

Distinct patterns of all-*trans* retinoic acid dependent expression of *HOXB* and *HOXC* homeogenes in human embryonal and small-cell lung carcinoma cell lines

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Abstract The expression patterns of the class I homeogenes *HOXB* and *HOXC* clusters in the presence of retinoic acid (RA) were studied in two human small-cell lung cancer (SCLC) cell lines and compared to that of NT2/D1 embryonal carcinoma cells. Contrasting with the sequential 3'-5' induction of the *HOX* genes observed after RA treatment of embryonic NT2/D1 cells, in the SCLC cells the responding genes (induced or down-regulated) were interspersed with insensitive genes (expressed or unexpressed), while no genomic alteration affected the corresponding clusters. These findings imply that *HOX* gene regulatory mechanisms are altered in non-embryonic SCLC cells, perhaps reflecting their ability to respond to more diversified stimuli, in relation with their origin from adult tissues.

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Key words: Retinoic acid; *HOX* gene; Small-cell lung cancer

1. Introduction

Homeobox containing genes are a family of evolutionarily highly conserved transcription factors (reviewed in [1,2]). The class I homeogenes encode proteins containing a homeodomain closely related to that of the archetypal *Drosophila antennapedia* domain. In human, the 39 members of the *HOX* family are tandemly arranged in four clusters of 90–120 kilobases borne by chromosomes 7 (*HOXA*), 17 (*HOXB*), 12 (*HOXC*) and 2 (*HOXD*) [3]. Within the clusters, each gene can be assigned to one of 13 paralogous groups, with successively higher numbered paralogous groups being located more 5' within each cluster. The expression of class I homeogenes is strictly regulated temporally and spatially during embryonic development [4]. The genes are expressed in overlapping domains, displaying unique anterior boundaries of expression for each gene, colinear with their order within the cluster.

All-*trans* retinoic acid (RA) is both an important signaling molecule in embryonic development and cell differentiation [5,6] and a useful drug for the treatment of several types of cancer [7]. In embryonal carcinoma (EC) cells, the expression of class I homeogenes can be modulated by RA [6,8–10]. *HOX* genes are activated by RA in a sequential order, colinear with their 3'-5' localization in the cluster: genes in 3' respond early to treatment, whereas upstream genes respond progressively later, a situation corresponding to the anterior to posterior pattern of expression in the embryo (temporal

colinearity). Similar sequential responses were obtained after inhibition of *HOX* gene expression by antisense oligodeoxynucleotides [11]. These data suggest a cascade mechanism in the regulation of *HOX* gene expression, operating on extended regions, with 3'-5' polarity [8].

Two types of RA binding proteins mediate RA effects: the cellular RA binding proteins, CRABP1 and 2, and the RA receptors, RAR α , β and γ . The roles of CRABP1 and CRABP2 are only partially understood. It has been proposed that CRABP1 sequesters RA in the cytoplasm and prevents its nuclear uptake [12]. RARs are ligand dependent inducible transcription factors (reviewed in [13]). The RARs can activate gene expression directly through RA responsive elements (RAREs) localized in their target genes. Functional RAREs are currently known for only a few *HOX* genes [8].

After embryogenesis, *HOX* genes continue to be transcribed according to a tissue specific pattern of expression. In cancer cells, alterations of this pattern, as compared to the corresponding normal mature tissues, have been described (reviewed in [2,14]). However, few data are available on *HOX* gene regulation in adult tissues.

Using two small-cell lung cancer (SCLC) cell lines, we report here that, in these mature tissue derived cells, the sequential 3'-5' induction of the *HOX* genes by RA is not observed. In contrast, the responding genes (induced or down-regulated) are interspersed with insensitive genes (expressed or unexpressed).

2. Materials and methods

2.1. Cell culture and treatments

The human EC cell line NT2/D1 was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in DMEM medium supplemented with 10% fetal calf serum. SCLC cell lines were obtained from human xenografted SCLC previously characterized [15,16]. The SCLC cells were grown in RPMI medium supplemented with 10% fetal calf serum. A stock solution (10 mM) of RA was prepared in ethanol and appropriately diluted with culture medium at optimal concentrations, according to each cell line's responsiveness. The culture medium containing RA was changed every 2 days with freshly prepared RA containing solution. We checked that the addition of ethanol alone did not influence the expression of the studied genes. RNAs were prepared using a Trizol kit according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD, USA). Two successive Trizol extractions were performed.

2.2. Gene expression analysis

The primers used for reverse transcription-polymerase chain reaction (RT-PCR) analysis of the *HOXB1-9* [16], *HOXB13* [17], *RAR α* , β and γ [18] and *CRABP2* [19] expression have previously been reported. The oligonucleotides used for RARs did not discriminate between the various isoforms. The primers synthesized for the present

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study are listed in Table 1. Human sequences were available only for *HOXC4*, *HOXC5* and *HOXC6*. For the other genes of the *HOXC* locus, mouse sequences were available. For these latter genes, the primers selected on the mouse sequence were used with human cDNA. The RT-PCR products were cloned and sequenced by the dideoxynucleotide method to confirm the specificity of the primers. Sequence analyses were performed using the computer facilities of the Bisanse server [20]. Homologies between human and mouse sequences were in the 95–98% range. The nucleotide sequences have been deposited with the GenBank/EMBL Data Libraries (accession numbers: X99630, X99631, X99679, X99680, X99681, X99682, X99683, X99684, X99685). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was unaffected by RA treatment and served as a control of expression using primers purchased from Clontech (Palo Alto, CA, USA). When the amplified fragments did not include any intron, control experiments were performed in which the reverse transcription step was omitted to ensure the absence of contaminating DNA. Reverse transcription was performed using the Life Technologies kit with 2 µg of total RNA, 200 ng of oligo-d(T) and 0.5 mM of each dXTP. One-tenth of the preparation was used for PCR amplification. The buffer conditions were those recommended by the Taq-polymerase supplier (Appligene-Oncor, Illkirch, France). The amplification procedure involved denaturation at 94°C for 1 min, annealing for 1 min at the appropriate temperature and extension at 72°C for 1 min during 38–40 PCR cycles. Reaction products were analyzed on a 2% agarose gel, labelled with 1 µg/ml of ethidium bromide and revealed under UV illumination. The images were captured by a CCD camera and the intensity of the bands quantified using Image software in the range of linearity of the signal.

In order to obtain comparable levels of detection for the expression of the *HOX* genes, the number of PCR cycles necessary to detect 100 molecules of target segments was determined for each gene. The threshold of detection was defined as the electrophoretic specific band having an intensity of five times the background level. The target segments were prepared by RT-PCR, purified (GeneClean III, Bio101, Vista, CA, USA) and quantified on gel by comparison with commercially available quantified molecular weight markers (Life Technologies). Genomic DNA was also used when no intron was present in the amplified DNA. Using 38 cycles for B6, B9, 39 cycles for B1, B2, B7 and 40 cycles for the remaining genes, the same threshold of detection could be obtained for each *HOX* gene in the experimental conditions used. The reproducibility of the band intensities varied by a factor of 1–2 taking into account all the experimental variations. The RA treatments of the cells were independently repeated 5 (SCLC-6), 3 (SCLC-10) and 2 (NT2/D1) times. In each case, 2–3 reverse transcriptions followed by at least 2 PCR amplifica-

tions for each point were performed. Similar results were obtained in each series of experiments.

2.3. Flow cytometry

The percentage of dead cells was determined by measuring the decreased ability to stain DNA with propidium iodide [21].

3. Results

RA induction of *HOXB* and *HOXC* gene expression in the EC cell line NT2/D1 was analyzed by RT-PCR. In the absence of RA (Fig. 1), only *HOXC12* and *HOXC13* were expressed at a low level. In the continuous presence of 10 µM RA, a 3'-5' induction of *HOX* expression was observed. In cluster B, *HOXB1* and *HOXB2* mRNA were detected after 2 h of treatment whereas *HOXB9* was switched on only after more than 150 h. *HOXB13* remained silent. In locus C, 3'-5' induction was observed for *HOXC4*, *HOXC5* and *HOXC6*. The four genes from *HOXC8* to *HOXC11* were insensitive to RA treatment whereas the expression of *HOXC12* and *HOXC13* did not change. When the RA concentration was decreased, the same 3'-5' induction was observed with an increase in the response time: after 168 h, the induction was detectable up to *HOXB8* and *HOXB4* in the presence of 1 µM and 0.1 µM RA, respectively, whereas the *HOXC* locus genes were not induced (not shown). These patterns of expression are in full accordance with those previously obtained using Northern blot or RNase protection assay [9,10]. The single discrepancy concerns *HOXC12* which was found to be constitutively expressed in our study.

In RA free medium, the cell doubling time was about 40 h and 60 h for SCLC-6 and SCLC-10, respectively. The proliferation rate of SCLC-10 cells was unaffected in the presence of 1 µM RA whereas a 20% decrease was observed in SCLC-6 cells cultivated in 0.1 µM RA containing medium. Exposure to RA did not affect the morphology of SCLC-10 cells, which grew as floating aggregates. In contrast, SCLC-6 cells, which normally grew as monolayers, detached from the plastic surface after about 7 days in the presence of 0.1 µM RA,

Table 1
Gene specific primers used to analyse gene expression by RT-PCR

Gene	Primers ^a	Species ^b	T (°C) ^c	Size ^d	Intron ^e	Ref. ^f
<i>HOXC4</i>	S: 5'-CAGTATAGCTGCACCAGTCTCCAGG-3' A: 5'-GATCTGCCTCTCAGAGAGGCACAGC-3'	H	65	406	+	[29]
<i>HOXC5</i>	S: 5'-TGGATGACCAAACTGCACATGAGC-3' A: 5'-CAAGTTGTTGGCGATCTCTATGCG-3'	H	62	149	+	[30]
<i>HOXC6</i>	S: 5'-CACCTTAGGACATAACACACAGACC-3' A: 5'-CACTTCATCCGCGGGTCTGGAACC-3'	H	62	317	+	[29]
<i>HOXC8</i>	S: 5'-CCACGTCCAAGACTCTTCCACGGC-3' A: 5'-CACTTCATCCTTCGATTCTGAAACC-3'	H	60	449	+	[31]
<i>HOXC9</i>	S: 5'-TGTTTCAGACCCGGAGGATCAAG-3' A: 5'-GGAAGAGAACGCAGTTTCTCTCC-3'	M	60	436	—	[32]
<i>HOXC10</i>	S: 5'-CTACCGCCTGGAACAACCTGTTGG-3' A: 5'-ATGGTCTTGCTAATCTCCAGGCG-3'	M	60	662	+	[33]
<i>HOXC11</i>	S: 5'-AGAAGCGCTGCCCTATTTCG-3' A: 5'-ATACTGCAGCCGGTCTCTGC-3'	M	60	191	—	[33]
<i>HOXC12</i>	S: 5'-AAAGAAGGCGCAAGCCGTATTCGAAG-3' A: 5'-AGACGTTGCTCCCTCAGCAGAAGTC-3'	M	60	192	—	[33]
<i>HOXC13</i>	S: 5'-TGCCCTATACCAAGGTGCAG-3' A: 5'-TAGATTTGCTGACCACCTTT-3'	M	60	173	—	[33]
<i>CRABP1</i>	S: 5'-AAGATGCGCAGCAGCGAGAATTTTCG-3' A: 5'-AAGTTCATCGTTGGCCAGCTCACG-3'	H	60	333	+	[34]

^aSelected sense (S) and antisense (A) oligodeoxynucleotides. ^bHuman (H) or mouse (M) origin of the known gene sequence. ^cHybridization temperature. ^dSize in base pairs of the fragment generated by RT-PCR. ^ePresence (+) or absence (—) of an intron in the corresponding genomic DNA. ^fReferences of the sequences used.

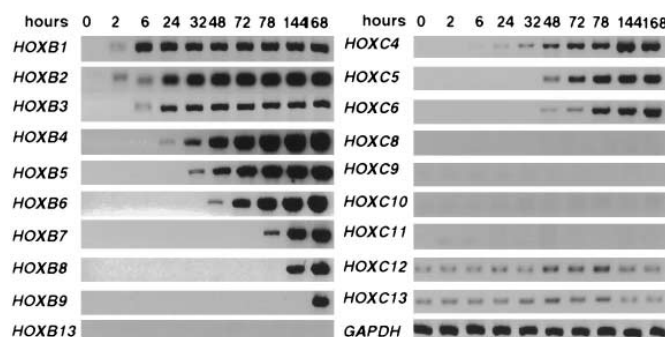


Fig. 1. RT-PCR analyses of *HOXB* and *HOXC* gene expression in the NT2/D1 cell line. Cells were treated with 10 μ M RA for 2–168 h. RNAs were extracted from treated and untreated cells. RT-PCR was performed using primers specific for each *HOX* gene. GAPDH expression was used as control. The reaction products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The images were captured by a CCD camera and processed using Image software.

although they were still dividing. In 1 μ M RA, the transition of SCLC-6 cells from attached to unattached was achieved after 2 days. RA toxicity differed between the two SCLC cell lines. SCLC-6 was highly sensitive: after 10 days in 0.1 μ M RA, the percentage of dead cells was 20% while it was 3% in the untreated cell controls. SCLC-10 cells were more resistant to RA with about 10% of dead cells after 10 days in the presence of 1 μ M RA, compared to 8% in the absence of RA. The data presented below were obtained from experiments performed at RA concentrations and for treatment periods for which more than 80% of the cells were alive.

Expression of *RAR* α , β and γ and of *CRABP1* and *CRABP2* genes was studied as a function of time and RA concentration by RT-PCR (Fig. 2). *RAR* α and γ were constitutively expressed and *RAR* β unexpressed in both cell lines. In the presence of RA, SCLC-6 and SCLC-10 cells produced *RAR* β mRNA, while the levels of expression of *RAR* α and *RAR* γ remained unchanged. When RA was removed, expression of *RAR* β returned to its initial level after few days (not shown). *CRABP2* was expressed in both cell lines, *CRABP1* in SCLC-10 only. Their pattern of expression did not change during RA treatment.

In SCLC-6 cells cultivated in RA free medium, *HOXB4* and *HOXC6* to *HOXC9* were strongly expressed whereas *HOXC11* mRNA was detectable at a low level (Figs. 3 and 4). The *HOXB* locus was completely switched off in SCLC-10 cells whereas in locus C, five genes (*HOXC6* to *HOXC9* and *HOXC12*) were expressed, the level of expression of *HOXC6* being low. The pattern of expression was characteristic of these two cell lines and remained unchanged as a function of the passage numbers in culture.

In SCLC-6 cells, transcripts from genes *HOXB3*, *HOXB5*, *HOXB8* and *HOXB9* appeared after 2 days of culture in 0.1 μ M RA containing medium (Fig. 3), and accumulated progressively as a function of the time of treatment. *HOXB2*, *HOXB6* and *HOXB7* gene expression became detectable after 4 days. Neither *HOXB1* nor *HOXB13* transcripts were observed even after 10 days. In the presence of 1 μ M RA, similar but accelerated expression patterns were seen for *HOXB3*, *HOXB5*, *HOXB8* and *HOXB9*, which were detectable from day 1 of treatment, with *HOXB2*, *HOXB6* and *HOXB7* being detected on day 2. Again, *HOXB1* and *HOXB13* were not detectable, even after 3 days (not shown). In SCLC-6 cells, *HOXB1* could be slightly switched on after 7 days in the presence of 10 μ M RA; such a treatment induced a high level

of cellular death (not shown). The expression level of *HOXB4* was unaffected by RA treatment.

In SCLC-10 cells cultured in 0.1 μ M RA containing medium, no change in *HOXB* gene expression was observed for up to 10 days (not shown). When the RA concentration was increased to 1 μ M, *HOXB3*, *HOXB5*, *HOXB6*, *HOXB8* and *HOXB9* transcripts were progressively observed after day 7, and no transcripts of *HOXB1*, *HOXB2*, *HOXB4*, *HOXB7* and *HOXB13* were present even after 10 days.

The expression of several *HOXC* genes was unaffected by RA treatment independently of their constitutively expressed or unexpressed status: *HOXC4*, *HOXC5*, *HOXC8*, *HOXC9* and *HOXC13* in both cell lines; *HOXC6* in SCLC-6 cells; *HOXC10* and *HOXC11* in SCLC-10 cells (Fig. 4). One constitutively unexpressed gene was weakly induced (*HOXC10* in SCLC-6), while two initially weakly expressed genes (*HOXC6* in SCLC-10 and *HOXC11* in SCLC-6) were slightly induced. *HOXC12* was very sensitive to RA: in SCLC-6, the constitutively unexpressed gene was induced, whereas in SCLC-10, the constitutively expressed gene was down-regulated. In both cell lines, when RA containing medium was removed and replaced

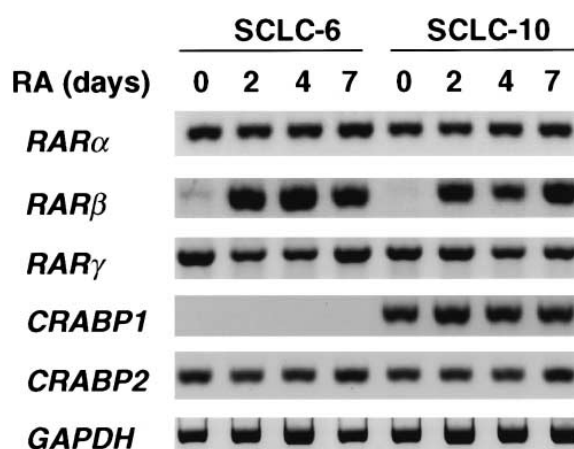


Fig. 2. RT-PCR analyses of *RAR* and *CRABP* expression. Response to RA treatments of SCLC-6 and SCLC-10 cell lines. Cells were treated with RA for 2, 4 and 7 days at 0.1 μ M for SCLC-6 and 1 μ M for SCLC-10. RNAs were extracted from treated and untreated cells. RT-PCR was performed using primers specific for the three forms of *RAR* and the two forms of *CRABP*. *GAPDH* expression was used as control. The reaction products were analyzed as described in the legend to Fig. 1.

by RA free medium, the induced expressions of all the *HOX* genes were retained for 3 days and then returned to their initial status by 10 days (not shown).

Fluorescent in situ hybridization of chromosome specific libraries and Southern blot analysis of *HOX* specific probes has shown that the genomic organization of the four *HOX* clusters was not altered in the SCLC cell lines ([16,22] and not shown).

4. Discussion

In embryonic cells, *HOX* gene expression is colinear with the organization of the genes: the genes at the 3' end of the locus are induced rapidly, whereas the time and concentration of RA required for induction of gene expression increase with increasing distance from the 3' end. This has been previously shown by Northern blot and RNase protection assay [9,10]. The same behavior can be evidenced by RT-PCR in rigorously controlled conditions as demonstrated here (Fig. 1). Several models have been proposed to explain this behavior [8,23]. A single RARE localized at the 3' end of the locus could sequentially activate the entire *HOX* cluster. Alternatively, multiple functionally distinct RAREs could coordinate *HOX* gene activity from 3' to 5' in the cluster. The *HOX* gene could also regulate their expression: the product of the gene at the 3' end could activate the neighboring gene in the 5' direction and so on through the cluster. The RAREs present in particular around *HOXA1* and *HOXB1* allow a complex regulation of the expression of these genes [24,25] and data have shown the regulation of the *HOX* gene expression by *HOX* proteins (reviewed in [1,6]). However, the exact mechanisms of RA activation are not yet understood, even if it is known that the maintenance of proper timing and polarity is assessed by the synthesis of short half-life proteins [10].

Regulation of *HOX* gene expression by RA in the two SCLC cell lines derived from mature tissues differs from that described in EC cells. In particular, the sequential 3'-5'

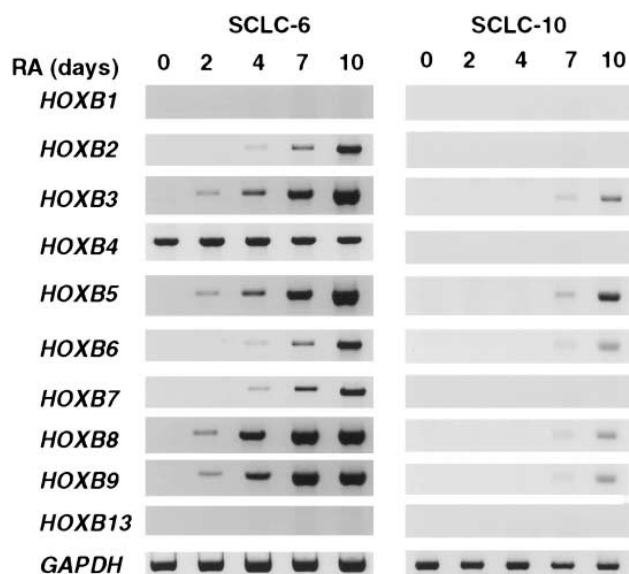


Fig. 3. RT-PCR analyses of *HOXB* gene expression in RA treated SCLC-6 and SCLC-10 cell lines. The cells were grown in the presence of 0.1 μ M RA (SCLC-6) or 1 μ M RA (SCLC-10) for 2, 4, 7 or 10 days.

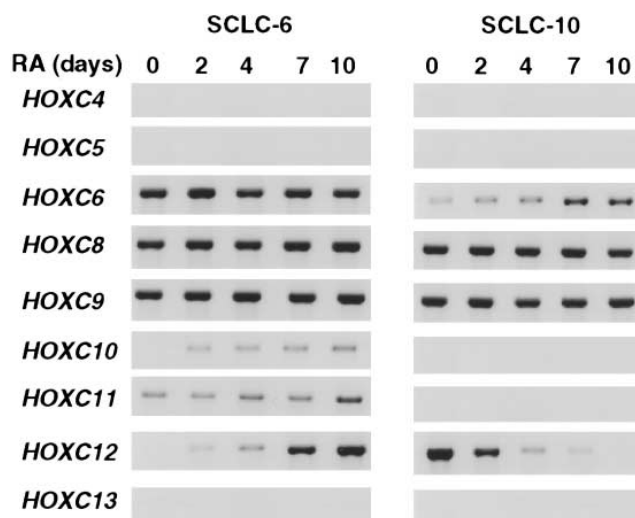


Fig. 4. RT-PCR analyses of *HOXC* gene expression in RA treated SCLC-6 and SCLC-10 cell lines. The cells were grown in the presence of 0.1 μ M RA (SCLC-6) or 1 μ M RA (SCLC-10) for 2, 4, 7 or 10 days.

activation is not found. Inside a cluster, very different responses to RA treatments were seen in the expression patterns of neighboring genes, each gene seeming to be induced independently. This property is well illustrated by cluster B in SCLC-6 cells: *HOXB1* and *HOXB13* localized respectively at the 3' and 5' ends of the cluster are not induced whereas gene expression along the cluster is induced with various timing (Fig. 3). This cannot be related to an alteration of the genomic organization of the clusters. The models proposed to explain the RA induction of the *HOX* genes in EC cells and in particular the master function of the 3'-most gene of the cluster cannot be applied to these cells: in SCLC cells, the powerful mechanism which switches off *HOXB1* does not prevent the activation of the other genes in the cluster.

These constitutively expressed genes are insensitive to RA treatment excepted *HOXC12* which appears to be regulated in a more complex manner. It can be activated or repressed by similar conditions of RA treatments depending on its constitutive expression status: in SCLC-6, the formerly unexpressed genes were induced, whereas in SCLC-10 the constitutively expressed genes were down-regulated. In the absence of data on the regulation region of this gene, it is currently impossible to propose a mechanism of regulation. However, it could be associated with RA/AP-1 antagonism (reviewed in [26]).

Another difference between EC and SCLC cells concerns the reversibility of the RA effects. In SCLC cells, the maintenance of the *HOX* gene response depended on the continuous presence of RA in the medium, whereas with EC cells the induced *HOX* genes continue to be expressed after removal of RA provided they had been exposed to the inducer for a sufficient time [10]. The beginning of the differentiation process observed in EC cells after RA treatment must induce an irreversible reprogramming of the *HOX* locus regulatory mechanisms lacking in the mature tissue derived SCLC cells.

Lack of 3'-5' sequential activation of the *HOX* genes has also been observed in other SCLC cell lines and in cell lines derived from tumors of mature tissues such as breast and colon (not shown).

The two SCLC cell lines used responded to RA with different extent and behavior: SCLC-10 viability was unaffected by the highest dose used (1 μ M), whereas SCLC-6 cell viability was reduced by a relatively low concentration of RA (0.1 μ M). These differences in sensitivity may be related to *CRABP1* expression, only found in SCLC-10 cells. It has been proposed that *CRABP1* acts as a buffer in the cytoplasm by limiting the availability of RA to its nuclear receptors [12]. It has also been shown that high levels of *CRABP1* reduced the expression of several but not all of the RA responsive genes in F9 teratocarcinoma cells [27]. The *CRABP1*-RA complexes are substrates for RA metabolizing enzymes [28]. Toxicity of RA in SCLC-10 cells might be attenuated by the high level of *CRABP1*. The role of *CRABP1* as an autocrine inhibitor of RA effects could also explain the poor response of these cells to RA in term of *HOX* pattern expression as compared to SCLC-6 cells.

The mechanisms of regulation of the *HOX* genes, active during embryonic development, seem to be no more effective in SCLC cells derived from mature tissues, indicating that the regulatory mechanisms may have been reprogrammed. In this new context, the *HOX* genes are not regulated colinearly, reminiscent of their role during embryonic development, but in a more individual manner corresponding to diversified abilities to respond to external stimuli arising from normal or pathological situations.

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