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Retinoic Acid Resistance at Late Stages of Human Papillomavirus Type 16-Mediated Transformation of Human Keratinocytes Arises Despite Intact Retinoid Signaling and Is Due to a Loss of Sensitivity to Transforming Growth Factor-β

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In our *in vitro* model of human cell carcinogenesis, normal human foreskin keratinocytes (HKc) transfected with human papillomavirus type 16 DNA (HKc/HPV16) progress toward malignancy through several phenotypically defined and reproducible "steps" that include immortalization, growth factor independence (HKc/GFI), differentiation resistance (HKc/DR), and ultimately malignant conversion. While HKc/HPV16 are very sensitive to growth inhibition by all-*trans*-retinoic acid (RA) at early passages, they lose their sensitivity to RA during progression in culture. However, gel mobility shift assays using the retinoid response elements DR1 and DR5 showed no changes in binding activity of nuclear extracts obtained from HKc/HPV16 at different stages of *in vitro* progression. Similarly, Western blot analyses for retinoic acid receptor γ-1 and the retinoid X receptors failed to reveal any decreases in the levels of these retinoid receptors throughout progression. In addition, luciferase activity driven by the SV40 promoter with a DR5 enhancer element was activated following RA treatment of HKc/DR that were resistant to growth inhibition by RA. Since RA induces transforming growth factor-β2 (TGF-β2) in normal HKc and HKc/HPV16, we investigated whether this response changed during progression. Again, RA induced TGF-β2 mRNA in early and late passage HKc/HPV16, HKc/GFI, and HKc/DR approximately to the same extent, confirming that the RA signaling pathways remained intact during *in vitro* progression despite the fact that the cells become resistant to growth inhibition by RA. We then investigated the sensitivity of HKc/HPV16 to growth inhibition by TGF-β. While early passage HKc/HPV16 were as sensitive as normal HKc to growth inhibition by TGF-β1 and TGF-β2, the cells became increasingly resistant to both TGF-β isotypes during *in vitro* progression. In addition, while both RA and TGF-β produced a decrease in the levels of mRNA for the HPV16 oncogenes E6 and E7 in early passage HKc/HPV16, this effect was also lost at later stages of progression. Finally, blocking anti-TGF-β antibodies partially prevented RA inhibition of growth and E6/E7 expression in early passage HKc/HPV16. Taken together, these data strongly suggest that inhibition of growth and HPV16 early gene expression in HKc/HPV16 by RA is mediated by TGF-β and that a loss of RA sensitivity is linked to TGF-β resistance rather than alterations in RA signaling.

INTRODUCTION

DNA from the oncogenic human papillomaviruses (HPVs), especially HPV types 16 and 18 (see Pfister, 1996, for a review), immortalize cultured human keratinocytes (HKc/HPV16) and human cervical cells (Durst *et al.,* 1987; Kaur and McDougall, 1988; Pirisi *et al.,* 1987, 1988; Woodworth *et al.,* 1988, 1989), thus providing a powerful model for studying the molecular mechanisms of cervical carcinogenesis. HKc/HPV16 first undergo premalignant progression in a series of phenotypically defined and reproducible "steps" *in vitro,* including growth factor independence (HKc/GFI) and differentiation resistance (HKc/DR) (Pirisi *et al.,* 1988; Zyzak *et al.,* 1994). HKc/DR, but not HKc/HPV16, are then susceptible to malignant conversion following transfection with either a viral *ras* oncogene or herpes simplex virus type 2 DNA (DiPaolo *et al.,* 1989, 1990). We have previously shown that HKc/HPV16 are initially more sensitive than normal HKc to growth and differentiation control by all-*trans*-retinoic acid (RA), an active metabolite of vitamin A (Pirisi *et al.,* 1992; Khan *et al.,* 1993; Creek *et al.,* 1994). In addition, RA treatment of HKc/HPV16 reduced steady-state levels of the HPV16 oncogenes E6 and E7 mRNA and protein (Pirisi *et al.,* 1992; Khan *et al.,* 1993; Creek *et al.,* 1994) and physiologic levels of RA (1 nM) inhibited HPV16-mediated immortalization of normal HKc by about 95% (Khan *et al.,* 1993; Creek *et al.,* 1994). Based on our previous finding that RA treatment induces the synthesis and secretion of the growth inhibitor transforming growth factor-β (TGF-β) (Shipley *et al.,* 1986; Coffey *et al.,* 1988) in normal HKc and HKc/HPV16, we have formulated the hypothesis that RA inhibition of growth is mediated by TGF-β (Batova *et al.,* 1992). This concept is supported by the findings of Woodworth *et al.* (1990) that TGF-β inhibits E6 and E7

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transcription. Therefore resistance to RA in HKc/HPV16 may be attributed to disruptions in either the RA or the TGF-β signaling pathways.

Retinoids, including RA, have shown promise in the chemoprevention and treatment of various malignancies, including cervical cancer (Meyskens and Manetta, 1995; Lippman *et al.,* 1994; Hong *et al.,* 1995). Cervical cancer, the second most common cancer among women worldwide, is a progressive disease that is preceded by precancerous lesions of the cervix called cervical intraepithelial neoplasia (CIN). While most CIN I will spontaneously regress, some lesions continue to progress through CIN II, CIN III (carcinoma *in situ*), and ultimately invasive cervical cancer. Phase I and phase II studies with RA determined that RA applied topically to the cervix can reverse or suppress CIN (Meyskens and Manetta, 1995; Meyskens *et al.,* 1983; Graham *et al.,* 1986). For example, a phase II trial found that RA suppressed CIN in 50% of patients (Graham *et al.,* 1986). However, a more recent randomized phase III trial found that while topical RA was effective in promoting regression in patients with CIN II, it was not effective in patients with more advanced dysplasias (Meyskens *et al.,* 1994). This suggests RA resistance as a feature of more advanced disease and is consistent with the findings of retinoid resistance in HPV16-immortalized endocervical cells (Sarma *et al.,* 1996) or lung cancer (Geradts *et al.,* 1993; Kim *et al.,* 1995).

Retinoids exert their biological effects through ligand-activated nuclear receptors consisting primarily of heterodimers of α, β, or γ isotypes of the RA receptors (RARs) and the retinoid X receptors (RXRs) (Mangelsdorf *et al.,* 1994). These nuclear receptor complexes bind to enhancer elements located in the regulatory regions of target genes, termed RA response elements (RAREs). RAREs that bind RAR/RXR heterodimers are usually composed of two direct repeat DNA half-sites separated by a 2- or 5-bp spacer element (DR2 and DR5, respectively). Retinoid receptors bound to these elements generally enhance rates of transcription upon ligand binding. RXR homodimers preferentially bind to DR1 elements in target genes. Altered levels of nuclear retinoid receptors have been well documented in a variety of tumors and cancer cell lines, suggesting cell- or cancer-specific decreases in RAR isotype expression as a common event during tumorigenesis (Geisen *et al.,* 1997; Geradts *et al.,* 1993; Kim *et al.,* 1995; Monzon *et al.,* 1997; Widschwendter *et al.,* 1997; Xu *et al.,* 1997a,b). A selective loss of RAR-β expression has been described in cervical carcinoma cells (Bartsch *et al.,* 1992), premalignant oral lesions (Lotan *et al.,* 1995), and non-small-cell lung cancer (Xu *et al.,* 1997a) and has been directly linked to RA resistance in human lung cancer cell lines (Geradts *et al.,* 1993). The molecular basis for the loss of sensitivity to RA treatment during later stages of CIN is, however, uncertain.

In the present study we have determined that the growth inhibitory effects of RA treatment in HKc/HPV16 at early stages of premalignant progression are directly attributed to the increased production and secretion of TGF-β. In fact, anti-TGF-β antibodies counteract growth inhibition by RA and RA inhibition of HPV16 gene expression in early passage, RA- and TGF-β-sensitive cells. However, HKc/HPV16 lose sensitivity to RA during *in vitro* progression despite the presence of functional retinoid receptors and intact RA signaling. Retinoid refractoriness is accompanied by a concurrent loss of sensitivity to the growth inhibitory effects of TGF-β. Thus, these studies directly link a loss of sensitivity to growth control by RA to TGF-β resistance.

RESULTS

Effect of RA on proliferation of HKc/HPV16 during *in vitro* progression

To explore the effects of RA on HKc/HPV16 proliferation during *in vitro* progression, we used a clonal growth assay coupled with computerized image analysis. Representative dishes from a single clonal growth assay are shown in Fig. 1. The growth of normal HKc was stimulated 40 to 60% at low RA concentrations (1 and 10 nM) but was inhibited about 50% at 1 μM RA. Confirming what we have reported previously (Creek *et al.,* 1994; Khan *et al.,* 1993; Pirisi *et al.,* 1992), low-passage HKc/HPV16 were much more sensitive than normal HKc to inhibition of clonal growth by RA, showing marked growth inhibition by 10 nM RA and almost complete inhibition by 1 μM RA (Fig. 1). In contrast, growth of high-passage HKc/HPV16 was not inhibited by 10 nM RA and was inhibited by only 20% at 1 μM RA (Fig. 1). The effects of RA on proliferation of HKc/HPV16 at different stages of premalignant progression were also investigated using mass culture conditions. Again, low-passage HKc/HPV16 (passages 16 to 25) were about sixfold more sensitive to growth inhibition by RA than high-passage HKc/HPV16 (passages 146 to 180), HKc/GFI, or HKc/DR (data not shown). Taken together, these data demonstrate that RA sensitivity of HKc/HPV16 dramatically decreases with continued passaging and is an early event during *in vitro* progression.

RARE binding activity is retained in RA-resistant HKc/HPV16

Loss of RA sensitivity in a variety of cancer cells has been attributed to lost or reduced RAR expression (Geisen *et al.,* 1997; Geradts *et al.,* 1993; Kim *et al.,* 1995; Monzon *et al.,* 1997; Widschwendter *et al.,* 1997; Xu *et al.,* 1997a,b). Therefore, we employed electrophoretic mobility shift assays to assess relative nuclear protein binding activity to the DR1 and DR5 RAREs in HKc/HPV16 at various stages of progression. DR1 (Fig. 2A) and DR5

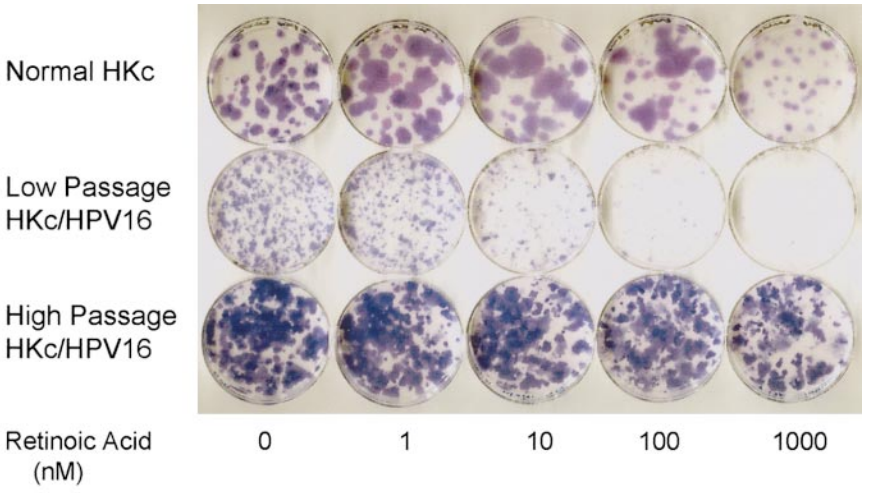

FIG. 1. Clonal growth of normal HKc and low- and high-passage HKc/HPV16 treated with RA. Normal HKc, low-passage HKc/HPV16d-1 (passage 14), and high-passage HKc/HPV16d-1 (passage 174) were plated in CM at a density of 1000 cells per 60-mm tissue culture dish. The following day the cells were refed with 8 ml of CM containing the indicated concentrations of RA in DMSO. Control dishes received DMSO only (final DMSO concentration 1%). The cells were treated as above 6 days after plating and stained with Giemsa solution 5 days later. A representative dish of three dishes per experimental condition is shown.

(Fig. 2B) binding activity was measured in nuclear extracts obtained from the HKc/HPV16d-4 line and its corresponding HKc/GFI and HKc/DR lines. Except for a modest decrease in HKc/DR, no significant change in binding activity was observed during progression of HKc/HPV16d-4 (Figs. 2A and 2B, lanes 5–8). Similarly, no decrease in DR1 or DR5 binding was demonstrated in the HKc/HPV16d-1 line or its HKc/GFI and HKc/DR counterparts (data not shown). The specificity of the shifted complex was demonstrated by competition with a 100-fold excess of cold DR1 (Fig. 2A, lane 4) or DR5 probe (Fig. 2B, lane 3). As expected, mutations at both DNA

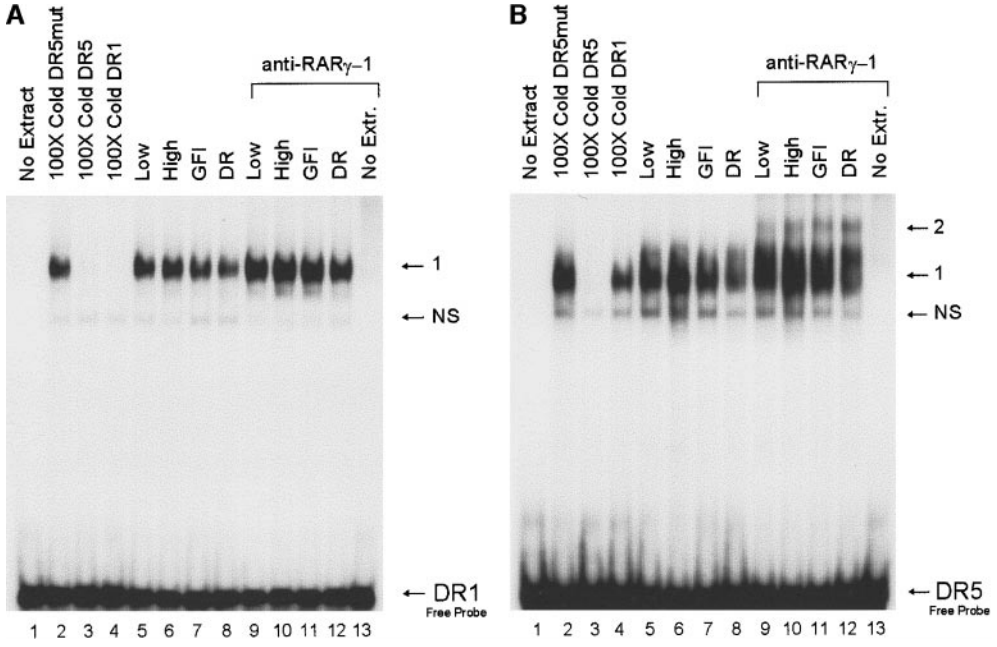

FIG. 2. DR1 and DR5 binding activities in HKc/HPV16d-4 during *in vitro* progression. Nuclear extracts (10 μg protein) obtained from HKc/HPV16d-4 were incubated with ^{32}P-labeled DR1 (A) or DR5 (B) oligonucleotide probes in an electrophoretic mobility shift assay. Endogenous binding activity of HKc/HPV16d-4 nuclear extracts to the DR1 and DR5 oligonucleotides was assessed during *in vitro* progression, from passage 22 (Low) to passage 114 (High), to growth factor independent (GFI) to differentiation resistant (DR). The specific shifted complex in both panels is indicated by arrow 1 and a nonspecific band is labeled NS. Preincubation of the nuclear extracts for 20 min at room temperature with an isoform-specific RARγ-1 polyclonal antibody produced a supershifted complex (arrow 2) with the DR5 (B, lanes 9–13), but not the DR1 oligonucleotide (A, lanes 9–13).

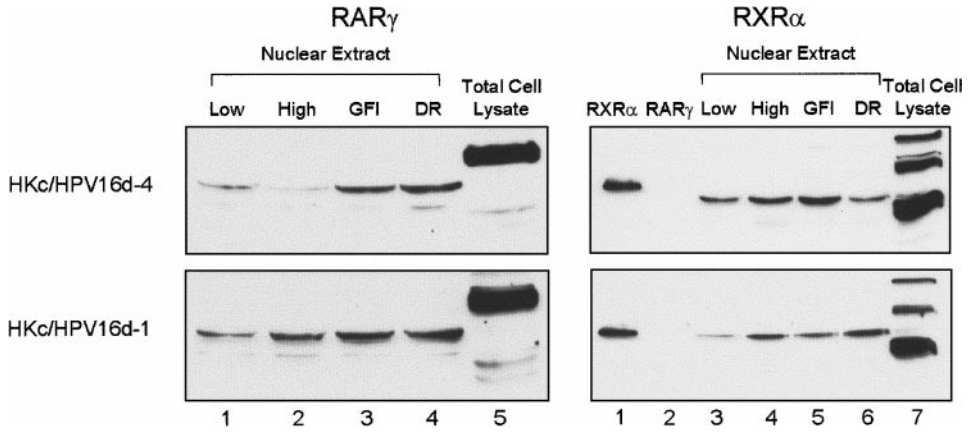

FIG. 3. *In vitro* progression of HKc/HPV16 is not associated with decreased RAR or RXR protein levels. Western blot analysis was performed using an isoform-specific polyclonal antibody against RARγ-1, or an anti-RXRα polyclonal antibody that cross-reacts with all RXR isotypes, on nuclear extracts from the HKc/HPV16d-4 and HKc/HPV16d-1 lines at low passage (Low) and high passage (High) and from their corresponding growth factor-independent (GFI) and differentiation-resistant (DR) cells. A similar amount of a total cell lysate from low-passage HKc/HPV16d-1 (lane 5) was included to assess for enrichment of RXR and RAR with nuclear isolation. RXRα, a human RXRα ligand binding domain–GST fusion peptide (55 kDa; lane 1 of right panels); RARγ, a full-length human RARγ–GST fusion peptide (75 kDa; lane 2 of right panels).

binding half-sites on the DR5 probe (DR5mut) failed to compete for binding to either labeled probe (Figs. 2A and 2B, lanes 2). These results suggest that changes in DR1 or DR5 binding activity are not consistently associated with loss of RA sensitivity in these cells. Anti-RARγ antibodies, when added during the binding reaction, caused a partial supershift of the DR5- but not the DR1-bound complex, suggesting that the DR1 element is recognized primarily by RXR homodimers (Figs. 2A and 2B, lanes 9–13). Importantly, the levels of DR5 supershifted complex did not change with *in vitro* progression (Fig. 2B, lanes 9–13), further supporting the finding that RA resistance in HKc/HPV16 was not due to a loss of RAR expression. Finally, preincubation of nuclear extracts from HKc/HPV16 at different stages of progression with 0.1 nM to 1.0 μM all-*trans*-retinoic acid or 9-*cis*-retinoic acid for 30 min prior to the binding reaction did not affect the levels of protein binding to either the DR1 or the DR5 probes (data not shown).

Protein levels of RARs are not decreased in RA-resistant HKc/HPV16

The retinoid receptors RARγ and RXRα are the predominant RARs and RXRs expressed in skin (Fisher *et al.,* 1994). Therefore, Western blot analysis was performed using nuclear extracts obtained from HKc/HPV16d-1 and d-4 to investigate whether decreased RARγ or RXR protein levels paralleled the loss of RA sensitivity during *in vitro* progression. We used either a polyclonal antibody that reacts with all RXR isotypes (but not with RARγ) or an isotype-specific anti-RARγ-1 polyclonal antibody. As shown in Fig. 3, RXR and RARγ-1 are clearly detectable at all stages and do not decrease during *in vitro* progression. Taken together, these results demonstrate that the decreased sensitivity of high-passage HKc/HPV16, HKc/GFI, and HKc/DR to RA is not due to reduced expression or binding activities of the nuclear retinoid receptors during *in vitro* progression.

RA induction of a RA-responsive DR5 reporter construct is maintained during *in vitro* progression of HKc/HPV16

The electrophoretic mobility shift assays using DR1 and DR5 RAREs, as well as the Western analyses for the nuclear RARs and RXRs, suggested that reduced sensitivity to growth control by RA during progression of HKc/HPV16 could not be ascribed to a loss of the nuclear retinoid receptors. To more directly assess overall retinoid signaling in HKc/HPV16, we employed a luciferase reporter construct in which three copies of the DR5 RARE were used as an enhancer element upstream of the SV40 promoter ($DR5_3$-SV40-Luc). The reporter was electroporated into HKc/HPV16d-1 or d-4 at various stages of *in vitro* progression and the ability of RA to induce luciferase expression was then determined. As shown in Fig. 4, RA induced luciferase expression over 10-fold in low-passage HKc/HPV16. Interestingly, not only did the ability of RA to induce luciferase remain intact during progression, but the levels of induction actually increased to 30- to 45-fold by the HKc/DR stage (Fig. 4). Thus, RA signaling clearly remains intact during progression despite the fact that the cells no longer respond to growth inhibition by RA.

RA induces TGF-β2 expression in RA-resistant HKc/HPV16

We have previously reported that RA treatment of normal HKc and HKc/HPV16 enhanced the secretion of latent TGF-β and increased the steady-state levels of TGF-β1 and TGF-β2 mRNA by about 3- and up to 50-fold,

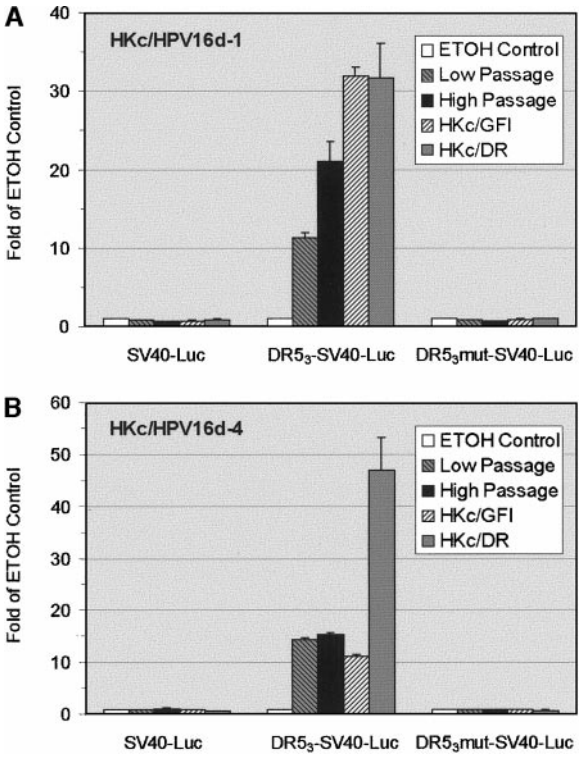

FIG. 4. RA-mediated activation of the reporter construct $DR5_3$-SV40-Luc is maintained throughout progression. HKc/HPV16d-1 (A) or HKc/HPV16d-4 (B) at each stage of premalignant conversion were electroporated with the luciferase reporter construct SV40-Luc (pGL3-Promoter), $DR5_3$-SV40-Luc, or $DR5_3$mut-SV40-Luc and were treated for 48 h with 10 nM RA (in ethanol, 0.1% final concentration) or 0.1% ethanol, with refeeding at 24 h. Luciferase activity was then determined in cell lysates. Luciferase activity is expressed as the fold change above the paired ethanol-treated control and represents the mean ± SEM of three independent experiments.

respectively (Batova *et al.,* 1992). In view of the fact that there appeared to be no loss of nuclear retinoid receptors or RA signaling capabilities during progression, we wished to investigate whether RA could still induce TGF-β2 in cells resistant to RA-mediated growth inhibition. Therefore, we performed RNase protection assays with a riboprobe for TGF-β2 using RNA extracted from low- and high-passage HKc/HPV16 and their corresponding HKc/GFI and HKc/DR lines. As shown in Fig. 5, RA promotes a marked increase in TGF-β2 mRNA at all stages of progression. Densitometric scanning of the autogradiogram shown in Fig. 5 found no significant differences in the extent of induction (about 8-fold) of TGF-β2 mRNA by RA during *in vitro* progression. Thus, the RA-mediated increase of TGF-β2 remains intact in HKc/HPV16, even when the cells are no longer sensitive to growth inhibition by RA.

Anti-TGF-β antibodies partially reverse RA inhibition of cell proliferation in low-passage HKc/HPV16

The finding that RA induces TGF-β production has led us to suggest that RA regulates proliferation of retinoid-

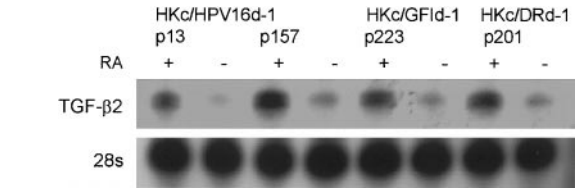

FIG. 5. RA induction of TGF-β2 mRNA is not lost during *in vitro* progression. RPAs were conducted using riboprobes specific for either TGF-β2 or 28S rRNA, with RNA isolated from HKc/HPV16d-1 at low (passage 13) and high passage (passage 157), and its corresponding HKc/GFI (passage 223) and HKc/DR (passage 201) lines, each treated for 72 h in the absence (−) or in the presence (+) of 100 nM RA.

sensitive HKc/HPV16 by enhancing the production of TGF-β that, after activation at the cell surface, could inhibit cellular proliferation in an autocrine/paracrine manner (Batova *et al.,* 1992). If this mechanism is correct, then neutralizing anti-TGF-β antibodies added to the culture medium of RA-treated HKc/HPV16 should at least partially reverse inhibition of DNA synthesis by RA. Therefore, we examined the effect of anti-TGF-β antibodies that recognize and neutralize both TGF-β1 and TGF-β2 on inhibition of [^{3}H]thymidine uptake in low-passage HKc/HPV16 treated with 100 nM RA. As shown in Fig. 6, RA alone inhibited [^{3}H]thymidine uptake about 40%, while anti-TGF-β antibodies (2.5–20 μg/ml) partially blocked RA inhibition of [^{3}H]thymidine uptake in a dose-dependent manner. At the highest concentration of anti-TGF-β antibody used (20 μg/ml), [^{3}H]thymidine uptake was 90% of that observed in the absence of RA and

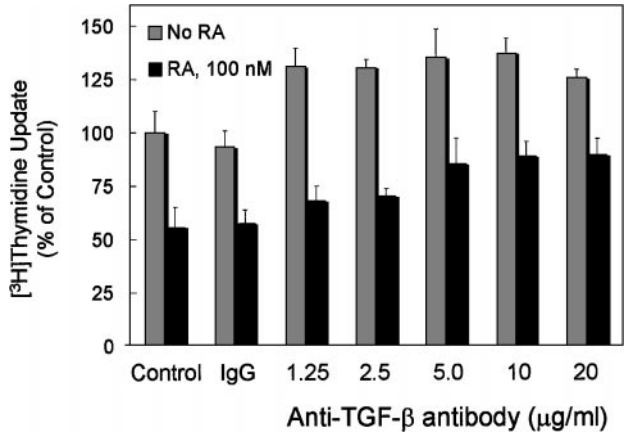

FIG. 6. Anti-TGF-β antibodies partially reverse RA inhibition of [^{3}H]thymidine uptake in HKc/HPV16. Low-passage HKc/HPV16d-1 (passage 38) were plated in CM (10,000 cells/well) into 24-well clusters. The following day the cells were refed with BPE-free CM containing either 0.1% DMSO (▨) or 100 nM RA (■) in DMSO (final DMSO concentration 0.1%), without any antibody (control), with nonspecific rabbit IgG (IgG), or with the indicated concentrations of anti-TGF-β antibodies (1.25–20 μg/ml). Cells were refed in the same way at 48 and 72 h and then pulsed with 1.0 μCi/ml [^{3}H]thymidine for 16 h, and [^{3}H]thymidine uptake was determined as described under Materials and Methods. The data are expressed as means ± SD of at least triplicate determinations. The experiment was conducted twice with similar results.

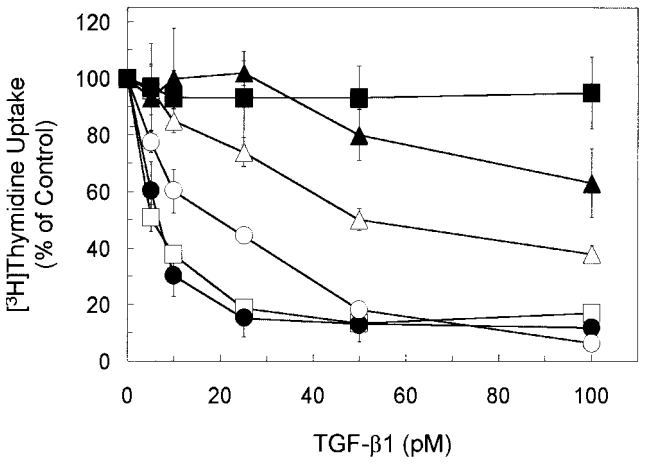

FIG. 7. HKc/HPV16 lose sensitivity to TGF-β1 during *in vitro* progression. Normal HKc (□), HKc/HPV16d-1 passages 7 to 26 (●), HKc/HPV16d-1 passage 40 (❍), HKc/HPV16d-1 passages 91 to 141 (△), HKc/HPV16d-1 passages 187 to 190 (▲), and HKc/DRd-1 (■) were plated into 24-well clusters in CM, with the exception of HKc/DR, which were plated in basal MCDB containing 5% FBS and 1.0 mM calcium chloride. The following day the cells were refed with 1.0 ml per well of CM containing the indicated concentrations of TGF-β1 (performed in triplicate) and 24 h later 0.5 μCi per well of [^{3}H]thymidine was added for 16 to 18 h. [^{3}H]Thymidine uptake was then determined. The data included for HKc/HPV16d-1 passages 7 to 26 represent the average of six separate experiments conducted at passages 7, 14, 15, 17, 24, and 26. The data included for HKc/HPV16d-1 passages 91 to 141 represent the average of two separate experiments conducted at passages 91 and 141. The data included for HKc/HPV16d-1 passages 187 to 190 represent the average of four separate experiments conducted at passages 187, 188, 189, and 190. The data included for HKc/DRd-1 represent the average of three separate experiments. The data are expressed as means ± SD.

anti-TGF-β antibody (Fig. 6). Control IgG had no effect on [^{3}H]thymidine uptake in the absence or in the presence of RA. Anti-TGF-β antibodies alone (i.e., in the absence of RA) consistently increased [^{3}H]thymidine uptake about 30%, most likely by blocking the antiproliferative effects of endogenous TGF-β secreted by the cells (Fig. 6). However, this effect was not dose-dependent, being maximal at all anti-TGF-β antibody concentrations used in these studies.

Loss of TGF-β responsiveness during *in vitro* progression of HKc/HPV16

Since HKc/HPV16 become increasingly resistant to the growth inhibitory actions of RA during *in vitro* progression, and since growth inhibition by RA is mediated by TGF-β, we reasoned that HKc/HPV16 should also become correspondingly resistant to the growth inhibitory effects of TGF-β. Thus, we compared the sensitivity of normal HKc and increasing passages of HKc/HPV16 (from passages 7 to 190) and HKc/DR to either TGF-β1 or TGF-β2 treatment. Early passage HKc/HPV16 (passages 7 to 26) were very sensitive to the antiproliferative effects of TGF-β1 (Fig. 7). By passage 40, HKc/HPV16 began to show a decreased sensitivity and from passage 91 to 190 they became increasingly TGF-β resistant (Fig. 7). By the HKc/DR stage of premalignant conversion, proliferation was virtually unaffected by TGF-β1 (Fig. 7). Treatment with TGF-β2 produced similar results: low-passage HKc/HPV16 (passages 14 to 20) were highly sensitive, high-passage HKc/HPV16 (passages 187 to 190) progressively lost sensitivity, and HKc/DR were completely resistant to the growth inhibitory effects of TGF-β2 (data not shown).

Resistance to growth inhibition by either TGF-β or RA is associated with resistance to inhibition of HPV16 E7 expression

We have previously shown that RA treatment of low-passage HKc/HPV16 causes a decrease in mRNA and protein levels of the HPV16 early ORFs E6 and E7 (Pirisi *et al.,* 1992; Khan *et al.,* 1993; Creek *et al.,* 1994) and others found that TGF-β is a potent inhibitor of HPV16 early gene transcription (Woodworth *et al.,* 1990). RNase protection analyses, using a riboprobe specific for HPV16 E7, demonstrated that, unlike low-passage HKc/HPV16, TGF-β treatment of high-passage HKc/HPV16 and HKc/DR no longer fully suppressed E7 mRNA expression (Fig. 8). Similarly, RA treatment of high-passage HKc/HPV16, HKc/GFI, and HKc/DR failed to reduce the steady-state levels of E7 mRNA (data not shown). Overall, loss of sensitivity to RA parallels a loss of sensitivity to TGF-β-mediated inhibition of E7 expression. In addition, anti-TGF-β antibodies partially reversed the decrease of E7 mRNA levels caused by RA treatment of low-passage HKc/HPV16 (data not shown). These observations further support the interpretation that the effects of RA on proliferation and HPV16 gene expression in low-passage HKc/HPV16 are mediated through TGF-β.

DISCUSSION

In this report we show a parallel loss of sensitivity to RA and TGF-β as HKc/HPV16 progress *in vitro* toward the

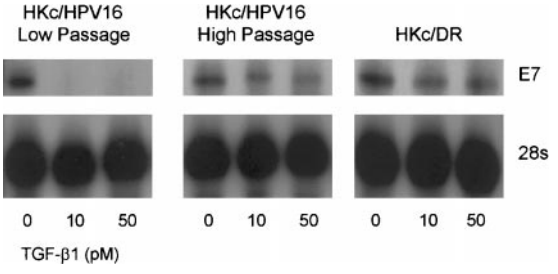

FIG. 8. Loss of sensitivity to TGF-β-mediated inhibition of E7 expression in high-passage HKc/HPV16 and HKc/DR. Low-passage HKc/HPV16d-1, high-passage HKc/HPV16d-1, and HKc/DRd-1 were cultured in CM until about 70% confluence and then treated for 24 h with the indicated concentrations of TGF-β1 prior to RNA extraction. RPAs were conducted using probes for HPV16 E7 and for 28S rRNA, as described under Materials and Methods.

HKc/DR phenotype, provide evidence that RA exerts its growth suppressive effects through TGF-β, and directly link RA resistance to TGF-β resistance. Furthermore, we demonstrate that retinoid refractoriness in HKc/HPV16 occurs despite the presence of functional retinoid receptors and intact RA signaling.

Previous studies have provided strong evidence for a loss of RARs, especially RAR-β, in cancers of the lung (Xu *et al.,* 1997a), cervix (Geisen *et al.,* 1997), and breast (Widschwendter *et al.,* 1997; Xu *et al.,* 1997b), suggesting loss of response to retinoids as an important event in the transformation process. In addition, some of these studies have linked RA resistance to a loss of RAR expression. We thus investigated in detail whether a loss in the expression of the RARs or RXRs or changes in retinoid signaling accompanied resistance to RA-mediated inhibition of growth during *in vitro* progression of HKc/HPV16. We obtained several lines of evidence that retinoid receptors are present and RA signaling remains functional in late-stage, RA-resistant HKc/HPV16: (1) Gel shift assays with DR1 and DR5 elements demonstrated similar levels of retinoid receptor binding activity using nuclear extracts prepared from retinoid-sensitive and -resistant HKc/HPV16, (2) Western blots showed comparable levels of RARγ and RXR in RA-resistant and -sensitive cells, (3) a RA reporter construct was activated by RA throughout progression, and (4) RA induction of TGF-β2 mRNA is conserved in RA-resistant HKc/HPV16. These results led us to conclude that altered expression of, or signaling by, the predominant RAR or RXR isotypes found in skin are not the mechanism leading to retinoid resistance in our *in vitro* model of HPV16-mediated transformation of HKc. Similar to our findings, Kim *et al.* (1995) reported that retinoid refractoriness can occur during lung carcinogenesis despite functional retinoid receptors. Therefore, resistance to growth control by RA in tumor cells may occur with or without a loss of nuclear retinoid receptors.

Since RA induces TGF-β synthesis and secretion in both normal HKc and HKc/HPV16, we have previously suggested that RA inhibition of growth in early passage HKc/HPV16 may be mediated, at least in part, by TGF-β (Batova *et al.,* 1992). This mechanism is strongly supported by our current studies that demonstrate that anti-TGF-β antibodies partially prevent RA inhibition of cell proliferation in HKc/HPV16 and by studies of Glick *et al.* (1989), who found that antibodies to TGF-β2 could partially reverse RA-induced inhibition of DNA synthesis in mouse keratinocytes. If growth inhibition by RA is mediated by TGF-β, one would predict that RA resistance should be accompanied by TGF-β resistance. Indeed we found that *in vitro* progression of HKc/HPV16 is accompanied by a parallel resistance to both RA and TGF-β. Overall, our results are in agreement with studies by Hietanen *et al.* (1998), who found that late passage HPV-positive cell lines derived from vaginal intraepithelial neoplasias are less sensitive to the anti-proliferative effects of retinoids and TGF-β than early passage cells and that neutralizing anti-TGF-β antibodies can block retinoid inhibition of cell proliferation in early passage cells.

In summary, using an HPV16-initiated model of multistep carcinogenesis of human cells *in vitro,* we have found that progression toward malignancy is associated with an early and progressive loss of sensitivity to both RA and TGF-β. Loss of sensitivity to RA was found to be the direct result of a loss of sensitivity to TGF-β. Based on these findings, we explored the molecular basis for TGF-β resistance during *in vitro* progression of HKc/HPV16. As shown in the accompanying paper, TGF-β resistance is accompanied by reduced expression of the TGF-β receptor type I (Mi *et al.,* 2000), an essential component of the TGF-β signal transduction pathway (Attisano *et al.,* 1994; Miyazono *et al.,* 1994; Wrana *et al.,* 1994). A partial loss of RA and TGF-β sensitivity in HKc/HPV16 usually occurs prior to any detectable loss of TGF-β receptor type I mRNA. This observation suggests that loss of TGF-β sensitivity during progression of HPV16-immortalized cells may be the result of a rather complex and multiple-component mechanism, with reduced TGF-β receptor type I expression being responsible for the complete TGF-β resistance observed at the HKc/DR stage. E7 expression has been previously linked to TGF-β resistance in HKc transformed by a plasmid expressing HPV16 E6/E7 under the control of a human β-actin promoter (Pietenpol *et al.,* 1990). However, our early passage HKc/HPV16, which express enough E7 to support continuous growth, are as sensitive as normal HKc to growth inhibition by TGF-β. Similarly, Braun *et al.* (1990) reported that nontumorigenic HPV16-immortalized human epithelial cells were sensitive to TGF-β, whereas two cervical carcinoma cell lines (Caski and Siha) were resistant. There are several possible reasons for this apparent paradox between our current studies and those of Pietenpol *et al.* (1990). First, the HPV16-immortalized cells used by Pietenpol *et al.* (1990) had been selected in high-calcium and serum-containing medium, and one could argue that they have the same phenotype as our late stage premalignant HKc/DR cells. Second, HKc/HPV16 used in the present studies were immortalized by the full-length HPV16 DNA, and E7 is expressed under the control of the HPV16 upstream regulatory region, which is extremely sensitive to inhibition by TGF-β (Woodworth *et al.,* 1990). In contrast, Pietenpol *et al.* (1990) used a human β-actin promoter to drive E6/E7 expression. It is possible that in early passage HKc/HPV16, in which TGF-β signaling mechanisms are intact, TGF-β treatment reduces dramatically E7 expression and therefore is growth inhibitory because E7 expression is required to maintain continuous growth. However, as the cells progress, TGF-β signaling is compromised and all mechanisms of growth inhibition by TGF-β (including inhibition of E6/E7 expression) are lost or greatly

diminished. It will be of extreme interest to explore further the possible connections between the expression of E6 and E7 and TGF-β resistance, including the loss of TGF-β receptor type I, in our model of HPV16-mediated transformation of HKc.

MATERIALS AND METHODS

Materials. RA was from Eastman Kodak Co. or Sigma Chemical Co. [^{3}H]Thymidine (sp act 65 Ci/mmol) was purchased from ICN. A rabbit anti-porcine platelet TGF-β1 antibody (purified IgG) was from R&D Systems. This antibody cross-reacts and neutralizes both human TGF-β1 and TGF-β2. Porcine TGF-β1 and TGF-β2 were also obtained from R&D Systems.

Cell culture and cell lines. Normal HKc were isolated from newborn foreskins as described previously, except the epidermis was separated from the dermis by incubation overnight at 4°C in 0.25% trypsin (Gibco BRL) instead of collagenase (Pirisi *et al.,* 1987, 1988, 1992). Isolation and characterization of the immortalized HKc/HPV16 lines have been described in detail in previous publications (Creek *et al.,* 1994; Khan *et al.,* 1993; Pirisi *et al.,* 1987, 1988, 1992; Zyzak *et al.,* 1994). These cell lines were obtained by transfecting normal HKc strains, each derived from a different individual, with the plasmid pMHPV16d; a head-to-tail dimer of the full-length HPV16 DNA cloned into the *Bam*H1 site of the vector pdMMT-neo, which carries a gene for resistance to the antibiotic G418 (Pirisi *et al.,* 1987, 1988). The different immortalized lines were selected with G418 and were designated HKc/HPV16d-1 to d-5.

Growth factor-independent HKc lines were established by maintenance of the various HKc/HPV16 lines in complete medium (see below for medium composition) lacking bovine pituitary extract (BPE) and epidermal growth factor (EGF). The establishment and growth characteristics of the HKc/GFI lines have been described previously (Pirisi *et al.,* 1988; Zyzak *et al.,* 1994). Differentiation-resistant HKc/HPV16 were selected from HKc/HPV16 by maintenance in medium containing 5% fetal bovine serum and 1 mM calcium chloride (Pirisi *et al.,* 1988; Zyzak *et al.,* 1994).

Normal HKc and HKc/HPV16 were cultured in serum-free MCDB153-LB medium, supplemented with hydrocortisone (0.2 μM), insulin (5 μg/ml), transferrin (10 μg/ml), triiodothyronine (10 nM), $CaCl_2$ (0.1 mM), EGF (5 ng/ml), and BPE (35–50 μg protein/ml) with medium changes every 48 h (Pirisi *et al.,* 1987, 1988; Zyzak *et al.,* 1994). This medium will be referred to as complete MCDB153-LB medium (CM). HKc/GFI were maintained in CM without EGF and BPE, which will be referred to as growth factor-depleted medium (GFDM). HKc/DR were cultured in CM containing 5% fetal bovine serum (FBS) and 1 mM calcium chloride. Media were changed every 48 h. Cells were routinely split 1:10 so that each passage represents three to four population doublings.

Clonal growth assay. Normal HKc and different passage HKc/HPV16d-1 were plated at low density (1000 cells/60-mm culture dish) in CM. Cells were fed 1 and 6 days after plating with 8 ml/dish of CM containing various concentrations of RA. RA was added to the medium in dimethyl sulfoxide (DMSO) and the controls contained DMSO only. The final DMSO concentration was 0.1%. Colonies were fixed in methanol and stained with Giemsa solution 11 days after plating. The total area of the colonies, relative to the area of the dish, was determined by computerized image analysis.

Mass culture growth assay. HKc/HPV16d-1 at different passages and its HKc/GFI and HKc/DR derivatives were plated at a density of 20,000 cells/35-mm culture dish in their respective media and refed 24 h after plating in media containing DMSO only (0.1%) or RA (25–500 nM) in DMSO. Cell number was determined in triplicate dishes for each RA concentration 6 days after plating, and every other day up to about 2 weeks in culture, by trypsinizing and counting cells in a hemocytometer.

[^{3}H]Thymidine uptake assay. Normal HKc and different passage HKc/HPV16d-1 or HKc/DR were plated (50,000 cells/well) into 24-well clusters (Costar) in their respective media. At 24 h after plating, the cells were refed with CM (1 ml) containing the indicated concentrations of TGF-β1 or TGF-β2, and 24 h later the cells were pulsed for 16 h with [^{3}H]thymidine (0.5 μCi/well). The cells were then washed three times with 1 ml of CM, followed by two washes with 1 ml of ice-cold 10% trichloroacetic acid. The cells were solubilized in 0.5 ml of 1 N sodium hydroxide containing 0.1% sodium dodecyl sulfate. [^{3}H]Thymidine present in 0.4 ml of each sample was determined in 5 ml of a water-compatible scintillation cocktail containing 0.4 ml of 1 N HCl. For experiments with RA and anti-TGF-β antibodies, the same procedures were followed with the modifications described in the figure legends.

RNA analysis. The levels of mRNA for TGF-β2 and HPV16 E7 were measured by RNase protection assays (RPAs) using an RPAII kit (Ambion) according to the instructions provided by the manufacturer. A HPV16 E7-specific riboprobe was synthesized on the template of a fragment of the HPV16 E7 ORF (nucleotides 653–846), cloned into the plasmid pCRII (Invitrogen). The template for the synthesis of a riboprobe specific for TGF-β2 (nucleotides 1204–1365) (Madisen *et al.,* 1988) was produced by PCR and cloned into pGEM-T Easy (Promega). Molecular size standards and housekeeping control templates for the RPAs were obtained from Ambion.

Preparation of nuclear extracts. Nuclear extracts were prepared using modifications of the methods of Dignam *et al.* (1983) and Fisher *et al.* (1994) and all procedures were performed at 4°C. HKc/HPV16 cultures were washed twice with ice-cold Dulbecco's phosphate-buff-

ered saline (PBS) and then scraped into PBS containing 1 mM EDTA using a cell lifter, pelleted by low-speed centrifugation for 5 min at 200 *g,* and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9 at 4°C, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) at a concentration of 2×10^7 cells/ml. The cell pellet obtained through a subsequent low-speed centrifugation was immediately resuspended in hypotonic buffer as described above, incubated for 10 min on ice, and then lysed by 10 strokes of a Dounce B homogenizer. Release of intact nuclei from >95% of the cells was confirmed through microscopic inspection. Nuclei in the homogenate were collected by centrifugation for 10 min at 1500 *g*. The resulting crude nuclear pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9 at 4°C, 25% (v/v) glycerol, 500 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) in a volume equivalent to 3 ml per 10^9 cells. Extraction of nuclear protein was performed for 30 min at 4°C with gentle mixing and the extracted nuclei were subsequently removed by a 30-min centrifugation at 16,000 *g.* The resulting supernatant was dialyzed in a Slide-A-Lyzer cassette (10-kDa cut-off; Pierce) against 400 vol of dialysis buffer (20 mM HEPES, pH 7.9 at 4°C, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, pH 8.0, 0.5 mM DTT, and 0.2 mM PMSF) for 45 min. Precipitates were removed by centrifugation for 20 min at 16,000 *g* and small aliquots of the soluble nuclear extracts were immediately frozen and stored at −80°C for subsequent use in gel shift assays and Western blot analyses. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad) using bovine serum albumin as a standard.

Electrophoretic mobility shift assays (EMSA). EMSA were performed essentially according to the methods of Rochette-Egly *et al.* (1991) with slight modifications. Double-stranded oligonucleotide probes containing the wild-type DR5 retinoid response element in the promoter region of the retinoic acid receptor β-2 (RARβ-2) gene, as described by Mader *et al.* (1993), and a DR1 retinoid response element (5′-GTAGGGTTCAGAGTTCACTCGC-3′) were 5′-end-labeled with T4 polynucleotide kinase (Promega) in the presence of [γ-^{32}P]ATP. A DR5mut double-stranded oligonucleotide containing mutations at both DNA binding motifs (5′-GTAGGCTTACCCGAATTTTCACTCGC-3′) was used as a cold nonspecific probe in competition of binding. Nuclear extracts (10 μg protein) were preincubated at room temperature for 20 min in a 20-μl binding reaction mix containing 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0, 0.5 mM DTT, 10% (v/v) glycerol, 1 μg poly(dI–dC), 1 μg sonicated herring sperm DNA, and cold competitor oligonucleotides when required. For antibody supershifts, 2 μl of isoform-specific RA receptor γ-1 (RARγ-1) rabbit polyclonal antibody (Affinity Bioreagents) was also included during this preincubation period. The ^{32}P-end-labeled probe (20 fmol) was subsequently added and the samples were incubated at room temperature for an additional 15 min. Resulting protein:DNA complexes were resolved by nondenaturing electrophoresis through a 5% polyacrylamide, 0.13% bisacrylamide gel in 1× Tris–borate/EDTA buffer for 2.5 h at 150 V and 20°C. The gels were dried and the radiolabeled complexes were visualized by autoradiography.

Western blot analysis. Equal amounts of nuclear extract (20–30 μg protein) were resolved on a 12% SDS–polyacrylamide gel in a miniunit/Tris–glycine buffer system (25 mM Tris, 192 mM glycine, and 0.1% SDS). Proteins were transferred onto Trans-Blot nitrocellulose (Bio-Rad; 0.2 μm) by electroblotting for 3.5 h (120 V) at 4°C in 25 mM Tris, 192 mM glycine, and 20% methanol. Air-dried membranes were rinsed briefly in several changes of PBS and blocked overnight at 4°C in blocking solution containing 5% Carnation skim milk powder and 0.05% Tween 20 (Sigma) in PBS. A polyclonal rabbit anti-retinoid X receptor antibody (anti-RXR-α) (Santa Cruz Biotechnology) that cross-reacts with all RXR isotypes or an isoform-specific anti-RARγ-1 was diluted 1:1000 in fresh blocking solution and incubated with the blot for 1 h at room temperature with gentle rocking. Following extensive washings in PBS containing 0.05% Tween 20, blots were subsequently incubated with a 1:5000 dilution of goat anti-rabbit biotin-conjugated secondary antibody (Sigma) in fresh blocking solution for 1 h at room temperature. After being washed as described above, blots were then incubated for 1 h at room temperature with a 1:1000 dilution of streptavidin–horseradish peroxidase conjugate (Amersham) in Western Blocking Reagent (Boehringer Manneheim) diluted to 1× with PBS containing 0.05% Tween 20. A final series of washes was followed by enhanced chemiluminescence detection using SuperSignal (Pierce). Membranes were exposed to X-OMAT AR film (Eastman Kodak) for 5–10 s and autoradiograms were quantified densitometrically. A RXRα ligand binding domain–glutathione *S*-transferase (GST) fusion peptide (55 kDa; 10 ng) and a full-length RARγ–GST fusion peptide (75 kDa; 10 ng) (Santa Cruz Biotechnology) were used to demonstrate the specificity of the immunoreaction. Parallel gels stained with Coomassie brilliant blue R-250 were used to confirm relative protein loading, and reprobing of the blots (stripped for 30 min at 60°C in 2% SDS, 62.5 mM Tris–Cl, pH 6.8, and 100 mM β-mercaptoethanol) with a rabbit anti-actin primary antibody (Sigma) was used to demonstrate a similar degree of nuclear protein purification.

Electroporation and luciferase reporter activity. Synthetic oligonucleotides of head-to-tail trimers of the DR5 or DR5mut gel shift probe sequence (see above) were cloned into the *Eco*RV site of pBluescript SK(+) (Stratagene), restriction digested with *Bam*HI and *Kpn*I, and then subcloned into the compatible *Kpn*I and *Bgl*II sites

of pGL3-Promoter (Promega) to serve as an enhancer element upstream of the SV40 promoter in the luciferase reporter. The constructs are referred to as $DR5_3$-SV40-Luc or $DR5mut_3$-SV40-Luc. Approximately 1.2×10^7 cells per sample were electroporated with 10 μg plasmid DNA. To produce paired samples with identical electroporation efficiencies, two electroporation samples were combined and then were equally plated between 2× 100-mm dishes. At 10.5 h postplating, the paired samples were treated for 48 h with either 10 nM RA in ethanol (0.1% final concentration) or 0.1% ethanol with refeeding at 24 h. HKc/HPV16 and HKc/DR were treated in CM and HKc/GFI were treated in GFDM. Following treatment, cells were lysed by sonication and luciferase activity was determined using the Luciferase Assay System (Promega). Luciferase activity is expressed as the fold change relative to the paired ethanol control group.

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