ARTICLE
Journal of Cellular Biochemistry 111:1437–1444 (2010)

Journal of Cellular Biochemistry

Expression of Homeobox Genes in Oral Squamous Cell Carcinoma Cell Lines Treated With All-*Trans* Retinoic Acid

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ABSTRACT

Oral squamous cell carcinoma (OSCC) may arise from potentially malignant oral lesions. All-*trans* retinoic acid (atRA), which plays a role in cell growth and differentiation, has been studied as a possible chemotherapeutic agent in the prevention of this progression. While the mechanism by which atRA suppresses cell growth has not been completely elucidated, it is known that homeobox genes are atRA targets. To determine if these genes are involved in the atRA-mediated OSCC growth inhibition, PCR array was performed to evaluate the expression of 84 homeobox genes in atRA-sensitive SCC-25 cells compared to atRA-resistant SCC-9 cells following 7 days with atRA treatment. Results showed that the expression of 8 homeobox genes was downregulated and expression of 4 was upregulated in SCC-25 cells but not in SCC-9 cells. Gene expression levels were confirmed for seven of these genes by RT-qPCR. Expression of three genes that showed threefold downregulation was evaluated in SCC-25 cells treated with atRA for 3, 5, and 7 days. Three different patterns of atRA-dependent gene expression were observed. ALX1 showed downregulation only on day 7. DLX3 showed reduced expression on day 3 and further reduced on day 7. TLX1 showed downregulation only on days 5 and 7. Clearly the expression of homeobox genes is modulated by atRA in OSCC cell lines. However, the time course of this modulation suggests that these genes are not direct targets of atRA mediating OSCC growth suppression. Instead they appear to act as downstream effectors of atRA signaling. J. Cell. Biochem. 111: 1437–1444, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ORAL SQUAMOUS CELL CARCINOMA; RETINOIC ACID; HOMEOBOX

Oral squamous cell carcinoma (OSCC) is the most common oral malignancy. Despite the fact that significant effort has been devoted to the identification of disease and prognostic markers, and to the improvement of treatment options, 5-year survival rate remains around 50% [Kim and Califano, 2004]. A major problem associated with OSCC treatment is related to the development of second primary tumors (SPT), which presents a risk of 2–7% per year in long-term survivors. With the aim of improving survival rates, researches have focused on the use of chemoprevention agents, such as all-*trans* retinoic acid (atRA), α-tocopherol, and β-carotene to block or reverse the oral carcinogenesis process [Hayashi et al., 2001; Ralhan et al., 2006]. Previous studies using OSCC cell lines have shown that OSCC cell growth can be suppressed by treatment with atRA [Le et al., 2000, 2002]. Therefore, atRA treatment of OSCC cell lines may represent a good in vitro model to study the molecular mechanism of retinoid-mediated OSCC growth suppression. Identification of cellular and molecular mechanisms triggered following atRA treatment of OSCC may lead to the development of new strategies to reduce toxicity and disease relapse, thereby improving patient treatment and survival rates [Wrangle and Khuri, 2007].

Retinoids are a large family of natural and synthetic derivatives of retinol (vitamin A) that promote differentiation and regulate cell growth in both embryonic and adult tissues. The ability to affect cell growth and differentiation of various cell types is related to the ability of atRA to alter the expression of a wide variety of gene types. This is likely to be the reason why no single common mechanism of atRA action has been identified [Soprano and Soprano, 2002; Mongan and Gudas, 2007].

One important mechanism by which atRA exerts its action is mediated by homeobox genes. Homeobox genes represent a large

Additional supporting information may be found in the online version of this article.

Grant sponsor: NIH; Grant numbers: DK070650, CA64945; Grant sponsor: CAPES (Brazil) Scholarship; Grant number: PDEE 0138-08-8.

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Received 26 August 2010; Accepted 30 August 2010 • DOI 10.1002/jcb.22871 • © 2010 Wiley-Liss, Inc.
Published online 9 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

family of transcription factors that regulate cell fate and differentiation. In mammals, over 200 homeobox genes have been described and are divided into two families, clustered homeobox genes, also called HOX, and non-clustered homeobox genes. HOX genes participate in positional specification of the anterior–posterior axis in the developing embryo. They are expressed in a manner such that genes located at the 3′ end of the gene cluster are expressed at the anterior portion of embryo and earlier in development, while genes located at the 5′ end are expressed at posterior areas and later during development [Abate-Shen, 2002]. It has been shown that atRA can partially influence this expression pattern. Studies of embryonal carcinoma cell lines treated with various atRA concentrations for different periods of time demonstrated that HOX genes located near the 3′ end of the gene cluster are induced extremely rapidly by low atRA concentrations. It was also observed that as the distance from the 3′ end increases, time and atRA concentration required for gene induction also increase. Genes located at the 5′ end of the gene cluster, generally do not have their expression activated by atRA [Simeone et al., 1991; Langston and Gudas, 1994]. Additional evidence that atRA is an important regulator of homeobox gene expression is suggested by the fact that retinoic acid responsive elements (RARE) have been identified in the promoter region of HOXA1, HOXB1, HOXD4, CDX1, and MSX1 [Shen et al., 1994; Morrison et al., 1996; Langston et al., 1997; Lickert and Kemler, 2002]. The importance of homeobox genes for cell differentiation has raised the question whether these genes might have an altered expression during carcinogenesis. Studies have already demonstrated the relationship between several types of cancer such as breast, lung, endometrial, and OSCC and altered expression of these genes [Zhu et al., 2004; Hassan et al., 2006; Tommasi et al., 2009].

In order to evaluate if homeobox genes mediate atRA-dependent OSCC growth arrest, we compared the expression of 84 homeobox genes in RA-sensitive versus atRA-resistant OSCC cell lines following treatment with atRA. Our results show that the expression of a limited number of homeobox genes are differentially regulated in OSCC cells growth inhibited by atRA. However, the time course of homeobox gene expression suggests that these genes do not directly mediate cell differentiation and growth arrest by atRA but instead are downstream effectors of atRA signaling in these OSCC cell lines.

METHODS

CELL CULTURE

For this study, human squamous cell carcinoma of tongue cell lines SCC-9 and SCC-25 were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cell lines were cultivated in 100 mm cell culture dishes with a 1:1 mixture of Dulbecco's modification of Eagle's medium and Ham's F-12 (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Cellgro, Herndon, VA), 2 mM L-glutamine (HyClone, Thermo Fisher Scientific, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech Cellgro) at 37°C in a humidified atmosphere of 5% CO_2. Cell counting was performed using a hemocytometer chamber. Trypsinized cells were mixed with 0.4% trypan blue (GIBCO, Invitrogen Corporation) in a ratio of 1:1 and 10 μl of the cell suspension was loaded in the chamber. All cell counts were performed in triplicate.

CELL PROLIFERATION

Powder form of atRA was kindly provided by Hoffmann-LaRoche, Inc. (Nutley, NJ). Stock solutions (10^{-3} M) were prepared in ethanol and stored at −20°C. For quantitative assays of cell proliferation following atRA treatment of SCC-9 and SCC-25 cell lines, 1×10^5 cells were seeded in 100 mm plates in regular growth medium and treated with atRA at the concentration of 10^{-6} M as previous described [Le et al., 2000]. Cell medium was changed every 2 days and atRA at 10^{-6} M was replaced. Control cultures were treated with an equivalent amount of ethanol for the same time period. Cell counting was performed on days 1, 3, 5, and 7 in triplicate. All procedures involving atRA were performed under subdued light.

PCR ARRAY

After atRA treatment for 7 days, SCC-9 and SCC-25 cells were rinsed twice with 1× PBS and detached from the culture plate using 1× trypsin–EDTA (GIBCO, Invitrogen Corporation). RNA isolation was performed with the RT^2 qPCR-Grade RNA Isolation Kit (SABiosciences, Frederick, MD), as recommended by the PCR Array protocol. RNA amount and purity was determined using a spectrophotometer (Beckman Du 640). OD260/OD280 ratio for all samples was between 1.9 and 2.1. RNA samples were stored at −70°C. cDNA synthesis was performed using RT^2 First Strand Kit (SABiosciences) and 2 μg of RNA, according to the manufacturer's protocol. For each sample, RT product was diluted in 91 μl of double distilled water and kept at −20°C. PCR array plates were purchased from SABiosciences for 84 homeobox genes. The complete gene list is included in Supplementary Data 1. PCR array cocktail was prepared with 2× SABiosciences RT2 qPCR master mix (SABiosciences) according to manufacturer protocol and 25 μl of the cocktail was distributed into each of the 96 wells of the PCR Array plates. Gene expression profile was determined using the Applied Biosystems 7500 PCR System (Applied Biosystems, Foster City, CA) with cycling conditions indicated by the manufacturer. For all PCR array plates, controls for genomic DNA contamination and reverse transcription were performed in accordance with optimal conditions required for success of PCR array experiments as suggested by the supplier.

RT-QPCR VALIDATION AND TIME-COURSE ANALYSES

SCC-9 and SCC-25 cell lines were treated with atRA or ethanol for 3, 5, and 7 days and RNA isolation was performed with RNA-Bee solution (Iso-Tex Diagnostics, Inc., Friendswood, TX), following the instructions provided by the manufacturer. RNA amount and purity were evaluated as previously described and samples were stored at −70°C. cDNA was synthesized using RT-PCR first strand cDNA synthesis kit (BD Biosciences, Clontech Laboratory, Inc., Mountain View, CA) using 1 μg of total RNA according to the manufacturer's protocol. Each cDNA reaction was further diluted with 80 μl of DEPC-treated water to obtain a final volume of 100 μl and stored at −20°C. Real-time quantitative PCR was performed in a 7500 PCR System (Applied Biosystems) in 96-well plates using the SYBR green method. Primer sequences used in this work are listed in Table I. Sequences were obtained from Primer Bank [Wang and Seed, 2003;

TABLE I. Primers Used for qPCR Analyses

Gene	Accession no.	Primer 5′ → 3′	Position	Product size
ALX1	NM_006982	F: AATGTCTCCCGTGAAAGGGAT	313–333	102
		R: GTTCGGTGCCTCCGTTTCTTA	414–394	
DLX3	NM_005220	F: TTACTCGCCCAAGTCGGAATA	463–483	173
		R: AGTAGATTGTACGCGGCTTTC	635–615	
DLX6	NM_005222	–[a]	–	66
HLX	NM_021958	F: CAGGTCCCTATGCTGTGCTC	1,212–1,231	141
		R: GCTTGGTCACGTACTTCTGAAT	1,352–1,331	
HOPX	NM_139211	F: GACAAGCACCCGGATTCCA	226–244	147
		R: GTCTGTGACGGATCTGCACTC	372–352	
HPRT	NM_000194.1	F: TTCTTTGCTGACCTGCTGG	305–323	119
		R: TCCCCTGTTGACTGGTCAT	423–405	
SIX3	NM_005413	F: AAGAGTTGTCCATGTTCCAGC	431–451	171
		R: GGATCGACTCGTGTTTGTTGA	601–581	
TLX1	NM_005521	F: CACGCAGAGCCCATTAGCTT	1,055–1,074	103
		R: GGCCGTATTCTCCGTCCTG	1,157–1,139	

F, forward; R, reverse.
[a]Primer pairs purchased from SABioscience.

Spandidos et al., 2008]. DLX6 primers were purchased from SABiosciences (catalogue number is PPH15032A). All primer sets yielded a dissociation curve with a single peak and a single DNA product of the appropriate size when separated by electrophoresis on an acrylamide gel. For each sample, a mix containing 15 μl of cDNA, 50 μl of SYBR green PCR Master Mix (Applied Biosystems), 200 nM of each primer and double distilled water up to a volume of 100 μl was prepared and mixed. Thirty microliters of the mix was distributed in three different wells. RT-qPCR reactions were incubated at 50°C for 2 min, followed by an initial denaturation step at 95°C for 10 min. This was followed by 40 amplification cycles that consisted of a 15 s denaturation step at 95°C, a 30 s annealing step at 60°C, and a 40 s extension step at 68°C. All procedures were performed with three separately isolated RNA samples.

DATA ANALYSES AND STATISTICS

PCR array data were normalized using the average of five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB) included in the PCR array plate. Individual qPCR reactions were normalized using hypoxanthine phosphoribosyltransferase 1 (HPRT1). All analyses were conducted using relative quantification analyses based on the $2^{-\Delta\Delta}$ method. Fold changes were calculated between ethanol and RA groups for each cell line. All experiments were performed in triplicate and results presented as the mean ± standard deviation. Student's *t*-test was used to determine statistical significance. *P*-values <0.05 were considered statistically significant.

RESULTS

EFFECT OF ATRA ON OSCC CELL PROLIFERATION

The effect of atRA treatment on OSCC cell growth was determined. Direct cell counting was used to generate 7-day cell growth curves for two OSCC cell lines, SCC-9 and SCC-25, following treatment with atRA (10^{-6} M) or ethanol. As can be seen in Figure 1a, SCC-9 cells exhibited similar cell numbers over the 7-day treatment period when treated with either atRA or ethanol. Figure 1b plots the data as percentage of cell growth inhibition. Since a similar cell number was observed following treatment with either atRA or ethanol at each time point, no growth inhibition was observed. Thus, this cell line was considered resistant to atRA treatment (Fig. 1b). In contrast, SCC-25 cells treated with atRA exhibited a lower growth rate compared to cells treated with ethanol (Fig. 1a). This growth inhibition increased over time and showed maximum inhibition on day 7, when an inhibition rate of 42% was observed ($P < 0.01$). Based on these results the SCC-25 cell line was considered sensitive to atRA treatment (Fig. 1b). The mean inhibition rate obtained in three independent sets of cells used for the PCR array was 6.5% (±5.7%) for SCC-9 cells and 43.3% (±3.2%) for SCC-25 cells.

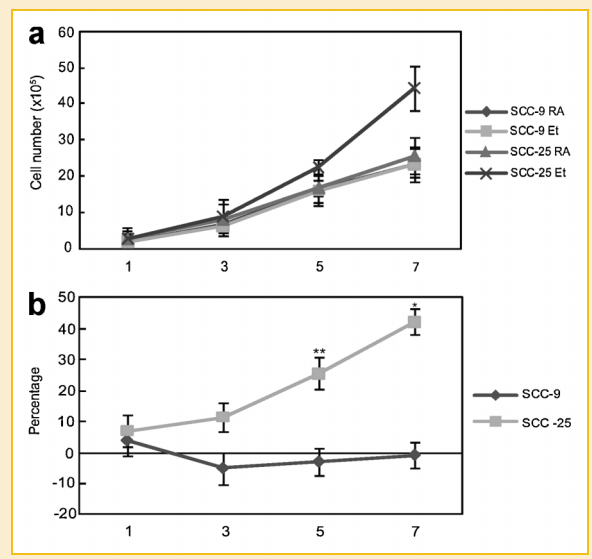

Fig. 1. Effects of atRA treatment on cell proliferation of oral squamous cell carcinoma cell lines SCC-9 and SCC-25. a: Cell number was monitored by direct cell counts using a hemocytometer after 1, 3, 5, and 7 days of treatment. SCC-9 cells treated with the vehicle ethanol (■) and atRA (◆) had a similar cell number at all time points evaluated. SCC-25 cells treated with atRA (▲) exhibited a lower cell number when compared to ethanol (×) on days 3, 5, and 7 and this difference increased over time. b: Inhibition rate for both cell lines was calculated at all time points. SCC-9 cells (◆) were not growth inhibited by atRA treatment while SCC-25 (■) showed an increasing level of inhibition under the same conditions (*P-value <0.02; **P-value <0.03).

PCR ARRAY

To determine if homeobox gene expression mediates atRA effects on OSCC cells growth, a commercially prepared PCR array containing 84 homeobox genes was used. SCC-25 cells sensitive to atRA and SCC-9 cells not sensitive to atRA, were treated with either ethanol or atRA (10^{-6} M) for 7 days. On day 7, total RNA was isolated from each treatment group.

PCR array results were analyzed according to the $2^{-\Delta\Delta}$ method, using as an internal normalizing control the average of five housekeeping genes for each PCR array experiment. All analyses were performed comparing atRA and ethanol-treated samples. Results with twofold difference were considered positives. Among the 84 genes evaluated, only two genes were found to exhibit upregulated expression in SCC-9 cells (HOXD12 and PROX1) and two genes exhibited downregulated expression (MSX2 and MEOX1) following atRA treatment. When the same analysis was performed for SCC-25 cells, nine genes showed a downregulation greater than twofold following RA treatment (ALX1, ALX4, DLX3, DLX6, HLX, HOPX, MEOX1, SIX3, and TLX1), while the expression of four genes was upregulated (EN2, HOXB3, HOXD1, and VAX1) by atRA. Fold induction, standard deviation and *P*-value for these genes are summarized in Table II.

VALIDATION BY RT-QPCR

Genes exhibiting a twofold downregulation in expression in atRA-sensitive SCC-25 cells, and not showing significant changes in expression in atRA-resistant SCC-9 cells, were considered to be the best candidates for atRA target genes mediating growth suppression. Genes fulfilling this criterion were selected for validation by RT-qPCR using samples treated for 7 days. A total of seven genes were analyzed (ALX1, DLX3, DLX6, HLX, HOPX, SIX3, and TLX1). Analysis of expression in SCC-9 cells showed that none of the genes exhibited a fold change >2 (Fig. 2a). These results confirm the PCR array results which also showed no significant difference in the expression of these genes in SCC-9 cells following atRA treatment. In contrast, results for RT-qPCR analysis of SCC-25 cell samples confirmed that the expression of each of the seven genes was downregulated and fold change values were similar to those observed in the PCR array analysis (Fig. 2b). Statistical analysis showed that fold difference was statistically significant for six of the genes (ALX1, DLX3, HLX, HOPX, SIX3, and TLX1). The only gene that did not show a statistically significant downregulation of expression was DLX6. These results confirm that these genes were downregulated during growth inhibition mediated by atRA treatment in OSCC cells.

TABLE II. Summary of Homeobox Genes That Displayed Up- or Downregulation in Expression After Treatment of SSC-9 Cells and SSC-25 Cells With RA by PCR Array Analysis

Gene	Fold change	Standard deviation	*P*-value
SCC-9			
HOXD12	3.07	0.23	0.0883
MEOX1	−6.15	0.16	0.0427
MSX2	−2.97	0.34	0.0542
PROX1	2.89	1.05	0.0617
SCC-25			
ALX1	−7.66	0.10	0.0205
ALX4	−2.12	0.33	0.0583
DLX3	−2.44	0.08	0.0009
DLX6	−2.48	0.06	0.0264
EN2	2.22	0.67	0.0031
HLX	−2.25	0.07	0.0020
HOPX	−2.28	0.29	0.0249
HOXB3	2.07	1.70	0.1504
HOXD1	2.72	0.41	0.0002
MEOX1	−3.06	0.11	0.0294
SIX3	−2.23	0.39	0.0821
TLX1	−2.38	0.48	0.2075
VAX1	3.97	0.75	0.1081

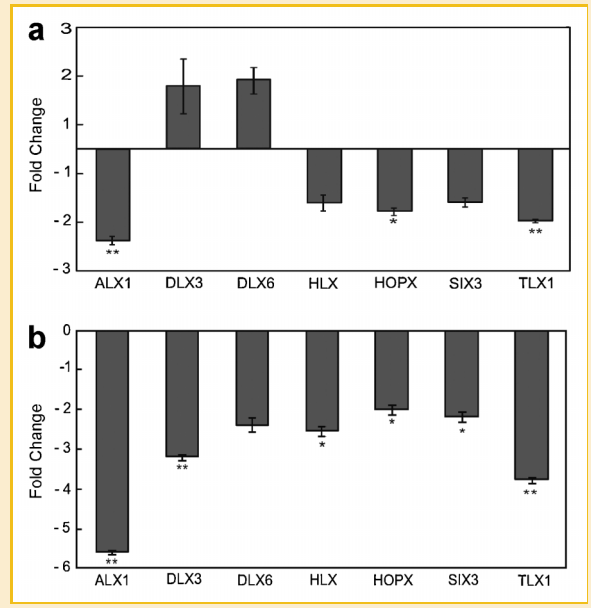

Fig. 2. Fold change in expression of genes identified in the PCR array after validation by RT-qPCR. a: Gene expression in SCC-9 cells confirmed results obtained by PCR array since none of the seven genes studied presented a fold change higher than two (*P-value <0.05; **P-value <0.03). b: Fold change values for SCC-25 cells confirmed that all genes are downregulated under treatment with atRA for 7 days and the results obtained are similar to the ones observed for PCR array (*P-value <0.05, **P-value <0.02).

TIME-COURSE ANALYSIS

To determine if the downregulation of homeobox genes observed in the PCR array experiment, and confirmed by RT-qPCR, represented a growth regulatory or growth regulated molecular event, a time-course analysis was performed. Growth regulatory events are likely to mediate growth suppression while growth regulated events usually occur as a result or consequence of growth inhibition. We reasoned that atRA modulated homeobox gene expression would most likely represent a growth regulatory event if it was observed early following atRA treatment, while modulated expression late after atRA treatment was most likely a result of the fact that cell growth was inhibited. Expression of homeobox genes that showed threefold or more downregulation (ALX1, DLX3, and TLX1) was analyzed by qPCR on SCC-25 cell samples treated with atRA for 3, 5, and 7 days. Figure 3 shows that ALX1 gene downregulation occurred on day 7 following atRA treatment but not on days 3 and 5. A significant and similar level of downregulated expression of DLX3 was observed on day 3 and on day 5 following atRA treatment. A

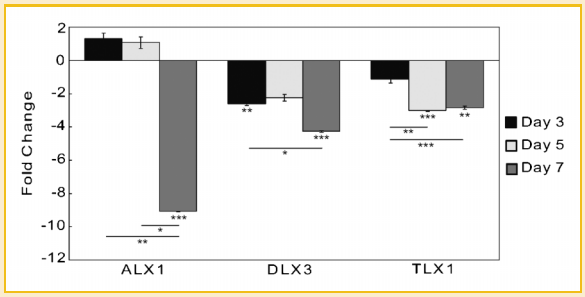

Fig. 3. Time-course analysis of homeobox genes treated with retinoic acid. Fold change values in expression of ALX1, DLX3, and TLX1 in SCC-25 cell samples treated with atRA for 3, 5, and 7 days ($^{*}P$-value <0.05, $^{**}P$-value <0.03, and $^{**}P$-value <0.01).

slightly greater reduction in expression was observed on day 7. TLX1 did not show a significant downregulation on day 3, but exhibited a significant downregulated expression on days 5 and 7 (Fig. 3).

DISCUSSION

This study evaluates for the first time the expression of members of the homeobox gene family in OSCC cells that are growth suppressed following atRA treatment. Our results showed that, from the 84 homeobox genes studied, 13 were differentially expressed following atRA treatment. Among them, nine were downregulated and four were upregulated. A more detailed analysis of the gene expression following treatment of OSCC cells with atRA for various periods of time was performed for three genes (ALX1, DLX3, and TLX1). Results of this experiment suggest that downregulation most likely represented an indirect response to atRA treatment. Therefore, these results suggest that homeobox gene downregulation occurred as an indirect result of growth inhibition mediated by atRA.

RA has been previously shown to promote growth inhibition of OSCC cell lines such as SCC-15, SCC-25, and KB. A number of studies have shown that OSCC growth inhibition by atRA is principally related to cell-cycle arrest during G1 phase, does not involve induction of apoptosis or cell death, and is accompanied by an increase in cytokeratin 13 expression and alkaline phosphatase activity, suggesting that RA induces cell differentiation [Yang et al., 2001; Kuroda et al., 2005; Xiao et al., 2006; Zhao et al., 2009]. In the present work, SCC-25 cells exhibited a growth inhibition rate of ~42% after 7 days of RA treatment, consistent with previous reports from our laboratory [Le et al., 2000, 2002]. In contrast, SCC-9 cells did not exhibit growth inhibition following atRA treatment. Differences in SCC-9 cell response to atRA observed by our laboratory [this study and previous reports by Le et al., 2000, 2002] versus a previous report by Yang et al. [2001] may reflect cell culturing differences. Nevertheless, this has no implications on the results of the present work, since in all steps of the present work SCC-9 cells behaved as a resistant cell line to atRA growth suppression.

PCR array results were analyzed using the $2^{-\Delta\Delta}$ method and revealed that both cell lines exhibited genes with two or more fold change in expression. SCC-9 cells exhibited four genes in this category, two upregulated (PROX1 and HOXD12) and two downregulated (MEOX1 and MSX2). Considering that this cell line did not show changes in cell growth following atRA treatment, it is likely that these four genes might be related to other, non-growth regulated cell process. In fact MSX2 and PROX1 expression have been associated with cell adhesion during tumorigenesis. For example, mouse mammary epithelial cells in which MSX2 was overexpressed exhibited downregulation of E-cadherin, upregulation of vimentin and *N*-cadherin, and showed higher invasive capacity [di Bari et al., 2009]. Likewise, PROX1 has been shown to contribute to tumor progression by suppressing cell adhesion and regulating actin cytoskeleton in colorectal cancer [Petrova et al., 2008].

The SCC-25 cell line expressed nine genes that were downregulated and four genes that were upregulated following atRA treatment. Homeobox genes, especially the HOX family, have been described as atRA inducible during embryonic development and RAREs have been identified in the promoter sequences of five of these genes (HOXA1, HOXB1, HOXD4, CDX1, and MSX1). For this reason, we expected to identify a number of genes with upregulated expression following atRA treatment. The results presented here; however, show that from the 84 genes evaluated, only 13 were differentially expressed and none of these genes were those with previously identified RAREs. Another interesting aspect from our data is the fact that the majority of genes differently expressed were downregulated instead of upregulated. This expression profile might be related to the homeobox expression pattern during carcinogenesis. Homeobox genes are known to be master regulators of development whose aberrant expression in cancer can present three patterns. In the first two patterns, homeobox genes exhibit a gain of expression in the tumor compared to the tissue that gives rise to the tumor; however, in one scenario the gene is expressed in the respective embryonic tissue and in the other it is not. The third pattern presents a loss of gene expression in tumor cells compared to the cells in the fully differentiated state [Abate-Shen, 2002]. The common characteristic among the three patterns is that the aberrant expression of homeobox genes leads cells to a less differentiated and embryonic-like state with enhanced proliferation and cell survival. Under this aspect, it is likely that the up- or downregulation observed after atRA treatment represents the return of homeobox gene expression to a pattern more similar to the one observed in fully differentiated oral epithelium, since it is known that OSCC undergoes differentiation following atRA treatment. It is also important to note the fact that more downregulated genes were identified. This suggests that the gain of homeobox expression during oral carcinogenesis may be a more usual mechanism than the loss of expression. Future studies comparing expression of homeobox genes identified by this study in normal oral mucosa, dysplasia, and more and less aggressive tumors would provide strong support for this hypothesis.

Another interesting aspect is that from the 13 genes differentially expressed, 10 showed fold-change values between two and three. If homeobox gene expression plays a major role mediating atRA action in OSCC, it would be expected that a higher percentage of genes would be differently expressed accompanied by a greater fold

change in expression values for some of the genes, similar to what was observed for ALX1 and VAX1. These data suggest that homeobox genes may not be a primary target of atRA in OSCC. However, it has been demonstrated that homeobox expression induced by atRA can be modulated by other cell signaling pathways. For example, treatment of neural crest cells with atRA induces HOXA2 expression. However, cell stimulation with BIO, a glycogen synthase kinase-3 (GSK-3) inhibitor that activates canonical Wnt signaling, reduces HOXA2 expression [Ishikawa and Ito, 2009]. Mechanisms such as the one described can be particularly important in OSCC development, since several cell pathways may be altered, and they could possibly interfere with atRA signaling and homeobox expression.

It is interesting to note that MEOX1, which participates in somite development, has been shown to be overexpressed in Wilm's tumor and predicted to be involved in mesenchymal–epithelial transition, was the only gene found to be downregulated in both, SCC-9 and SCC-25, with fold change of approximately −6 and −3, respectively [Bard et al., 2008]. Since the SCC-9 cell line is not growth inhibited by atRA, MEOX1 downregulation observed here is probably related to some mechanism other than cell differentiation and cell-cycle arrest promoted by atRA. Since MEOX1 downregulation has been shown to be trigged by ALR (MLL2) knockdown in a cervical cancer cell line and is followed by cytoskeleton reorganization and a decrease in tumorigenicity, it is possible that MEOX1 may be involved in cytoskeleton reorganization [Issaeva et al., 2007].

Since the PCR array method is based on the use of qPCR technology to screen genes differently expressed under experimental conditions, it is more quantitative and reproducible than microarray. Once qPCR is used in this method, one could argue that validation is not necessary; however, this step was chosen to certify that results from the PCR array were valid and reproducible. Since downregulation of homebox genes with atRA seemed to be an important aspect in regulating growth and differentiation in OSCC, we focused only on the downregulated genes identified in the array. From the nine genes in this category, genes with the greatest downregulation were selected for further study. MEOX1, which was also downregulated in SCC-9 cells, was excluded from this evaluation. Seven genes were selected for validation (ALX1, DLX3, DLX6, HLX, HOPX, SIX3, and TLX1). Results confirmed that in atRA-resistant OSCC cells, these genes did not show significant changes in expression (Fig. 2a) while in the atRA-sensitive OSCC cells, all these genes showed downregulated expression greater than twofold (Fig. 2b).

Genes downregulated threefold were submitted to a time-course analyses (ALX1, DLX3, and TLX1). During this step, expression levels were evaluated in SCC-25 cell samples treated with RA for 3, 5, and 7 days. ALX1, also called CART1, is mostly related to skeletogenesis, especially from head, and neural tube development. In cancer development, its expression was described only in a chondrosarcoma cell line, not in any epithelial tumor [Qu et al., 1999]. The present work observed that ALX1 did not exhibit a significant fold change on days 3 and 5 of RA treatment of OSCC; however, on day 7 it was downregulated by ~9-fold ($P = 0.0036$). This indicates that the downregulation of ALX1 after atRA treatment occurs later after cell treatment and suggests that reduction in ALX1 expression following atRA treatment is mediated by other factors.

DLX3 (distal-less homeobox 3) is a homeobox gene from the distal-less sub-family and is located on the long arm of chromosome 17 (17q21) [Merlo et al., 2000]. The role of DLX3 in humans is mostly related to osteogenesis and craniofacial development and its expression has also been described in placenta, epidermis, tooth, and hair folicule [Hassan et al., 2004]. One major pathway in which DLX3 participates involves p63 regulation. p63 plays an important role in development and maintenance of stratified epidermis. There are six p63 isoforms known that result from the presence (TA) or absence (ΔN) of N terminal transactivation domain and alternative splicing at the C terminus (α, β, and γ). It has been demonstrated that p63 isoforms TAp63α and TAp63γ, respectively, activates or represses DLX3 transcription. Interestingly, DLX3 induces Raf1 phosphorylation, which leads to phosphorylation followed by degradation of ΔNp63α (Fig. 4). The role played by DLX3 in p63 isoform regulation might be relevant in oral carcinogenesis, since ΔNp63α, the p63 isoform degraded by DLX3, has been described in OSCC and its loss is associated with an increase in cell invasion capacity [Higashikawa et al., 2007; Radoja et al., 2007; Di Costanzo et al., 2009]. In this study, DLX3 fold change values were >2 at all time points evaluated. Additionally, it was observed that on days 3 and 5, fold change values were, respectively, −2.59 ($P = 0.0247$) and −2.23 ($P = 0.0945$). However, on day 7 a further increase in downregulation was detected, when fold change value was −4.24 ($P = 0.0055$). This indicates that in contrast to our results with ALX1, DLX3 expression presents a more rapid and consistent downregulation. In the literature only one study correlated DLX3

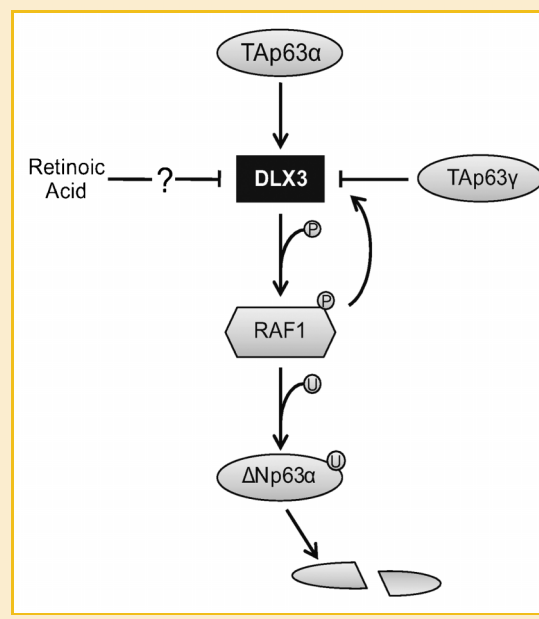

Fig. 4. Mechanisms of DLX3 activation and interaction. p63 isoforms TAp63α and TAp63γ, respectively, activates and repress DLX3 transcription. atRA is also predicted to repress DLX3 transcription. DLX3 promotes Raf1 activation which leads to ubiquitinization and degradation of ΔNp63α. Raf1 in a feedback loop, activates DLX3.

and atRA. Shimizu and colleagues evaluated gene expression in limb development after atRA treatment and showed that DLX3 exhibited downregulation, a result which is consistent with our data [Shimizu et al., 2007].

TLX1 (HOX11) is a homeobox gene whose expression in mouse embryo has been described in organs and tissues derived from the three germ cell layers but its role in cancer development is still unclear. It was initially described as a proto-oncogene in acute lymphoblastic leukemia where translocation on chromosome 10 leads to TLX1 overexpression [Hatano et al., 1991]. However, methylation-driving gene silencing of TLX1 has been demonstrated to be an early event in breast cancer development contributing to tumor development [Tommasi et al., 2009]. Our data demonstrated that TLX1 exhibits significant downregulation on day 5 (-3.01; $P = 0.0031$) and 7 (-2.82; $P = 0.0209$), but not on day 3 (-1.13; $P = 0.1620$). This result suggests that TLX1 is not downregulated until later after RA treatment (day 5) but this reduced expression is sustained over time. Since this loss of expression occurs later after atRA treatment, it is likely that it is mediated by an indirect mechanism or as a result of changes in the growth of the cells.

These analyses indicate that the modulation of the three genes by atRA is mostly likely through an indirect effect. However, expression of DLX3, which showed a significant downregulation on day 3, should be evaluated in future studies at earlier time points following atRA treatment. It is possible that downregulation of DLX3 might occur more rapidly following atRA treatment and therefore might represent a direct target of atRA. If DLX3 is in fact identified as a direct target of atRA it is more likely to represent an important mediator of atRA growth inhibition.

In summary, our studies show that homeobox genes, including those previously described as RA responsive, are not direct targets of atRA responsible for mediating atRA-dependent OSCC growth suppression but instead act as downstream effectors of the atRA signaling pathway.

ACKNOWLEDGMENTS

This work was supported by NIH Grants DK070650 and CA64945 and CAPES (Brazil) Scholarship PDEE 0138-08-8.

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