Cervical keratinocytes containing stably replicating extrachromosomal HPV-16 are refractory to transformation by oncogenic H-Ras

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Abstract

Ras expression in human epithelial cells with integrated HPV genomes has been shown to cause tumorigenic transformation. The effects of Ras in cells representing early stage HPV-associated disease (i.e., when HPV is extrachromosomal and the oncogenes are under control of native promoters) have not been examined. Here, we used human cervical keratinocyte cell lines containing stably replicating extrachromosomal HPV-16 and present the novel finding that these cells resist transformation by oncogenic H-Ras. Ras expression consistently diminished anchorage-independent growth (AI), reduced E6 and E7 expression, and caused p53 induction in these cells. Conversely, AI was enhanced or maintained in Ras-transduced cervical cells that were immortalized with a 16E6/E7 retrovirus, and minimal effects on E6 and E7 expression were observed. Ras expression with either episomal HPV-16 or LXSN-E6/E7 was insufficient for tumorigenic growth suggesting that other events are needed for tumorigenic transformation. In conclusion, our results indicate that Ras-mediated transformation depends on the context of HPV oncogene expression and that this is an important point to address when developing HPV tumor models.
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Introduction

High-risk human papillomavirus (HPV) infection is a causative agent of cervical cancer, with nearly all malignancies containing HPV DNA (Walboomers et al., 1999). During the natural course of infection, HPV is first established as an extrachromosomally replicating genome (often referred to as episomal replication) in the basal cells of stratifying epidermis. The association of the E6 and E7 oncogenes with several cellular factors, like the tumor suppressor p53, the hTERT catalytic component of telomerase, and the retinoblastoma (Rb) protein, promotes cellular immortalization and transformation (Mantovani and Banks, 2001; Munger et al., 2001).

Progression to malignancy is commonly associated with transcriptional upregulation of E6/E7 as a result of integration of the viral genome with preferential deletion of the E1 and E2 ORFs (Andersson et al., 2005; Romanczuk and Howley, 1992). Many studies have relied on the use of cloned HPV E6 and E7 genes artificially expressed from a retroviral or CMV promoter to mimic HPV-infected cells and the use of extensively cultured cell lines that contain integrated HPV to model advanced cervical disease. Models of HPV transformation based on these systems, however, do not consider that a significant number of cervical lesions, even some high grade, continue to maintain extrachromosomal copies of the viral genome. Arias-Pulido et al. showed that integration most commonly occurs in the presence of a multitude of HPV episomes (Arias-Pulido et al., 2006), and in light of a recent report, viral genes expressed episomally, namely E2, likely influence expression from integrated HPV DNA in a natural infection (Pett et al., 2006). The maintenance of

extrachromosomal genomes and its effect on oncogene regulation, both in cis and trans, are important considerations for understanding how high-risk HPV malignancies develop.

Examination of the early stages of HPV infection and transformation has relied on the establishment of viral replication in vitro. These studies have been hindered by the tendency of viral genomes to integrate during cell culture, although a handful of reports have demonstrated stable episomal replication of HPV-11, -16, -18, -31, and -45 in foreskin keratinocytes (Flores et al., 1999; Frattini et al., 1996, 1997; McLaughlin-Drubin et al., 2003; Thomas et al., 2001) and HPV-18 in cervical keratinocytes (Meyers et al., 1997). Study of episomal HPV-16 specifically in cervical keratinocytes has relied on the use of W12 cells and its subclonal derivations. W12 cells were isolated from a pre-existing CIN I lesion (Stanley et al., 1989), and early passage cells from the parental cell line maintain episomal copies of HPV-16 which are susceptible to integration over time (Jeon et al., 1995). We previously reported the in vitro establishment of episomally replicating HPV-16 in isogenic cell lines derived from primary human cervical keratinocytes by transfection of HPV-16 genomes isolated from W12 clone E or by a novel adenoviral vector (Lee et al., 2004; Sprague et al., 2002). The cell lines were isolated from independently derived clones soon after introduction of HPV into primary cells. Extrachromosomal replication of HPV-16 in these cells is quite stable when cultured with irradiated feeder fibroblasts (Sprague et al., 2002). Thus, the HPV-positive cell lines generated in vitro provide a model of early stage cervical disease to study malignant progression of HPV-16-infected cells by factors such as oncogenic Ras.

Ras is a cellular GTPase that signals through MAPK, PI3K, and Ral-GEF pathways (Katz and McCormick, 1997). Ras expression in primary fibroblasts induces growth arrest and a flattened, enlarged phenotype characteristic of premature senescence (Mason et al., 2004; Serrano et al., 1997). Murine keratinocytes (Lin et al., 1998), human ovarian surface epithelial cells (Nicke et al., 2005), and human esophageal keratinocytes (Takaoka et al., 2004) react similarly. However, it has been reported that human thyroid epithelial cells proliferate upon introduction of Ras (Skinner et al., 2004), suggesting that the effects of Ras on proliferation are cell type specific. In cells where Ras mediates senescence, it is generally accepted that senescence is caused by activation of the ARF-p53 and p16^{INK4a} pathways which trigger cell cycle arrest in G1 (Ferbeyre et al., 2002; Lin et al., 1998; Serrano et al., 1997). This response is considered a tumor suppressing mechanism to prevent aberrant cell signaling and proliferation. Unlike rodent cells, human diploid fibroblasts require disruption of both p53 and p16^{INK4a} pathways to overcome senescence (Serrano et al., 1997). Esophageal keratinocytes lacking a functional p53 pathway (Takaoka et al., 2004) or hTERT-immortalized human adenoid epithelial cells lacking p14^{ARF} (Farwell et al., 2000) undergo Ras-induced senescence, further supporting that disruption of the ARF-p53 pathway alone is insufficient for human cells to escape growth arrest. The contribution of p16^{INK4a} in Ras-induced senescence in epithelial cells is less

clear. In one study, overexpression of Cdk4 (which counteracts p16^{INK4a}) in human epidermal keratinocytes was sufficient for Ras-mediated transformation (Lazarov et al., 2002) while hTERT-immortalized human foreskin keratinocytes deficient for p16^{INK4a} remained sensitive to Ras oncogene expression (Farwell et al., 2000).

Constitutive activation of the Ras signaling pathway is an important component of malignant progression for a number of different cancers. Overexpression or mutation of Ras has been identified to varying degrees in cervical cancers depending on the experimental design (reviewed in Mammas et al., 2005). Several studies indicate an association of Ras with high-grade cervical lesions (Alonio et al., 2003; Bernard et al., 1994; Riou et al., 1988; Sagae et al., 1990). Alonio et al. detected mutated H-Ras in 20% of CIN III cases and 41% of in situ carcinomas (Alonio et al., 2003). In another study, elevated Ras expression was detected in 17.9% of CIN I lesions as compared to 53.9% of CIN III (Sagae et al., 1990). There are also reports that Ras alterations rarely occur in cervical cancer, however, and these studies conclude that there is no correlation between disease severity and Ras expression (Ngan et al., 1999; O'Leary et al., 1998; Van Le et al., 1993). Thus, it is unclear at what stage Ras overexpression or mutation occurs during malignant transformation and whether HPV episome-containing cells tolerate constitutive activation of the Ras pathway, since there was no distinction between episomal or integrated HPV in the studies mentioned.

It is understood that HPV infection alone is not sufficient for tumor formation and that carcinogenesis is a multistep process (reviewed in Hanahan and Weinberg, 2000). In rodent models, Ras has been shown to cooperate with oncogenes from HPV in vivo by inducing tumor growth in HPV-16 E6/E7 transgenic mice (Schreiber et al., 2004). In human cells, understanding the contribution of Ras to cellular transformation in the context of the full HPV genome has been limited to studies using constructs which integrate. Integrated HPV constructs have been shown to cooperate with H-Ras or K-Ras in cervical keratinocytes (DiPaolo et al., 1989), foreskin keratinocytes (Durst et al., 1989, 1991), and prostate epithelial cells (Rhim et al., 1994) by inducing tumor growth in mice. Retroviral transductions using only the 16-E6/E7 oncogenes, however, along with H-Ras and hTERT in fibroblasts and HEK cells did not result in anchorage independence or tumor growth (Hahn et al., 2002; Morales et al., 1999), indicating other events or the full HPV genome are necessary for malignant transformation in those tumor models.

Because there is a lack of understanding of cancer progression based on Ras and cervical keratinocytes maintaining HPV-16 DNA extrachromosomally, we utilized clonal cell lines generated in our laboratory to examine whether oncogenic Ras cooperates with stable episomal HPV-16, specifically during the early stages of transformation. Since we also generated clonal cell lines derived from the same genetic background as the episomal cells using LXSN-16E6/E7 retrovirus, we were able to compare the effects of Ras between two different contexts of oncogene expression. While Ras enhanced or maintained anchorage independence of E6/E7-transduced cells, our results demonstrate that Ras actually caused a loss in transformation potential of cells containing HPV-16 episomes.

Our findings suggest that HPV-infected cells are refractory to Ras-induced transformation commensurate with reduction of E6 and E7 and induction of p53.

Results

Ras expression does not alter episome copy number

Since no in-depth study has analyzed whether Ras and episomal HPV-16 cooperate in transformation of human cervical cells, we grew clonal cervical keratinocyte cell lines containing HPV-16 episomes, called HPV_{EP} cells, and transduced them with H-Ras^{G12V}. We also used isogenically derived clonal cell lines transduced with LXSN-16E6/E7, called E6/E7$_{LX}$ cells, as well as primary human cervical keratinocytes (HCKs). Ras protein expression was confirmed by Western blot (Fig. 1A). The H-Ras^{G12V} construct used in our studies produces a functional protein capable of stimulating the Ras pathway in both HPV_{EP} and E6/E7$_{LX}$ cells, as evidenced by increased phosphorylation of MEK and MAPK effector proteins (Fig. 1B). The reason for higher basal levels of phosphorylated p44/42 MAPK in the E6/E7$_{LX}$ cells is unknown but may have to do with higher expression levels of E6 in these cells (Chakrabarti et al., 2004). To verify that Ras did not disrupt episome copy number control or cause integration, Southern analysis was performed (Fig. 1C). Even in the presence of high Ras expression, HPV_{EP} cells maintained stable episomal DNA
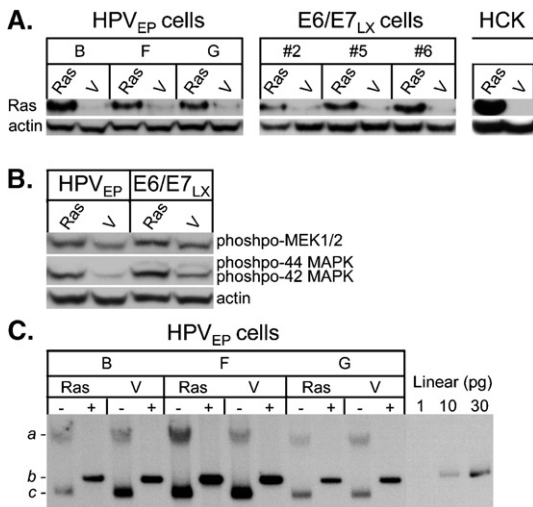

Fig. 1. (A) Western blot verifying Ras protein expression one passage after retroviral transduction of pBabe-puro H-Ras^{G12V} (Ras) or pBabe-puro vector (V). Cell lines used are HPV_{EP} clones (B, F, and G), E6/E7$_{LX}$ clones (#2, #5, and #6), or primary HCKs. (B) Western blot verifying functionality of the H-Ras^{G12V} construct by activation of downstream effectors via phosphorylation of MEK1/2 and 44/42 MAPK proteins in HPV_{EP} and E6/E7$_{LX}$ cells. (C) Southern blot displaying viral DNA present in HPV_{EP} clonal populations expressing Ras or vector. Cells were cultured for 10 passages (~20 population doublings) after Ras-retrovirus infection and DNA was digested either with BglII (− lanes) or BamHI (+ lanes). BglII cleaves cellular genomic DNA, leaving the HPV genome as an intact supercoiled episome (c) or nicked-circular plasmid (a). BamHI linearizes the HPV genome (b). Picogram quantities of linearized HPV-16 plasmid were used to estimate copy number as previously described (see Materials and methods). The 30 pg lane is equivalent to 5 copies per cell.
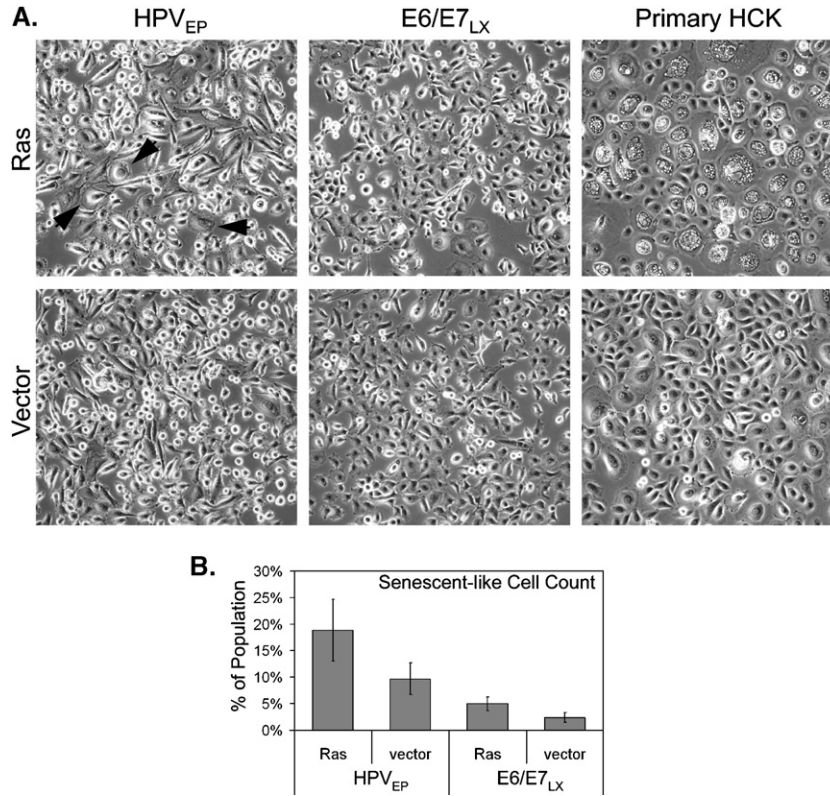
for at least 20 population doublings after Ras transduction with equivalent copies between Ras-transduced cells and vector controls. We have also observed episome maintenance with no evidence of integration in untransduced HPV_{EP} cells even when grown for ~30 population doublings on plastic without irradiated feeders, thought necessary for episome stability, in serum-free keratinocyte media (data not shown). This finding along with Fig. 1C demonstrates the notable stability of episomes in the HPV_{EP} cells created from primary cervical keratinocytes.

Effects of Ras on cell growth

Because Ras is capable of inducing senescence in some cells and stimulating growth in others (Skinner et al., 2004), we first examined whether expression of mutated Ras induced morphological changes indicative of senescence in the HPV_{EP} and E6/E7$_{LX}$ cells. Functionality of the H-Ras^{G12V} retroviral construct used in our study was further confirmed in primary HCKs as indicated by the presence of enlarged, highly vacuolated cells typical of senescence-associated morphology (Fig. 2A). Ras-HPV_{EP} cells did not exhibit as dramatic of an effect, although there were sporadic populations of enlarged, senescent-like cells (Fig. 2A). Differences between Ras and vector transduced E6/E7$_{LX}$ were not readily apparent (Fig. 2A). We stained for senescence-associated beta-galactosidase (SA-β-gal) activity, a marker that is commonly observed in senescent fibroblasts, but detected only weak SA-β-gal staining in late passage senescent primary keratinocytes and Ras-expressing clones (data not shown), suggesting that this is not a reliable marker for senescence in keratinocytes. To quantify the senescent-like cells, a blinded count of cells with an enlarged morphology was performed (Fig. 2B). Senescent-like cells were more frequently found in the Ras-HPV_{EP} cultures than vector control. To a lesser extent, a small fraction of senescent-like cells was also counted in the Ras-E6/E7$_{LX}$ cultures as compared to vector. We also examined the cells for changes in cell cycle profile and did not see dramatic differences between Ras and vector controls in either cell type (data not shown). We next examined the effect of Ras on population doubling (PD) time based on growth assays in monolayer culture (Fig. 3). Ras lengthened the PD time of two HPV_{EP} clones by 4% in clone B and 8% in clone F, yet had no effect in clone G. Two of three E6/E7$_{LX}$ clones had decreased PD time upon Ras expression (i.e., a 3% decrease in PD time for Ras-E6/E7$_{LX}$ clone #6 and only a 1% decrease for Ras-E6/E7$_{LX}$ clone #5). No difference in PD time was observed for E6/E7$_{LX}$ clone #2. In summary, Ras expression had a negative but small effect on growth of HPV_{EP} cells in monolayer culture, and growth of E6/E7$_{LX}$ clones was either enhanced or unchanged.

Ras reduces transformation potential of HPV_{EP} but not E6/E7$_{LX}$ cells

Growing cells in soft agar is a common in vitro transformation assay that measures anchorage-independent (AI) growth (i.e., the ability to grow without substrate attachment). To test if Ras altered anchorage independence of the HPV_{EP} and E6/E7$_{LX}$

Fig. 2. (A) Microphotographs of morphological changes induced by H-Ras^{G12V}. Photographed cultures of HPV$_{EP}$ clone G, HPV$_{EP}$ clone #2, and HCKs were grown without irradiated feeders. Black arrows point to enlarged cells in Ras-expressing HPV$_{EP}$ cells, which are rarely found in E6/E7$_{LX}$ cultures. HCKs demonstrate typical morphology induced by oncogenic Ras in primary epithelial cells. (B) Blinded counts of enlarged and flattened cells, indicative of senescence, from triplicate plates of either HPV$_{EP}$ clone G or HPV$_{EP}$ clone #2 are expressed as a percentage of the total number of cells counted. Data are expressed as the mean±SD.

cervical cell lines, we assayed cell growth in a modified soft agar assay. Two experiments were performed (Table 1) using cell lines generated from two independent transductions with Ras and vector. For visualization purposes, the results of the first experiment are shown graphically (Fig. 4). AI growth of both the HPV$_{EP}$ and E6/E7$_{LX}$ clones was heterogenous, with vector controls ranging from 1 to 16% CFE (Fig. 4). More striking than the growth changes induced by Ras in monolayer culture
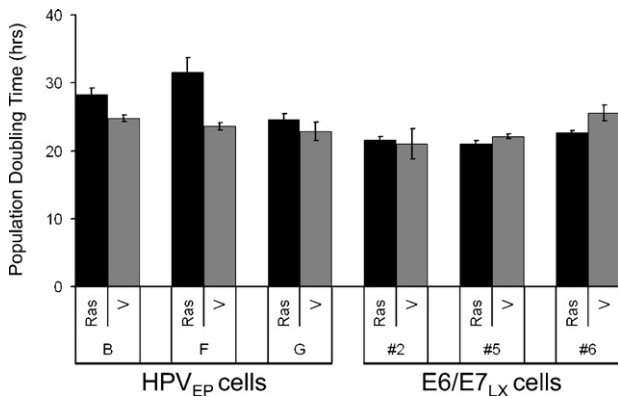

Fig. 3. Population doubling (PD) time was determined over a 3-day period from absorbance data generated from crystal violet staining of cultures (see Materials and methods). H-Ras^{G12V} increased PD time in two of three HPV$_{EP}$ clones. No change in PD time was observed for two E6/E7$_{LX}$ clones, #2 and #5, while Ras slightly decreased PD time in one cell line, clone #6. Data are expressed as the mean±SD of triplicate data points.

Table 1
The effect of H-Ras^{G12V} on colony-forming efficiency in soft agar and tumor growth

Cell line	Clone construct	Anchorage independence (Experiment #1)[a]	Anchorage independence (Experiment #2)	No. of mice with tumors
		% CFE±SD	% CFE±SD	
HPV$_{EP}$	B-Ras	9.5±1.8	5.2±1.0	0/3
	B-vector	14.6±0.6	12.0±1.0	0/3
	F-Ras	3.4±0.9	1.0±0.7	0/3
	F-vector	6.3±0.8	9.6±1.4	0/3
	G-Ras	2.3±0.3	2.1±0.1	0/3
	G-vector	15.8±3.4	6.8±2.8	0/3
E6/E7$_{LX}$	#2-Ras	7.1±0.6	2.5±0.5	0/3
	#2-vector	3.7±0.5	0.9±0.2	0/3
	#5-Ras	8.1±0.8	7.0±0.7	0/3
	#5-vector	8.4±1.1	7.8±0.7	0/3
	#6-Ras	22.1±2.2	12.8±1.0	0/3
	#6-vector	11.8±1.3	3.7±0.6	0/3
Control	HPV-18 tumor[b]	N/D±N/D	N/D±N/D	3/3
	C33A	7.1±0.5	N/D±N/D	N/D
	HCK	0±0	0±0	0/3

[a] Experiment #1 is graphed in Fig. 4.
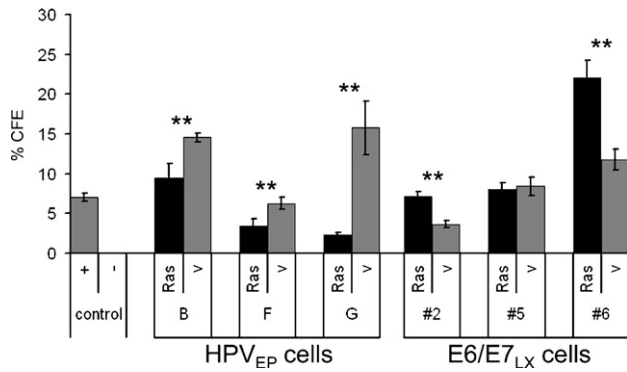[b] 1811-NMU cells; N/D=not determined.

Fig. 4. H-Ras^{G12V} significantly reduced anchorage-independent (AI) colony formation of all HPV$_{EP}$ clones (**$p < 0.001$) and stimulated growth of two E6/E7$_{LX}$ clones (**$p < 0.001$). Percent colony forming efficiency (%CFE) is reported as the percentage of colonies that grew in soft agar per number of cells initially seeded. HPV-negative C33A cells (+) control; primary HCKs (−) control. Data are expressed as the mean±SD of triplicate data points.

(Fig. 3), we observed profound effects on growth in soft agar. Oncogenic Ras significantly limited AI growth of all three HPV$_{EP}$ cell lines ($p < 0.001$) (Fig. 4). In contrast, two E6/E7$_{LX}$ clones (#2 and #6) showed significant growth enhancement upon Ras expression ($p < 0.001$) while one cell line (#5) showed no difference (Fig. 4). The lack of enhanced anchorage independence by Ras in E6/E7$_{LX}$ clone #5 likely reflects heterogeneity in E6/E7$_{LX}$ cell clones. Overall, our results indicate that Ras can cause different effects on AI growth depending on whether cells contain episomal HPV-16 or the 16E6/E7 retroviral construct.

Since AI growth does not necessarily correlate to an ability to form tumors in vivo, full transformation of human cells is typically determined by injecting cells into immuno-compromised mice. Therefore, we tested whether Ras could cooperate with episomal HPV or LXSN-E6/E7 to form tumors in athymic nude mice after subcutaneous injection. After 100 days post-injection, no tumorigenic growth was observed for HPV$_{EP}$ or E6/E7$_{LX}$ cells expressing Ras or vector (Table 1), indicating that Ras and expression of E6/E7 are insufficient for causing tumorigenic transformation.

Effects of Ras on HPV oncogene expression

To determine whether differential oncogene expression is responsible for the observed differences between the HPV$_{EP}$ and E6/E7$_{LX}$ cells, we used real-time RT-PCR to measure the abundance of E6 and E7 mRNA. For reference, mRNA levels were calculated relative to W12 cells. Vector control HPV$_{EP}$ cells, which contain W12-E genomes, had comparable amounts of E6 and E7 to the parental W12 cell line (Figs. 5A and C). Clearly, vector control E6/E7$_{LX}$ cells expressed more E6 than the HPV$_{EP}$ vector control cells (13-fold more on average) (Figs. 5A and B; note different scales). Interestingly, the HPV$_{EP}$ and E6/E7$_{LX}$ vector control cell lines had comparable amounts of E7 mRNA with E6/E7$_{LX}$ vector controls expressing only 1.6-fold more on average (Figs. 5C and D). In agreement with previous reports, this supports the finding that the dual LXSN-E6/E7

retroviral construct expresses E7 inefficiently (Halbert et al., 1991). These results indicate that E6 expression is one key distinction between HPV$_{EP}$ and E6/E7$_{LX}$ cell types.

Interestingly, we found that Ras reduced E6 mRNA levels in the HPV$_{EP}$ clones (Fig. 5A). However, Ras expression affected E6 transcripts in only one of three E6/E7$_{LX}$ clones (#6), where it actually increased levels (Fig. 5B). Similar results were attained for E7. Ras reduced E7 transcripts in the HPV$_{EP}$ clones (Fig. 5C) but did not have a significant effect on E7 transcript levels in E6/E7$_{LX}$ clones #2 and #5 and marginally increased levels in E6/E7$_{LX}$ clone #6 (Fig. 5D). Since we observed changes in E7
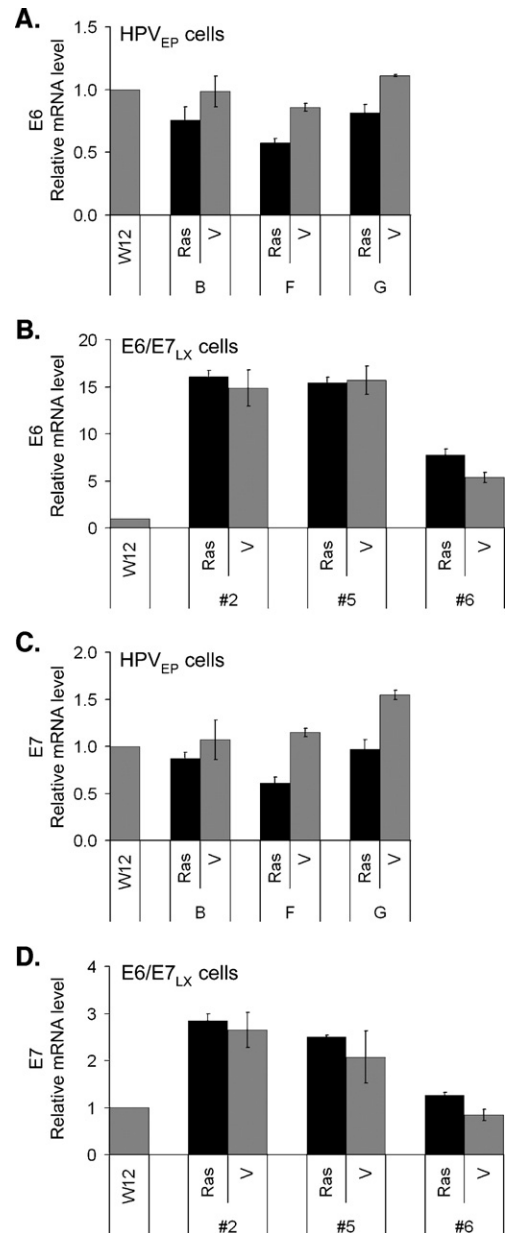

Fig. 5. Real-time RT-PCR for E6 transcripts in Ras or vector-transduced HPV$_{EP}$ (A) or E6/E7$_{LX}$ cells (B) and E7 transcripts in HPV$_{EP}$ (C) or E6/E7$_{LX}$ cells (D) all standardized to 18S mRNA levels. HPV$_{EP}$ cells contain the full genome derived from W12-E cells. E6/E7$_{LX}$ cells dually express E6 and E7 from a single retroviral promoter. For reference, RNA levels were calculated relative to W12 cervical cells, the cell line containing episomal HPV-16 from which the W12-E clone was derived. Data are expressed as the mean±SD of triplicate data points.
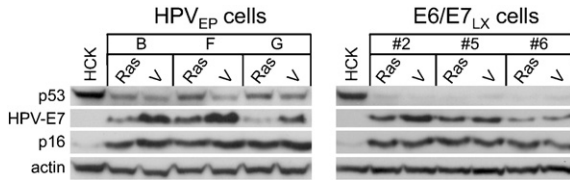
Fig. 6. Western blot of p53, HPV16-E7, and p16^{INK4a} proteins in HPV$_{EP}$ and E6/E7$_{LX}$ clonal populations. Primary HCKs were used for reference. Lanes consist of lysates collected one passage after transduction and selection for H-Ras^{G12V} or vector (V).

transcripts in Ras-expressing cells, we analyzed E7 protein levels (Fig. 6). In HPV$_{EP}$ cells, Ras reduced E7 protein and this corresponds to the reduction of E7 mRNA shown in Fig. 5B. With the E6/E7$_{LX}$ clones, some reduction of E7 protein was observed upon Ras expression but, overall, this was not as great as that observed for the HPV$_{EP}$ cells. The reason for the slight differences between E7 transcript (Figs. 5B and D) and protein levels (Fig. 6) is unknown. Since reliable antibodies for E6 are not available, we were unable to assay for E6 protein in our cells. Overall, however, our real-time RT-PCR and Western data provide evidence that Ras represses expression of E6 and E7 in HPV$_{EP}$ cells but has minimal effects in E6/E7$_{LX}$ cells.

Oncogenic Ras induces p53 but not p16^{INK4a} in cells with episomal HPV-16

Mutant Ras has been shown to upregulate p53 and p16^{INK4a} in normal human fibroblasts, resulting in growth inhibition and senescence (Serrano et al., 1997). Since we observed senescent-like cells, reduction in AI growth, and changes in E6 and E7 transcript levels upon Ras transduction we examined expression of p53 and p16^{INK4a} in our different cell lines. Western analysis was performed using protein lysates from early passage cells (i.e., within 1 to 2 passages after transduction of vector or Ras). As expected from basal levels of E6 mRNA (Figs. 5A and B), HPV$_{EP}$ cells have higher p53 basal expression than E6/E7$_{LX}$ cells (Fig. 6). An increase in p53 protein was detected in Ras-HPV$_{EP}$ cells (Fig. 6), corresponding to a decrease in E6 mRNA. Changes in p53 were not evident between Ras-E6/E7$_{LX}$ and vector controls and this is likely related to higher levels of E6 mRNA in these cells. Since disruption of the p16^{INK4a} pathway is sufficient to overcome Ras-induced senescence in some epithelial cell types (Lazarov et al., 2002) but not in others (Farwell et al., 2000), we also examined p16^{INK4a} protein levels. p16^{INK4a} in either cell type was unchanged upon Ras expression, indicating that induction of p16^{INK4a} was not associated with inhibition of AI growth by Ras in HPV$_{EP}$ cells.

Discussion

The use of cervical cell lines with stably replicating HPV-16 extrachromosomal genomes allowed us to examine the effects of Ras in cells that more closely resemble early HPV infection. The finding that episomal HPV-16 does not support Ras-mediated transformation in human keratinocytes indicates that

HPV genome status has a significant impact on transformation capacity. HPV E6 and E7 oncogenes are essential for transformation and the downregulation of both E6 and E7 by Ras may partially explain why HPV$_{EP}$ cells have reduced AI growth when Ras is introduced. Because p53 closely regulates cell growth and can induce senescence, the significant inhibition of AI growth is likely related to p53 upregulation by Ras. Together, increased p53 and decreased E6 and E7 may limit the growth of HPV$_{EP}$ cells in tissue culture and in soft agar. The results with HPV$_{EP}$ cells contrast with those obtained from cells expressing E6/E7 from a retroviral construct. The E6/E7$_{LX}$ cells were able to maintain or enhance anchorage independence likely because Ras expression did not profoundly affect p53, E6, and E7 levels. Because of the skewed oncogene expression favoring E6 transcription from the heterologous promoter in the E6/E7$_{LX}$ cells, we speculate that it was artificial overexpression of E6 and not E7 that specifically contributed to maintenance of the transformed phenotype in the presence of Ras. Therefore, we conclude that the context of oncogene expression may be a determinant for transformation by oncogenic Ras.

Not uncommon for some transformed cells (Bihani et al., 2004), our cell lines may retain properties of primary cells which trigger tumor suppressing mechanisms, as in p53 induction in Ras-HPV$_{EP}$ cells. It has been previously shown that oncogenic Ras synergizes with integrated HPV-18 in HeLa cells by stimulating cell cycle progression (Cordova-Alarcon et al., 2005), but it was also noted in a related paper that a subpopulation of Ras-transduced HeLa cells have aberrant, giant cell morphology and underwent "mitotic death" (Miranda et al., 1996). Thus, even HeLa cells may still retain some normal responses to Ras. Interestingly, it has been noted, using reporter constructs and HeLa cells, that Ras can mediate activation of the HPV-18 viral upstream regulatory region (URR) (Medina-Martinez et al., 1997). Regarding integrated HPV-16, one report showed increased E6/E7 RNA expression in CX16-2 cells with a human H-Ras oncogene (Gaiotti et al., 2000) while another study reported no change in E6/E7 protein levels in the same CX16-2 cells using a viral H-Ras homolog (DiPaolo et al., 1989). We conclude that the effects of Ras on E6 and E7 RNA and protein levels vary depending on HPV type, source of oncogenic Ras, and most importantly, the context of oncogene expression either from integrated HPV, integrated HPV constructs containing non-HPV DNA sequences, or extra-chromosomal genomes as shown here. A mechanism of how Ras regulates E6/E7 is not fully understood but may involve regulation of AP-1 transcription factors such as c-Jun and c-Fos or other factors (Medina-Martinez et al., 1997).

As with p53, p16^{INK4a} is another protein associated with senescence (Serrano et al., 1997). Although we have observed weak p16^{INK4a} induction by Ras in primary cervical keratino-cytes (unpublished data), Ras did not alter p16^{INK4a} expression in the HPV$_{EP}$ or E6/E7$_{LX}$ cells (Fig. 6). This is likely due to the fact that Rb inactivation by E7 causes misregulation of the p16^{INK4a} pathway leading to high accumulation of p16^{INK4a} protein (Giarre et al., 2001), thus overshadowing any effect by Ras. Therefore, while growth inhibition of HPV$_{EP}$ cells may be in part due to p53 induction, it does not appear to be due to Ras

induction of p16^{INK4a}. It should be noted that other functions of E6 and E7, besides inactivation of p53 and pRb, may play a role in Ras-mediated effects on AI growth. Both E6 and E7 bind to a plethora of cellular proteins and these interactions may be important for maintaining or even promoting cell growth upon Ras activation (Mantovani and Banks, 2001; Munger et al., 2001). Further work is necessary to determine what functions of E6 and/or E7 are important for allowing transformation by Ras.

The answer to why Ras reduces transformation of cells with episomal HPV-16 may also not be as simple as changes in expression levels of E6 and E7. In our studies, E6 and E7 mRNA levels do not strictly correlate to %CFE in the AI growth assays. For example, there is a notable increase in AI of E6/E7$_{LX}$ clone #6 by Ras (Fig. 4) yet this clone expresses lower amounts of oncogenes than the other E6/E7$_{LX}$ clones (Fig. 5). There may be a heterogeneous response to Ras in E6/E7$_{LX}$ cells or any number of other unknown cellular defects that could have occurred in this cell line. In addition to expression levels, whether Ras inhibits or enhances AI growth may also depend on spliced E6 mRNA species. Transcription of the E6 ORF produces alternatively spliced E6* in addition to full-length E6. The function of E6* is unclear but may regulate E6/E7 expression (Sedman et al., 1991) and inhibit degradation of p53 by unspliced E6 (Pim et al., 1997). Studies have shown that cells with extrachromosomal HPV genomes contain more spliced E6 than unspliced (Doorbar et al., 1990; Hummel et al., 1992). This could account for the lower levels of full-length E6 detected in the W12 and HPV$_{EP}$ cells in our assays. The overabundance of full-length E6 in the E6/E7$_{LX}$ cells suggests that the LXSN-16E6/E7 construct is partially dysfunctional in its ability to make the spliced forms. Enhancement of AI growth by Ras in E6/E7$_{LX}$ and not HPV$_{EP}$ cells could also be due to genetic variations in the oncogenes. HPV$_{EP}$ cells contain HPV-16 genomes derived from W12-E (GenBank accession no. AF125673) whereas LXSN-16E6/E7 contains sequences from the prototype genome (GenBank accession no. K02718). Sequence analysis reveals a deviation in W12-E from the prototype—a codon change at amino acid 83 in E6. E6 variants occur in nature and are epidemiologically linked to progression of high-grade lesions to invasive cancers (Zehbe et al., 1998). The E6 amino acid 83 variant (or E6 L83V) does not differ from prototype in ability to degrade p53 but does enhance MAPK signaling (Lichtig et al., 2006; Zehbe et al., 1998), and this may affect the ability of cells to be transformed by Ras.

Certainly, other HPV proteins besides E6 and E7 could play a role in the response of HPV$_{EP}$ cells to Ras. It is possible, for example, that loss of E2 by integration of the HPV genome constructs in previous studies with Ras (DiPaolo et al., 1989; Durst et al., 1989, 1991; Rhim et al., 1994) or lack of E2 in our E6/E7$_{LX}$ cells played a role in Ras-transformation. Gammoh et al. showed that E2 inhibits transformation by HPV-16 E7 and Ras in rodent cells (Gammoh et al., 2006). E2 may similarly effect the ability of E6 to cooperate with Ras via transcriptional regulation as well as protein interaction and mislocalization (Grm et al., 2005). In our HPV$_{EP}$ cells, E2 may be limiting the transformation potential of E6 and E7 through protein–protein

interactions, mislocalization, and/or repression of transcription, thereby rendering cells more susceptible to Ras-induced growth inhibition than cells without E2.

Several epidemiological studies have reported higher levels of Ras in advanced cervical lesions (reviewed in Mammas et al., 2005) and experimental data show that Ras stimulates malignant transformation of human cells containing integrated high-risk HPV (DiPaolo et al., 1989; Durst et al., 1989, 1991; Rhim et al., 1994). Since high-grade lesions are often associated with integration and upregulated oncogene transcription while low-grade lesions more commonly contain extrachromosomal HPV, mutations in Ras may only be tolerated by cells expressing high levels of E6 and E7. Since advanced lesions harboring integrated DNA likely also contain episomal DNA (Arias-Pulido et al., 2006) and also since episomal E2 expression can affect regulation of integrated viral sequences (Pett et al., 2006), episomal HPV may still prevent Ras-mediated transformation even when integrated HPV is present. These hypotheses would require further testing using similarly derived episomal and integrated cell lines. Since our experiments were performed using a retroviral construct that expresses high levels of H-Ras^{G12V}, we also cannot rule out the possibility that lower levels of mutated H-Ras from the endogenous promoter could lead to enhanced transformation in HPV-infected cells (Hua et al., 1997).

Not surprisingly, cervical cells expressing Ras with episomal HPV-16 or overexpressed 16E6/E7 do not form tumors in mice. In HPV tumor models, Ras has only been shown to induce tumor growth of cells containing integrated genomes (DiPaolo et al., 1989; Durst et al., 1989, 1991; Rhim et al., 1994) or by crossing Ras transgenic mice with E6/E7 transgenic mice (Schreiber et al., 2004). Our analysis of cervical keratinocytes agrees with Morales et al. and Hahn et al. who demonstrated that E6/E7 overexpression, hTERT, and Ras were insufficient for tumor growth of human cells in mice (Hahn et al., 2002; Morales et al., 1999). The addition of SV40-small t antigen was required for tumorigenesis (Hahn et al., 2002; Uren et al., 2005). SV40-small t antigen is known to affect the MAP kinase pathway and also inhibit protein phosphatase 2A. Since our cell lines already have active telomerase (Sprague et al., 2002), it would be of interest to determine whether expression of small t might be sufficient for tumor growth in conjunction with Ras expression. Furthermore, in some cervical cancer specimens, Myc, ErbB-2, PTEN, and EGF-R proteins are found to be altered (Pinion et al., 1991; Spandidos et al., 2000) and promote malignant transformation in experimental tumor models (Boehm et al., 2005; Goessel et al., 2005). These are other cellular changes that may be required for full transformation of the HPV$_{EP}$ and E6/E7$_{LX}$ keratinocytes.

In conclusion, we have demonstrated that human cervical keratinocytes containing stable HPV episomes resist transformation by H-Ras^{G12V}. We are currently developing a model of how early cervical lesions progress to malignancy using the HPV$_{EP}$ cells in conjunction with other cervical cancer markers. These studies will be important for determining the types of events necessary for HPV-associated transformation and may point to strategies for inhibiting HPV-related cancer.

Materials and methods

Cell culture

Cell cultures were maintained with post-mitotic, γ-irradiated J2 3T3 feeder fibroblasts in E-media: 3 parts Dulbecco's modified Eagle's media (Invitrogen, Carlsbad, CA) and 1 part HAMs F-12 nutrient mixture (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin–streptomycin (Invitrogen), 1.36 ng/ml tri-iodo-thyronine (Sigma, St. Louis, MO), 0.5 μg/ml hydrocortisone (Sigma), 8.4 ng/ml cholera toxin (Sigma), 5.0 μg/ml transferrin (Sigma), 5.0 μg/ml insulin (Sigma), and 4.5 ng/ml human-EGF (Invitrogen). Primary human cervical keratinocytes were isolated as previously described (Blanton et al., 1991). Prior to splitting and sample collection, irradiated fibroblasts were removed using a mixture of 0.05% trypsin, 50 mM HEPES buffer in Hanks balanced salt solution (Invitrogen). Keratinocytes were removed by incubation in 0.05% trypsin/EDTA (Invitrogen) and passed at a 1:4 ratio.

Cell lines

We previously generated human cervical keratinocyte cell lines containing stable HPV-16 episomes (Sprague et al., 2002), named HPV_{EP} cells in this paper. Briefly, primary keratinocytes were co-transfected with circular HPV-16 genomes derived from W12 subclone E cells (W12-E) and a selectable marker (W12-E DNA provided by Paul Lambert, University of Wisconsin). After selection and dilution plating, colonies were isolated by ring cloning to establish isogenic cell lines (labeled B, F, and G). The same primary cells were used for transductions with the retroviral construct LXSN-16E6/E7 (obtained from Denise Galloway, Fred Hutchinson Cancer Research Center (Halbert et al., 1991)) by a method previously described (Farwell et al., 2000). Isogenic cell lines were generated from ring-cloned colonies (labeled #2, #5, and #6) and named $E6/E7_{LX}$ cells. Both HPV_{EP} and $E6/E7_{LX}$ cells have been passaged over 40 times, equivalent to greater than 100 PD at which point we consider them to be immortalized. Due to the premise of the paper, we used cells at an earlier passage. Cells were used at passage 15 (∼30 PD) for retroviral transductions. The W12 parental cell line (positive for HPV-16 episomes), 1811-NMU-T cells (Klingelhutz et al., 1993), and C33A cells were used for various controls.

Constitutively active Ras expression was achieved using the pBabe-puro H-Ras^{G12V} retroviral construct (obtained from Scott Lowe, Cold Spring Harbor Laboratory). The vector was sequenced to ensure functionality. The concentration for neomycin selection for LXSN transductions was 100 μg/ml G418 (Invitrogen). Puromycin (Sigma) was used for three days at 1 μg/ml after infection of pBabe-puro retroviruses. All collections and experiments with Ras were performed within the first 3 passages after final puromycin selection.

Senescent cell count

HPV_{EP} and $E6/E7_{LX}$ cells were plated in triplicate 60-mm dishes in E-media without feeders and grown to subconfluency. Cell culture microphotographs were taken using a Nikon Eclipse TE2000 inverted microscope. Enlarged and flattened cells, indicative of senescence, were tabulated from a blinded count of triplicate microphotographs. The number of senescent-like cells was calculated as a percentage of the total number of cells counted in each field.

Crystal violet assay

Cells were plated in triplicate in a 96-well format at a concentration of 2000 cells per 150 μl media and media changed daily. At specific time points, wells were washed with 1× PBS and stained with a solution of 0.5% crystal violet (w/v) in 20% methanol for 15 min. Wells were washed five times with distilled water and allowed to air-dry for 45 min or overnight. The cell-associated dye was extracted with 100 μl of 10% acetic acid and absorbance was read at $\lambda = 595$ nm. Population doubling time was calculated as $[\text{time in hours}]/[(\log N - \log N_O)/\log 2]$; $N = $ absorbance value at day 5 and $N_O = $ value at day 2.

Southern analysis

Southern analysis was performed as previously described (Sprague et al., 2002). Briefly, whole cell DNA was isolated from later passage clonal cultures using the QIAamp DNA BloodMini Kit (Qiagen, Valencia, CA) and digested either with BamHI or BglII. DNA was resolved on 1% agarose gels, depurinated in 0.25 M HCl, and blotted onto positively charged Hybond-XL nylon membranes (Amersham Biosciences Corp., Piscataway, NJ) by alkaline transfer with 0.4N NaOH. The different forms of HPV DNA were detected using radioactive probes of PCR-amplified segments of HPV-16 (nt 6226–3873/4471–6000). Known quantities of HPV DNA linearized by BamHI were included to estimate the viral load.

Western analysis

Protein from subconfluent cultures was collected in lysis buffer as previously described (Foster and Galloway, 1996) and quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Western blots were prepared using 30 μg of protein transferred to polyvinyl difluoride membranes. Blots were probed with primary antibodies for H-Ras (sc-29, Santa Cruz), p-MEK1/2 (Ser217/221; #9121, Cell Signaling Technology, Danvers, MA), p-44/42 MAPK (Thr202/Tyr204; #9101S, Cell Signaling Technology), p53 (Ab-6, Oncogene Research), HPV-16 E7 (#28-0006, Zymed Labs, South San Francisco, CA), p16 (#554070, Pharmingen) or beta-actin (I-19, Santa Cruz) and with the appropriate HRP-conjugated secondary antibodies, either goat anti-mouse IgG (Jackson Immuno-Research Laboratories), goat anti-rabbit IgG (Santa Cruz), or donkey anti-goat IgG (Santa Cruz). Chemiluminescence was detected using the Western Lightning kit (Perkin-Elmer Life Sciences). All films were scanned with a Hewett-Packard PrecisionScan 5300 and Adobe Photoshop software. Membranes were stripped by a 30 min, 50 °C incubation in a solution

of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl at pH 6.7.

Soft agar assay

Polyester membranes of transwell inserts (12-mm diameter, 0.4-μm pore; Costar, Corning, NY) were coated with 100 μl 1% noble agar. After counting cells using a hemacytometer, 1 part of 3.3% noble agar at 45 °C was diluted with 10 parts cell suspension for a final concentration of 700 cells per 400 μl. Triplicate wells for each cell line expressing Ras or vector were seeded with 700 cells. After agar solidification, media were added to the outer wells. After 3 weeks, transwell inserts were photographed using an Olympus stereoscope. Colonies approximately 0.19 mm in diameter or greater were tabulated. For statistical analysis, we compared the overall proportion (Z-statistic) of soft agar colonies between the Ras-expressing and vector conditions.

Tumorigenicity

Cells (1×10^6) in 0.2 ml KSFM were injected subcutaneously into 3-week-old female athymic (nu/nu) mice (Harlan-Sprague-Dawley, Madison, WI). Three mice were used for each Ras-expressing and each vector control cell line. Mice were housed in a pathogen-free barrier facility at The University of Iowa and handled in compliance with federal and institutional regulations.

Real-time RT-PCR

Total RNA was collected using TRIzol reagent (Invitrogen) and treated with 1U per μg RNA of DNase I AMP-grade (Invitrogen) per manufacturer's instructions. The RT reaction consisted of 1.7 μg RNA, 2 μl random hexamers, and RetroScript kit reagents (Ambion, Austin, TX) according to instructions. RNA was reverse transcribed for 1 h at 42 °C with inactivation at 92 °C for 10 min. Real-time PCR was performed on cDNA equivalent to 150 ng RNA in triplicate 25 μl reactions using SYBR Green PCR master mix (#4309155, Applied Biosystems, Foster City, CA) containing 250 nM forward and reverse primers for E6 or E7. Unspliced HPV16-E6 was detected as described (Lanham et al., 2001). *E6 forward*: 5′-CAA ACC GTT GTG TGA TTT GTT AAT TA-3′; *E6 reverse*: 5′-GCT TTT TGT CCA GAT GTC TTT GC-3′ (Integrated DNA Technologies, Coralville, IA). Sequences for HPV16-E7 primers were provided by Sara Isabel Pai (Johns Hopkins Medical Institution, MA) (Walboomers et al., 1999). *E7 forward*: 5′-CCG GAC AGA GCC CAT TAC AA-3′; *E7 reverse*: 5′-CGA ATG TCT ACG TGT GTG CTT TG-3′ (Integrated DNA Technologies). Standardization was based on 18S levels from reactions using primers at 75 nM final concentration. *18S forward*: 5′-CCT TGG ATG TGG TAG CCC GTT T-3′; *18S reverse*: 5′-AAC TTT CGA TGG TAG TCG CCG-3′ (Sigma-Genosys, The Woodlands, TX). PCR amplification began at 94 °C for 10 min prior to 30 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 1 min.

Real-time PCR data were analyzed using the ABI 7000 Sequence Detection System and ABI Prism 7000 SDS software (Applied Biosystems). Fold differences in oncogene mRNA levels were calculated relative to the W12 cell line by using the equation:

$$2^{\wedge}(\Delta\Delta C_T) \text{ where } \Delta\Delta C_T = \Delta C_T^{W12} - \Delta C_T^{cell\ line}.$$

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