

# Normal HC11 and ras-transformed mouse mammary cells are resistant to the antiproliferative effects of retinoic acid

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## Abstract

The objective of the present study was to determine the effects of retinoic acid on the growth of the mouse mammary cells HC11 and HC11ras, which are a model for *in vitro* breast cancer progression. The expression of the two classes (RARs and RXRs) of retinoic acid receptor mRNAs was determined by Northern blot analysis. Receptor functional integrity was determined by testing whether RAR  $\beta$  mRNA could be induced by retinoic acid. The effects of a 72-h exposure to 50  $\mu$ M 13-*cis* retinoic acid on HC11 and HC11ras cell proliferation and HC11 cell differentiation were investigated by flow cytometric cell cycle analysis, and by determination of  $\beta$ -casein mRNA expression, respectively. The possibility that retinoic acid would induce the expression of the vitamin D receptor and synergize with vitamin D, a known inhibitor of HC11 cell growth, was also investigated. HC11 cells expressed higher mRNA levels of both RAR  $\alpha$  and RAR  $\gamma$  when compared to HC11ras cells. In contrast, RAR  $\beta$ , as well as RXR  $\alpha$ ,  $\beta$  and  $\gamma$  expression was low in both HC11 and HC11ras cells. In addition, RAR  $\beta$  mRNA was induced by retinoic acid treatment in both cells. In spite of these observations, no effects were seen on cell proliferation or differentiation upon exposure to retinoic acid. Neither vitamin D receptor induction nor synergy with vitamin D on growth inhibition was observed. We conclude that the RAR expression profile could be related to the transformed state in HC11ras cells and that the retinoic acid resistance observed merits further investigation.

## Key words

- Retinoic acid receptors
- Mouse mammary cells
- HC11ras cells
- Carcinogenesis

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## Introduction

Murine carcinogenesis in several organs, including the breast, is inhibited by retinoids (1). Data showing the growth inhibiting effect of retinoids on murine breast tumors induced by N-nitromethylurea (2) have in-

spired clinical protocols like the human breast cancer chemoprevention trial using the retinoid fenretinide (3). It has recently been shown that the retinoid LGD 1096 suppresses estrogen receptor- (ER) negative tumor development in virus Erb-b2 transgenic mice (4).

Retinoids bind to nuclear receptors, of

which there are two classes, RARs and RXRs, with three subtypes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) in each class. RARs form heterodimers with RXRs, which control gene transcription, binding to specific DNA regions known as retinoid responsive elements (RAREs and RXREs) or interfere with the function of the AP-1 transcriptional factor, which binds to other DNA sites (5). RAR  $\beta$  expression tends to be very low or absent in breast cancer cells when compared to senescing cells (6). ER-positive breast cancer cell lines express higher levels of RAR  $\alpha$ , compared to ER-negative lines (7).

The exact molecular mechanisms underlying the chemopreventive effects of retinoids are unknown, but probably involve growth regulation and induction of differentiation. These compounds are capable of inhibiting the proliferation of several breast cancer cell lines (8) by inhibiting G1 transition in the cell cycle (9).

An interesting *in vitro* model of breast cancer progression, in which the action of retinoid can be investigated, is the mouse mammary cell line HC11 (10), and HC11ras, obtained by the stable transfection of a mutated oncogene Ha-ras into HC11 cells (11). HC11 cells isolated from the normal mammary glands of a midpregnant mouse retain important normal features like the capacity to differentiate and express as a marker the milk protein  $\beta$ -casein, after lactogenic hormone induction. HC-11 cells present mutations in both alleles of the p53 tumor suppressor gene (12), which could explain the immortalized phenotype of these cells. HC11ras cells, in contrast, do not differentiate upon lactogenic hormone exposure and are tumorigenic when injected into immunosuppressed mice (13). A previous study by our group has shown that vitamin D inhibits the proliferation of parental HC11 cells, but not of Ha-ras-transformed HC11 cells (14).

The objective of the present investigation was to determine the effects of retinoic acid on the growth of the mouse mammary

cells HC11 and HC11ras. The expression of the two classes (RARs and RXRs) of retinoic acid receptor mRNAs was measured by Northern blot analysis. Receptor functional integrity was studied by testing whether RAR  $\beta$  mRNA could be induced by retinoic acid, since RAR  $\beta$  itself is a retinoid transcriptional target (5). The effects of a 72-h exposure to 50  $\mu$ M 13-*cis* retinoic acid on proliferation of HC11 and HC11ras cells and differentiation of HC11 cells were investigated by flow cytometric cell cycle analysis and by determination of  $\beta$ -casein mRNA expression, respectively. The possibility that retinoic acid would induce the expression of the vitamin D receptor (VDR) and synergize with vitamin D, a known inhibitor of HC11 cell growth, was investigated, since the VDR promoter contains a candidate retinoic acid-responsive element (15).

## Material and Methods

### Cell culture

HC11 cells (donated by Dr. Nancy Hynes, Friedrich Meischer Institute, Basel, Switzerland) were seeded at an initial cell density of  $2 \times 10^4$  cells/cm<sup>2</sup> and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 5  $\mu$ g/ml insulin and 2 mM glutamine. When exposed to 100 nM vitamin D (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA), 50  $\mu$ M 13-*cis* retinoic acid or 10  $\mu$ M 9-*cis* retinoic acid (Sigma, St. Louis, MO, USA), cells were maintained under the same conditions, except for a 24-h preculture in 10% charcoal-adsorbed FCS.

### Flow cytometric DNA content determination

Cells were assessed for DNA content using the DNA intercalating agent propidium iodide (16). Analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the

percent of cells in the G0/G1, S and G2/M phases was determined by the ModFit software (Becton Dickinson).

#### Vitamin D receptor evaluation using a specific monoclonal antibody

HC11 cells were grown for 48 h with or without 10  $\mu$ M 9-*cis* retinoic acid. The latter was chosen due to its action on both RXRs and RARs, which could possibly imply a greater capacity to trans-activate the target VDR (5). VDR expression was evaluated in indirect immunofluorescence assays using a specific murine monoclonal antibody. Cells were fixed in 70% cold ethanol and maintained at -20°C for at least 12 h, washed twice in PBS and incubated with 13  $\mu$ g/ml anti-VDR (VD2F12) (17) for 60 min. Positivity for the marker was identified by enumerating the fraction of cells located above the channel where 1% positivity was obtained for the background stain. Fluorescence intensity, which reflects the number of antigen molecules/cell, was evaluated on the basis of the mean fluorescence channel.

#### RNA isolation and Northern blot assays

Total RNA from HC11 cells was isolated using the TRIZOL reagent (Gibco-BRL, Rockville, MD, USA). Twenty-microgram samples were electrophoresed on 1% agarose-3% formaldehyde gels and the RNA was transferred to Hybond N nylon filters (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) which were hybridized in 50% formamide, 5X SSPE, 0.2% SDS, 5% dextran sulfate, 5X Denhardt's solution containing 100  $\mu$ g/ml salmon sperm DNA, and a 3 x 10<sup>6</sup> cpm/ml [ $\alpha$ <sup>32</sup>P]-dCTP (Amersham) oligo-labeled specific probe using the random primer labeling technique (Klenow fragment of *E. coli* DNA polymerase; Gibco-BRL, Gaithersburg, MD, USA) for 20 h at 42°C. The following fragments were used as probes: a 1.9-kb *Eco*RI,

1.4-kb *Sac*I/*Bam*HI and 1.5 kb *Eco*RI fragments of human RAR  $\alpha$ ,  $\beta$  and  $\gamma$ , respectively (5), and 4.8-kb *Eco*RI, 1.7-kb *Eco*RI/*Pst*I, 1.67-kb *Asp*718/*Bam*HI fragments for RXR  $\alpha$ ,  $\beta$  and  $\gamma$ , respectively (18), and a 2.1-kb fragment of human VDR cloned at the *Eco*RI site of pGEM (19). Membranes were washed for 15 min, twice at room tempera-

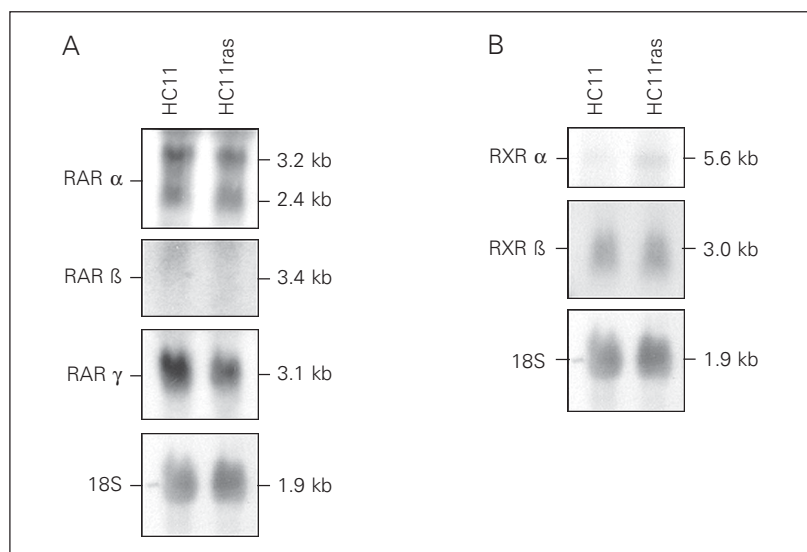


Figure 1. Expression of RAR and RXR in HC11 and HC11ras cells. Total RNA was subjected to Northern blot analysis and filters were sequentially hybridized with [<sup>32</sup>P]-labeled probes for RAR  $\alpha$ ,  $\beta$  and  $\gamma$  (A) and RXR  $\alpha$ ,  $\beta$  and  $\gamma$  (B) and 18S rRNA as a control for RNA loading.

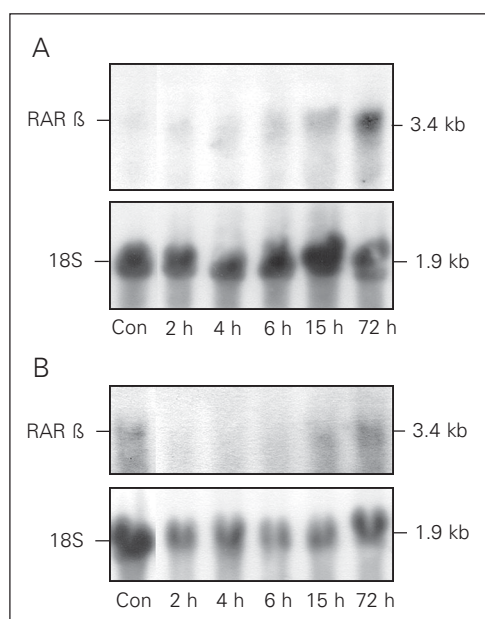


Figure 2. RAR  $\beta$  receptor mRNA expression in retinoic acid-induced HC11 (A) and HC11ras (B) cells. Cells were exposed or not (Con) to 50  $\mu$ M 13-*cis* retinoic acid for different times. Total RNA was extracted and subjected to Northern blot analysis and filters were sequentially hybridized with [<sup>32</sup>P]-labeled probes for RAR  $\beta$  and 18S rRNA as a control for RNA loading.

ture in 2X SSPE, 0.1% SDS, once in 1X SSPE, 0.1% SDS, once in 0.2X SSPE, 0.1% SDS, and finally for 30 min at 52°C in 0.1X SSPE and 0.1% SDS. Hybridization with the 18S ribosomal RNA probe, a 1.9-kb fragment cloned at the *SalI/EcoRI* site of plasmid pBR322 (20), was subsequently performed to check for equivalence of RNA loading. Band intensities in autoradiograms were quantified by densitometric scanning (UltraScan XL, Pharmacia LKB Biotechnology, Uppsala, Sweden) and data are reported as the ratio of specific mRNA to 18S rRNA. All solutions were prepared as described in Ref. 21.

#### Differentiation assay

HC11 cells were induced to differentiate and synthesize the milk protein  $\beta$ -casein by growing and maintaining the cultures with

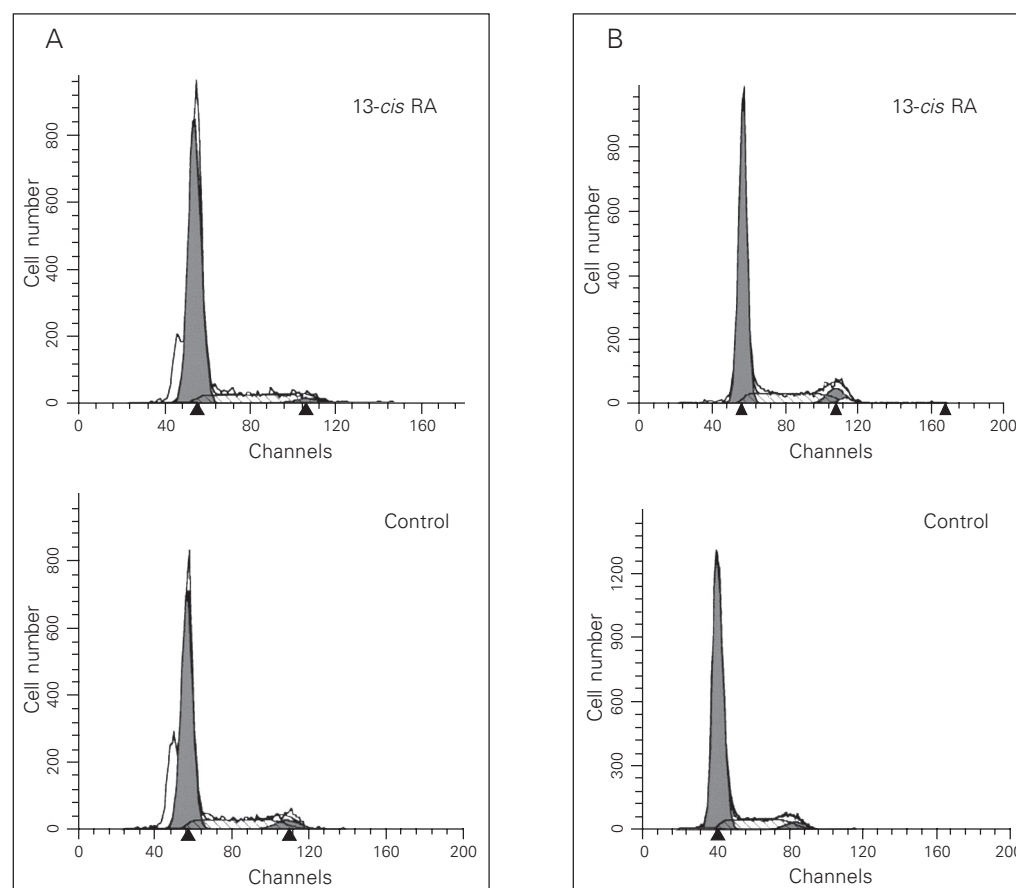
10 ng/ml murine EGF (Sigma). After 3-4 days, EGF was removed and the competent cultures were treated for 3 days with RPMI medium supplemented with 1  $\mu$ M dexamethasone, 5  $\mu$ g/ml insulin and 5  $\mu$ g/ml prolactin (DIP).

## Results

#### Retinoic acid receptor expression

We started by determining the mRNA of retinoic acid receptors in HC11 and HC11ras cells in order to obtain evidence for a possible responsiveness to retinoids. HC11 cells express approximately two times more mRNA of both RAR  $\alpha$  and RAR  $\gamma$ , when compared to HC11ras cells. RAR  $\beta$  expression, in contrast, was low in both cells (Figure 1A). RXR  $\alpha$ ,  $\beta$  and  $\gamma$  expression was low in both HC11 and HC11ras cells (Figure

Figure 3. Effect of retinoic acid on cell cycle distribution. HC11 (A) and HC11ras (B) cells were grown without (control) or with 50  $\mu$ M 13-*cis* retinoic acid (RA) for 72 h, harvested, permeabilized and labeled with propidium iodide. DNA content was evaluated by fluorescence intensity, and appears on the x-axis as channel numbers. Cell number is depicted on the y-axis of the histograms.



1B). In addition, RAR  $\beta$  mRNA was induced after a 72-h treatment with 50  $\mu$ M 13-*cis* retinoic acid, in both HC11 (Figure 2A) and HC11ras (Figure 2B) cells, by 50- and 2-fold, respectively.

### S-phase assessment

We determined the effect of retinoic acid on cell proliferation, on the basis of the cell cycle distribution measured by flow cytometric analysis. Exposure to 50  $\mu$ M 13-*cis* retinoic acid for 72 h failed to affect the S-phase fraction in both HC11 and HC11ras cells. Representative histograms show an S-phase fraction of 21 and 23% in control and induced HC11 cells, respectively. Similarly, other representative histograms show an S-phase fraction of 19 and 20% in control and induced HC11ras cells, respectively. These results indicate that both HC11 (Figure 3A) and HC11ras (Figure 3B) cells are resistant to the growth-inhibiting effects of high levels of 13-*cis* retinoic acid.

### Differentiation assessment

Expression of the milk protein  $\beta$ -casein mRNA was used as a marker of the differentiation of HC11 cells. Exposure to 50  $\mu$ M 13-*cis* retinoic acid for 72 h failed to induce  $\beta$ -casein mRNA expression (Figure 4), indicating that retinoic acid had no effect on cell differentiation, in contrast to cells differentiated by exposure to DIP.

### Investigation of the combined effect of retinoic acid and vitamin D

Retinoic acid receptors may be functional in these cells, since there was an induction, albeit late (72 h) of de RAR  $\beta$  mRNA after treatment with 13-*cis* retinoic acid. Thus, we wondered whether there could be a sensitization to the growth-inhibiting effect of vitamin D on HC11 cells by a possible induction of the VDR after exposure to retinoic acid.

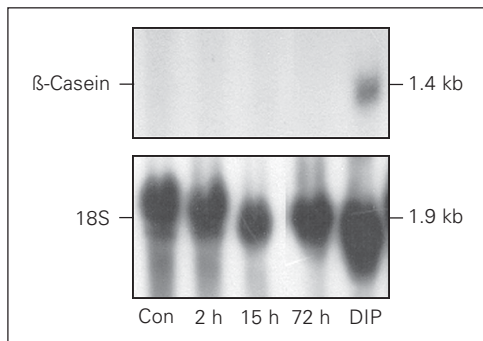


Figure 4.  $\beta$ -Casein mRNA expression in HC11 cells induced with 50  $\mu$ M 13-*cis* retinoic acid for different times and exposed to 1  $\mu$ M dexamethasone, 5  $\mu$ g/ml insulin and 5  $\mu$ g/ml prolactin (DIP). The control (Con) was HC11 cells which were not induced. Total RNA was subjected to Northern blot analysis and filters were sequentially hybridized with [ $^{32}$ P]-labeled probes for  $\beta$ -casein and 18S rRNA as a control for RNA loading. Only DIP treatment induced  $\beta$ -casein expression, in contrast to retinoic acid, which failed to differentiate the cells.

Therefore, we studied the expression of VDR protein by monoclonal antibodies and flow cytometric analysis. There was no increase in the expression level of the VDR protein after a 48-h induction with 10  $\mu$ M 9-*cis* retinoic acid. VDR expression was detected in more than 80% of both control and retinoic acid-induced cells (Figure 5). Accordingly, retinoic acid failed to potentiate the antiproliferative effect of vitamin D. The S-phase fraction was 12.2 and 10.0% after a 72-h induction with 100 nM vitamin D alone or 100 nM vitamin D plus 50  $\mu$ M 13-*cis* retinoic acid, respectively (Figure 6).

### Discussion

The expression of retinoic acid receptor mRNAs was initially determined in parental and ras-transformed HC11 cells. We found that HC11 cells expressed higher mRNA levels of both RAR  $\alpha$  and RAR  $\gamma$  as compared to HC11ras cells. In contrast, RAR  $\beta$ , as well as RXR  $\alpha$ ,  $\beta$  and  $\gamma$  expression, was low in both HC11 and HC11ras cells. RAR  $\beta$  expression was shown to be very low in the breast cancer cell lines MCF-7, T-47D, MDA-MB-361 and BT-474, as determined at the mRNA level, in contrast to RAR  $\alpha$  and RAR  $\gamma$  expression, which was variable (6,7). In breast cancer samples, RAR  $\beta$  expression, as determined by *in situ* hybridization, was lower compared to normal adjacent tissue, in contrast to RAR  $\alpha$ , RAR  $\gamma$  and RXR  $\alpha$  which were expressed equally by tumor and normal tissues (22,23). Our results showing a low

Figure 5. Effect of retinoic acid on vitamin D receptor (VDR) protein expression in HC11 cells. VDR content was evaluated with a monoclonal antibody by flow cytometry analysis. In the histograms, the cell number is shown on the y-axis and the fluorescence channel number on the x-axis. The open area represents the nonspecific staining and the filled area represents cells specifically labeled with anti-VDR antibody. HC11 cells were grown for 48 h without (control) or with 10  $\mu$ M 9-*cis* retinoic acid (RA).

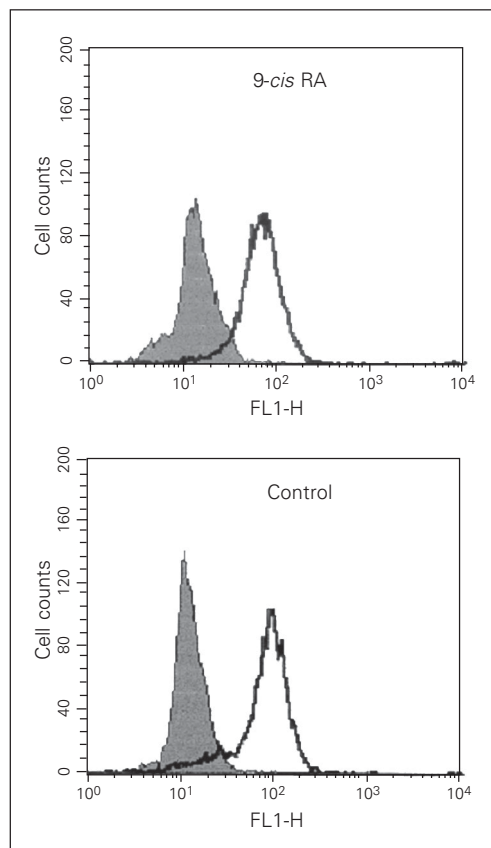
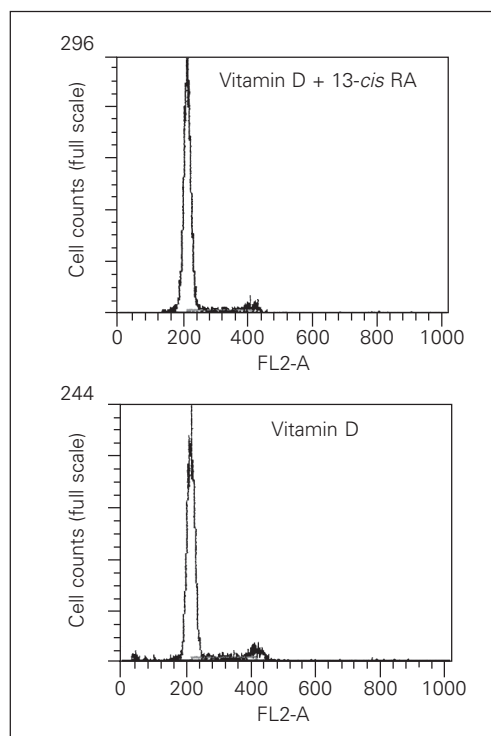


Figure 6. Effect of retinoic acid plus vitamin D on the cell cycle distribution. Cells were exposed to 100 nM vitamin D alone or 100 nM vitamin D plus 50  $\mu$ M 13-*cis* retinoic acid (RA) for 72 h, harvested, permeabilized and labeled with propidium iodide. DNA content was evaluated by fluorescence intensity, and appears on the x-axis as channel numbers. Cell number is indicated on the y-axis of the histograms. Two assays were performed with similar results.



RAR  $\beta$  expression in parental HC11 cells, which present several normal features, contrast with those of another study in which RAR  $\beta$  was highly expressed in benign breast lesions (24). We would suggest that the concomitant loss of p53 and RAR  $\beta$  might be a marker of progression in the process of carcinogenesis. The RAR  $\beta$  induction seen in HC11 and HC11ras cells after retinoic acid exposure suggests the presence of functional retinoic receptors, since one of the transcriptional targets of RAR  $\beta$  is the RAR  $\beta$  gene itself (5). The modest level of induction (2 times) observed in HC11ras cells compared to that seen in HC11 cells (50 times) could be due to the lower basal expression of RAR  $\alpha$  and RAR  $\gamma$  of HC11ras cells. Although capable of expressing apparently functional retinoid receptors, both HC11 cells and HC11ras cells were resistant to the antiproliferative effects of retinoic acid. A possible explanation for these observations is the low RAR  $\beta$  basal expression, since this specific receptor could be critical to mediate the growth-inhibiting effects, as previously suggested (6). There are, however, conflicting reports concerning the relative importance of these receptors, showing the critical role played either by RAR  $\alpha$  (25,26) or by RAR  $\gamma$  (27) as mediators of the biological effects of retinoic acid. Another factor possibly associated with resistance to the antiproliferative effect of retinoic acid is the lack of expression of ER, both in HC11 and HC11ras cells. The ER-negative breast mammary cell line MDA-MB-231, which is retinoic acid resistant, becomes sensitive when stably transfected with ER, although the underlying molecular mechanism remains to be elucidated (28).

We also investigated the effect of retinoic acid on HC11 cell differentiation using  $\beta$ -casein milk protein mRNA expression as a marker. Our results showed no induction of  $\beta$ -casein upon long term exposure to retinoic acid. Our findings are in contrast to another study, in which retinoids were found to be

capable of inducing differentiation in the breast cancer cell line SKBR3 by regulation of the cadherin adhesion molecule expression and function (29). We cannot rule out the possibility of partial differentiation since  $\beta$ -casein is a marker of milk production and thus indicates a final stage of differentiation of HC11 cells.

Our own data (14) had shown that vitamin D inhibits the proliferation of HC11 but not of HC11ras cells. Since vitamin D induces hypercalcemia, it would be of potential clinical interest to use this compound at lower doses. Thus, we determined whether the addition of retinoic acid to vitamin D could have a synergistic effect on HC11 cells. The S-phase inhibition was similar in both the combination and vitamin D only treatment. These findings are in contrast to those of others, in which this combination was found to be synergistic in MCF-7 and T-47D mammary cells (30,31). The cooperative effects of vitamin D and retinoic acid could be explained by the formation of VDR/RXR heterodimers and by the enhancement of the trans-activating capacity (32). Another possible mechanism of synergy could be the induction of VDR by retinoic acid (15). The lack of synergy, alternatively, could be explained by the competition of RAR and

VDR for the partner RXR (33). Thus, there could be either synergy or antagonism with the combination of retinoic acid and vitamin D depending on the cellular context.

We conclude that the RAR expression profile could be related to the transformed state in HC11ras cells. A key unknown component in the retinoic pathway may be altered in both parental and ras-transformed HC11 cells. A possible candidate could be the recently identified retinoid target transcriptional factor SOX9, which seems to mediate growth inhibition in breast cancer cell lines (34). The resistance to retinoic acid described here merits further investigation.

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