have presented evidence that RARβ directly regulated HOXA5 expression in breast cancer cells (Fig. 2*D*). To test whether HOXA5 induction is functionally important for RA-mediated cellular growth inhibition, we generated a MCF7 breast cancer cell line stably transfected with *HOXA5* shRNA. In scrambled shRNA-transfected cells, HOXA5 expression was strongly induced by RA. In contrast, in *HOXA5*-specific shRNA transfected cells, induction of HOXA5 expression was greatly decreased (Fig. 4*A*). Blockage of HOXA5 induction also partially inhibited RA-induced apoptosis. Treatment for 6 days with 1 μmol/L of RA resulted in apoptosis of 8.1% of scrambled shRNA-transfected cells and 3.8% of HOXA5 shRNA-transfected cells (Fig. 4*B*). Consistent with this finding, clonogenic assays showed that *HOXA5* shRNA-transfected cells survived significantly better than scrambled shRNA-transfected cells in response to RA treatment (Fig. 4*C*). Collectively, these results suggest that HOXA5 is a direct target of *RARβ* and is partially responsible for RA-mediated apoptosis and cellular growth inhibition.

Discussion

Previous studies from our laboratory have shown that HOXA5 expression is lost in >60% primary breast carcinomas, and that introduction of the *HOXA5* gene into breast cancer cells induced apoptosis through both p53-dependent and p53-independent mechanisms (27, 29). By identifying upstream activators of HOXA5 expression, it might be possible to devise targeted treatment strategies. Based on findings that HOXA5 expression is induced in embryonic stem cells and developing mouse lung by RA (25, 42), we hypothesized that HOXA5 expression is regulated in breast cells by the RARs and/or RXRs. As predicted, we found that HOXA5 expression is induced by RA in the majority of *RARβ*-positive breast cells. Further, for the first time, we defined the RARE in the HOXA5

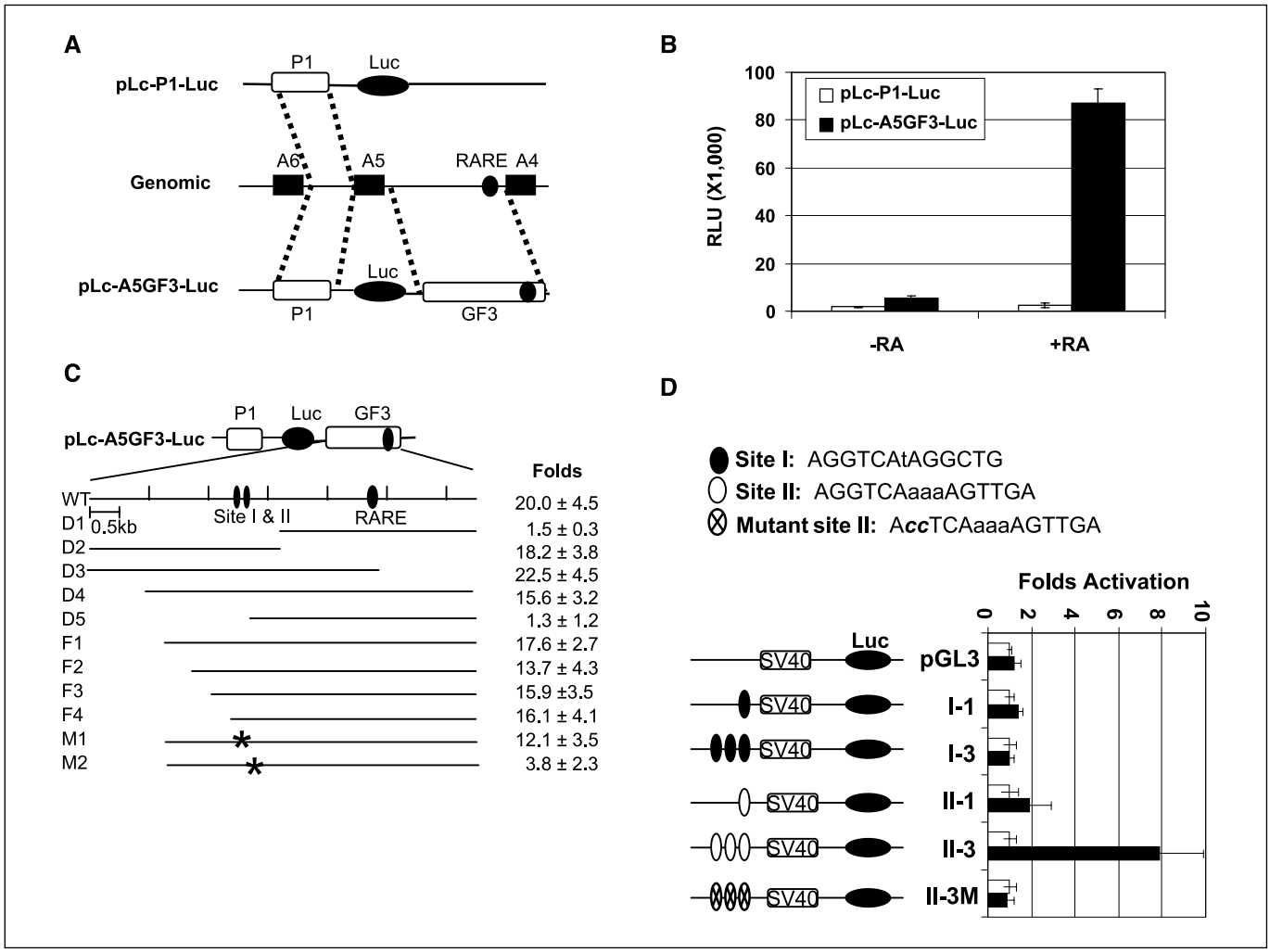

Figure 2. Identification of the *HOXA5*-specific RARE. *A,* schematic presentation of constructs pLc-P1-Luc and Plc-A5GF3-luc. The P1 fragment represents the *HOXA5* promoter located in the intergenic region between the genes *HOXA5* and *HOXA6*. A5GF3 represents a 6.5-kb genomic DNA sequence downstream of the *HOXA5* coding region. *B,* the RA-responsive element is located in the 3′-end fragment. MCF7 cells were transiently transfected with pLc-P1-Luc and pLc-A5GF3-Luc and then treated with RA for 24 h before harvesting for luciferase assay. *C,* deletion analysis of the 3′-end fragment (*GF3*). A series of deletion constructs were generated as described in Materials and Methods. The responsiveness of each deletion construct was examined by transient transfection assays. The M1 and M2 construct contain two point mutations introduced into the first half of the putative RA binding motif of sites I and II, respectively (AGGTCA 6AccTCA). RARE represents the known RA binding site for HOXA4. *D,* site II is a functional RARE in MCF7 cells. Transient transfections were done to test the responsiveness of site I and II to RA.

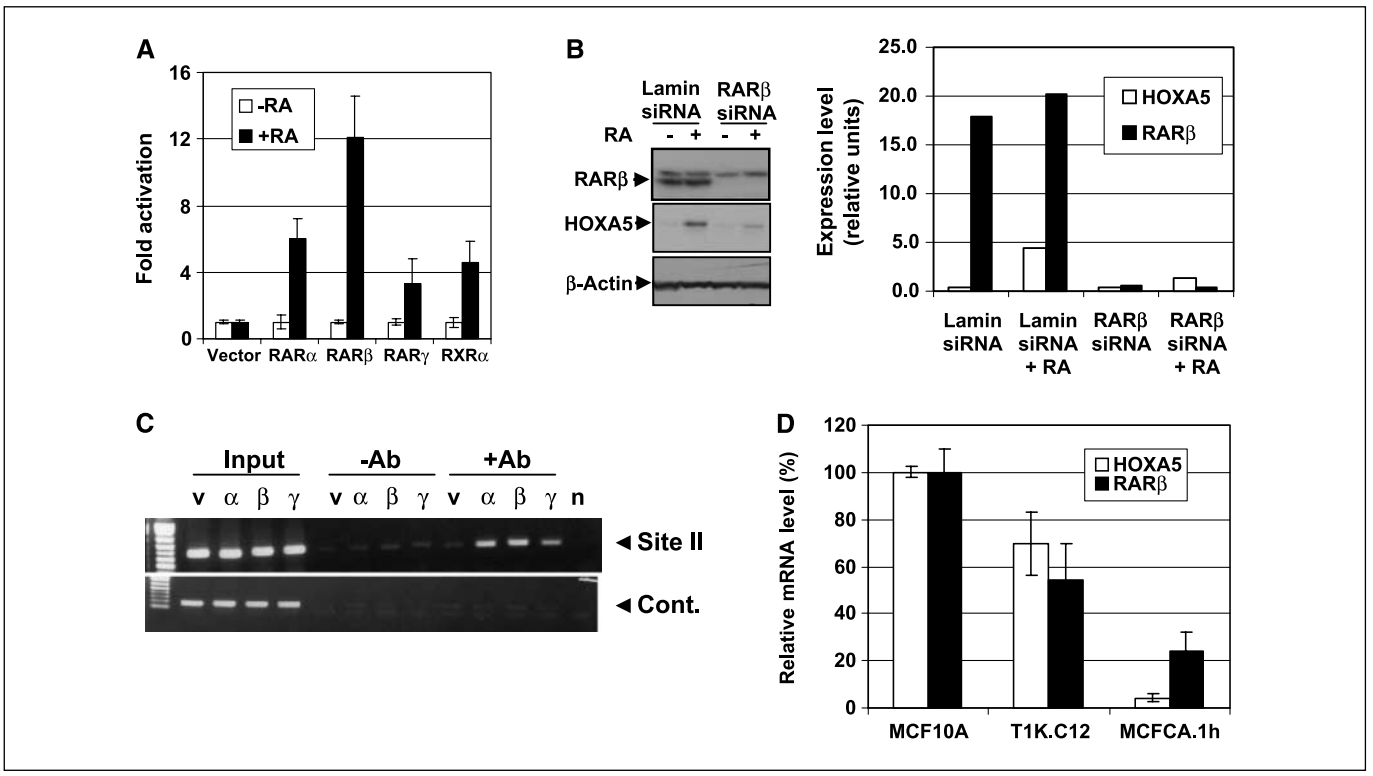

Figure 3. *HOXA5* acts directly downstream of RARβ. *A,* the pLc-A5GF3-Luc plasmid was cotransfected into CV-1 cells with RAR-expressing plasmids (pRShRARα, pRShRARβ, and pRShRARγ), RXRα-expressing plasmid (pRShRXRα), or vector (pRSV-0). The transfected cells were treated with 1 μmol/L of RA for 24 h before being harvested for luciferase analysis. *B,* knockdown of RARβ expression blocked the induction of HOXA5 expression by RA. Breast cancer cell line Hs578T cells were transiently transfected with siRNA to RARβ. Then, the cells were treated with RA for 24 h before being harvested for Western blot analysis of HOXA5 and RARβ expression. Quantitative analysis of the Western blot was done using IPlab software. *C,* examination of the binding affinities of RAR receptors to the site II region *in vivo*. MCF7 cells were transiently transfected with RAR-expressing plasmids (pRARα-Myc, pRARβ-Myc, and pRARγ-Myc) and vector, and then harvested for ChIP analysis with Myc-Tag antibody. *n,* no DNA; *Cont.,* control region. *D,* coordinated loss of HOXA5 and RARβ expression in a series of MCF10A cells model. The expression of HOXA5 and RARβ was examined by real-time PCR analysis in epithelial cell lines—MCF10A, the parental (benign); MCF10A transformed with *Ras* gene, T1K.C12 (premalignant); and MCFCA.1h (malignant). The mRNA expression level of HOXA5 and RARβ are shown relative to parental MCF10A cells (represented as 100%).

coding region, and showed that among the RARs, RARβ is the critical determinant of induction of HOXA5 and subsequent apoptosis induced by RA.

***HOXA5* is a direct target of RARβ in breast cancer cells.** We identified a specific RARE in the 3′ end of the *HOXA5* coding region. The RARE was a direct repeat site with a 3-bp spacing (DR3); the first half site contained a consensus RA binding motif (AGGTCA) to which, potentially, all of RARs can bind. Our transient transfection and ChIP assays together showed that RA induced HOXA5 expression preferably via RARα and RARβ, but other RA receptors (RARγ and RXRα), when overexpressed, can also functionally activate HOXA5 expression. It is important to note that regardless of the expression status of other RARs in breast cells, HOXA5 expression was induced only in RARβ-positive cells. This observation led us to reason that RARβ may be the limiting factor for HOXA5 induction or that this specificity may be controlled by other cofactors that display preference for RARβ in breast cancer cells. In either scenario, it seems that RARβ expression is the critical determinant of induction of HOXA5 expression. Consistent with this hypothesis, knockdown of RARβ expression significantly blunted the induction of HOXA5 expression by RA. In addition, the expression of HOXA5 and RARβ was coordinately lost during tumor progression and malignant transformation of MCF10A cells. These findings of an apparent preference for RARβ, however, do not exclude the possibility that, under certain conditions, HOXA5 expression can be regulated by RA via other RAR or RXR isoforms. Also, although *HOXA5* is the direct target of RARβ, we found that high expression of RARβ alone may not predict the ability of RA to induce HOXA5 expression. For instance, MDAMB435 cells express high levels of RARβ but failed to induce HOXA5 expression after RA treatment (Fig. 1*A*). Similarly, we could not restore HOXA5 induction by reintroducing *RARβ* into RARβ-negative MDAMB231 cells (data not shown), implying that additional cellular factors are required for HOXA5 induction in breast cancer cells.

Role of HOXA5 in RA-induced anticancer effects. Retinoids are effective inhibitors of breast cancer cell growth. Compared with the relatively ubiquitous expression of RARα and RARγ in breast cancer cells, RARβ expression is lost in majority of breast cancer cell lines and primary breast carcinomas (33, 43), suggesting that RARβ may play an important part in breast tumorigenesis. Several studies have shown that *RARβ* behaves as a tumor-suppressor gene in breast cancer and that induction of RARβ mediates RA-induced growth inhibition and apoptosis (12, 15, 44–47). However, the mechanism by which RARβ mediates cellular growth inhibition and apoptosis is largely unknown. In this study, we have shown for the first time that *HOXA5* is one of the direct targets of RARβ in breast cancer cells.

Our previous studies have shown that HOXA5 induces apoptosis in a p53-dependent (27), as well as a caspase-dependent, pathway

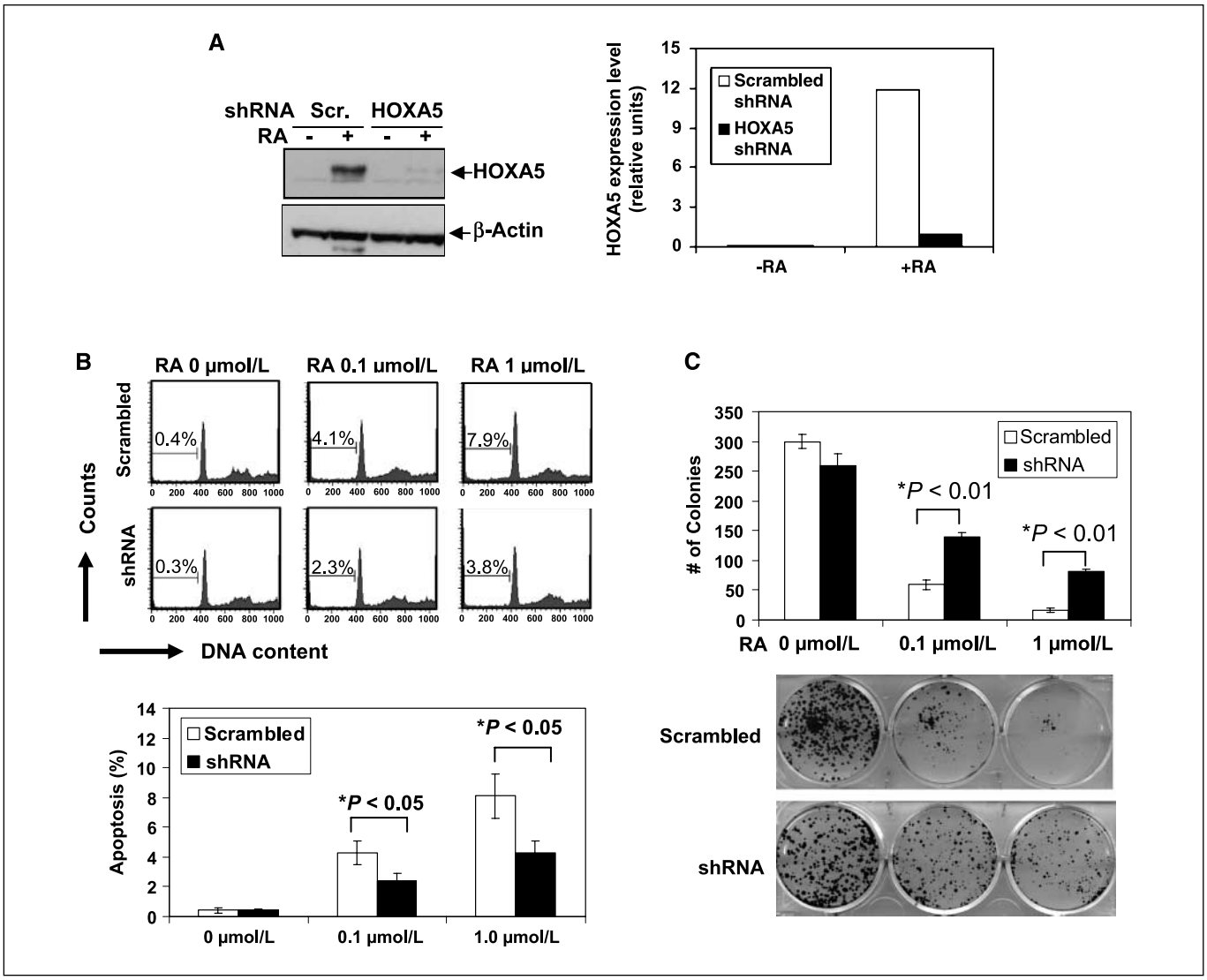

Figure 4. HOXA5 mediates RA-induced apoptosis and cellular growth inhibition. *A,* establishment of *HOXA5* shRNA stably transfected cell line. *HOXA5* shRNA and scrambled shRNA-expressing plasmids were stably transfected into MCF7 cells. The G418 selected cells were treated with 1 μmol/L of RA for 24 h. The expression of HOXA5 was examined by Western blot analysis. *Columns,* quantitative analysis of the Western blot. *Scr.,* scrambled shRNA. *B,* blockage of HOXA5 induction inhibits the RA-induced apoptosis. Stably transfected cells expressing *HOXA5* shRNA or scrambled shRNA were treated with different concentrations of RA for 6 d. The percentages of apoptotic cells was assessed by cell cycle analysis. The sub–G_0-G_1 population represents the apoptotic cells in this assay. *C,* blockage of HOXA5 induction promotes cell survival after RA treatment. Clonogenic assay was repeated twice. The average numbers are presented.

(29) in breast cancer cells. It is interesting to speculate that HOXA5 induction in breast cancer cells may bridge the RA signaling pathway and apoptotic signaling pathways. Consistent with this notion, when HOXA5 induction was blunted by shRNA in breast cancer MCF7 cells, RA-induced apoptosis was also significantly blocked.

RA-induced gene expression in the HOX clusters. RA is not only a cancer prevention and therapeutic agent for cancer but is also a key morphogen in vertebrate development. Retinoids induce body axis formation in different animal systems while specifically regulating the *HOX* genes (48, 49). Although *HOX* genes have been known to be induced by RA shortly after they were identified in the vertebrate, RAREs have been found only in two genes of *HOX* paralog group 1 (*HOXA1* and *HOXB1*) and three genes from group 4 (*HOXA4, HOXB4* and *HOXD4*; 21, 22). The RAREs for the first paralog *HOX* genes are located in the 3′-end of the coding region, whereas the RAREs for the fourth paralog genes are located in the 5′-end of the coding regions (21–24). Despite the fact that few RAREs have been found in *HOX* genes, the entire cluster of *HOX* genes are sequentially activated by RA in embryonic stem cells. Several models, including direct and indirect regulation of *HOX* gene expression by RA, have been proposed to explain this sequential activation phenomenon. In one model, the *HOX* genes are activated via an RA-responsive "locus control region," and binding of RA to the RARE leads to opening up the chromatin structure of this region. In a second model, the *HOX* genes located at 3′-end of the cluster are activated directly via the RARE enhancers; the protein products of these genes activate the next 5′-end genes in the clusters. In a third model, multiple RAREs control the response of *HOX* genes with differing affinities for RA (50). These models do not necessarily exclude each other. Identification of *HOXA5* RARE in this study may support the third model although more RARE sites for other

HOX genes are yet to be identified. Much remains to be understood regarding the precise role of *HOX* genes in RA-mediated cell death.

Acknowledgments

Received 4/16/2007; revised 6/6/2007; accepted 6/19/2007.

Grant support: Department of Defense fellowship (DAMD17-02-1-0426; H. Chen) and grants from the Department of Defense Center of Excellence (W81XWH-04-1-0595) and AVON Foundation (S. Sukumar).

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We thank Ronald M. Evans (Salk Institute, San Diego, CA) for generously sharing with us the RAR-expressing plasmids (pRShRARα, pRShRARβ, and pRShRARγ), RXRα-expressing plasmid (pRShRXRα), and vector (pRSV-0); Karen Swisshelm (University of Washington, Seattle, WA) for providing the plasmid pGL2-RARE; Lorraine Gudas (Cornell University, New York, NY) for generously sharing cells and reagents; Hiroyuki Kagechika for providing RARβ antagonist LE135; and Alan Rein for critically reviewing the manuscript.

References

1. Evans TR, Kaye SB. Retinoids: present role and future potential. Br J Cancer 1999;80:1–8.
2. Lippman SM, Kessler JF, Meyskens FL, Jr. Retinoids as preventive and therapeutic anticancer agents (part I). Cancer Treat Rep 1987;71:391–405.
3. Niles RM. Recent advances in the use of vitamin A (retinoids) in the prevention and treatment of cancer. Nutrition 2000;16:1084–9.
4. Decensi A, Serrano D, Bonanni B, Cazzaniga M, Guerrieri-Gonzaga A. Breast cancer prevention trials using retinoids. J Mammary Gland Biol Neoplasia 2003;8:19–30.
5. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell 1995;83:841–50.
6. Zhang XK, Hoffmann B, Tran PB, Graupner G, Pfahl M. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature 1992;355:441–6.
7. Heyman RA, Mangelsdorf DJ, Dyck JA, et al. 9-*Cis* retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 1992;68:397–406.
8. de The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H, Dejean A. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. Nature 1990;343:177–80.
9. Widschwendter M, Berger J, Daxenbichler G, et al. Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer. Cancer Res 1997;57:4158–61.
10. Youssef EM, Lotan D, Issa JP, et al. Hypermethylation of the retinoic acid receptor-β(2) gene in head and neck carcinogenesis. Clin Cancer Res 2004;10:1733–42.
11. Suh YA, Lee HY, Virmani A, et al. Loss of retinoic acid receptor β gene expression is linked to aberrant histone H3 acetylation in lung cancer cell lines. Cancer Res 2002;62:3945–9.
12. Liu Y, Lee MO, Wang HG, et al. Retinoic acid receptor β mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. Mol Cell Biol 1996;16:1138–49.
13. Si SP, Lee X, Tsou HC, Buchsbaum R, Tibaduiza E, Peacocke M. RAR β2-mediated growth inhibition in HeLa cells. Exp Cell Res 1996;223:102–11.
14. Houle B, Rochette-Egly C, Bradley WE. Tumor-suppressive effect of the retinoic acid receptor β in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 1993;90:985–9.
15. Seewaldt VL, Johnson BS, Parker MB, Collins SJ, Swisshelm K. Expression of retinoic acid receptor β mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. Cell Growth Differ 1995;6:1077–88.
16. Toma S, Raffo P, Nicolo G, et al. Biological activity of *all-trans*-retinoic acid with and without tamoxifen and α-interferon 2a in breast cancer patients. Int J Oncol 2000;17:991–1000.
17. Marshall H, Morrison A, Studer M, Popperl H, Krumlauf R. Retinoids and Hox genes. FASEB J 1996;10:969–78.
18. Krumlauf R. Hox genes in vertebrate development. Cell 1994;78:191–201.
19. Simeone A, Acampora D, Arcioni L, Andrews PW, Boncinelli E, Mavilio F. Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature 1990;346:763–6.
20. Flagiello D, Gibaud A, Dutrillaux B, Poupon MF, Malfoy B. Distinct patterns of all-trans retinoic acid dependent expression of HOXB and HOXC homeogenes in human embryonal and small-cell lung carcinoma cell lines. FEBS Lett 1997;415:263–7.
21. Langston AW, Thompson JR, Gudas LJ. Retinoic acid-responsive enhancers located 3′ of the Hox A and Hox B homeobox gene clusters. Functional analysis. J Biol Chem 1997;272:2167–75.
22. Morrison A, Ariza-McNaughton L, Gould A, Featherstone M, Krumlauf R. HOXD4 and regulation of the group 4 paralog genes. Development 1997;124:3135–46.
23. Marshall H, Studer M, Popperl H, et al. A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. Nature 1994;370:567–71.
24. Popperl H, Featherstone MS. Identification of a retinoic acid response element upstream of the murine Hox-4.2 gene. Mol Cell Biol 1993;13:257–65.
25. Kim C, Nielsen HC. Hoxa-5 in mouse developing lung: cell-specific expression and retinoic acid regulation. Am J Physiol Lung Cell Mol Physiol 2000;279:L863–71.
26. Fibi M, Zink B, Kessel M, et al. Coding sequence and expression of the homeobox gene Hox 1.3. Development 1988;102:349–59.
27. Raman V, Martensen SA, Reisman D, et al. Compromised HOXA5 function can limit p53 expression in human breast tumours. Nature 2000;405:974–8.
28. Raman V, Tamori A, Vali M, Zeller K, Korz D, Sukumar S. HOXA5 regulates expression of the progesterone receptor. J Biol Chem 2000;275:26551–5.
29. Chen H, Chung S, Sukumar S. HOXA5-induced apoptosis in breast cancer cells is mediated by caspases 2 and 8. Mol Cell Biol 2004;24:924–35.
30. Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 1996;148:313–9.
31. Chen H, Rubin E, Zhang H, et al. Identification of transcriptional targets of HOXA5. J Biol Chem 2005;280:19373–80.
32. Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci U S A 2002;99:5515–20.
33. Roman SD, Clarke CL, Hall RE, Alexander IE, Sutherland RL. Expression and regulation of retinoic acid receptors in human breast cancer cells. Cancer Res 1992;52:2236–42.
34. Zhao Z, Zhang ZP, Soprano DR, Soprano KJ. Effect of 9-*cis*-retinoic acid on growth and RXR expression in human breast cancer cells. Exp Cell Res 1995;219:555–61.
35. Fanjul AN, Delia D, Pierotti MA, Rideout D, Yu JQ, Pfahl M. 4-Hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. J Biol Chem 1996;271:22441–6.
36. Umemiya H, Fukasawa H, Ebisawa M, et al. Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers. J Med Chem 1997;40:4222–34.
37. Li Y, Hashimoto Y, Agadir A, Kagechika H, Zhang X. Identification of a novel class of retinoic acid receptor β-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. J Biol Chem 1999;274:15360–6.
38. Moreau J, Jeannotte L. Sequence analysis of a Hoxa4-5 intergenic region including shared regulatory elements. DNA Seq 2002;13:203–9.
39. Laflamme L, Hamann G, Messier N, Maltais S, Langlois MF. RXR acts as a coregulator in the regulation of genes of the hypothalamo-pituitary axis by thyroid hormone receptors. J Mol Endocrinol 2002;29:61–72.
40. Hauksdottir H, Privalsky ML. DNA recognition by the aberrant retinoic acid receptors implicated in human acute promyelocytic leukemia. Cell Growth Differ 2001;12:85–98.
41. Peng X, Wood S, Bratescu L, Shilkaitis A, Christov K. Retinoids suppress premalignant MCF10AT but not malignant MCF10CA1a breast epithelial cells *in vivo*. Role of retinoic acid receptor β2 expression. Cancer Lett 2005;222:153–63.
42. Murphy SP, Garbern J, Odenwald WF, Lazzarini RA, Linney E. Differential expression of the homeobox gene Hox-1.3 in F9 embryonal carcinoma cells. Proc Natl Acad Sci U S A 1988;85:5587–91.
43. Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R. Down-regulation of retinoic acid receptor β in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. Cell Growth Differ 1994;5:133–41.
44. Treutin PM, Chen LI, Buetow BS, et al. Retinoic acid receptor β2 inhibition of metastasis in mouse mammary gland xenografts. Breast Cancer Res Treat 2002;72:79–88.
45. Seewaldt VL, Kim JH, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ. *All-trans*-retinoic acid mediates G_1 arrest but not apoptosis of normal human mammary epithelial cells. Cell Growth Differ 1997;8:631–41.
46. Xu XC, Sneige N, Liu X, et al. Progressive decrease in nuclear retinoic acid receptor β messenger RNA level during breast carcinogenesis. Cancer Res 1997;57:4992–6.
47. Wu Q, Dawson MI, Zheng Y, et al. Inhibition of *trans*-retinoic acid-resistant human breast cancer cell growth by retinoid X receptor-selective retinoids. Mol Cell Biol 1997;17:6598–608.
48. Tabin C. The initiation of the limb bud: growth factors, Hox genes, and retinoids. Cell 1995;80:671–4.
49. Papalopulu N, Clarke JD, Bradley L, Wilkinson D, Krumlauf R, Holder N. Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in *Xenopus* embryos. Development 1991;113:1145–58.
50. Langston AW, Gudas LJ. Retinoic acid and homeobox gene regulation. Curr Opin Genet Dev 1994;4:550–5.

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Cancer Res 2007;67:8007-8013.