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

Hexin Chen,1 Huiping Zhang,1 Jishin Lee,1 Xiaohui Liang,1 Xinyan Wu,1 Tao Zhu,1 Pang-kuo Lo,1 Xiaokun Zhang,2 and Saraswati Sukumar1

1The Breast Cancer Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland and
2The Burnham Institute, La Jolla Cancer Research Center, La Jolla, California

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 all-trans 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 HOX5 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

Requests for reprints: Saraswati Sukumar, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB 1 Room 143, Baltimore, MD 21231-1000. Phone: 410-614-2479; Fax: 410-614-4073; E-mail: saras@jhmi.edu.

©2007 American Association for Cancer Research.

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

www.aacrjournals.org


Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 2007 American Association for Cancer Research.
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, TIK,C12, and MCFCA11h were obtained from Fred Miller and Robert Pupey (Karmanos Cancer Institute, Detroit, MI) and were cultured in MCF10A medium (30).

**Plasmids and transient transfection.** The pLE-P1-Luc was constructed by replacing the cytomegalovirus promoter sequence of the reporter plasmid pLE-Luc with HOXA5 promoter sequence, which was amplified with PCR using forward primer 5'-AGAGTCTAACTCCTTGGCCTGCC-3' and reverse primer 5'-CAAGCTTTGTGGCTCCTGGTCTTGT-3'. The pLE-ASGF3-Luc was generated by inserting 6.5 kb of HOXA5 downstream sequence into pLE-P1-Luc vector. To construct the deletion plasmid D1, pLE-ASGF3-Luc was digested with (KpnI) and (EcoRI) to release a small fragment of the downstream sequence followed by filling with Klengon enzyme and self-ligation with T4 ligase. Similarly, pLE-ASGF3-Luc was cut with EcoRI/NdeI to construct D2, BsaBI/NdeI to construct D3, KpnI/AfII to construct D4, and KpnI/MluI to construct D5. The deletion sequences in F1 to F4 was PCR amplified with primers (F1 forward primer 5'-AGGTACCATGAGACACATCCGCTCCA-3'; F2 forward primer 5'-AGTTACCATGAGAGTCGAGACATCC-3'; F3 forward primer 5'-AGTTACACTGCTGTCATGCCCTAA-3'; F4 forward primer 5'-AGTTACCGTGTCCTAAGAAAGGGTGATCAT-3') and reverse primer 5'-ACATGTGCAACACTAGGGTTCAC-CTG-3' and cloned into the D1 at the KpnI/MluI sites. The oligonucleotides containing one or three copies of either site I or II were synthesized and inserted into pG3-Promoter vector to generate 1-1, 1-3, 1-1, and 1-3 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'-AGCTTAAGGGCCGAGGCTGAGATCCCTTGTG-3' and oligo 2a: 5'-AGCTTACGGCCGACGCTGATCTCTTGTG-3' and oligo 2b: 5'-AATTTCAAAAAGGATCTACGGCCGCTGAGATCCCTTGTG-3') into a modified vector pBBS/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'-TCCTCGTGGCTCCTTGAACCTTTG-3' and reverse primer: 5'-CATTAGGACACAGAGATGACGGA-3' and RARβ forward: 5'-CTGTAGATTTGTGCTCTGACT-3' and reverse primer: 5'-CATGTGAGGCTCTGTCCTGTC-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. RARB small interfering RNA (siRNA) was purchased from Dharmacon, Inc. Transient transfection experiments were done using standard procedure with antibodies to HOXA5 (Zymed) and RARβ expressing plasmids. The PCR primers included site II-specific forward primer 5'-AGAACAGGGGATCCTGAGA-3' and reverse primer 5'-CACCCAC-TAGGTTACACTTG-3', and the control forward primer 5'-TGGTGGCTCGGCTGCTGTTTG-3' and reverse primer 5'-CTCGCGGTGTTGTGCTGTC-3'.

**Apoptosis analysis.** Cells (1 × 10^6) in six-well plates were treated with the desired concentration of RA and vehicle for 6 days. A total of 5 × 10^6 cells were used for cell cycle distribution analysis as described previously (29). The sub-G1-G0 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. 1A). 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 MDA MB231 cells, responded to RA treatment (Supplementary Fig. S1). A few cell lines, such as ZR75.1 and MDMB435, 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 RARx 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, MDMB435, and HS578T cells; Fig. 1B). Interestingly, HOXA5 expression was induced by RA in these three 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-IPR, which selectively binds to RARy, very weakly activated HOXA5 expression (35; Fig. 1C). Further, RA-induced HOXA5 expression was competitively inhibited by RARβ-specific antagonist, LE135 (Fig. 1D; 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. 2A). Transient transfection assays showed that the upstream DNA sequence failed to confer RA responsiveness (Fig. 2B). 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
of HOXA5 coding region, we added the 6.5 kb 3’-end DNA sequence to our construct (pLc-A5GF3-Luc; Fig. 2A). The new construct showed a strong response to RA treatment, indicating that the RARE is located in this 3’-end fragment (Fig. 2B).

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. 2C). 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. 2C).

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 μM) 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 alone or in combination with LE135 for 24 h before harvesting for Western blot analysis. * nonspecific band.

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 RXRa-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 RXRa 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. 3A). Conversely, knocking down of RARβ expression in breast cancer cell line Hs578T cells using siRNA blunted the induction of HOXA5 expression by RA (Fig. 3B). 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. 3C). 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. 3D). 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 hyperplastic 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. 3D). 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. 2D). 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. 4A). 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. 4B). 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. 4C).

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 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.

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.
coding region, and showed that among the RARs, RAR\(\beta\) is the critical determinant of induction of HOXA5 and subsequent apoptosis induced by RA.

**HOXA5 is a direct target of RAR\(\beta\) 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 RAR\(\beta\). We also showed that this induction can be disturbed by the presence of RXR\(\alpha\)-Myc, which acts directly downstream of RAR\(\beta\), and pRShRXR\(\alpha\)-Myc, which behaves as a tumor-suppressor gene 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 RAR\(\beta\)-positive cells. This observation led us to reason that RAR\(\beta\) may be the limiting factor for HOXA5 induction or that this specificity may be controlled by other cofactors that display preference for RAR\(\beta\) in breast cancer cells. In either scenario, it seems that RAR\(\beta\) expression is the critical determinant of induction of HOXA5 expression. Consistent with this hypothesis, knockdown of RAR\(\beta\) expression significantly blunted the induction of HOXA5 expression by RA. In addition, the expression of HOXA5 and RAR\(\beta\) was coordinately lost during tumor progression and malignant transformation of MCF10A cells. These findings of an apparent preference for RAR\(\beta\), however, do not exclude the possibility that, under certain conditions, HOXA5 expression can be regulated by RA via other RAR or RXR isofoms. Also, although HOXA5 is the direct target of RAR\(\beta\), we found that high expression of RAR\(\beta\) alone may not predict the ability of RA to induce HOXA5 expression. For instance, MDAMB435 cells express high levels of RAR\(\beta\) but failed to induce HOXA5 expression after RA treatment (Fig. 1B). Similarly, we could not restore HOXA5 induction by reintroducing RAR\(\beta\) into RAR\(\beta\)-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 RAR\(\alpha\) and RAR\(\gamma\) in breast cancer cells, RAR\(\beta\) expression is lost in majority of breast cancer cell lines and primary breast carcinomas (33, 43), suggesting that RAR\(\beta\) may play an important part in breast tumorigenesis. Several studies have shown that RAR\(\beta\) behaves as a tumor-suppressor gene in breast cancer and that induction of RAR\(\beta\) mediates RA-induced growth inhibition and apoptosis (12, 15, 44–47). However, the mechanism by which RAR\(\beta\) 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 RAR\(\beta\) 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.
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 is 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 would need to be identified to test this hypothesis.

**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-G0-G1 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.
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).

References

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

Hexin Chen, Huiping Zhang, Jishin Lee, et al.