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Mutation of the endogenous p53 gene in cells transformed by HPV-16 E7 and EJ c-*ras* confers a growth advantage involving an autocrine mechanism

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Rat embryo fibroblasts transformed with the HPV-16 E7 gene and the activated c-H-*ras* gene fall into two distinct phenotypic classes. At high cell density, clones of one class form colonies in methylcellulose supplemented with low serum; at low cell density, these cells display responsiveness to mitogenic factors present in serum-free conditioned medium from rat embryo fibroblasts. In contrast, clones of the second class exhibit an absolute dependency on growth factors present in serum at all cell densities in the methylcellulose colony assay and fail to respond to conditioned medium. We find that the status of the endogenous p53 gene is tightly correlated with these two classes of clones. Clones of the first class contain missense mutations in the p53 gene and have lost the wild-type allele. Clones of the second class express wild-type p53 protein. The importance of mutant p53 expression in reducing the growth factor dependency of transformed clones was confirmed in a separate series of experiments in which rat embryo fibroblasts were transformed with three genes, E7 + *ras* + mutant p53. The growth behaviour of these triply transfected clones was similar to that of the E7 + *ras* clones expressing endogenous mutant p53. We demonstrate that the enhanced proliferation of E7 + *ras* clones expressing mutant p53 protein involves an autocrine mechanism.

Key words: autocrine growth/HPV/p53/*ras*/transformation

Introduction

The common occurrence of p53 gene mutations in human and animal tumours, together with the observation that wild-type p53 suppresses oncogene-mediated transformation, support the classification of p53 as a tumour suppressor gene (Levine *et al.*, 1991). In gene transfer experiments, expression of mutant forms of p53 protein leads to the immortalization of early passage rat cells and, in co-operation with activated *ras* proteins, to cellular transformation (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984; Rovinski and Benchimol, 1988). In addition, expression of mutant p53 enhances the transformation process mediated by other pairs of oncogenes (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Peacock *et al.*, 1990; Crook *et al.*, 1991a; Taylor *et al.*, 1992).

Two very different hypotheses have been proposed to explain the oncogenic potential of mutated p53 alleles in transformation assays. One model proposes that the transformation-proficient mutants encode dominant negative polypeptides. The inactive proteins encoded by the dominant negative alleles would retain key properties enabling them to act as *trans* dominant repressors of endogenous wild-type p53 protein in normal recipient cells (Eliyahu *et al.*, 1988; Finlay *et al.*, 1988; Hinds *et al.*, 1989; Lane and Benchimol, 1990; Munroe *et al.*, 1990). Disruption of wild-type function might occur by competitive inhibition or by the formation of functionally inactive heteroligomers. p53 protein is multimeric (Kraiss *et al.*, 1988; Sturzbecher *et al.*, 1992; Friedman *et al.*, 1993) and immunochemical evidence for the formation of p53 heteroligomers between wild-type and mutant p53 polypeptides has been obtained (Eliyahu *et al.*, 1988; Rovinski and Benchimol, 1988; Gannon *et al.*, 1990; Milner *et al.*, 1991). The second hypothesis proposes that certain mutant p53 polypeptides have acquired novel or aberrant functions that promote growth and cellular transformation through a mechanism that is more elaborate than the removal or inactivation of normal endogenous p53 protein (Deppert, 1989; Dittmer *et al.*, 1993). Persuasive evidence in support of this idea is accumulating. For example, in gene transfer experiments, expression of an exogenous mutant p53 allele in a p53-negative cell line, L12, increased the tumorigenicity of these cells (Wolf *et al.*, 1984; Shaulsky *et al.*, 1990); expression of a transfected mutated human p53 allele in the p53 non-producer cell line Saos-2 increased the saturation density of these cells in culture (Chen *et al.*, 1990) and their plating efficiency in agar (Dittmer *et al.*, 1993); the finding of a recurrent missense mutation that results in the same amino acid change from arginine to serine at codon 249 in human hepatocellular carcinoma (Bressac *et al.*, 1991; Hsu *et al.*, 1991) represents another example of a mutated p53 allele whose expression (rather than the complete absence of p53 protein) contributes to malignancy.

We reported previously that co-transfection of mutated p53 alleles with the HPV-16 E7 gene and the human EJ c-*ras* gene enhanced transformation. Both quantitative and qualitative differences in the efficiency of transformation were detected. The number of transformed foci generated was greater than the sum of the foci obtained when mutant p53 or E7 was co-transfected separately with *ras*. In addition, the foci had a distinct morphology and the transformed cells exhibited a reduced dependency on serum for growth (Peacock *et al.*, 1990). In this study, we found that cells transformed by HPV E7 + *ras* occasionally sustained recessive mutations in their p53 alleles and acquired a selective growth advantage in culture. The resulting clones could be distinguished from E7 + *ras* transformed clones that retained a wild-type p53 gene by their marked growth factor independence. In this regard, E7 + *ras* transformed clones bearing an endogenous p53 gene mutation resembled E7 + *ras* transformed clones expressing an exogenous mutant p53 allele. We demonstrate that the reduced serum

requirement of these clones is mediated through an autocrine mechanism.

Results

Transformation of REFs with HPV-16 E7 and EJ c-ras

Expression vectors carrying the HPV-16 E7 gene and the human EJ c-*ras* gene were introduced into early passage rat embryo fibroblasts (REFs). Foci of morphologically transformed cells that overgrew the monolayer culture were picked and established as cell lines by repeated passaging in tissue culture medium consisting of α-MEM and 10% fetal calf serum (FCS). Numerous cell clones were readily established in this way; all of 21 foci sampled were successfully established. In control experiments, transfection of the EJ c-*ras* gene alone resulted in a small number of foci, very few of which could be established in long-term culture; transfection of the E7 gene alone resulted in no foci. In another series of transformation experiments with E7 and *ras*, transfected cultures containing multiple foci per dish were trypsinized and cell lines were established by repeated passaging of the cells in α-MEM containing 1% FCS. From 14 independent transfection experiments, giving rise to 14 cultures of pooled foci, 11 cultures became established under the restrictive conditions imposed by the low serum. Single-cell clones were subsequently isolated from the surviving cultures. Independently derived transformed REF clones obtained by both strategies were used in the studies described below. HPV E7 protein synthesis was confirmed in a number of these cell lines by metabolic labelling and immunoprecipitation analysis.

Assessment of endogenous p53 protein expression and p53 gene status in transformed clones

When human or murine wild-type p53 cDNA is transfected along with E7 and *ras*, there is a sizeable and significant repression in the number of transformed foci generated (Peacock *et al.*, 1990; Crook *et al.*, 1991a; Slingerland *et al.*, 1993), similar to the repression seen when wild-type p53 is co-transfected with adenovirus E1A + *ras* or with c-*myc* + *ras* (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). In all of these studies, the wild-type p53 gene was supplied exogenously and it is, therefore, pertinent to consider what the role of the endogenous wild-type p53 protein might be during oncogene-mediated transformation of normal recipient cells. Our collection of E7 + *ras* transformed REF clones provided an opportunity to address this question.

p53 protein synthesis and steady-state level were measured in the transformed clones using a 1 h metabolic labelling and immunoprecipitation assay, or a combined immunoprecipitation and Western blot procedure, respectively. With the metabolic labelling assay, rat p53 protein synthesis was detected with PAb421 (Harlow *et al.*, 1981) in all of the clones examined including normal REFs (data not shown). In the two-step Western blot procedure, lysates were prepared and p53 protein was immunoprecipitated with PAb421. The immunoprecipitated proteins were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose filters for visualization and quantitation with PAb421 and [^{125}I]protein A. The steady-state level of p53 protein was found to be highly variable in the different clones (Figure 1). In some clones, p53 protein was overexpressed and readily detected, whereas in other clones p53 protein could only be

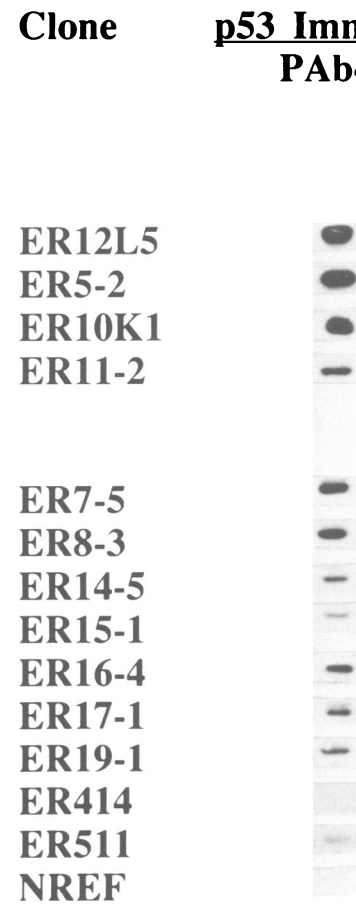

Fig. 1. p53 protein expression in rat embryo fibroblasts transformed by HPV E7 + *ras*. Clones were screened for p53 protein expression using a combined immunoprecipitation and Western blot procedure. Protein lysates were prepared from 1 × 10^7 cells. Total protein (1.0 mg) was reacted with monoclonal antibody PAb421 and immune complexes were resolved by SDS–PAGE and electroblotted onto a nitrocellulose membrane as described in Materials and methods. The nitrocellulose membrane was incubated with PAb421 followed by [^{125}I]protein A to detect p53 protein. NREF, normal rat embryo fibroblasts.

detected by the more sensitive metabolic labelling procedure and not by the Western blot procedure. In four clones that expressed high levels of p53 protein, the p53 protein was recognized by PAb240 (Gannon *et al.*, 1990) which reacts preferentially with mutant p53 polypeptides (data not shown).

PAb240 reactivity is suggestive of missense point mutation in the p53 coding sequence. In addition, an increased steady-state level of p53 protein usually reflects an extended protein half-life which could also result from missense mutation in the coding sequence. Consequently, we proceeded to generate and sequence p53 cDNA from clones expressing PAb240+ p53 protein and from clones expressing PAb240– p53 protein. The entire coding sequence of p53 cDNA was examined.

First-strand cDNA was synthesized and used as a template for amplification by polymerase chain reaction (PCR) primed with oligonucleotides to the rat p53 cDNA nucleotide sequence (Soussi *et al.*, 1988). Amplification products were sequenced directly and/or after cloning into plasmid vectors. cDNA from ER10K1 had a single nucleotide substitution consisting of a C-to-T transition that converted the alanine codon at position 136 to a valine codon. cDNA from ER12L5 contained a C-to-T transition at position 276,

Table I. Growth behaviour and p53 gene expression in E7 + *ras* transformed REF clones

	Cell clones	Endogenous p53 protein		Endogenous p53 gene status	Growth in methylcellulose with low serum	Response to 12L5-CM
		PAb421	PAb240			
A.	ER7-5	+	−	wt	−	−
	ER8-3	+	−	wt	−	−
	ER14-5	+	−	wt	−	−
	ER15-1	+	−	wt	−	−
	ER16-4	+	−	wt	−	−
	ER17-1	+	−	wt	−	−
	ER19-1	+	−	wt	−	−
	ER414	+	−		−	−
	ER416	+	−		−	−
	ER511	+	−		−	−
B.	ER10K1	+	+	Ala136(GCG)→Val(GTG)	+	+
	ER12L5	+	+	Pro276(CCT)→Ser(TCT)	+	+
	ER11-2	+	+	Ala201(GCT)→Asp(GAT)	+	+
	ER5-2	+	+	Cys174(TGC)→Phe(TTC)	+	+
C.	ERM-1				+	+
	ERM-2					+
	ERM-4				+	+
	ERM-104				+	+
	ERM-115				+	+

converting a proline codon to a serine codon. These mutations, initially detected in individual cDNA clones, were confirmed by complete sequence analysis of pooled cDNA clones derived from independent first-strand cDNA and PCR reactions. In no case (6/6 ER10K1 clones and 7/7 ER12L5 clones) was wild-type rat p53 cDNA sequence identified, indicating that the mutations were likely accompanied by loss of heterozygosity. cDNAs from ER5-2 and ER11-2 were sequenced directly, and each was found to contain a single nucleotide substitution. In ER5-2, a G-to-T transversion at position 174 converted the cysteine codon to phenylalanine. In ER11-2, a C-to-A transversion converted the alanine codon at position 201 to an aspartic acid codon. Only the mutated cDNAs were detected by this procedure. The mutation in ER5-2 was confirmed by sequencing of individual plasmid cDNA clones. Direct sequencing of the PCR amplified cDNA from seven other E7 + *ras* transformed REF clones expressing PAb240− p53 protein showed only the wild-type p53 cDNA sequence. As a result of this genetic analysis, REF clones were classified into one of two groups. Members of group A express PAb240− and wild-type p53 protein, while members of group B contain a mutated p53 allele and express a PAb240+ p53 protein (Table I).

Cloning efficiency in methylcellulose as a function of cell density

We next wished to determine if the two groups of clones could be distinguished phenotypically and, if so, whether p53 protein expression might account for any difference detected. Anchorage-independent growth and enhanced sensitivity to growth factors are features often associated with transformed cells. Consequently, a methylcellulose colony assay was employed to investigate the growth of E7 + *ras* transformed REF clones from group A and from group B as a function of cell density and concentration of serum. Cells were cultured in triplicate in methylcellulose-containing medium in 10% or 1% serum, and the mean number of colonies was determined 2 weeks later. The results were then used to calculate the slope of the logarithm of these values. The results presented in Figure 2 (A, B) in the form of a log−log plot indicate that in 10% serum all the clones tested gave rise to colonies with slope values of ~1.0 (Table II), indicative of a linear relationship between input cell number and colonies recovered. Clones ER414, ER416 and ER15-1 (group A) gave rise to no or few (<5) colonies in the presence of 1% serum even at very high cell density (50 000 cells/dish). The inability of E7 + *ras* transformed REF clones expressing wild-type p53 protein to form colonies in methylcellulose supplemented with 1% serum was confirmed with several other independently derived clones (Table I). Clones ER10K1, ER12L5 and ER5−2 (group B), however, gave rise to colonies in the presence of 1% serum with slope values of ~2.0 (Table II), indicative of a non-linear relationship between the number of cells cultured and the number of colonies formed. A slope of >1 indicates that a factor associated with cell crowding, possibly cell-to-cell contact or the release of a growth stimulatory factor, plays a role in colony formation.

Our initial attempts to plate ER11-2 cells on methylcellulose containing 1% serum failed. However, when the methylcellulose culture medium was modified by replacement of the α-MEM with Iscove's MEM, and a new source of methylcellulose was used, ER11-2 cells formed colonies in the presence of 1% serum with >100-fold increased efficiency compared with ER414 cells from group A. While we cannot readily account for the difference between α-MEM and Iscove's MEM on cell plating efficiency, these data do show that clone ER11-2, like clones ER10K1, ER12L5 and ER5-2, has a reduced requirement for serum at high cell density and, hence, is phenotypically different from E7 + *ras* transformed REF clones from group A.

Growth behaviour of REF clones transformed with HPV E7, EJ c-ras and mutant p53

The data presented thus far demonstrate a correlation between endogenous p53 gene mutation in REF clones

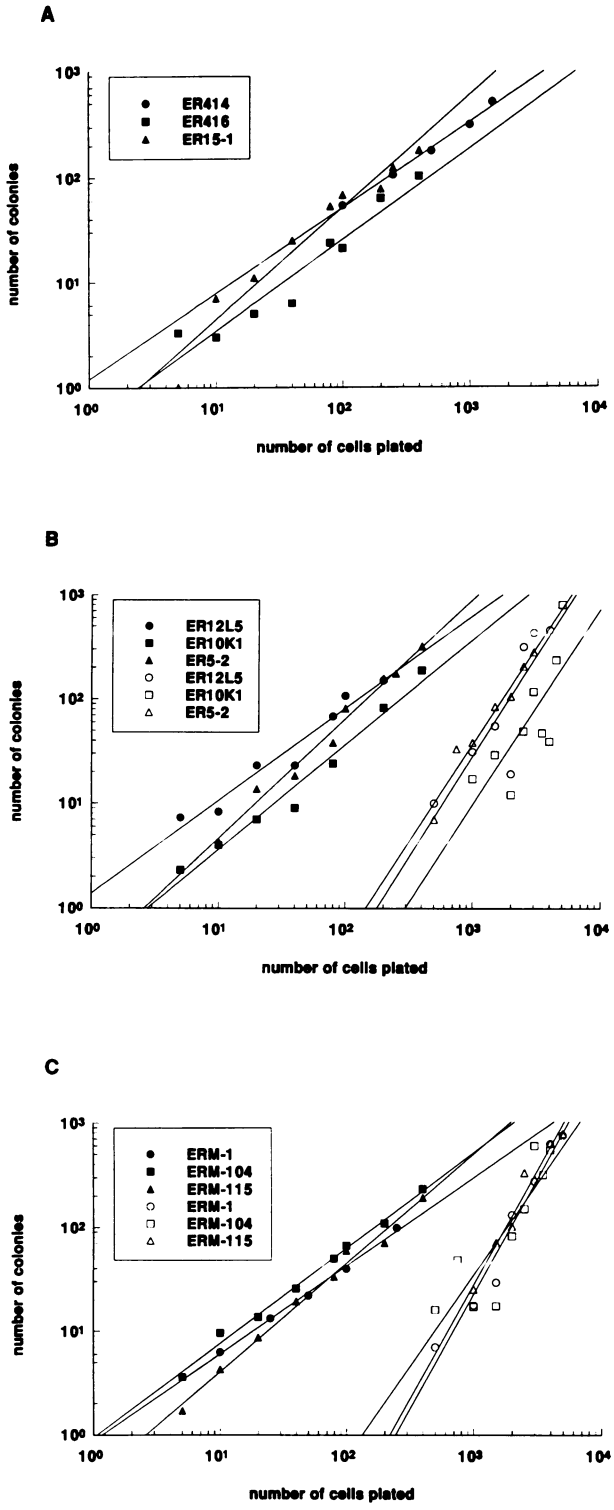

Fig. 2. Relationship between input cell number and colony formation in methylcellulose. Cells were plated in methylcellulose culture medium supplemented with 10% serum (closed symbols) or 1% serum (open symbols) and the number of colonies obtained at different cell densities is plotted on a log−log scale. (**A**) Clones derived from rat embryo fibroblasts transformed by HPV E7 + *ras* (ER414, ER416, ER15-1); these clones were unable to form colonies in 1% serum. (**B**) Clones derived from rat embryo fibroblasts transformed by HPV E7 + *ras* that express endogenous mutant p53 protein (ER10K1, ER12L5, ER5-2). (**C**) Clones derived from rat embryo fibroblasts transformed by HPV E7 + mutant p53 + *ras* (ERM-1, ERM-104, ERM-115). Curves were fitted to the data by linear regression analysis.

Table II. Colony-forming efficiency of transformed REF clones

Clone	Slope[a] (10% serum)	Slope (1% serum)
ER414	0.8	–[b]
ER416	0.9	–
ER15-1	1.1	–
ER10K1	1.0	1.9
ER12L5	0.9	1.9
ER5-2	1.2	1.8
ERM-1	0.8	2.2
ERM-104	0.9	1.8
ERM-115	1.0	2.2

[a]The slopes were determined by linear regression analysis of the data presented in Figure 2, relating colony number to the number of cells plated in methylcellulose.
[b]ER414, ER416 and ER15-1 did not give rise to colonies in methylcellulose supplemented with 1% serum.

transformed by E7 + *ras*, and the growth of these clones in methylcellulose culture medium containing low serum. To test this correlation, normal REFs were transformed with HPV-16 E7, EJ c-*ras* and a murine mutant p53 allele. The murine p53 allele contains a missense mutation at codon 193 that converts arginine to proline. This allele was shown previously to be transformation competent and may, therefore, act through a dominant negative mechanism. p53 and E7 protein synthesis was confirmed by metabolic labelling and immunoprecipitation analysis. A number of triple transfected clones (hereafter referred to as ERM clones) were characterized in the methylcellulose colony assay (Figure 2C). In the presence of 10% serum, the relationship between the number of cells cultured and the number of colonies developing 2 weeks later was linear. The slope of the logarithm of these values was close to 1.0 (Table II). In 1% serum, all the clones tested gave rise to colonies. The relationship between input cell number and colonies formed was non-linear with a slope value of ~2.0, similar to that observed in E7 + *ras* transformed REF clones containing an endogenous p53 gene mutation and assigned to group B.

Detection of growth-promoting activity in conditioned medium

The cell density-dependent growth of ER10K1, ER12L5, ER5-2 and of the ERM clones in medium containing low serum indicated a contribution by the cells themselves or soluble factors derived from them. To distinguish between these possibilities, conditioned medium (CM) from high-density ER12L5 cultures was collected and tested for mitogenic activity on ER12L5 cells and ERM-115 cells. CM was collected from ER12L5 cells growing in the absence of serum since we had observed that growth of these cells (and also ER10K1 and ERM-115) was completely serum independent at high cell density. ER12L5 and ERM-115 cells were plated at varying densities in 24-well tissue culture dishes (15 mm) in 2 ml α-MEM ± 40% CM, and the proportion of wells showing cell growth was determined by visual inspection. The results are presented in Table III and show that CM from ER12L5 cells contained one or more factors that supported the growth of ER12L5 and ERM-115 cells when these cells were plated in serum-free medium at a low, normally non-permissive, cell density.

The mitogenic response of other E7 + *ras* transformed

Table III. Growth response (proportion of wells showing cell growth[a]) of ERM-115 and ER12L5 cells to conditioned medium

Cell number plated	ER12L5		ERM-115	
	− CM[b]	+ CM	− CM	+ CM
3000	0/24	13/24	0/24	5/24
5000	10/24	24/24	0/24	10/24
8000	14/24	24/24	4/24	24/24
10 000	13/24	24/24	2/24	24/24

[a]Serum-free conditioned medium from ER12L5 cells at a final concentration of 40% was tested for its ability to stimulate the proliferation of ER12L5 and ERM-115 cells plated at varying density in multi-well dishes (15 mm) in a volume of 2 ml. Positive wells were identified by visual inspection under a microscope.
[b]CM, conditioned medium.

REF clones and ERM clones to ER12L5-CM was tested. Cells were plated at the normally non-permissive densities of 5000 cells/well and 10 000 cells/well in 24-well tissue culture dishes in serum-free medium ± 40% ER12L5-CM, and the proportion of wells showing cell growth was determined. The data corresponding to 5000 cells/well are summarized in the histogram presented in Figure 3. Each of the four E7 + *ras* transformed clones from group B that expressed endogenous mutant p53 protein and each of the five ERM clones demonstrated responsiveness to the mitogenic factor(s) present in ER12L5-CM. In contrast, none of 10 E7 + *ras* clones from group A showed a proliferative response at either cell density.

REF clones transformed with mutant p53 + *ras* were also obtained and these, too, showed a dependency on serum at all cell densities tested in both the methylcellulose colony assay and the multi-well assay (data not shown). Like the REF clones transformed by E7 + *ras* that we have assigned to group A, REFs transformed by mutant p53 + *ras* did not show a proliferative response in serum-free medium supplemented with ER12L5-CM. Hence, in the absence of E7 expression, mutant p53 expression is insufficient to render REF cells less dependent on serum or responsive to ER12L5-CM.

In the next series of experiments, CM was collected from a variety of transformed REF clones that had been cultured for 48 h in the absence of serum, and tested for mitogenic activity on low-density ER12L5 cells in serum-free medium. All the CM scored positively, albeit with variable efficiency (data not shown). These included CM from REF clones transformed by E7 + *ras* (groups A and B), REF clones transformed by *ras* alone, and ERM clones. In addition, CM collected from cultures of normal REFs sustained the proliferation of low-density ER12L5 cells. REF-CM was as effective as ER12L5-CM in supporting the growth of ER12L5 cells in serum-free medium. Collectively, the data presented in Figures 2 and 3, and in Table I, support the idea that mutant p53 expression in REF clones transformed by E7 + *ras* confers upon these cells the ability to respond to one or more cell-derived factors and to proliferate in serum-free medium through an autocrine mechanism.

Discussion

In this study, we have addressed the role of the endogenous p53 protein during transformation mediated by the HPV E7

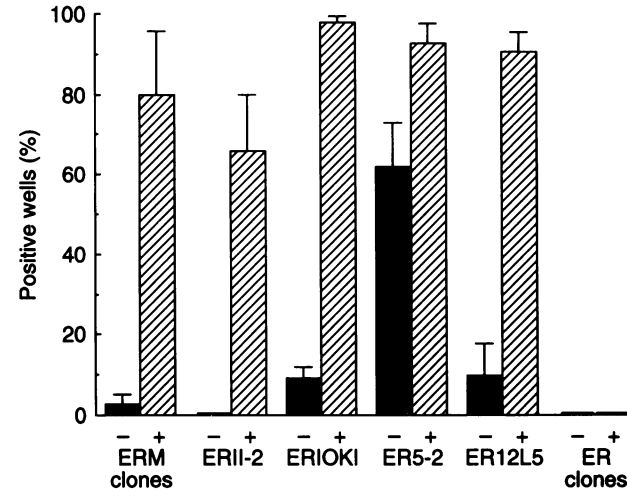

Fig. 3. Cell growth in serum-free medium in response to conditioned medium from ER12L5 cells. A total of 5000 cells were plated on 24-well dishes (15 mm) in a final volume of 2 ml α-MEM with (+) or without (−) 40% CM. CM was prepared as described in Materials and methods. Wells were scored for cell growth visually under the microscope 7−9 days after plating and the number of positive wells is expressed as a percentage. The values shown represent the means and the SEM. The mean values for ER11-2, ER10K1, ER5-2 and ER12L5 were obtained from at least four experiments. The mean value for the ERM clones represents an aggregate derived from the five independent clones shown in Table I each tested once. The mean value for the ER clones represents an aggregate derived from the 10 independent clones shown in Table I each tested once.

and EJ c-*ras* oncogenes. This combination of genes, when introduced and expressed in early passage REFs, gives rise to morphologically transformed foci. Clonal cell lines derived from these foci are immortal, capable of anchorage-independent growth and are highly tumorigenic (Storey *et al.*, 1988; Peacock *et al.*, 1990; R.G.Bristow and R.P.Hill, personal communication). In most of the E7 + *ras* transformed REF clones we examined, no mutations in the p53 gene were detected and all the clones expressed p53 protein. We noted that the amount of wild-type p53 protein present in these clonal cell lines varied from clone to clone. These findings are similar to those reported by Lu *et al.* (1992) who determined that wild-type p53 was expressed in mouse prostate carcinoma cells transformed by *myc* + *ras*. Immunofluorescence staining of these cells showed heterogeneity of p53 staining with 5−10% of the cells showing intense nuclear staining for p53. One conclusion that can be drawn from the two studies is that p53 mutation is not required during immortalization and transformation mediated by E7 + *ras*, or by *myc* + *ras*.

In four E7 + *ras* clones, however, a single missense mutation in the p53 coding sequence was detected. In all four clones, mutation of one p53 allele was accompanied by loss of the homologous wild-type allele. Loss of wild-type p53 expression and/or acquisition of mutant p53 expression appears to have provided these four clones with a strong selective advantage in culture. The generation of p53 gene mutations during cell culture has been observed previously. Harvey and Levine (1991) and Rittling and Denhardt (1992) have shown that p53 gene alterations (primarily missense mutations) are involved in the spontaneous immortalization of mouse embryo cells. Ulrich *et al.* (1992) have shown that loss or mutation of p53 is correlated with the immortalization of v-*src* transformed

chick embryo fibroblasts and v-*erb*B/v-*erb*A transformed chicken erythroblasts. These three studies demonstrate that p53 mutations accompany cell growth in culture and they indicate an involvement of endogenous p53 in the immortalization process. These findings are consistent with earlier studies (Jenkins *et al.*, 1984; Rovinski and Benchimol, 1988) showing that expression of foreign mutant p53 alleles promoted immortalization of rodent fibroblasts. Although the present study demonstrates that establishment and transformation of REFs by E7 + *ras* can occur in the absence of p53 gene mutation and in the presence of continued wild-type p53 protein synthesis, recessive mutations in the p53 gene, nevertheless, do occur in culture.

Our results indicate that REFs transformed by E7 + *ras* fall into two phenotypically distinct classes which correlate precisely with the p53 genotype of the cells. E7 + *ras* transformed REF clones expressing mutant p53 protein displayed: (i) reduced serum requirement for colony formation in methylcellulose; (ii) cell density-dependent clonogenic growth in methylcellulose containing low serum; and (iii) responsiveness in serum-free medium to mitogenic factors present in conditioned medium from normal or transformed REF cells. In contrast, E7 + *ras* transformed REF clones expressing wild-type p53 protein exhibited an absolute dependency on growth factors present in serum at all cell densities tested in the methylcellulose colony assay, and failed to respond to conditioned medium. This was also seen in malignant REF clones transformed by mutant p53 and *ras*. Thus, while mutant p53 protein expression appears to be necessary, it is not sufficient to render cells less dependent on exogenous growth factors present in serum. The growth phenotype described above requires the concerted action of E7, mutant p53 and *ras* proteins.

The importance of mutant p53 expression in reducing the growth factor dependency of transformed clones was confirmed in a separate series of experiments in which REFs were transformed with three genes, E7 + *ras* + mutant p53, giving rise to the ERM series of clones. The growth behaviour of the ERM clones was identical to that of the E7 + *ras* clones expressing endogenous mutant p53. We provide evidence showing that the enhanced proliferation of E7 + *ras* transformed cells expressing mutant p53 protein (encoded either by the endogenous p53 gene or by an exogenous p53 allele) occurs through an autocrine mechanism. The identity of the secreted factor present in the conditioned medium remains to be determined.

We have considered the possibility that expression of mutant p53 protein is indirectly associated with the autocrine growth of cells transformed by E7 and *ras*. For example, one could imagine that the autocrine pathway is active in all E7/*ras* transformed cells and that it becomes evident only in those cells that are spared from cell death following serum deprivation. A number of studies have suggested a role for wild-type p53 expression in programmed cell death or apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992). Hence, it is possible that loss of wild-type p53 expression by mutation of the endogenous alleles or by ectopic expression of a dominant negative p53 allele abrogates the apoptotic response, permitting cell proliferation through an existing autocrine loop. While we cannot rule out this model, we consider it to be unlikely for the following reason. At low, non-permissive cell densities, cells transformed by E7 and *ras*, and expressing mutant p53 protein, are unable to form colonies in the methylcellulose assay supplemented with low serum, and are unable to proliferate in the absence of serum or conditioned medium in the multi-well assay. We have investigated the fate of these non-proliferating cells and find intranucleosomal DNA fragmentation and chromatin condensation, two hallmarks of apoptosis (data not shown). This response is similar to that of serum-deprived, non-proliferating, E7/*ras* transformed clones expressing wild-type p53 protein. Thus, apoptosis can occur in the presence or absence of wild-type p53 protein. In this regard, we note that expression of p53 protein is not required for the androgen ablation-induced programmed cell death since this process occurs to the same extent in prostatic glandular cells obtained from wild-type and p53-deficient mice (Berges *et al.*, 1993). Furthermore, glucocorticoid- or calcium ionophore-induced programmed death occurs normally in thymocytes from p53-deficient mice (Clarke *et al.*, 1993; Lowe *et al.*, 1993).

It is pertinent to consider the selection pressures which may have existed during cell culture to favour the outgrowth of cells bearing p53 mutation. Clones ER10K1, ER12L5, ER5-2 and ER11-2 were derived from E7 + *ras* transformed REF cultures grown in 1% serum. Hence, the sub-optimal growth conditions resulting from low serum may have provided a selection pressure for the outgrowth of cells carrying p53 mutation. However, seven of the 10 E7 + *ras* transformed REF clones expressing wild-type p53 protein (Table I, group A) were generated in the same way. It is important to note that these seven clones, although capable of growth in monolayer cultures in 1% serum, were unable to form colonies in methylcellulose supplemented with 1% serum and were unable to proliferate at low cell density in serum-free conditioned medium. The other three clones assigned to group A were isolated in 10% serum. Clones expressing high steady-state levels of p53 protein were isolated under both high (10%) and low (1%) serum conditions.

An important question that arises from these studies is whether the endogenous wild-type p53 protein present in E7 + *ras* transformed REF clones is functionally active. This question has profound implications with regard to the involvement of p53 in suppressing transformation and tumorigenicity. There is now abundant evidence that p53 protein activity can be neutralized by non-genetic mechanisms (Moll *et al.*, 1992). The binding of cellular (mdm-2) and viral proteins (SV40 large T-antigen, HPV-16 E6 protein, Ad2 E1B 55 kDa protein) to the p53 protein, moreover, has been shown to block its ability to function as a transcription factor (Farmer *et al.*, 1992; Mietz *et al.*, 1992; Momand *et al.*, 1992; Yew and Berk, 1992). The absence of functional p53 protein in all the E7 + *ras* transformed clones would support the idea that p53 protein acts to suppress transformation and tumour formation, and that it must be inactivated during the initial stages of oncogene-mediated transformation to generate foci of morphologically transformed and tumorigenic cells. On the other hand, the presence of functional p53 protein in certain clones and non-functional p53 protein only in clones bearing a p53 gene mutation would indicate that mutations in the p53 gene represent secondary events that are not involved in the initial stages of cellular transformation. The correlation seen in this study between endogenous p53 gene status and the growth behaviour of E7 + *ras* transformed REF clones suggests that the wild-type p53 protein expressed in E7 + *ras* transformed clones (group A) differs functionally from

the mutant p53 protein expressed in the E7 + *ras* transformed clones in group B. These data support the latter hypothesis and provide, moreover, one explanation for the selective growth advantage experienced by cells that have acquired a mutation in the p53 gene.

The involvement of p53 protein in ano-genital cancers is particularly informative in this context. Somatic p53 gene mutations are very rare in HPV-positive tumours, but can be detected frequently in HPV-negative tumours (Crook *et al.*, 1991b, 1992; Scheffner *et al.*, 1991; Kaelbling *et al.*, 1992). HPV E6 protein has been shown to bind p53 protein and to enhance its degradation through a ubiquitin-dependent pathway (Scheffner *et al.*, 1990; Werness *et al.*, 1990; Crook *et al.*, 1991c). These findings indicate that the loss of wild-type p53 function following HPV E6 expression is sufficient to allow the development of a primary neoplasm. Crook and Vousden (1992), however, have shown that p53 gene mutations can be detected in metastatic lesions resulting from HPV-positive primary cervical cancers. These studies suggest that p53 protein retains partial function even in the presence of E6 protein and that this function must be completely disrupted by mutation to allow metastatic spread of the disease. An alternative explanation is that loss of wild-type p53 protein mediated by the E6 protein is required for the formation of the primary tumour and that mutant p53 protein acquires a novel function that promotes or facilitates the metastatic spread of the disease.

In a number of other human malignancies, p53 mutations are more frequently seen in the later stages of the disease. The extensive analysis of p53 in colorectal tumours indicates that p53 mutation occurs late in the development of this disease (Baker *et al.*, 1990; Fearon and Vogelstein, 1990). In a study of 43 hepatocellular carcinomas, p53 mutations were restricted to advanced-stage disease (eight of 22 samples showing mutation) with none of 21 early, primary tumours revealing mutation (Murakami *et al.*, 1991). p53 mutations were also found exclusively in late-stage gastric carcinomas (Yamada *et al.*, 1991). In a study of eight paired glioma specimens obtained at the time of initial tumour resection and at recurrence, histologic progression had occurred in four cases and was accompanied by p53 mutation in three of these (Hayashi *et al.*, 1991). In a separate study in which low-grade, histologically benign brain tumours from three patients recurred with a malignant phenotype, p53 mutations were detected in a small subpopulation of tumour cells present in two primary samples. In the recurrent tumours, cells containing the same mutations and having lost the wild-type allele were predominant in the tumour sample. A third sample, containing a heterozygous p53 mutation at presentation, acquired a second mutation in the homologous allele during progression. In the recurrent tumour, cells bearing the compound heterozygous mutations dominated the tumour population (Sidransky *et al.*, 1992). In chronic myelogenous leukaemia, p53 gene rearrangements and point mutations are rarely seen in the chronic phase of the disease, but do occur in myeloid blast crisis (Ahuja *et al.*, 1989, 1991; Kelman *et al.*, 1989; Mashal *et al.*, 1990; Feinstein *et al.*, 1991). These data, obtained from clinical specimens, indicate that wild-type p53 blocks late rather than early steps in tumour formation, and that loss of wild-type p53 protein and/or gain of mutant p53 protein expression promotes tumour progression. Our data, obtained with cells transformed in culture, similarly indicate a role for p53 in the later stages of cell transformation.

Materials and methods

Recombinant plasmids

Mp53pro193 contains a 16 kilobase (kb) *Eco*RI fragment in pUC18 carrying the entire mouse p53 gene with a mutation at codon 193 that converts an arginine residue to proline (Rovinski and Benchimol, 1988; Munroe *et al.*, 1990). HPV E7 coding sequence was present on pJ4Ω16.E7 plasmid (Storey *et al.*, 1988). Activated c-Ha-*ras* sequences were present on pEJ6.6 (Shih and Weinberg, 1982).

Transformation assay

REFs were prepared from 14-day Fisher rat embryos as described previously (Rovinski and Benchimol, 1988). Cells to be transfected were thawed and plated, giving rise to secondary cultures. These were then trypsinized and replated at 3.0×10^5 cells/60 mm diameter dish, giving rise to tertiary REF cultures which were transfected the following day. Transformation assays were performed as described previously (Munroe *et al.*, 1990; Slingerland *et al.*, 1993) and contained 10 μg of NIH 3T3 carrier DNA in addition to one or more of the following as indicated in the text: 2 μg pEJ6.6 (EJ c-H-*ras*), 1 μg pJ4Ω16.E7 (HPV E7) and 4 μg Mp53pro193. Approximately 24 h after transfection, the cells were trypsinized and replated in fresh medium.

Cell lines

All of the ER clones were obtained from REFs transformed by pEJ6.6 (EJ c-*ras*) and pJ4Ω16.E7 (HPV E7). ER414, ER416 and ER511 were derived from single, independent foci which were picked and established in culture by repeated passage in α-MEM supplemented with 10% FCS. The remaining ER clones were derived from pooled foci of transformed REF cells. After 10 sequential passages in α-MEM supplemented with 1% FCS, single-cell clones were obtained in one of two ways. Cells were plated at low cell density in α-MEM containing 10% FCS and single colonies were isolated with cloning cylinders and expanded in α-MEM supplemented with 10% FCS (ER12L5 and ER10K1). Alternatively, cells were plated in methylcellulose containing 10% FCS and individual colonies were picked and expanded in α-MEM supplemented with 10% FCS. Each clone was derived from a single dish of transfected cells to ensure its independent derivation.

ERM-1, ERM-2, ERM-4, ERM-104 and ERM-115 were obtained from REFs transformed by E7, Mp53pro193 and EJ c-*ras*. Single, independent foci were picked from transfected cultures and established by repeated passage in α-MEM supplemented with either 1% FCS (ERM-1, ERM-2 and ERM-4) or 10% FCS (ERM-104 and ERM-115).

Methylcellulose colony assay

Viable cells were serially diluted and added at various densities to α-MEM supplemented with either 20% or 2% FCS. Viable cells were then mixed by vortex with an equal volume of 0.8% α-MEM−methylcellulose. One hour later, 2 ml of each cell cocktail were plated in triplicate onto 35 mm diameter Lux (Nunc, Inc., Naperville, IL) suspension culture dishes. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. After 10−14 days, individual colonies of >40 cells were counted under the microscope.

Multi-well cell culture assay

Cells were plated on 24-well dishes (15 mm diameter) in a final volume of 2 ml α-MEM ± 40% CM. Conditioned medium was prepared in the following way. Cells were grown to subconfluence on a 175 cm^2 flask in α-MEM containing 10% FCS. The medium was removed and the cells were washed with phosphate-buffered saline (PBS) followed by fresh α-MEM lacking serum, and cultured for a further 48 h in 200 ml α-MEM. The resultant CM was collected, centrifuged to remove cells, filtered through a 0.45 μm Nalgene filter unit, and diluted to 40% concentration with fresh α-MEM prior to use. Wells were scored for cell growth visually under the microscope 7−9 days after cell plating.

Metabolic labelling and immunoprecipitation

To monitor HPV E7 protein expression, cells (5.0×10^6) were labelled for 90 min at 37°C with 0.5 mCi [^{35}S]cysteine and 0.2 mCi [^{35}S]methionine in 1 ml of α-MEM lacking methionine and cysteine (ICN Biomedicals Inc.). Cells were lysed for 30 min on ice in 1.0 ml of buffer A [150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris (pH 7.2), 1% NP40, 1 μg/ml aprotinin]. The cell debris was removed by centrifugation and the remaining supernatant was retained, and incubated overnight with 5 μg non-specific IgG2a mouse monoclonal antibody. These were then mixed with 0.5 ml of a 10% suspension of formalin-treated *Staphylococcus aureus* cells (Immunoprecipitin; BRL), which were then removed by centrifugation. The pre-cleared supernatant

was immunoprecipitated with 100 μl HPV-16 E7 monoclonal antibody (Triton Diagnostics, Almeda, CA) or mouse IgG overnight at 4°C. Immunoprecipitates were then incubated with 2 μl rabbit anti-mouse antibody for 1 h on ice. Immune complexes were recovered with 100 μl protein A–Sepharose (Pharmacia) and incubated for 1 h at 4°C. Complexes were collected by centrifugation and washed twice with lysis buffer A and then eluted into 30 μl of sample buffer [2% SDS, 0.1% bromophenol blue, 10% glycerol, 25 mM Tris (pH 6.8), 0.1 M dithiothreitol] by heating at 90°C for 10 min. Samples were loaded onto a 13% polyacrylamide gel in the presence of SDS. Electrophoresis was at 45 mA. Dried gels were exposed to Eastman Kodak XAR-5 film.

p53 protein synthesis was assessed by metabolic labelling with [^{35}S]methionine and immunoprecipitation as described previously (Slingerland *et al.*, 1993).

Western immunoblot analysis of p53 protein

Cell extracts were prepared from 1 × 10^7 cells. Cells were lysed for 30 min on ice with 0.5 ml lysis buffer B [1% NP-40, 150 mM NaCl, 20 mM Tris (pH 8.0), 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin]. Lysates were cleared by centrifugation for 5 min. Cell lysates containing 1.0 mg of total protein were immunoprecipitated with monoclonal antibody PAb421 (Harlow *et al.*, 1981). Immune complexes were resolved by SDS–PAGE and transferred to nitrocellulose paper by electroblotting at 7–8 V/cm for 6 h in a solution containing 75 mM glycine, 10 mM Tris and 20% methanol. Filters were incubated overnight in Tris–saline [10 mM Tris (pH 7.4), 0.9% NaCl] containing 5% bovine serum albumin (BSA). Filters were subsequently incubated for 90 min at room temperature in Tris–saline–BSA supplemented with PAb421 antibody. Filters were then washed with Tris–saline solution without BSA and again with the same solution containing 0.05% NP-40. The final wash was performed in Tris–saline and filters were then incubated in a solution of Tris–saline containing 5% BSA and 5 × 10^5 c.p.m./ml [^{125}I]protein A for 30 min at room temperature. Finally, filters were washed as described above, dried and exposed to XAR-5 film (Kodak) at −70°C.

Amplification and sequencing of p53 coding sequences

Total RNA was prepared from ER cell lines with guanidinium isothiocyanate (Maniatis *et al.*, 1982) and polyadenylated RNA was selected on an oligo(dT)-cellulose column (Collaborative Research). For the analysis of ER10K1 p53 coding sequence, first-strand cDNA was prepared, and amplified in separate reactions that were directed to 5′ and 3′ regions of the p53 coding sequence. Full-length, first-strand cDNA was generated using 2 μg poly(A)$^+$ RNA (Amersham Corp.), primed with a 3′-untranslated region derived, rat p53-specific antisense oligonucleotide R-53.1 (GGTGATGGGGACAGGATG). The cDNA was then used as a template for amplification by the PCR catalysed by the thermostable *Taq* polymerase (Perkin-Elmer Cetus Corp.). 5′-End p53 cDNA was amplified by using p53-specific antisense oligonucleotide AB12 (AACACGAACCTCAAAGCT) and R-H53.2 sense oligonucleotide (ATGCAAGCTTCCCCTGAAGACTGCATAAC) which includes a *Hin*dIII restriction site adjacent to 5′-untranslated rat p53-specific sequence. 3′-End p53 cDNA was amplified using a nested 3′-antisense oligonucleotide, R-B53.3 (CGACGGATCCCAGAGGCTGTCAGTCTGAG) that includes a restriction site for *Bam*HI and R53.4 (GCTGCCCCCACCATGAGCG), a rat p53-specific sense oligonucleotide spanning codons 173–178. Thirty cycles of denaturation (94°C, 1 min), annealing (56°C, 2 min) and elongation (72°C, 3 min) were carried out in a thermal cycler apparatus (Perkin-Elmer Cetus Corp.). The expected PCR products of 839 (5′-end) and 682 (3′-end) bp overlap by 299 bp and contain a single, common *Dra*III restriction site within the overlap. PCR products were purified by agarose gel electrophoresis, restriction endonuclease digested with either *Hin*dIII and *Dra*III for 5′-end sequences, or *Bam*HI and *Dra*III for 3′-end sequences, and ligated into the plasmid vector pHβAPr-1-neo (Gunning *et al.*, 1987) previously digested with *Bam*HI and *Hin*dIII.

First-strand cDNA synthesis of ER12L5 RNA was performed as for ER10K1, except that a single PCR amplification reaction was performed with oligonucleotide pairs R-B53.3 and R-H53.2. The expected PCR product of 1207 bp was agarose gel purified, restriction endonuclease digested with *Hin*dIII and *Bam*HI, and ligated into the pHβAPr-1-neo plasmid vector. Two independent clones from each ligation were isolated and direct sequencing from purified plasmid DNA was performed using the dideoxy-chain termination method (Sequenase, US Biochemical Corp.). The entire coding sequence was analysed using a series of p53-specific oligonucleotides as sequencing primers. Missense mutations were confirmed by sequencing the entire coding sequence of a pool of either five (ER12L5) or four (ER10K1) independent clones derived from an independent first-strand cDNA reaction and PCR amplification.

The nucleotide sequence of p53 cDNA from the other ER clones was obtained directly from PCR amplified DNA as described by Winship (1989). Briefly, p53 cDNA was amplified in two separate reactions directed to the 5′ and 3′ regions of the coding sequence as described above. Double-stranded DNA fragments produced after 40 cycles of amplification were eluted from agarose gels by centrifugation over glass wool. An aliquot of the eluate was mixed with rat p53-specific oligonucleotides as sequencing primers, frozen in dry ice, dried in a centrifugal evaporator (Savant SpeedVac), redissolved in the buffer described by Winship (1989) and subjected to the sequencing reaction as described above. The mutation in ER5-2 cDNA, initially detected by direct sequencing, was confirmed after cloning ER5-2 cDNA and sequencing individual plasmid clones.

Statistics

Linear regression and determination of the slopes were computed using the method of least squares (Minitab, Inc.).

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