

# The role of differentiation in the suppression of malignancy\*

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Although, as far as I am aware, there is as yet no journal wholly devoted to it, nor any burgeoning biotechnology company committed to its commercial exploitation, the genetic suppression of tumour formation is a subject that has now moved to centre stage in the intricate melodrama of contemporary cancer research. This event has been a long time in the making. It is more than twenty years since the discovery was made that normal cells contain genes that have the ability to suppress the malignant phenotype (Harris *et al.* 1969). The experiment had two parts. First, it was shown, in an assay that defined malignancy as the ability of a cell to grow progressively and kill its host, that hybrids formed by fusing malignant and non-malignant mouse cells were initially non-malignant; and second, that when certain specific chromosomes were eliminated from the hybrid, the malignant phenotype reappeared (Harris, 1971). The second part of the experiment was no less important than the first, for without the reappearance of malignancy in the hybrid cell populations, we would have had no plausible explanation for its disappearance. As it was, the conclusion was drawn, rightly it turned out, that the non-malignant character of the hybrids between malignant and non-malignant cells was due to the activity of normal genes that had the ability to suppress malignancy in a reversible fashion. As is usual, these initially heterodox ideas met with a good deal of resistance, but with the passage of the years evidence in support of them, derived from experiments with many different types of tumour cell and involving several animal species including man, continued to accumulate; and I do not think that anyone now resists the idea that malignancy is a suppressible phenotype and that the normal cell does contain genes that suppress tumour formation. We now call these genes tumour suppressor genes. (For a recent review, see Harris (1988).)

Interpreted in Mendelian terms, these experiments with hybrid cells indicated that whatever genetic lesions were responsible for conferring malignancy on a cell, and, however many of them might have to accumulate before this phenotype was finally expressed, they were all recessive to the wild-type tumour suppressor genes in the genome of the normal cell. Or, to put it more simply, no cell becomes malignant until the tumour suppressor genes are put out of action. These ideas received strong support from investigations on two other, very different, systems. Gateff and Schneiderman (1967) discovered that a mutation in *Drosophila* that mapped at the lethal 2 giant larvae (*l(2)gl*) locus, first described by Bridges (Bridges and Brehme, 1944), gave rise to an apparently malignant tumour of the nervous system. At that time there was no evidence that genuine malignant tumours occurred in invertebrates, and the early work on this mutant was

concerned with demonstrating that the growth in the nervous system was indeed a malignant tumour and not simply a malformation. It was subsequently shown that the mutation gave rise to the tumour only when present in the homozygous condition (Gateff and Schneiderman, 1969). Some 16 such homozygous recessive mutations are now known to produce tumours in *Drosophila*, in a range of different organs (Gateff and Mechler, 1989). The second line of investigation supporting the idea that malignant tumours can be generated by recessive mutations was the epidemiological work of Knudson. Knudson proposed, originally for retinoblastoma, that where there was a strong genetic predisposition to develop a particular tumour, a recessive mutation was present in the germ line in the heterozygous condition, but that the tumour arose only when a cell sustained a second genetic lesion that rendered that mutation homozygous (Knudson, 1971). The further exploration of these two lines of work has had a profound influence on our understanding of how malignancy is generated, and I shall discuss them both in more detail presently. For the moment, I should like to stay with hybrid cells.

Once it was established that tumour suppressor genes were present in the genome of the normal cell, one might have thought that it would be a fairly routine matter to determine at least their approximate location. For a number of technical reasons, the mapping of these genes in hybrid cells proved to be unexpectedly difficult, but solid chromosomal assignments have now been made in a number of cases. There is strong evidence that, where the normal cell is a mouse fibroblast, a critical suppressor gene is located on chromosome 4 in the region between A4 and C3 (Jonasson *et al.* 1977; Evans *et al.* 1982). This gene has the ability to suppress malignancy in any of the wide range of different tumour cells with which the mouse fibroblast has been fused. Apparently similar genes have been found in genetically homologous regions on rat chromosome 5 and human chromosome 1 (Islam *et al.* 1989; Stoler and Bouck, 1985). Tumour suppressors have also been located on human chromosomes 11 and 6 (Stanbridge *et al.* 1981; Kaelbling and Klinger, 1986; Trent *et al.* 1990). In the case of human chromosomes 1, 6 and 11, the assignments are supported by experiments in which the normal chromosome has been shown to suppress cell multiplication, either *in vitro* or *in vivo*, when it alone is inserted into the tumour cell (Sugawara *et al.* 1990; Trent *et al.* 1990; Saxon *et al.* 1986). Sustained efforts are

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now being made to isolate and characterize the genes that suppress malignancy in hybrid cells for two particular reasons: first, these genes do actually suppress the malignant phenotype whereas, in the case of several other genes that have been classified as tumour suppressor genes, the nature of their involvement in the emergence of malignancy is for the moment obscure; and second, the study of hybrid cells has provided some evidence concerning the mechanism by which this suppression is brought about. It is the question of mechanism that I now wish to discuss.

My present ideas on this subject have their origins in a histological observation made by Stanbridge and Ceredig (1981). They found that when cells of a line derived from a human carcinoma were fused with normal human fibroblasts, hybrids in which malignancy was suppressed acquired a different morphology *in vivo* from segregants in which the malignant phenotype had reappeared as a consequence of chromosome loss. The malignant segregants grew progressively as undifferentiated epithelial tumours; but the hybrid cells in which malignancy was suppressed assumed an increasingly elongated, fibrocytic shape and gradually ceased to multiply. Now this is just what normal fibroblasts do in, for example, a healing wound. The cells are at first induced to multiply, but as they secrete and organize their characteristic collagenous extracellular matrix, they gradually elongate and stop multiplying. The end result is an immature scar in which an orderly array of quiescent fibrocytes lies embedded in the collagenous matrix that they have themselves elaborated. The observations of Stanbridge and Ceredig thus suggested that the hybrids in which malignancy was suppressed were executing, at least in part, the differentiation programme of a normal fibroblast, whereas the malignant segregants were not.

It was obviously of interest to explore this possibility further. A range of different mouse tumour cells were fused with normal mouse fibroblasts and a histological study made of the behaviour *in vivo* of hybrids in which malignancy was suppressed and malignant segregants derived from them. In all cases in which malignancy was suppressed, the hybrid cells executed the differentiation programme of the normal fibroblast: they became progressively more elongated, secreted a copious extracellular matrix in which the collagen gradually became more cross-linked, and ceased to multiply (Harris, 1985). Malignant segregants did not elongate, made no organised collagenous matrix detectable by histological methods, and continued to multiply. The morphology of the malignant segregants showed some variation. In most cases, the segregant closely resembled the malignant parent cell, for example, a carcinoma, melanoma or lymphoma, but intermediate forms with some sarcomatous characteristics were also seen. No malignant segregants completed the fibroblast differentiation programme. This is true not only for hybrids between normal fibroblasts and malignant cells of other differentiation lineages, but also for hybrids between normal fibroblasts and cells of the same histotype such as fibrosarcomas.

It was obviously necessary to reduce these histological observations to molecular terms. An analysis was therefore made of some of the main extracellular components secreted by these hybrid cells *in vitro*. Normal mouse fibroblasts were fused with mouse melanoma cells and a comparison was made under the same conditions between hybrids in which malignancy was suppressed and malignant segregants derived from them (Copeman and Harris,

1988). It was established first that the melanoma cells produced very little collagen type I, but they did produce collagen type V and also released an active extracellular protease into the medium. Normal fibroblasts, on the other hand, produced large amounts of collagen type I, but no collagen type V and no active extracellular protease. Hybrids in which malignancy was suppressed produced the extracellular products characteristic of normal fibroblasts: they made large amounts of collagen type I, but very little collagen type V and no active protease. But when the elimination of the normal mouse fibroblast chromosomes 4 permitted the re-emergence of the malignant phenotype, the extracellular products characteristic of the normal fibroblast ceased to be produced and those characteristic of the melanoma reappeared: the malignant segregants secreted collagen type V and an active protease, but very little collagen type I. It was clear that the normal fibroblast imposed its own programme of differentiation on the hybrid cell *in vitro*, and *in vivo* this programme was carried through to its normal conclusion.

Essentially the same result was obtained when tumour cells were fused with normal keratinocytes. Terminal differentiation in the keratinocyte involves the synthesis of the protein involucrin, which is cross-linked by a keratinocyte-specific transglutaminase to form an insoluble envelope. Eventually the cell loses its nucleus and becomes a cornified squame. When hybrids between normal human keratinocytes and cells of a line derived from a human carcinoma were examined, it was found that those in which malignancy was suppressed continued to synthesize involucrin, but malignant segregants did not (Harris and Bramwell, 1987). When injected into the animal the non-malignant hybrids showed the characteristic histological features of terminally differentiating keratinocytes and ceased to multiply, whereas the malignant segregants continued to multiply as undifferentiated epithelial tumours (Peehl and Stanbridge, 1982). It appears that once again the suppression of malignancy in these cases involves the imposition on the hybrid cell of the terminal differentiation programme of the normal cell with which the tumour cell was fused.

This result raises the ancient question of whether it is the terminal differentiation that brings cell multiplication to a stop, or whether the cell differentiates terminally because it stops multiplying. I have long regarded this as a pseudo-question, for reasons that will soon become clear. Nonetheless, it seemed worthwhile to explore the matter further in hybrid cells because they lend themselves to certain direct approaches that are difficult to achieve in differentiating tissues. If the progressive multiplication of the malignant cell is suppressed by the terminal differentiation of the normal cell with which it is fused, then it should be possible to abrogate this suppression by impeding this differentiation programme in some way other than by the elimination of the specific chromosomes that determine it. It seemed reasonable to hope that this might be achieved by interfering directly with the expression of certain genes that were known to be necessary for the complete execution of the differentiation programme in question. In the case of fibroblasts, terminal differentiation requires the elaboration of a normal extracellular matrix. Fibronectin, which forms a functional bridge between the surface of the cell and the collagen that it secretes, is an essential component of this matrix. If, in a hybrid between a normal fibroblast and a malignant cell, fibronectin production could be abolished, a normal extracellular matrix could not be produced, and a

normal relationship between the cell and its matrix could not be established. If the suppression of malignancy in such a hybrid requires the production of a normal matrix and the establishment of a normal relationship between cell and matrix, then one might expect that this suppression would be abolished if fibronectin production could be inhibited. This was attempted in a series of experiments in which non-malignant hybrids between tumour cells and normal fibroblasts were transfected with expression vectors that incorporated sections of the fibronectin gene in the antisense configuration with respect to its promoter. Five clones were finally isolated in which fibronectin production was abolished or very greatly reduced. In four of these, the malignant phenotype had reappeared and the hybrid cells again produced rapidly progressive tumours when injected into appropriate animals (Steel and Harris, 1989). This result, preliminary though it is, encourages the belief that it is indeed the imposition of the terminal differentiation programme of the fibroblast that brings the progressive cell multiplication *in vivo* to a halt. This work is now being analysed in greater detail, and similar experiments have been initiated with hybrids between tumour cells and normal keratinocytes. In the latter case, expression vectors incorporating sections of the involucrin gene in the antisense configuration have been transfected into non-malignant hybrid cells and their subsequent behaviour *in vitro* and *in vivo* is now being studied.

While the results obtained with antisense constructs are as yet rather tentative, the analysis of tumorigenic mutations in *Drosophila* leaves little doubt that the critical step in the generation of a malignant tumour is indeed an impediment to normal differentiation, and not a direct stimulus to cell multiplication. To begin with, we know that homozygous recessive mutations at different loci in *Drosophila* produce tumours in different tissues (Gateff and Mechler, 1989). Since the mutant embryos may otherwise undergo more or less normal development, which involves phases of controlled cell multiplication, it is clear that the wild-type gene (the tumour suppressor gene) cannot be a general repressor of cell multiplication. The mechanism by which defective function or non-function of the suppressor gene produces tumours must involve some interaction with the differentiation processes of a particular target tissue. In the case of the lethal 2 giant larvae locus, where homozygous recessive mutations produce tumours of the nervous system, the evidence is compelling (Gateff and Mechler, 1989; Mechler *et al.* 1989a,b). First, it has been shown that there are two stages of development at which the product of this gene is normally synthesized. During the early development of the embryo, all cells synthesize this product, but during the larval period it is found only in the mid-gut, the salivary glands and the axonal projections of the neuropile of the central nervous system, tissues in which tumours do not occur. Second, by the use of genetic mosaics in which the gene is eliminated at different stages of development, it has been established that the tumours are produced only when the gene product is absent during early embryonic development. Its absence at later stages is not tumorigenic. And third, it has been found that the homozygous mutation produces not only tumours of the nervous system, but also non-tumorigenic abnormalities of terminal differentiation in other tissues. The wild-type product of the lethal 2 giant larvae gene is obviously not simply a repressor of cell multiplication; its essential function is to maintain cell fate and to regulate certain

forms of terminal differentiation. This gene acts as a tumour suppressor only in a particular type of tissue and only at one stage in the differentiation of that tissue.

Although recessive mutations are found in the homozygous condition in a wide range of human malignancies and have been shown to play a determinative role in some, as Knudson predicted, we know very little about the normal function of the corresponding wild-type genes or how they might act as tumour suppressors. Three such genes have been cloned and sequenced, the *Rb* gene, located on chromosome 13 q14 and implicated in the genesis of retinoblastoma (Friend *et al.* 1986; Lee *et al.* 1987), a gene on chromosome 11 p13 involved in the genesis of at least some sporadic Wilms' tumours (Call *et al.* 1990; Gessler *et al.* 1990), and the gene coding for the p53 protein, which is found in a mutated form in the nuclei of a wide range of tumours (Finlay *et al.* 1989; Baker *et al.* 1989). The product of the *Rb* gene is a nuclear protein that is phosphorylated during specific phases of the cell cycle (Buchkovic *et al.* 1989). It has been found to bind specifically to the products of certain viral genes: the E1A protein of adenovirus (Whyte *et al.* 1988), the large T antigens of simian virus 40 and JC virus (DeCaprio *et al.* 1988; Dyson *et al.* 1989a) and the E7 protein of the human papillomavirus (Dyson *et al.* 1989b). Since these viral genes are able to transform cells *in vitro* and act in some way as regulators of transcription, it has been proposed that the product of the *Rb* gene suppresses tumour formation by impeding the action of these transforming genes. But general models of this kind, even if they contain an element of the truth, will not provide a satisfactory explanation of the specificity of the effects produced by mutation of the *Rb* locus. It is known that the *Rb* gene is expressed in most normal tissues, yet homozygous events that inactivate this gene generate retinoblastomas and a very limited range of other tumours. The situation is, in its essentials, comparable to what one finds at the lethal 2 giant larvae locus in *Drosophila*, and no explanation of the role of the *Rb* gene as a tumour suppressor will be convincing if it does not concern itself with critical events in the differentiation of the retina. The putative Wilms' tumour gene specifies a zinc-finger polypeptide that may well be involved in the regulation of transcription. The gene is expressed in the kidney and, apparently, most strongly in the foetal kidney, but transcripts are also found in the spleen and in small amounts in the heart (Call *et al.* 1990). It has been proposed that the wild-type gene may be concerned with growth regulation in the metanephric blastema, the presumptive tissue of origin of the Wilms' tumour. This is, of course, plausible, but the analysis of the lethal 2 giant larvae mutation demonstrates that a high level of transcription of the gene in a particular tissue may have no connection with tumorigenesis, and indicates further that absence of the gene product or a defective gene product is likely to be of importance at only one critical stage in the development of the organ in which the tumour arises. Once again, we shall have to explore the programme of differentiation that leads to a kidney if we wish to understand how a homozygous recessive mutation at the Wilms' locus generates a malignant tumour there.

The history of research on the p53 protein is something of an object lesson. This nuclear phosphoprotein was first detected as a common feature in a range of human tumour cell lines (Crawford *et al.* 1981). Normal cells were found to contain very little. A genomic p53 clone and a number of p53 cDNAs were found to transform normal rodent cells

when these genes were transfected into the cells together with a mutated *ras* gene. Moreover, there was evidence that expression of the *p53* gene could enhance tumour growth *in vivo*. *p53* was therefore classified as a nuclear oncogene in the same category as *myc*, *myb* and *fos* (Lane and Benchimol, 1990). All this collapsed when it was found that the genomic clone used in the transfection experiments was in fact mutated; an unmutated genomic clone not only failed to transform in the presence of a mutated *ras* gene, but actually suppressed the transformation produced by the mutated *p53* gene (Finlay *et al.* 1989). Normal genomic *p53* was therefore re-classified: it ceased to be an oncogene and became a tumour suppressor gene, which remains its current status. Mutations and deletions in the *p53* gene are extremely common in human malignancies; they are found, for example, in some 40 % of mammary carcinomas and 30 % of colorectal carcinomas. In the case of colorectal carcinoma, which has provided the most detailed study of the *p53* gene in a human tumour (Vogelstein *et al.* 1989), it has been shown that both *p53* alleles are commonly affected, a situation at once reminiscent of what one finds in retinoblastoma and Wilms' tumour. Although, as far as I know, detailed sequence analysis of both alleles has so far been limited to this one tumour, it is to be expected that heterozygous mutations that result in accumulation of the mutated *p53* protein in the cell would also have phenotypic consequences, since the cell normally contains only minute amounts of the wild-type protein. What the *p53* wild-type gene does is obscure. The evidence from colorectal carcinoma indicates that *p53* mutations most frequently occur at the stage of transition from adenoma to carcinoma (Vogelstein *et al.* 1989). It has, as usual, been suggested that the *p53* gene is involved in some way in regulating mitosis. Since adenomas of the colon are generally diploid and carcinomas almost always aneuploid, my guess is that the accumulation of the mutated *p53* protein in the cell nucleus destabilizes the genome much as the gene products of simian virus 40 were shown to do some twenty years ago (Lehman and Defendi, 1970).

The object lesson provided by the *p53* gene is that transformation of cells resulting from the transfection of pieces of DNA does not identify dominant oncogenes. In defining the cardinal features of oncogenes, Robert Weinberg has written: 'The first of these is that oncogenes all act as agonists of cell growth; they appear to be hyperactive alleles of normal cellular growth-promoting genes. Consequently, oncogenic alleles act dominantly in relationship to the normal proto-oncogene alleles from which they arise' (Weinberg, 1989). If these really are the criteria that define this class of gene, then its membership may well be perilously close to zero. It is clear that whatever contribution oncogenes make to the malignant phenotype, this contribution is suppressible by the activity of normal genes. When tumour cells bearing mutated or translocated oncogenes are fused with normal fibroblasts, malignancy is suppressed, whether the oncogene remains active in the hybrid cell or not (Klein and Harris, 1972; Dyson *et al.* 1982; Geiser *et al.* 1986). If the phenotype we are scoring is the ability of a cell to produce a progressive tumour, no oncogene, as far as I am aware, has yet been shown to determine this phenotype in a genetically dominant fashion. On the contrary, evidence continues to accumulate in support of the view that oncogenes do not enhance cell function but, like the homozygous recessive mutations in retinoblastoma or Wilms' tumour, induce losses of cell function. Indeed, as the *p53* protein

illustrates, the distinction between an oncogene and a tumour suppressor gene is becoming hard to discern.

Among the losses of function that oncogenes have been shown to induce are defects in the execution of differentiation programmes. It has, for example, been shown that the transformation of fibroblastic cells by Rous sarcoma virus (Arbogast *et al.* 1977; Vaheri *et al.* 1978), by simian virus 40 (Kreig *et al.* 1980; Trueb *et al.* 1985) or by transfection with the H-*ras* or v-*mos* oncogenes (Liau *et al.* 1985; Schmidt *et al.* 1985) produces a severe impairment of one or more components of the extracellular matrix; terminal differentiation of erythroleukemic cells is blocked by the avian erythroblastosis virus (Beug *et al.* 1982) or by constitutive production of the c-*myc* oncogene protein (Coppola and Cole, 1986); transfection of bronchial epithelial cells by the H-*ras* oncogene renders them incapable of undergoing squamous differentiation (Yoakum *et al.* 1985) and several different oncogenes have been shown to impair the differentiation of myogenic cells (Holtzer *et al.* 1975; Falcone *et al.* 1985). In the cases of fibroblast and myoblast differentiation, substantial progress has been made in elucidating the molecular basis of the block to differentiation: the product of the v-*mos* oncogene binds specifically to the collagen gene promoter and inhibits the expression of the gene (Schmidt *et al.* 1985); the products of the mutated *ras* and *fos* oncogenes inhibit the expression of the *MyoD1* gene (Lassar *et al.* 1989). Nothing now argues against the view that oncogenes, insofar as they represent altered cellular genes, contribute to the genesis of malignancy by impeding normal differentiation.

The thesis I have been advancing is strongly supported by the observed effects of oncogenes in transgenic mice. (For a recent review, see Compere *et al.* (1988).) Three major conclusions can be drawn from these experiments. The first is that all malignant tumours that arise as a consequence of incorporating oncogenes into animals by transgenic procedures are clonal in origin and appear only after substantial latent periods. This is true even when two oncogenes, *ras* and *myc*, are introduced into the animal together (Sinn *et al.* 1987). Since the oncogenes are expressed in huge numbers of cells even when their expression is targeted to a particular tissue, it is clear that no oncogene determines malignancy in a dominant fashion against the genetic background of a normal cell. At least one other event, but possibly more, must take place in the genome of the cell before the malignant phenotype emerges. In the present climate of opinion, it is, of course, tempting to suppose that at least one of these subsequent events will be the inactivation of a tumour suppressor gene. The second conclusion is that not all tissues in which the integrated oncogene is expressed will generate malignant tumours, even in a stochastic fashion. For example, in some strains of animal the *myc* gene fused to the hormone-sensitive promoter of the mouse mammary tumour virus was expressed not only in breast tissue, but also in pancreas, lung, brain and salivary gland, but malignant tumours arose only in the breast (Leder *et al.* 1986). The contribution that the *myc* transgene makes to tumorigenesis thus hinges on the particular programme of differentiation that the host cell exhibits. And third, oncogenes active in transgenic animals may, like the lethal 2 giant larvae mutations, produce abnormalities of differentiation in certain tissues without producing tumours. For example, in three transgenic mouse strains, the c-*mos* gene, which transforms cells very efficiently *in vitro*, produced no tumours, but did produce aberrant

differentiation of the lens (Khillian *et al.* 1987). The findings that have emerged from transgenic animals are entirely consonant with the conclusions drawn from hybrid cells and *Drosophila* mutants.

Let me conclude with two trivial points that I should not have thought worth making were it not for the fact that they have been the occasion of some remarkably confused controversy. The first is that not all impediments to differentiation generate progressive cell multiplication. There are, of course, any number of aberrations that result in incomplete differentiation without generating a tumour. It is only at certain critical junctures that continuance of a differentiation programme and unabated cell multiplication are posed as alternatives; and it is only at these junctures that genetic impediments to differentiation have a bearing on the genesis of malignancy. The second point is that malignant tumours often contain subpopulations of cells that do complete their differentiation programmes. The erythrocytes overproduced in *polycythemia vera* do eliminate their nuclei, and squamous carcinomas of the skin do produce enucleate squames. This does not argue against a causative role for genetic impediments to differentiation in the origin of these tumours. It simply indicates that the mutations responsible for these impediments have a 'leaky' character: the mutants generate a mixed progeny in which some cells revert to the wild-type. It is not, of course, these fully differentiated cells that drive the tumour.

The idea that cancer is a disease of differentiation has a long history. Its early proponents had in mind epigenetic models of malignancy, which they advocated as alternatives to models based on somatic mutation. It would be very difficult to maintain that position now. But it does seem that cancer is, after all, a disease of differentiation, even if it is determined by random events that take place in genes.

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