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Genetics of Growth Arrest and Cell Death: Key Determinants of Tissue Homeostasis

P.A. Hall and D.P. Lane

INTRODUCTION: THE PROBLEMS OF BEING METAZOAN

BEING A multicellular organism poses considerable practical problems! In some way the information encoded as a linear array of four nucleotides within the genome must be transformed into a complex, dynamic arrangement of cell types that make up the three-dimensional complexity of organisms. This enigma can be broken down into three specific (but inter-related) problems. The first is the problem of cell type specification – the differentiation problem — which involves the processes that control the phenotype of cells. The second problem concerns the control of the number of these cell types — the quantity problem. And the final problem relates to the regulation of the spatial relationship of the various numbers of the different cell types — the morphogenesis problem. It is the second of these problems, the issue of regulation of cell number, that we will consider here. This is of obvious relevance to an understanding of cancer, since this set of diseases represents conditions in which the mechanisms that control cell number become deranged. It is also, however, of relevance to a wide range of non-neoplastic disease states.

In the past, considerable attention has focused upon those regulatory processes that control the generation of new cells i.e. the cell cycle and its control. In many cases, the mechanisms that have been studied have involved those factors that positively control proliferation including the many dominantly acting oncogenes and the pathways in which their products participate.

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However, consideration of the kinetic architecture of adult multi-cellular organisms indicates that the majority of cells are not proliferating. Furthermore, it is clear that even in those tissues in which there is continual cell proliferation, there is a highly orchestrated control of cessation of cell proliferation. In addition, the control of cell number must involve mechanisms for regulating cell loss. A second important concept is that tissues can respond to various external insults in an adaptive manner, and nowhere is this more apparent than in the regulation of cell number. Failure of adaptation has many consequences which are detrimental to the organism. Clearly then the controls that regulate cell number in the normal state and in response to external stimuli are likely to be very important to the maintenance of homeostasis. It is in this context that we will argue for the importance of mechanisms that control cell number by the regulation of cessation of cell proliferation and the control of population size by co-ordinated cell death. These ideas are central to our understanding of neoplasia. So much so that we suggest that the current paradigm of neoplasia should be revised with proliferation taking a subsidiary place to the primary regulation of growth arrest and cell death.

THE KINETIC ORGANISATION OF TISSUES

The organisation of tissues has a remarkable unity throughout metazoa. The autoradiographic observations of the spatial distribution of cell proliferation in adult rodent tissues made by Leblond [1], in which he defined three broad categories of tissue organisation (continually renewing or labile; conditionally renewing or stable; and static), are relevant to many (and probably all) metazoan organisms. Considerable attention has focused upon the cellular hierarchies that give rise to differentiated cell populations in the continually renewing tissues, as exemplified by vertebrate bone marrow, skin and gut epithelium. In such tissues, a small population of stem cells gives rise to the final differentiated populations via an intermediate cohort of transit amplifying cells (Figure 1)[2–5]. This form of cellular hierarchy is probably relevant to conditionally renewing or stable populations, and possibly to those developing cellular populations that become static in the adult organism.

The particular point that should be emphasised with regard to stem cell hierarchies is the remarkable diversity of potential control mechanisms that might determine the final number of cells derived from any given stem cell as outlined in Figure 1 [6]. The frequency of division of the stem cell and its cell cycle time (T_c) are pivotal determinants of events higher in the hierarchy. In addition, the probability that a given daughter of a stem cell division becomes committed to the differentiation pathway(s) or remains a stem cell (i.e. the probability of self-renewal or P_{sr}) or is deleted in some way, for example by apoptosis, will also be of critical importance. Further on in the hierarchy similar variables are of potential importance. For example, the number of rounds of division that occur within the transit population (N_1, N_2, N_3 . . . . etc.), the cell cycle time of such cells, the differentiation pathways chosen, the lifespan of any individual cell including its maturation time, and the possibility of loss of cells, particularly by apoptosis.

In situ analyses of cellular hierarchies indicate that there is very tight spatial regulation of proliferation, and it is easy to define proliferative and quiescent compartments in many tissues [1,3,6]. However, for any given tissue, the details of the cellular hierarchies involved in generating the final differentiated population, including the definition in cellular and molecular terms of the putative stem cell compartments, has proven very difficult. The intrinsic and extrinsic factors that regulate the transition from the proliferative to the quiescent compartments are poorly understood, and the determinants of how many rounds of division any given hierarchy undergo are unknown. Furthermore, the regulation of cellular lifespan are also largely a mystery. Perhaps only in the case of haemopoietic development is there any clear view of how cellular populations develop and the mechanisms of control involved, and in no case can we

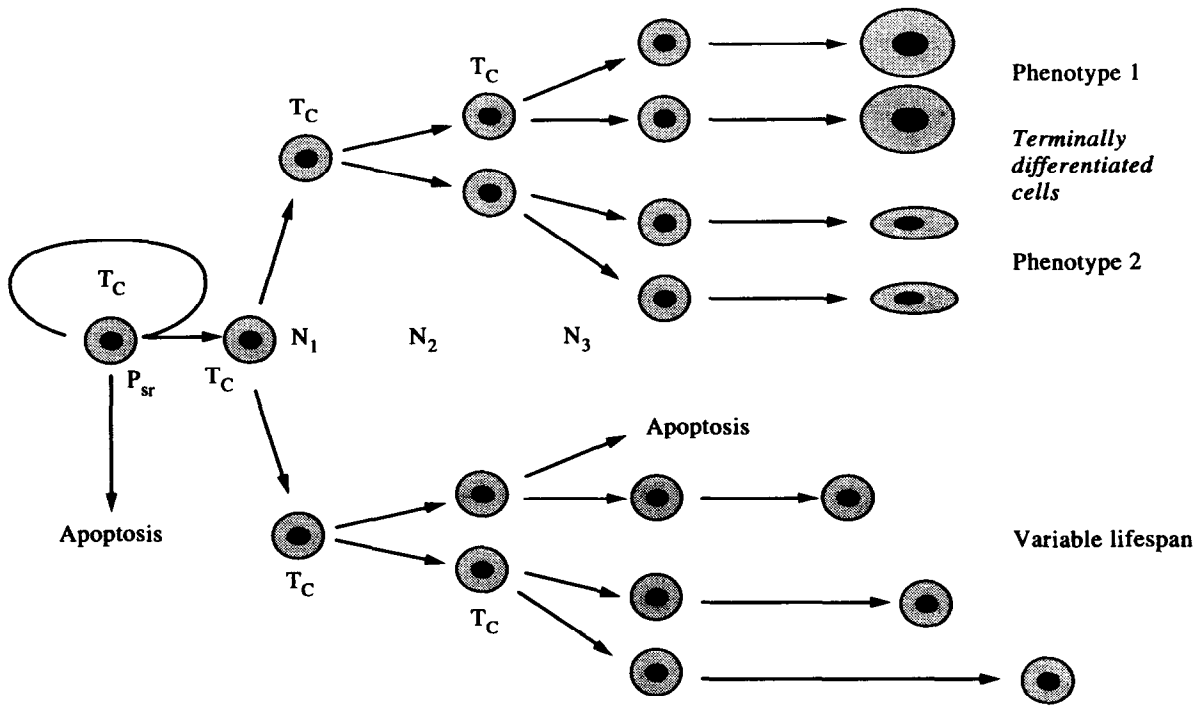

Figure 1. Regulatory events in controlling stem cell hierarchies (adapted from Ansari and Hall [6]). See text for explanation.

specify with certainty many of the parameters outlined in Figure 1. Nevertheless, it is apparent that these variables and the mechanisms that control them are essential to our understanding of how tissues develop, are maintained and respond to perturbing influences.

CLASSICAL APPROACHES TO THE STUDY OF POPULATION CONTROL

The traditional approaches to defining the regulation of cell number in tissues has focused almost entirely upon the analysis of cell proliferation in tissues using mitosis counting and labelling strategies of various sorts, coupled with the definition of various kinetic parameters and the use of mathematical models [3,5,7]. Such descriptive studies have been of inestimable value in defining the parameters that control population size, particularly in epithelia such as skin, gut and liver. However, while very important, these studies have failed to provide mechanistic insights into the cellular and molecular control of population size, nor have they allowed an easy integration of the diverse and extensive knowledge of cell proliferation control *in vitro*.

Critical examination of many *in vivo* systems will demonstrate the central importance of apoptosis and growth arrest as key determinants of cell number. For example, consideration of the continually renewing tissues, such as bone marrow and skin, indicate that apoptosis, or processes mechanistically akin to it, must be continually occurring. What becomes of the neutrophils, monocytes, erythrocytes and lymphocytes continually being produced by the bone marrow? Loss of neutrophils by apoptosis is well established, although under normal circumstances neutrophil apoptosis is not easily demonstrable in tissues. Loss of monocytes and their cellular products, macrophages, is also not usually apparent by apoptosis, but this must be occurring. The process of erythrocyte loss probably begins 120 days before they are finally removed due to the loss of their nucleus by a process that is morphologically similar to apoptosis. Many differentiation processes are associated with profound growth arrest. Indeed, there is almost universally a close inverse relationship between differentiation and proliferation [4,8], and, in the case of keratinocytes and lens epithelial cells, the last phases of terminal differentiation are associated with loss of the nucleus in a manner analogous to that seen in erythrocytes, and which has many similarities with the nuclear condensation and fragmentation seen in apoptosis.

The increase in cell number that occurs as an adaptation to external stimuli (or hyperplasia) is a reversible process, and a tissue will revert to normality on the cessation of the invoking stimulus. The mechanism by which the population increases will involve altering some or all of the parameters outlined in Figure 1: the reversion to normality is more complex. At least in the hyperplastic adrenal gland and liver, the mechanism by which normality is established involves cessation of the proliferation (increased growth arrest) but also a dramatic increase in apoptotic cell death [3,9]. Of particular importance is the principle that small alterations in the apparent number of apoptotic bodies can account for enormous fluxes in population size due to the very transient nature of the morphological end point of the process, the apoptotic body [10–12]. Nevertheless, in some developmental situations the amount of apoptosis is very large, for example in the developing vertebrate nervous system [13], in the formation of digits or the alterations of amphibian metamorphosis [14].

Cell death and proliferation control have been studied in a wide range of *in vitro* systems. For example, the phenomenology of the classical models of growth arrest as induced by contact inhibition, anchorage independence and serum deprivation, and models of apoptosis involving heat shock and exposure to genotoxic agents are well defined. Unfortunately, the mechanistic basis of these processes are much less well defined. Furthermore, the direct relevance of many of these *in vitro* assays to the complex changes in population behaviour *in vivo* is not at all understood. This is particularly so if one considers the heterogeneity of response of the cells at different points in a given stem cell hierarchy. To what extent is this heterogeneity apparent *in vitro*? For the most part, we would suggest that they have scarcely been investigated. It is perhaps only in the context of myocyte differentiation that any of the mechanistic aspects of cell cycle exit have been analysed [8,15,16], and even these have not considered the issues of heterogeneity implicit in analyses of whole cell populations.

GASTROINTESTINAL EPITHELIUM AS A MODEL FOR CELL NUMBER REGULATION

The various anatomical regions of the gut show an essential unity of architecture, differentiation and kinetic organisation. The basic structural unit is a test tube-shaped gland or crypt with a spatially well defined proliferative compartment which contains multi-potential stem cells and the daughter transit populations. In the small intestine, the proliferative compartment is close to the bottom of the crypt and the predominant flux of cells is upwards, although a minor population moves downwards to the crypt base. The production of cells by any crypt is massive and continuous, perhaps 200–300 cells being produced every day by the murine small intestinal crypt [17]. Approximately 1200 cells a day move on to and cover finger-like projections or villi that dramatically increase the intestinal surface area and facilitate absorption. In the colon, the organisation is very similar, although the proliferative compartment extends to the base of the crypt and no villi are present. In the stomach, the proliferative compartment lies towards the middle of the mucosa, and there is a clear bi-directional flux of cells away from this zone. The gut epithelium contains a number of differentiated cell types which differ somewhat between stomach, small and large intestine. There is now overwhelming evidence that at any given anatomical site all are derived from a common stem cell population [4,5,18,19].

A range of insults alter the kinetics of the gastrointestinal epithelium in fairly stereotyped ways. For example, low doses of radiation or other genotoxic insults induce apoptosis in a particular sub-population of gastrointestinal epithelial cells [20,21]. Furthermore, a number of studies have defined the alterations in cell production that occur in a characteristic temporal and spatially regulated manner after genotoxic insults to the small intestine [3,6]. As discussed earlier, a number of critical issues underlie these basic facts: the differentiation problem, the quantity problem, and the morphogenesis problem. With regard to the quantity problem, two sets of data indicate the importance of cell death and growth arrest, and also the potential value of the epithelium of the mammalian gastrointestinal tract as a useful model of cell number regulation in normal and diseased states.

The traditional view of the gut epithelium is that the massive cell production is balanced by cell loss by shedding into the gut lumen [22] but the evidence for this is not compelling (reviewed in Hall *et al.* [12]). Examination of histological preparations shows that on any given small intestinal villus there is approximately one apoptotic body. Given that in the order of 1200 cells

enter a murine villus on any given day and that the same number are lost, it might at first sight seem implausible that apoptosis has any significant contribution to the overall flux. This is not in fact the case! The 'lifespan' of the morphologically definable apoptotic body is very short [10,11], perhaps being as short as an hour. Furthermore, a murine small intestinal villus is approximately 150 μm in diameter and a histological section is of the order of 3–4 μm. Consequently, the average of one apoptotic body per histological section reflects 24 per day per section, or between 1000 and 1250 per day per villus: approximately equal to the known cell loss. Therefore, while some shedding undoubtedly occurs, apoptosis accounts for the bulk of cell loss in the small intestine, and in other parts of the gut [12].

The response of the gastrointestinal tract to genotoxic insult also depends upon a co-ordinated response involving cell death and growth arrest. It has been known for many years that genotoxic insult will induce apoptosis in a highly stereotyped manner in a particular population of cells in the small intestinal crypt, and that this is associated with a profound and spatially and temporally well defined growth arrest [3,20,21]. As discussed below, the mechanism underlying apoptosis after genotoxic insult involves the product of the *TP53* gene [23,24]. The growth arrest seen appears not to be *TP53* dependent, and indeed is a G2 block not a G1 arrest [24]. The essential message of these two sets of data is that growth arrest and cell death are central determinants of homeostasis in the gastrointestinal epithelium, both in normal circumstances and after exogenous stress. In addition, it is important to recognise that the growth arrest and apoptotic responses are very highly regulated both temporally and spatially, and there is extreme heterogeneity within the epithelial populations. This heterogeneity of response is of considerable biological importance yet is very difficult to investigate experimentally in *in vitro* systems. However, it is the genetic mechanisms by which cell death and growth arrest are controlled that will provide insight into the maintenance of cellular homeostasis in tissues such as the gut.

THE GENETIC CONTROL OF APOPTOSIS

Rapid progress in several systems has allowed the definition of genes whose products are involved in apoptosis [25,26]. In particular, the power of genetic analysis in *Drosophila melanogaster* and in *Caenorhabditis elegans*, together with detailed morphological approaches, have combined to give profound insights into the mechanisms of cell death. Such strategies have allowed the definition of cascades of events and the identification of gene products critical for the various stages of apoptosis. For example, in *Drosophila* apoptotic cell death has been morphologically defined during development [27], and has been shown to be due in part to cell–cell interactions and also to the effects of hormonal influences. White and colleagues [28] have defined a gene, termed reaper (*rpr*), whose product is essential for apoptotic cell death, using a strategy based upon finding absent or reduced cell death in flies with a range of homozygous chromosomal deletions. This screen allowed the identification of a 85-kb region critical for normal apoptosis, although high dose radiation was found to induce apoptotic death. *rpr* was cloned from this region and was found to rescue apoptosis in a mutant organism (H99) with deletion of the 85-kb segment. These data point to the intact nature of the death programme in the *rpr* flies, with *rpr* being an upstream regulator of the apoptosis programme, but downstream of a range of initiating insults including cell–cell interactions, differentiation events, cell lineage specific factors and exogenous genotoxic insults including low dose irradiation [29]. These environmental and intrinsic determinants of the apoptotic pathway are outlined in Figure 2 and appear to be as relevant to mammalian apoptosis as to *Drosophila*.

A more detailed cascade of genetic elements involved in apoptosis has been defined in the nematode *C. elegans*, where the combination of powerful genetic techniques and the invariant nature of the developmental programme has allowed the genetic basis of regulation of apoptosis to be investigated. During its development, 131 cells are deleted by an invariant process morphologically and functionally analogous to apoptosis [30,31]. A series of mutants have been defined in which various components of this process are defective, and appropriate genetic analyses have allowed the genes involved to be ordered. In this genetic pathway, three genes (*ced3*, *ced4* and *ced9*) are involved in the execution of the cell. Two sets of genes (*ced1*, *ced6*, *ced7* and *ced8* in one group and *ced2*, *ced5* and *ced10* in the other) appear to be involved in parallel pathways involved in engulfment of the dead (or dying) cell. Finally, a nuclease encoded by *nuc1* is involved in degrading the DNA of the dead cell. In the presence of *nuc1* mutations, the DNA is not degraded but death still occurs.

Genetic analysis in *C. elegans* points to an important role for *ced3* in the induction of apoptosis [32]. ced3 has close homology with the *nedd-2* mouse protein and the human interleukin-1β-converting enzyme (ICE). Furthermore, the ced3 protein may act as a cysteine protease which initiates programmed cell death both in *C. elegans* and also in mammalian cells [33]. Direct evidence for this has come from the induction of apoptosis in mammalian fibroblasts by the overexpression of ICE [34]. It is of note that high levels of ICE have been observed in a number of disease states associated with apoptosis, and that manipulation of ICE activity may prove to be a therapeutic strategy in a range of diseases [34]. The structure and mechanism of ICE have recently been reported [35], and a number of recent studies indicate that a family of ICE-like proteins exist [36, 37]. It is

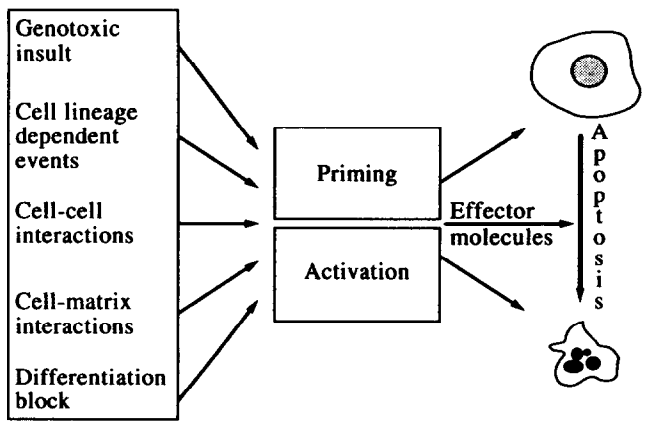

Figure 2. Events in the induction of apoptosis. A wide range of external and internal stimuli can induce and regulate the process of apoptosis (left). It may be that all cells are (to varying degrees) primed to undergo apoptosis, but that external events can fully prime a cell such that the elements of the apoptotic pathway are all in place. Subsequent events, again induced by external or internal events (and which may be the same or different from priming events), then lead to activation of the apoptotic programme. A range of effector molecules then induce the biochemical and structural events that lead to the characteristic morphological features of apoptotic cell death and subsequent clearance of the cellular fragments.

intriguing that at least one ICE/*ced3*-related gene encodes both positive and negative regulators of apoptosis, perhaps giving us a glimpse of the complexity of regulation of this fundamental biological process [37].

What regulates the activity of *ced3* in *C. elegans*? Further genetic analyses indicate a role for *ced9* whose action is to inhibit cell death since overexpression of this gene protects against all apoptotic cell death. There are both sequence and structural homologies between *ced9* and *bcl-2* and it has been found that overexpression of human *BCL-2* in *C. elegans* was found to mimic the effect of *ced9* [38]. The identification of *ced3* as a homologue of ICE and of *ced9* as a homologue of *BCL-2*, indicates that there may be extensive conservation of the mechanisms of apoptosis control throughout metazoa [38]. bcl-2 protein appears to block most types of apoptotic cell death as evidenced by inhibition of apoptosis after overexpression of this protein in transgenic mice [39] and in a range of other situations [40] including induction by ionising radiation [41], chemotherapeutic agents [42] and induction by *c-myc* [43]. Further evidence for the role of *bcl-2* in the regulation of apoptosis has come from the demonstration of massive and inappropriate apoptotic cell death in *bcl-2* null mice [44]. Furthermore, these *bcl-2* null animals raised the possibility of *BCL-2* having a role in a defence against oxidative stress since some of the pathological changes resembled those seen after metabolic poison-induced abnormalities of redox. The bcl-2 protein is localised to mitochondrial and nuclear membranes and also the endoplasmic reticulum, potential sites of oxygen free radical generation. Based upon these observations, Hockenbery and associates [45] investigated the possible role of *bcl-2* in the defence against oxidative stress. They have demonstrated that overexpression of bcl-2 protein can suppress lipid peroxidation, and have suggested that bcl-2 has a general role in regulating anti-oxidant stress at sites of free radical formation. However, it should be noted that a role for the product of the *bcl-2* gene in the defence against oxidative stress is by no means proven.

A number of mammalian genes with homology to *BCL-2* have recently been defined, including *bax*, and *bcl-x* with the latter existing as at least two isoforms [46,47]. The function of bcl-2 and the related molecules may be intimately linked. For example, it appears that heterodimers and homodimers of the various species can exist with differing effects on the process of apoptosis. bax/bax dimers appear to be death promoting, bcl-2/bcl-2 dimers are potent survival signals while bcl-2/bax heterodimers are intermediate. This is perhaps analogous to interactions between myc and max. Mutational analysis of human *BCL-2* has defined two conserved regions, BH1 and BH2, required for interaction with the *BCL-2* homologue bax which are functionally involved in apoptosis [48]. Similar observatons have been made in *C. elegans* where there is close homology of ced9 protein with bcl-2 [49]. bcl-2 has also been shown in the yeast two hybrid system to interact with the GTP binding protein R-ras, and thus an interaction with signal transduction pathways seems likely. This idea is amplified by the recent cloning of a Grb2 isoform with apoptotic properties [50]. It would seem highly likely that other signal transduction pathways will be important in regulating apoptosis, linking cell surface events including survival signals to the apoptotic machinary.

Currently, little is known of the range of cell surface receptors that can signal death or survival. Furthermore, many identified cell surface receptors considered to be involved in mitogenic pathways may also be components of survival systems. One cell surface receptor that has been identified as having a role in apoptosis is the fas antigen, also known as APO-1 in murine cells [51,52]. The interaction of this cell surface receptor with its ligand is a potent inducer of cell death. Furthermore, a family of receptors exist with close homology to *fas* [53] and a number possess a particular domain which signals apoptotic death [54]. Components of the TNF family and associated family of receptors may be involved in apoptosis in a wide range of situations, being essential components of normal lymphoid homeostatis and possibly being involved clinically important states such as cachexia [55]. Defects in the *fas* system have been shown to cause considerable functional defects in the immune system [56]. Moreover, point mutations in the 45-kDa fas ligand, a member of the TNF/NGF family [57,58], also lead to defective apoptosis that results in lymphoproliferation [59].

Presumably, there will be a number of families of factors which will signal survival or death, probably depending upon the overall balance of signals that a given cell receives. It may be that our previous cell biological assays and our general preoccupation with proliferation rather than growth arrest and cell death has blinded us to the existence of such regulatory factors. Indeed, it could be that a number of well known molecules have hitherto unexpected properties in the context of growth arrest and apoptosis. Finally, a particularly important insight into the environmental regulation of cell death has come from the recognition of the role of extracellular matrix in providing survival signals [60–62]. Given that apoptosis is regulated in a spatially highly constrained manner, this may prove to be of great biological relevance.

Nuclear regulators of apoptosis are also known. For example, the transcription factor c-rel will induce apoptosis in bone marrow cells when expressed at high levels, and may have a physiological role in the control of haemopoiesis [63]. The transcription factor c-fos is expressed immediately prior to apoptosis in many cells *in vivo* [64] and has been extravagantly termed 'the harbinger of death'. However, the greatest attention has been focused on c-myc. The realisation that c-myc played a role in apoptosis grew from the paradoxical behaviour of myc expressing cell lines [65] and the important studies of Evan and colleagues [66]. It was shown that in the presence of low serum levels c-myc overexpression led to dramatic apoptosis in cultured Rat-1 fibroblasts. Furthermore, the regions of the c-myc protein involved in apoptosis overlap with those involved in the processes of cellular transformation. Evan and colleagues [67] have proposed a model of c-myc function in which the levels of myc and the nature of external signals combine to determine whether a cell proliferates (high, myc, high growth factors), arrests its growth (low myc, low growth factors) or undergoes apoptosis (high myc but low growth factors). In addition, recent data point to the insulin-like growth factors as having a particularly potent role as potentiating growth factors [68]. These ideas are particularly relevant in the light of Raff's concept of continuous signalling to cells by survival factors in order to inhibit apoptosis [69].

Another nuclear protein that has a clear role in the induction of apoptosis is p53. This is of particular interest because the *TP53* gene is a potent tumour suppressor gene [70]. Inheritance of a germ line mutation in one allele of the gene is associated with a greatly elevated lifetime risk of developing cancer, and in man is the common genetic basis of the Li–Fraumeni cancer family syndrome. Both parental copies of the *TP53* gene are very frequently inactivated by mutation in human solid tumours [70]. The p53 gene product, a nuclear phosphoprotein, acts as a sequence specific DNA binding protein and as a transcription

factor. When high levels of p53 are introduced exogenously into cells, they can trigger growth arrest in the G1 phase of the cycle (but other phases may well be affected) or alternatively may induce an apoptotic response. The normal physiological role of this process was initially unclear, and this uncertainty was underscored by the production of mice by homologous recombination that lacked p53 function completely [71]. These "p53 knock-out" animals develop normally and therefore suggest that the normal apoptotic processes of development and tissue maintenance do not require p53. Nevertheless, these animals have a very high rate of cancer since essentially all will die of tumours within one year of birth [71]. The resolution of this enigma came from the realisation that p53 is an inducible regulator of apoptosis [72,73]. The gene product is normally produced in minute amounts, but when cells are exposed to DNA-damaging agents, such as common chemotherapeutic drugs or ionising radiation, p53 levels rise and the protein is activated as a transcription factor. Active p53 protein will then turn on downstream genes that will drive the cell into an induced death [72–74]. Thus, p53 can act to block the survival of cells that have sustained genetic damage. The p53 protein has been described as a "Guardian of the Genome" in this context [75]. Additional functions of p53 not involving transcriptional regulation are still a real possibility, and it may even be that p53 can act as a direct sensor of DNA damage [76,77].

The study of radiation-induced apoptosis in normal mice as compared to those in which the gene has been inactivated have proven the absolute importance of p53 function in this process [23,24,78,79,80]. The thymocytes and intestinal epithelial cells of the p53 minus mice are extraordinarily resistant to radiation-induced apoptosis. The induction of apoptosis by DNA damage thus appears to be critically dependent on this one gene function. Hence it could be predicted that p53 function would be central to determining the therapeutic response of cancer cells to therapy. These predictions have now been largely confirmed as the p53 pathway seems to be essential for a good therapeutic response to physiologically tolerable doses of radiation or chemotherapeutic drugs [81]. The action of p53 confers genetic stability under stress, and so loss of its function permits the division of cells with damaged chromosomes. These cells, it is now realised, form a pool of aberrant cells from which more malignant or drug-resistant cells may be isolated. So loss of this apoptotic pathway not only blocks apoptosis, but also gives rise to pools of aberrant surviving cells.

For any given amount of p53 protein in a cell, how is a decision to die or to arrest growth made? Obviously, we are not yet able to answer this question, but it seems highly likely that it is the overall cellular environment that is of key importance. Furthermore, there may be functional interactions between the p53 pathway and members of the bcl-2 family [82]. For example, p53 may act by altering the relative levels of bcl-2 and bax [83]. Again the stoichiometry of interactions may be of critical significance, rather than the absolute levels *per se*.

From this discussion, it is apparent that a complex set of systems has evolved in metazoa for eliminating unwanted cells in development, in the maintenance of normal populations in the adult, and in the context of external stimuli including viral infections and genotoxic challenge. Of note is the fact that even within a cell lineage, there are important, but poorly understood differences in the response of different cells to a given insult [23], and we understand little of how the normal background level of apoptosis that is continually occurring in populations *in vivo* is controlled. This is despite its fundamental importance to the regulation of population size and normal tissue homeostasis. However, progress in understanding apoptosis has been rapid, and it seems likely that the many disparate observations, some of which we have reviewed here, will eventually fall into place as a coherent explanation for the role of apoptosis in normal tissue regulation.

THE GENETIC CONTROL OF GROWTH ARREST

A range of different signals will induce growth arrest in a variety of systems. For example, *in vitro* deprivation of nutrients and/or growth factors, contact inhibition or exposure to a range of growth inhibitory molecules will induce cellular quiescence. In addition, many cells will respond to genotoxic insult by slowing or stopping the cell cycle. However, while these and related phenomena have been described for many years, the mechanisms by which these stimuli actually cause cell cycle arrest remain poorly defined. That specific genes and their products are actively associated with the process of growth arrest is demonstrated by a number of different observations: there is a highly regulated pattern of cell proliferation in most tissues, and the control of such patterns must depend upon negative as well as positive regulation [1,3,4,6]; somatic cell hybrid experiments with fusion of normal replicating cells with either quiescent or senescent cells have demonstrated that entry into the S phase of the cell cycle could be strongly inhibited by the non-replicating partner [84–86] and have shown suppression of tumorigenicity [87–89]; the observation that mRNA species obtained from growth-arrested cells can be microinjected into cycling cells and induce cessation of proliferation [90–92]; and finally, differences in protein or mRNA expression between cycling to non-cycling cells [93–100].

The properties and functions of the gene products whose expression is associated with growth arrest are diverse. Some appear to have structural roles, as in the case of lamins A and C [101] and gas-2 [102], while others are suggested to be involved in regulation of gene expression [103–105], differentiation [15], control of cell cycle progression [106] or appear to be involved in cell surface events or signal transduction [107,108]. However, our knowledge of the nature and function of molecules whose expression is associated with growth arrest and with negative growth control is still poor. Furthermore, the nature of the regulatory pathways that might link some of these elements are poorly understood, although recent progress has again been rapid.

The effective regulation of the cell cycle depends upon the correct ordering of the biochemical and structural events associated with this process. Hartwell and Weinert [109] proposed the existence of checkpoints through the cell cycle at which specific biochemical questions were asked. Only if the appropriate answers (again in terms of biochemical parameters) were provided could a cell pass further in the cycle. Such a framework allowed for the correct temporal ordering of the cell cycle, and also provides potential mechanisms for cell cycle (or growth) arrest. A diverse range of potential checkpoint controls exist and many may yet be discovered. The current state of our understanding of growth arrest should be considered in the context of cell cycle checkpoints, or cell cycle transitions, where feedback mechanisms operate to prevent premature entry into the subsequent phase of the cycle. In general, such checkpoints have been defined using both functional and genetic analyses in yeast. Comparable regulatory mechanisms also appear to exist in higher eukaryotes.

One important set of checkpoint mechanisms appear to

involve the inhibition of cyclin/cyclin dependent kinase (CDK) complexes, thus preventing some of the phosphorylation events essential for normal cell cycle progression. In mammalian cells, at least five inhibitors have now been described, p15, p16, p21, p24 and p27 (or p28) [106–112]. The first to be identified was a 21-kDa nuclear protein associated with cyclins A, D1 and E, all of which form complexes with Cdk2. This protein [variously known as Cip1 (Cdk-interacting protein [113], Sdil (senescent cell-derived inhibitor [114]), Waf1 (wild-type p53-activated fragment [115]) or Pic1 (p53-regulated inhibitor of Cdks [110]), is a potent inhibitor of the activity of the complexes formed between Cdk2 and these cyclins. Inactivation of cyclin-Cdk2 activity leads to a reduction in phosphorylation of the product of the retinoblastoma gene product (and possibly related proteins such as p107) which can in its hypophosphorylated state complex with and inhibit members of the E2F family of transcription factors, thus altering the transcription of many genes required for cell cycle progression (Figure 3). In this way, activity of $p21^{waf1}$ (our preferred name) causes G1 arrest and inhibits DNA synthesis. New members of this family of proteins continue to be recognised and the complexity of this process is very great [111,112].

It has been shown that p53 protein can be induced by genotoxic damage both *in vitro* [117–119] and *in vivo* [23,120], and this is associated with growth arrest [118,121]. This important function of p53 appears to be mediated, at least in part, by the induction of $p21^{waf1}$ since there is now good evidence

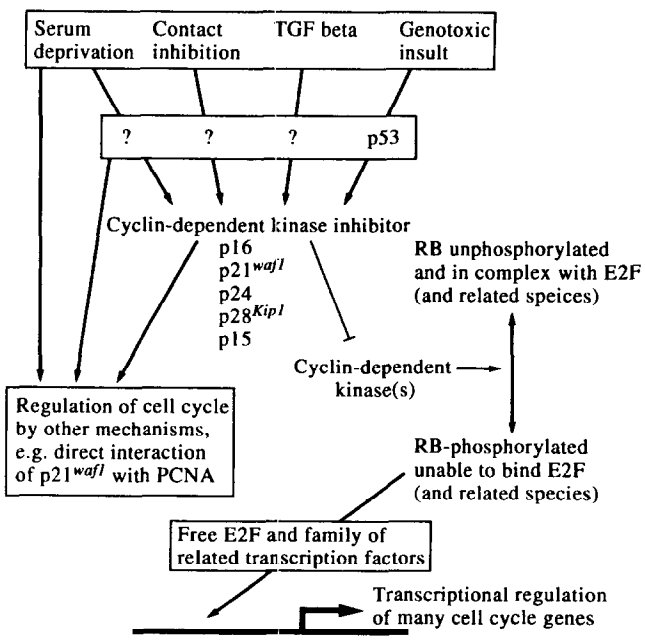

Figure 3. A range of stimuli can induce growth arrest in model systems including serum deprivation, contact inhibition, soluble proteins, such as TGF-β, and genotoxic insults. The means by which these stimuli lead to growth arrest are unclear for the most part. Some forms of genotoxic insult act via a p53-dependent pathway and p53 may have a role after other stimuli. Recent data indicate a role for inhibitors of cyclin-dependent kinases, which by altering the phosphorylation of Rb (and possibly other related species) alter the availability of E2F and related transcription factors to control the expression of key cell cycle genes. Other pathways are also probably involved including possible direct interactions of cyclin dependent kinases with other key cell cycle control proteins. Other pathways may also exist and the physiological relevance of all these systems in the control of growth arrest in tissues is unknown (adapted from Hall and Coates [116]).

that irradiation activates p53 protein function and this in turn induces transcription of $p21^{waf1}$ mRNA. This protein can then act as an inhibitor of cyclin-dependent kinases including the Cdk2–cyclin E complex [115,122]. In addition, recent data suggest that $p21^{waf1}$ can act in at least one other way. Using the SV40 *in vitro* replication assay, Waga and associates [123] have demonstrated that $p21^{waf1}$ can directly inhibit PCNA-dependent replication in the absence of cyclin/Cdk. This is particularly important since it suggests that the interaction of PCNA and p21 may be involved in co-ordinately regulating cell–cycle progression, DNA replication and repair of DNA damage. In addition, it may be the stoichiometry of the various components rather than the absolute levels of any single component that is paramount in regulating these complex processes.

The other Cdk inhibitors so far defined are much less well characterised at present but may act in a comparable manner, perhaps effecting the growth inhibitory signals from other external stimuli such as contact inhibition and negative regulatory factors, such as TGF beta (Figure 3). For example, Polyak and associates [124] have identified a 28kDa protein which acts as a Cdk inhibitor and appears to be involved in the growth inhibition after TGF beta treatment via an inhibition of cyclin B/Cdk2 activity [124–126]. Hengst and associates [127] have defined a related protein ($p28^{Ick}$) that inhibits Cdk/cyclin complexes from human cells. Another recently identified Cdk inhibitor is the 24-kDa human protein called Cdi1 (for Cdk interactor) or Cip2 [128]. This protein has protein phosphatase activity and can complex with cdc2 (now also called Cdk1), Cdk2 and Cdk3 but not with Cdk4. It is expressed at the G1/S transition, and when overexpressed it delays progression through the cell cycle [128]. A further protein called p16, which binds to and inhibits the catalytic activity of the Cdk4-cyclin D complex, has been identified. By inhibiting the Cdk4-cyclin D complex, it inhibits the phosphorylation of retinoblastoma protein and progression through the cell cycle [129]. Although the p16 gene product is an inhibitor of cyclin-dependent kinases and mutations in p16 have been found in many tumour derived cell lines [130,131], mutations appear to be rare in primary tumour samples.

A wide range of other genes are associated with growth arrest. For example, members of the interferon family [132], transforming growth factor-β [133], the growth arrest and DNA damage-inducible (*GADD*) genes [97]; the growth arrest-specific (*gas*) genes [16], TI1 [96], *c-abl* [134], prohibitin [92] and many others. It seems probable that there are many as yet unrecognised, growth inhibitory genes. Knudson's estimate of more than 50 [135] may prove to be an underestimate since negative regulators of systems involved in cell number control might act at a very wide range of sites. There may also be a range of novel strategies for regulating growth arrest. A highly novel mechanism of growth suppression has been described by Helen Blau and associates [136] who demonstrated that RNA from the 3′ untranslated region of the tropomyosin gene could suppress anchorage independent growth and tumorigenicity of NMU2 cells (a murine rhabdomyosarcoma cell line). The mechanism by which non-coding regions of messenger RNA can act as regulators of growth and differentation, and suppress tumour formation remains unclear, but this phenomenon, if further substantiated, may be of widespread biological relevance.

The issue of growth arrest may also have another implication relating to the longevity of cells and organisms since at its most extreme the process of cellular senescence may be viewed as a form of growth arrest. The mechanisms underlying this remain obscure, but a particularly attractive hypothesis proposes that

progressive telomere erosion may underpin senescence [137]. In support of this come the recent observations of Wynford-Thomas and associates indicating that mutant p53 can induce escape from senescence in human diploid fibroblasts [138]. These authors suggest that the role of p53 in senescence can be reconciled with the "guardian of the genome" hypothesis of p53 function by considering telomere erosion as a form of DNA damage. This is attractive since it is entirely consistent with the notion that immortalisation (or increased lifespan) of transformed cells involves loss of normal control of growth arrest signals. As with the molecular basis of apoptosis, our understanding of the regulation of growth arrest is sketchy, and we understand little of how this process is integrated with other processes.

INTEGRATION OF THE VARIOUS CONTROLS OF CELL NUMBER

We have seen that there is a diverse range of genetic controls of growth arrest and cell death which compliment the established controls of cell proliferation [69,139,140]. It is probable that there are many other relevant molecules and interactions to discover that will add to the complexity of control growth arrest and cell death. In order to regulate cell number in a physiologically relevant manner, these diverse controls must be integrated. That this occurs *in vivo* is obvious and there are a number of experimental systems in which some evidence for integration is available, for example muscle differentiation [8]. However, for the most part, we are largely ignorant of the molecular basis of this integration, although it is clearly of great significance. It does seem, however, that with regard to the integration of external stimuli and the regulation of proliferation, growth arrest and cell death, it is the combination and context of signals that is particularly important in determining their effect. Perhaps the area of our greatest ignorance is in the translation of the diverse mechanistic insights that we already have into an understanding of how cells at different points in a particular hierarchy respond by ceasing to proliferating or by undergoing apoptotic cell death. The subtlety of control that is seen *in vivo* is at present outside our analytical abilities.

CANCER AS A DEFECT IN NEGATIVE REGULATION PATHWAYS

What has all this to do with cancer? The answer is self-evident. The control of cell number is a critical event in the homeostasis of normal tissues, including their response to external stress. A simple consideration of the control of cell number in normal tissue indicates the exquisite control under which cell number is placed. Consider the skin: if the stem cell compartment divides twice as frequently as normal, or the transit amplifying population undergoes an extra round of division, then the epidermis would (all other things being equal) be twice as thick. External insults lead to hyperplastic responses in many situations — adaptive responses that facilitate the survival of the organism. If the external stress is genotoxic then there is a real need to ensure fidelity of replication and there is likely to be selective pressure in favour of systems that prevent the accumulation of damage and genetic error. Such a goal will be facilitated by mechanisms that allow effective repair of damage — a situation itself favoured by mechanisms that allow a cessation (or at least slowing) of the cell cycle to allow repair events. Likewise, if the damage is excessive (and beyond repair) then mechanisms that allow "altruistic suicide" of defective cells will be advantageous. Such mechanisms have clear evolutionary advantage to metazoa. Yet if they become deranged then there is a possibility of either the persistence of damaged cells (and the possibility of further genetic error) and/or deregulation of normal control mechanisms that determine cell number. Both these eventualities are well known to be components of the neoplastic process.

The widely used operational definitions of cellular transformation, including reduced serum requirements, enhanced growth rate, increased saturation density, loss of anchorage dependency and extended lifespan are usually explained on the basis of alterations in cell proliferation [141,142]. For example, the ability to grow in reduced serum conditions is argued to reflect the ability of cells to proliferate without the need for mitogenic stimulation. This is presumably as a consequence of constitutive activation of the relevant transduction pathways, perhaps because of 'gain of function' mutation in genes encoding components of this pathway. Similar arguments are often used for the other features of cellular transformation. We would argue, however, that these views are not correct.

The widely held view of neoplasia as a primary disorder of cell proliferation is being replaced as the importance of growth arrest and cell death in the regulation of cell number becomes accepted. This should have a profound impact upon our view of the genes involved in the genesis and progression of neoplasia. The assays that have led to the understanding of oncogenes and tumour suppressor genes are all biased to the identification of genes whose products increase cell number by failure of growth arrest and cell death control. For example, the NIH 3T3 focus formation assay is an assay that will detect absence of normal growth arrest effects. Similarly, the anchorage independence assay has a similar function and may also identify cells unable (or at least with reduced propensity) to undergo apoptosis. The known genetically recessive oncogenes (or tumour suppressor genes or anti-oncogenes) would act in such assays as suppressors of growth/inducers of death, and their function in neoplasia is then a consequence of loss of function. Such genes are then quite appropriately termed suppressor genes. Those genes identified by the classic transformation assays are in fact genes whose products suppress or in some way overcome growth arrest and/or reduce cell death. Such genes might be better termed anti-suppressors (Figure 4). A nomenclature based upon the effects of gene products on growth arrest and/or cell death more properly reflects the biological importance of these processes, the role of the gene products in neoplasia and avoids the confusing terminology currently employed. Furthermore, these

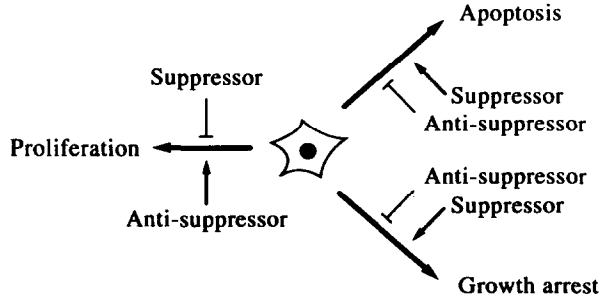

Figure 4. Cell number in tissues depends upon the balance of cell proliferation, growth arrest and cell death. The regulation of cell number may then be viewed as a competition between two large families of genes whose products, under normal circumstances, are either suppressors of cell number or anti-suppressors. This view should not obscure the role of other processes such as differentiation in neoplasia, although this process itself is intimately linked to cell number regulation.

terms more adequately reflect the nature of the commonly used assays of cell transformation, and also have the merit of being applicable to non-neoplastic disease states.

The utility of this nomenclature is further demonstrated by its use in the context of tumour viruses. The action of several viral and cellular oncogenes can be explained at least in part by their ability to block p53 function. For example, the viral oncogenes SV40 large T, adenovirus E1B 58 kDa protein and human papilloma virus E6 all act by physically binding p53 protein and blocking its function as a transcription factor. Similarly, the cellular protein MDM2 can bind and inactivate p53, and high levels of this gene product are found in many sarcomas where the *TP53* gene itself appears intact. Given the essential role of p53 as a protein that induces growth arrest or cell death (a suppressor), these oncogenes can thus be described quite accurately as anti-suppressors.

CONCLUSION: CELL NUMBER CONTROL AND CANCER

In this review, we have argued that the organisation and control of cell number in tissues is determined as much by growth arrest and cell death as it is by proliferation. This is of relevance to neoplasia as many of the molecular events that lead to the development and progression of cancer involve deregulation of the mechanisms of growth arrest and cell death. Clearly then, an understanding of the molecular and cellular basis of growth arrest and cell death must be important goals in our understanding of neoplasia, the more effective diagnosis of cancer, and ultimately its more effective prevention or, at least, treatment. We would argue that a new and more appropriate view of the relative contribution and importance of apoptosis and growth arrest (on one hand) and cell proliferation (on the other) is needed. Consequently, we propose a redefinition of the two broad classes of gene that contribute to the molecular basis of neoplasia in terms of their effects on growth arrest and cell death as suppressors or anti-suppressors.

Direct *in vivo* support for these ideas comes from recent important experiments using transgenic models of *TP53* and *RB* gene inactivation [143–145]. Morgenbesser and associates [144] examined the consequence on the cells of the murine lens of inactivating both the *TP53* genes and the *RB* genes in doubly null *TP53* −/− and *RB*−/− embryos. In animals containing functional *TP53* but no *RB*, there is extensive apoptosis in the lens leading to marked structural abnormality. In the doubly null embryos, this apoptosis is not seen, indicating the role of *TP53* as a critical suppressor of cell number, and the important relationship of *RB* and *TP53* in this pathway [145,146]. An even more dramatic illustration of this comes from the experiments of Symonds and associates [143]. In mice bearing a transgene with a fragment of SV40 large T antigen that inactivates Rb, but not p53, an abnormal hyperplastic proliferation of choroid plexus epithelium occurred. In crosses between the mice with the SV40 large T transgene and *TP53* −/− mice, there was a rapid emergence of multiple aggressive tumours, whereas in crosses with the hemizygous *TP53* +/− animals, there was a stochastic emergence of such tumours from this hyperplastic background and these were found to have inactivated the *TP53* gene. In the hyperplastic lesions, apoptosis was easily identified whereas in the aggressive tumours this was minimal. These important experiments provide conclusive *in vivo* evidence for the role of apoptosis, mediated by p53, as a critical regulator of cell number in neoplasia. Understanding the mechanistic basis of this will have important implications for the development of new therapeutic strategies in malignancy.

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Genetic Counselling in the Cancer Family Clinic

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INTRODUCTION

GENETIC COUNSELLING is the term historically used to describe the interview which occurs when an individual attends a genetic clinic, although this is only part of what actually happens when a patient visits a clinical geneticist. Counselling is important in genetics, and its non-directive nature, offering choices to patients, is the basis of the practice. However, much of the consultation, like any other outpatient appointment, is for diagnosis and management of disease, and this is carried out ordinarily using the history and examination of an affected individual. With genetic disease, it may be the family history that holds the clue to diagnosis, and in a Family Cancer Clinic, diagnosis of a genetic susceptibility to cancer may be largely determined by the family history. Patients are generally referred to the clinic by cancer physicians and surgeons, although a proportion are referred by their General Practitioner, or go of their own volition.

THE FAMILY HISTORY

Establishing the pedigree is an important part of the interview. This is standardised to include the family history of cancer, other diseases, developmental and congenital abnormalities, and a history of miscarriages. At least information about first and second degree relatives should be requested, and, where appropriate, the family history should be extended as far as possible [1].

The age at which cancer was diagnosed, the site(s), and the date of treatment/hospital involved should be ascertained. This will allow assessment of risks to relatives, and confirmation of diagnosis from hospital records. In addition, the diagnosis of a particular cancer syndrome may be possible from the pattern of cancers or associated nonmalignant problems. It is important that the clinician has the necessary background knowledge to recognise any significant pattern, and be able to assess the risks from pedigree analysis.

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ASSESSING THE RISK

As a general rule, the occurrence of the same cancer in three close blood relatives of a family is suggestive that there is a genetic susceptibility, particularly if they were affected at an early age.

If there are two close relatives with the same cancer, then the population risk of that cancer is an important guide as to the chance of a genetic susceptibility, i.e. if a cancer is rare, then two cases in a family are less likely to have occurred by chance.

Having a single relative with a particular cancer often does not greatly increase the risk to relatives. The exception to this is if the relative is young or had multiple primaries or a recognisable cancer syndrome. The risk of bowel cancer in the relatives of a single case illustrates the importance of age at diagnosis (Figure 1) [2].

Occasionally, a malignancy may be known to occur frequently as a result of a germ line mutation. An example would be retinoblastoma, a rare childhood malignancy of the eye, in which 40% of cases are due to a genetic susceptibility. Some children have multifocal disease, which is almost invariably due to the presence of a germline mutation, with the risk for children of individuals with bilateral disease approaching 50% [3].

Some cancer syndromes have phenotypes that can be diagnosed in an individual. Frequently, it is the premalignant phenotype, such as adenomatous polyps in familial adenomatous polyposis (FAP), that will enable the diagnosis to be made.

There is now published information on the risks for relatives of cancer patients, particularly for common cancers such as breast cancer and colorectal cancer [2, 4–6], these are particularly useful for genetic counselling, permitting visual demonstration of risk assessment to the patient. The likelihood of a genetic susceptibility can be calculated, combining information on the