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Cell biology of precancer☆

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Abstract

This article explores the possiblities of understanding the natural history of human cancers. In particular it attempts to understand *precancer* in cell biological or molecular rather than clinical or pathological terms. The questions discussed on the relevance of precancer in the neoplastic development are: are all cancers preceded by precancer? Is a precancer in the cell lineage characterised by hypermutability? Is there a direct DNA lineage from precancer to cancer? How many mutations have been addded as a function of a number of DNA generations in the process to neoplastic transformation? Is precancer reversible? Can analysis of precancer provide a short cut to assessment of carcinogenic risk? The present data addressing these questions are discussed and the still unexplained phenomena are highlighted.

Keywords: Precancer; Carcinoma-*in-situ*; Cell lineages; Clonality; Hypermutability; Mutation rates

1. Introduction

The scientific study of cancer has its roots in the clinic. No definition of cancer is entirely satisfactory from a cell biological point of view despite the fact that cancer is essentially a cellular disease. Whereas clinicians and pathologists, who usually encounter cancer at a biologically late stage, have had no principal doubts about a qualitative difference between malignant, i.e. cancer, and benign overgrowths, modern tumour biology has emphasised a quantitative model by which cells undergo gradual 'progressive' alterations, taking them from normal via less well understood proliferative or even inflammatory/degenerative intermediate stages to benign neoplasia to cancer-*in-situ* to locally invasive cancer to metastasising 'frank' malignancies. In this scheme, borders between hyperplasia, regeneration, precancer and cancer become rather arbitrary.

A discussion about precancer will lack substance unless a qualitative transition is assumed between precancer and cancer, at which a cell undergoes a permanent change which takes it to an irreversible state where there is not only net proliferation but also the kind of 'asocial' behaviour which makes the cells grow without formation of functionally or morphologically normal tissue. This will eventually lead to impairment of vital functions incompatible with life of the host organism. The transition to the malignant phenotype is conventionally termed 'malignant transformation'.

There is indirect evidence of malignant transformation in a qualitative sense. An enormous amount of empirical morphological evidence has been amassed showing that a malignant cell, or at least an assembly of such cells, can be distinguished in the microscope on the basis of cellular atypia and growth patterns distinct from those displayed by cells in hyperplasia, embryonic development or inflammation. This largely holds true also *in vitro*, with its strong correlation between focal non-inhibited irregular growth [1,2] and malignant behaviour upon transplantation to syngeneic hosts. It is remarkable that the best predictor of malignancy *in vivo* was cellular atypia in a study of spontaneous transformation of mouse fibroblasts [3,4]. The cellular or molecular biology behind the characteristic features of cellular atypia have hardly been discussed or investigated. They are of importance for any understanding of precancer and will therefore be dealt with here despite meagre experimental data.

Our task is to illuminate steps before malignant transformation, i.e. to attempt to understand precancer in cell biological or molecular rather than clinical or pathological terms. The emphasis will be on human

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cells, not only because of practical implications but also because of inherent genetic problems of animal cells, particularly those of rodent origin.

Crucial questions for any understanding of precancer are: Are all cancers preceded by precancer? Is a precancer cell lineage characterised by hypermutability? Is there a direct DNA lineage from precancer to cancer, and if so how long is it and how many mutations have been added as a function of number of DNA generations? Related questions concern the phenotypic changes that occur in a precancer–cancer lineage. Can they be morphologically and/or functionally discerned? Are basic features such as degree of aggressiveness and differentiation laid down already in precancer, i.e. before malignant transformation, or are such features added during progression after malignant transformation? Is precancer reversible? Can analysis of precancer provide a short cut to assessment of carcinogenic risk? Answers to these questions will differ from cancer to cancer and be dealt with in the relevant articles of this volume. This introductory chapter will explore possibilities of understanding precancer at a more general level and highlight unexplained phenomena. Until now, there has been such a heavy emphasis on cancer that we are only at a beginning in understanding precancer.

2. Definitions

Precancer is defined as any morphologically distinguishable (cellular atypia and impaired differentiation) proliferative lesion that statistically is known to be followed by cancer with significant likelihood. This likelihood is often below 100%. The formal proof, i.e. that a precancer lineage contains cells on a direct path to cancer, is rarely delivered.

Within the broad category of precancer, the following terms are applicable.

Carcinoma-in-situ is defined as a condition confined to epithelial cancers (carcinomas) with all morphological criteria of cancer except signs of invasive growth. There will consequently always be a degree of disorder among the constituent cells. In the breast and other exocrine glands intraductal carcinoma is synonymous with carcinoma-*in-situ*. A few non-epithelial cancers have analogous terms, for example melanoma-*in-situ*. Solid mesenchymal tumours, leukaemias and lymphomas do not have recognised analogues to carcinoma-*in-situ*. Malignant gliomas do not have any recognised counterparts to carcinoma-*in-situ*, possibly because of their peculiar pathology.

Dysplasia is an epithelial lesion with a degree of cellular atypia and disturbed differentiation. It is conventionally graded mild, moderate or severe. In contrast to carcinoma-*in-situ*, constituent cells are still ordered along physiological lines.

3. Clonality and cell lineage: relevance for precancer

A useful way to look at development of precancer and cancer is to concentrate on numbers of generations of newly synthesised cellular DNA rather than the cells themselves, which from a genetic point of view can be regarded as temporary phenotypic costumes of variable appearance, which the genome will modify and use as it multiplies.

At the very beginning, there will be one diploid genome of the fertilised egg. During development of the individual, branching chains or lineages of DNA will arise, all of which belong to the same clone and therefore carry along any mutations or polymorphisms delivered by the paternal and maternal haploid genomes. For lack of markers and the impossibility of experimental manipulation this maze of lineages and relay of genomes will presumably never be as completely disentangled as in the nematode worm *Caenorhabditis elegans* [5,6], but some main principles can be formulated for mammals, including humans.

DNA lineages of variable lengths measured in number of rounds of DNA replication will unfold. These lineages should not be defined in phenotypic terms but rather according to genotype, in order to facilitate understanding of, for instance, 'hypermutability' and 'multihit', as these terms are used in precancer and cancer. Lineages will have a very variable fate. Some will continuously propagate their DNA via rounds of replication throughout the lifetime of the individual, others will die out. Others will remain in limbo until reactivated by a call for regeneration, and still others will persist in an irreversible dormant stage from the point of view of potential to enter new rounds of DNA synthesis. Some may even continue as cripples with occasional new rounds of DNA synthesis.

The lineages of tumour biological interest are those that lead up to a point where precancer can be spotted or where malignant transformation of a single cell starts the growth of a cancer.

Fig. 1 surveys some general principles. Lineages are traced from the zygote to single cells in the peripheral semicircle, which either have transformed to invasive cancer (black centres), *in situ* cancer (dark purple centres) or dysplasia (light purple centres) or remained untransformed (unfilled centre). The snap-shot is taken when the first malignantly transformed cell appears in certain lineages. All lineages are depicted as running through developmental stages where embryonic cells, stepwise by epigenetic mechanisms, irreversibly lose their potency to differentiate into a multitude of directions [7]. The light green lineage, for instance, passes through rounds of omnipotence (bright red) via generations of multipotence (dark green) to monopotence (light green). DNA synthesis followed by asymmetrical divisions will create a genomic chain schematically

depicted as containing 14 rounds until dysplasia ensues. After six further rounds of DNA synthesis, carcinoma-*in-situ* develops in the light green lineage, in which after three additional rounds, one *in situ* cell transforms to invasive cancer. Each cell on the light green route will, as hinted at by two clusters of cells, give off numerous lineages that end with terminal differentiation after a few rounds. The dark blue lineage illustrates the possibility that *in situ* cancer develops without being preceded by dysplasia. It also demonstrates the possibility that daughter cells rapidly disappear in apoptosis. Single precancer cells may then continue multiplying without any increase in the size of the cell population. The purple line leads straight to dysplasia and the orange one straight to carcinoma-*in-situ*. The dysplastic and *in situ* cells on their respective lineages divide symmetrically and thus give rise to subclones with an increasing number of cells with retained 'stemcellness'. In the purple lineage, an *in situ* clone is seen to have emerged. The figure illustrates the important point that neoplastic lineages (orange and purple) may split into parallel lineages because more than half of the daughter cells retain stem cell character, i.e. are capable of infinite propagation of the property of self-renewal.

Fig. 1 also portrays the importance of point of time of any mutation. The purple, light blue and dark blue lineages show (arrows) how lineages may branch away from each other at stages before monopotence is reached. The earlier a mutation occurs the more lineages will carry it.

Fig. 2 shows estimates of the lengths of lineages measured as numbers of rounds of DNA synthesis. Units of such a lineage are referred to as stem cells, functionally defined by 'stemcellness', that is a capacity of the cellular genome, at each round of DNA synthesis, to give rise to at least one new genome which still possesses the original proliferative capacity to an undiminished degree. Fig. 2 demonstrates that human cancer can have variable but never very many rounds of cellular DNA synthesis before malignant transformation. For such a constantly regenerating tissue as epidermis, 4000 rounds of DNA synthesis are computed at age 55 years, including a stretch from precancer to cancer corresponding to about 1000 rounds from age 40 years. In this case, which may be representative also of gut epithelium, an average stem cell cycle time of about 5 days is assumed [8]. A retinoblastoma may have been preceded by as few as 15 rounds of DNA synthesis to fit with recorded rates of incidence and number of retinoblasts at risk [9]. Estimates of intervals between rounds of DNA synthesis in bone marrow stem cells illustrate the extreme difficulty of obtaining reliable results. The definition of stem cells is not unequivocal, but several investigators regard them as quiescent because of lack of evidence of ongoing DNA synthesis and one estimate of murine bone marrow stem cells gave a value of 1 month. Fig. 2 is based on about 35 days. In the healthy liver, where cellular turnover is slow [10], there may be only 600 rounds before the first hepatocellular cancer cell makes its appearance at age 55 years. As a corollary,

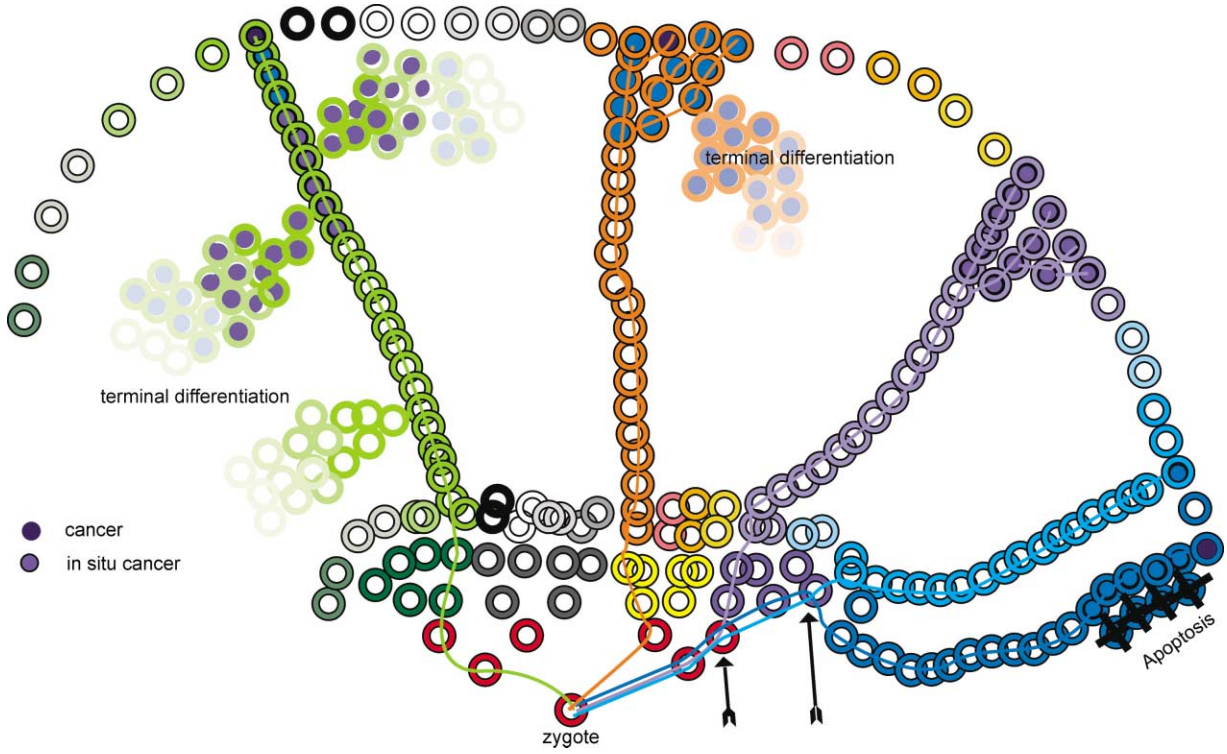

Fig. 1. Principles cell lineage formation. For details see text.

the influence of chronic liver cell regeneration is illustrated by the guess that 2900 rounds of DNA synthesis may constitute the lineage that ends with malignant transformation. Breast and cervix take intermediate positions whereas astrocytes of the brain may have undergone only 40 rounds before malignant transformation, as suggested by the virtual absence of DNA synthesis in putative precursor cells.

The estimates of number of cell cycle rounds of Fig. 2 are rough, based on imperfect data and intended only to illustrate principles, but they show that the genetic hits generally considered necessary for malignant transformation need to occur more frequently than intuitively expected. This paradox is, however, only apparent, because the number of potential lineages leading to malignant transformation is large in an individual. The denominator of risk of precancer (and cancer) is the total number of genomes at risk of crucial mutations on

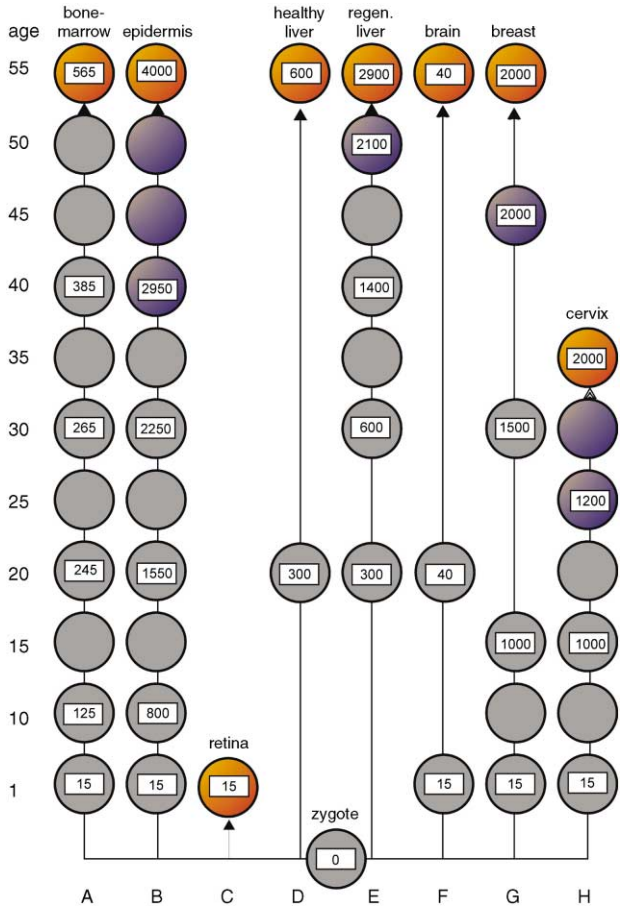

Fig. 2. Principal outline of cell lineages leading towards transformation to precancer (purple with yellow centre) and cancer (orange with yellow centre). A–H represent the tracks of rounds of genomic DNA synthesis among stem cells which end by malignant transformation in bone marrow, epidermis, retina, healthy liver, regenerating liver (e.g. in cirrhosis), brain, breast and cervix. Assumptions about the number of rounds of DNA synthesis (white labels) are expounded in the text. Age of an individual is given in the column to the left.

lineages able to end by transformation. The numerator will be the number of lineages that actually have ended in precancer and cancer, respectively. The latter is in turn reflected by incidence figures, as recorded in cancer registries. The essence of malignant transformation of a single cell is that its lineage has suffered mutations of crucial genes at a high rate. Below follows a discussion of whether this is so because of hypermutability [11] or whether it can be explained on the basis of a normal rate of spontaneous mutations in combination with selection [12].

A model of precancer and cancer can be formulated from the concept of genomic lineages and single cell origin of neoplasia. The following factors will determine whether any given cell will undergo transformation. (1) Frequency of fixed genetic 'hits' per round of DNA synthesis necessary to accomplish transformation, i.e. a progressive count of hits in alleles necessary to forge the 'malignant phenotype'. This implies not only a defined set of genes (oncogenes, tumour suppressor genes and other, unidentified genes) as targets but also that the hits occur at codons that alter encoded proteins in a critical manner. For most adult cancers, the number of necessary rate-limiting hits before malignant transformation of a cell lineage is conjectured to be of the order of 4–6. For carcinoma-*in-situ* it may be one fewer, i.e. 3–5 and for dysplasia two fewer, i.e. 2–4. Most data indicate that the order by which the hits accumulate is not important. If a mutation leads to genetic instability this will be reflected as an increased rate of hits. (2) Number of DNA rounds in the lineage that undergoes transformation. This number will vary depending on the proliferative status of the lineage and here is estimated to be between 15 and 4000 (Fig. 2). The more rounds of DNA synthesis the more likely that the necessary hits will accumulate. (3) Number of lineages susceptible to transformation. This number will vary considerably within an individual. For human bone marrow it may be as large as 10^8, in contrast to the cervix, where a narrow transformation zone is the target with perhaps as few as 1000 lineages. The number of lineages may be modified during embryonic, fetal and childhood development, for example by selective action of steroid hormones [13] or by regeneration. (4) The time pattern of acquisition of hits. Early hits will affect a larger number of lineages than late hits [14]. (5) The effect of the hits on the likelihood of self-renewal. If a mutation increases the probability of self-renewal (symmetrical mitoses with preserved 'stemcellness'), it will by branching increase the number of lineages and thus increase the number of target cells primed for transformation. (6) The effect of competing hits, i.e. the numerous mutations which in excess of the transforming ones affect the remaining genome. The majority are neutral but a fraction will cause changes in metabolism, cell structure and so on, often with a negative effect on viability but

sometimes, instead, rendering a positive selective value. The importance of this component has to a large extent been neglected. (7) The phenotype of the target lineage. Certain cells such as retinoblasts, haemopoietic stem cells, mesenchymal cells and endocrine and neuroendocrine epithelia behave as if they require fewer hits than the bulk of epithelial stem cells. (8) Species derivation of target cells. Certain factors co-operate, according to an obscure scheme, to ensure that a correlation exists between the lifespan of a species and the length of the cell lineage until malignant transformation occurs. In a comparison between species, the probability of cancer is roughly the same per individual but vastly different per target cell or lineage.

4. Are all cancers preceded by precancer?

4.1. Oligohit neoplasia

It is commonly assumed that all cancers are preceded by precancer. The reason for our failure sometimes to detect putative precancers would then be explained by the selective growth advantage of cancer cells, which permits them to over-grow and destroy any precursors.

Retinoblastoma was the first clear example of a human two-hit malignancy depending on homozygous obliteration of the function of both alleles of a tumour suppressor gene [15,16]. The tumour serves as a convincing demonstration of the qualitative nature of malignant transformation, the importance of correct target cells and absence of precancer in a two-hit suppressor gene scenario.

In Fig. 3, from a fictitious hereditary case of retinoblastoma, all cell lineages carry a non-functional *RB* allele. Lineages A–D have by epigenetic mechanisms differentiated to retinoblasts in the embryo. By chance, and with a likelihood estimated at about 10^{-7} mutations per locus per round of DNA synthesis [9,15,17], a second *RB* mutation has occurred in lineage B. The retinoblast responds by malignant transformation, in this particular kind of tumour predominantly characterised by profound inhibition of differentiation with rapid proliferation of blast cells. Some tumour cells may undergo sufficient numbers of rounds of DNA synthesis to accumulate more mutations (not illustrated), but there is no direct evidence that the mutation rate is increased in retinoblastoma, even if some reports have claimed that loss of *RB* function will in a moderate manner influence genetic stability negatively [18–20]. By and large, retinoblastomas behave in a uniform manner both morphologically and clinically, supporting the notion that the two original hits are sufficient to explain their neoplastic properties.

There is no indication that destruction of the function of only one *RB* allele has any phenotypic effect, i.e. that precancers exist. Retinoblasts in a child who has inherited a defective *RB* gene perform normally, i.e. the classical suppressor gene scenario is displayed. This part of the *RB* story is recapitulated in genetically manipulated mice, with the unexplained difference that pituitary adenomas rather than retinoblastomas are created after a second *RB* hit [21,22].

Fig. 3 illustrates some other consequences of Knudson's two-hit model. It is not excluded that the second *RB* mutation will occur after the retinoblasts have left their stem cell stage to become irreversibly committed to terminal differentiation. Then a few mature retina cells may carry homozygously destroyed *RB* genes but will fail to develop retinoblastoma (lineage D)—a prediction fully compatible with virtual absence of retinoblastoma once childhood is over.

A second possibility (not illustrated) is that cells resting in G_0 may acquire permanent mutations. Although interesting extrapolations from bacteria have been discussed [23], there is no convincing evidence that this

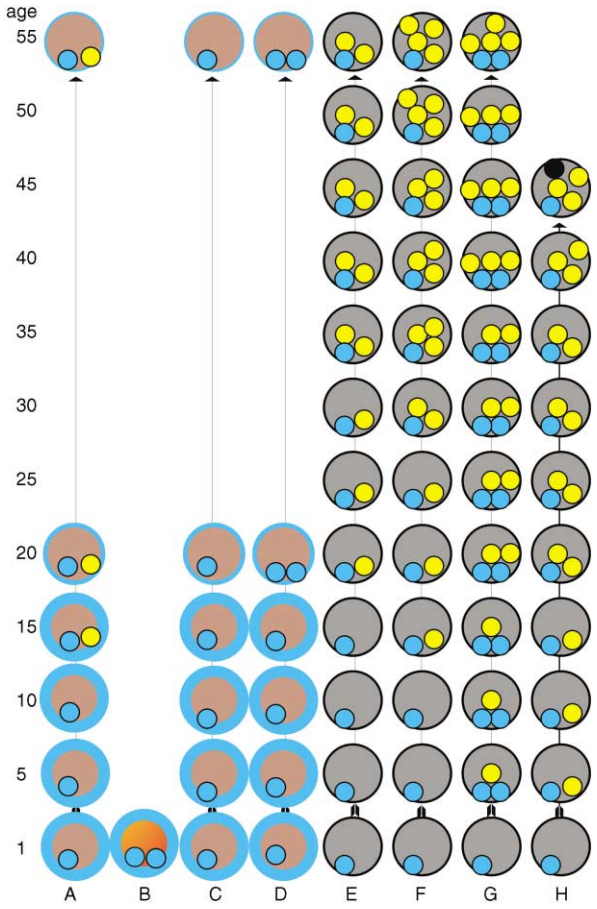

Fig. 3. Examples of lineages of cellular DNA synthesis in spontaneous retinoblastoma compared with non-transformed lineages. Thick blue rings denote retinoblasts. Orange-red indicates malignant transformation to retinoblastoma. Mutations are indicated by small circles: blue = *RB* allele, yellow = neutral allele and black = lethal allele.

ever happens in mammalian cells, at least on a scale of importance when DNA repair is untouched. The reasons for low or no mutability in resting cells are (a) that repair will have sufficient time to be effective and (b) that mutations caused by error-prone DNA polymerase cannot happen [24].

Epithelial stem cells outside of the retinoblastoma lineages (E–H, Fig. 3) will, according to theory, accumulate *RB* mutations at the same rate of 10^{-7} per DNA synthesis round. This will cause some lineages to suffer the same two *RB* hits as the retinoblast founder of a malignant eye tumour. No excessive risk for any tumours other than mesenchymal tumours, particularly osteosarcomas has been established for carriers of *RB* mutations [25]. This suggests that the stem cells involved in epithelial cancers can have homozygous destruction of two important suppressor genes without adverse effects. The earlier this happens the more cells will carry doubly defective *RB* alleles, because the homozygous state will be transmitted to larger numbers of daughter stem cells. One explanation for the inference that *RB* gene lesions can behave as neutral mutations might be that retinoblasts (and osteoblasts, etc.) cannot substitute for loss of *RB*, in contrast to other cells, which may be provided with backup systems and therefore require more than disruption of *RB* to undergo malignant transformation, as indicated by lineage G in Fig. 3, which remains normal in spite of two *RB* and four other mutations.

Fig. 4 illustrates the difference between hereditary and spontaneous retinoblastoma. For the latter to occur two spontaneous hits will have to inactivate both *RB* loci. This will take more rounds of DNA synthesis than the single second hit needed in hereditary cases. The likelihood of such a combination will be 10^{-14}, based on the standard figure of 10^{-7} [9]. To accommodate this, a large number of retinoblast lineages have to be at risk, i.e. spontaneous retinoblastoma will become a rare disease at the population level. Lineage H illustrates that non-retinoblastoma cells may suffer homozygous mutational inactivation of *RB* without undergoing transformation. Lineage G shows the possibility that malignant transformation may, at least theoretically, be irrelevant in a non-retinoblastoma cell, which in this case was transformed because of mutations of two other suppressor genes (red dots).

Basal cell cancer (BCC) has always puzzled observers by its apparent absence of precursors. This stands in sharp contrast to squamous cell cancer and melanoma, which have well recognised precursors to be dealt with in the article by Brash.

Recent results point at possibilities to explain the absence of 'pre-BCC' along the same lines as in retinoblastoma. The gene defect that causes a hereditary BCC syndrome with early onset of tumours was shown to involve the gene *patched*. This gene, which has a homologue in fruit flies and mice, is part of the 'hedgehog' signalling pathway, which regulates embryonic patterning, including the development of the nervous system [26–30]. Homozygous genetically based inhibition of the function of *patched* (*ptc*) may, from still limited data, be a *sine qua non* for development of hereditary and acquired BCC, which would then qualify as a two-hit tumour without any need to postulate a precursor [31–34]. The hereditary form is transmitted via point mutations or small deletions within *ptc*. In the tumours, the other allele will somatically be subject to inactivation typically detected as loss of heterozygosity (LOH) at chromosome 9q22.3, i.e. the site of *ptc*. Recent data suggest the possibility that *Gli1* (overexpression or mutation?) may substitute for damaged *ptc* [35]—a not unreasonable proposition in view of the involvement of both genes in the same control system.

Hereditary BCCs usually become manifest during the third or fourth decade, and the acquired form in the

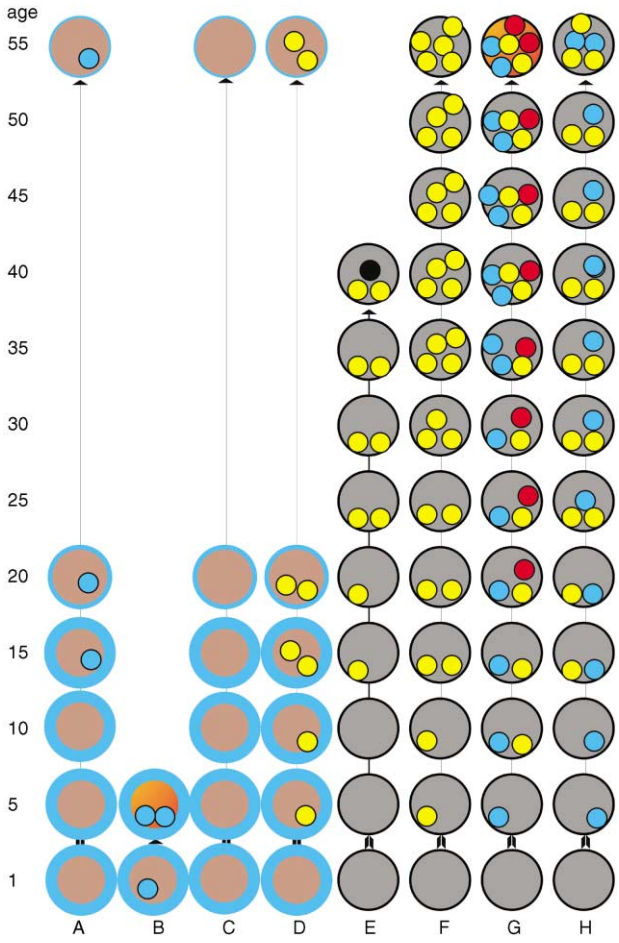

Fig. 4. Examples of lineages of cellular DNA synthesis in spontaneous retinoblastoma compared with non-transformed lineages. Thick blue rings denote retinoblasts. Organge-yellow indicates malignant transformation. Mutations are indicated by small circles: blue = *RB* allele, yellow = neutral allele, black = lethal allele and red = suppressor gene.

seventh or eighth decade. About 10^7 or 10^{14} interphase genomes at risk, respectively, would be required for accumulation of homozygous mutations in *patched*, in analogy with retinoblastoma. Spontaneous BCC would arise from a type of lineage indicated by B in Fig. 2. According to the model, 5500 rounds of DNA synthesis would be completed before malignant transformation to BCC at age 70 years, compared with only 15 for a retinoblastoma in a 1-year-old child. Such age of onset difference would be conceivable if the number of stem cell lineages for BCC were few compared with retinoblastoma lineages. In reality, a much higher incidence of BCC than of retinoblastoma suggests the opposite. These theoretical deliberations strongly suggest that more than two hits are required for BCC. The possibility that a *TP53* gene mutation is one of them will be discussed in the article by Brash and Pontén.

Sequencing of mutated *ptc* has, surprisingly, only shown the typical ultraviolet (UV) light involvement of dipyrimidines in one-third of the cases, in contrast to the spectrum of *TP53* mutations in the same tumours, which is of the order of 80% py–py involvement, hinting at indirect mechanisms for *ptc* damage by UV light [32,36].

From cytogenetic data, there is evidence that morphological absence of precancer in sarcoma, leukaemia, lymphoma and myeloma could be explained as a result of a requirement for only few hits to accomplish malignant transformation [37,38]. The existence of leukaemia/lymphoma/sarcoma already in childhood hints at two-hit neoplasia. Karyotypic and molecular analysis, particularly of chronic myelogenous leukaemia and follicular lymphoma, is compatible with a requirement of only a few hits. Since they are not childhood tumours it could—in analogy with the reasoning about BCC above—be suggested that the number has to be at least three.

Most forms of cancers in endocrine glands resemble mesenchymal malignancies by conforming to a concept of genetic stability. They are usually diploid or near diploid in DNA content and karyotypes [37,38]. Studies of extent of global point mutations do not seem to have been carried out. Endocrine cancers follow the pattern of most other epithelial cancers by having clearly defined precancers. This has been extensively studied in intestinal carcinoids, where one typically will see multiple precancers morphologically termed 'adenomas' without invasion, which often individually undergo malignant transformation to invasive metastasising carcinoids. Endocrine cancers look like oligohit, but in their case a single hit may have phenotypic effects as precancer in the form of hyperplasia and/or adenoma. Sequential genetic analyses of lineages from normal via precancer to (neuro)endocrine cancer could be extremely rewarding.

4.2. Precancer in a multihit scenario

The multihit concept of cancer and precancer has developed gradually. An early indication came through plotting incidence of cancer as a function of age. A log–log plot will form a straight line. The necessary number of rate-limiting hits can be derived from its slope [39]. This model was extended and elaborated on by Armitage and Doll [40], who concluded that four to six hits were required for clinical cancer to develop. Such statistics will reflect the combination of all cancers and cannot give any deeper insight into events at the single cell level. This is conspicuously illustrated by the fact that a similar curve for cancers in the rat, which of course develop within just a few years compared with eight human decades, has the same slope. To accommodate this, it is necessary to assume that the rate at which hits accumulate in the rat is very much higher than in the human—something for which no evidence exists (see Ref. [41]).

Demonstration of complete multihit sequences at the single-cell level has not succeeded. Stringent experiments, which would require sequential addition of defined mutations on a stable genetic background until the endpoint of precancer or malignant transformation is reached [42], have not been feasible to devise.

Murine cells in culture were originally employed in pioneering discoveries of oncogenes. After transfection with mutated *RAS*, 3T3 cells responded by malignant transformation as if this single hit was sufficient—a finding that seemed to violate the multihit theory of cancer [43–46]. Later research has made it likely that 3T3 and other similar cell lines are already primed for malignant transformation by several genetic hits. Transfection of mutated *RAS* will therefore supply only the last of a number of necessary hits and in this way give the false impression of being sufficient. Established lines such as 3T3 should be regarded as 'precancer' in a cell biological sense.

In spite of numerous attempts, it has never been possible to achieve malignant transformation of normal human cells in analogy with the *RAS*–3T3 system (see Refs. [41,47]). The explanation rests with the vast difference in sensitivity to transformation between the two species. One estimate claims that mouse fibroblasts are 10^9 times more susceptible to transformation than their human counterparts [48]. Such a huge difference has not been explained. Differences in mutability, DNA repair, effects of non-disjunction (chromosome error propagation), quality of cell-cycle checkpoints, epigenetic changes concerning methylation and telomere maintenance are the most commonly cited factors (see Refs. [41,49]).

A remarkable biological phenomenon with profound influence on our understanding of precancer, cancer and ageing still searches for an explanation. From the point of view of understanding precancer and progression to

cancer, extrapolation from findings in mice to human beings (and vice versa) will always be uncertain. This probably also applies to knockout mice and other forms of genetic manipulation where, additionally, omnipresent tumorigenic retroviruses may inadvertently be activated, as incidentally suggested by the common development of lymphomas rather than the intended malignancies.

4.3. Dynamics of multihit lineages

The cellular biology of multihit precancer (and cancer) has turned out to be complex and much more difficult to understand than the principally simpler oligohit models provided foremost by retinoblastoma [14,50]. To prove that precancer is a precursor of cancer, it is necessary to show that a stable marker in precancer prevails after malignant transformation to cancer, i.e. that the lineage to cancer passes through a recognisable stage of precancer (lineages B, E, G and H in Fig. 2). This approach would ideally require serial sampling from the same lesion—clearly unattainable in humans and hardly ever tried in experimental animals. A good substitute is, however, provided by genetically probing synchronously present precancer and cancer. A marker found in precancer that is also consistently present in a clone of cancer cells will prove that the cancer cells belong to a lineage already present in precancer.

Markers suitable for clonality determination operate at different levels. Historically, they were all linked to inactivation of the X chromosome in females [51,52]. Inactivation occurs in a way that, in most organs, produces a stable mosaic mixture of cells with suppression of either the paternal or maternal X. Inactivation is accompanied by methylation of genes, which alters their restriction enzyme cleavage pattern. In informative cases, where the two alleles of a particular gene differ in length, gel electrophoresis after enzyme cleavage will reveal whether an allele is intact, i.e. has not undergone methylation associated with inactivation. Three patterns are possible: (a) partial disappearance of both alleles, (b) disappearance of the paternal allele and (c) disappearance of the maternal allele. The two last patterns are interpreted as indicative of sampling a monoclonal cell population, whereas the first indicates polyclonality.

The clonal nature of colonic precancer (adenoma) was originally suggested by an X chromosome marker. Polyps consistently had either the paternal or the maternal X chromosome inactivated [53], but these early results could not exclude the possibility that the adenomas were derived from crypts with identical X chromosome inactivation [54]. Subsequent studies have with increasing likelihood demonstrated that this, indeed, seems to be the case. One patient with X0/XY mosaicism (estimated to occur by chance in 1 out of 100 million people), also had familial adenomatous polyposis [55]. Normal crypts were either X0 or XY. The patient's colon was studded by tubular adenomas, either monocryptic or slightly larger 'microadenomas'. The first variety was monoclonal, but the second scored polyclonal in excess of what could be statistically expected, suggesting a field effect possibly with symbiosis between adjacent originally monoclonal adenomas.

Clonality analysis by X chromosome inactivation is beset with difficulties, mainly founded on inadequate sampling, which reflects the difficulty of identifying proper controls. One needs to exclude the artefact that monoclonality scoring reflects multiple origins from a field of cells with identical X chromosome inactivation—a condition that is hard to meet unless microdissection of normal potential target cells is meticulous. Unfortunately, virtually all published control tissue has been composed of a mixture of epithelial and stroma cells, even including lymphocytes as in the normal gut. We have no way of knowing whether a polyclonal X chromosome inactivation signal in these circumstances reflects a mixture of cells of different developmental origin or a random mixture of X inactivated potential target cells for transformation.

If significant contamination by normal cells can be excluded, the finding of polyclonality in neoplasia convincingly indicates that the population in question must have originated by malignant transformation of at least two separate cells. Recording of a monoclonal type of electrophoretic X chromosome inactivation will, however, never be equally convincing because of the possibility of derivation from a cluster of cells with identical X chromosome inactivation, the possibility of statistical coincidence caused by origin from two cells with the same paternal or maternal X inactivation or the possibility that selection of one clone from an original mixture of several transformed clones had taken place before the time of sampling. This will be further discussed in connection with cervical cancer where precursors were found to score either as clonal or polyclonal cell populations.

No direct genetic link seems to have been proven between individual adenomas and cancer in the colorectum. The possibility remains that precancer (adenoma) develops independently, governed by its particular set of mutations, and that adenocarcinoma may develop *de novo* from a normal cell lineage, which has developed another set of mutations before malignant transformation. That set may never have forced cells to evolve the phenotype of adenoma-precancer cells.

There have been recent studies where clonality was based on somatic marker mutations rather than X chromosome inactivation. For skin and cervix, a few cases with direct genetic linkage between synchronous dysplasia, *in situ* and invasive cancer have been established [56,57], but the issue is not yet settled.

From a practical point of view, it will only be meaningful to eradicate individual precancers if such lesions are known precursors of invasive cancer and not separate neoplastic lesions that arise as parallel phenomena. The extent to which invasive cancers arise without visible precursors still remains to be elucidated.

4.4. Precancer and hypermutability

The concept of hypermutability has its root in microbiology, where it is rather straightforward to establish a 'mutator phenotype'. Increased formation of colonies in suitable selective media measures a mutation frequency above background. Mutations can be analysed at the level of the individual base pair. Analogous *in vitro* methods are of only limited value for animal cells. Normal cells are generally unsuitable because plating efficiency is too low. They will also be heterogeneous with respect to number of divisions remaining until the Hayflick senescence limit is reached [58,59] and thus carry an unknown number of chromosome lesions and point mutations. In such circumstances, cells may be unable to form a colony even if the index mutation has occurred. For these reasons, most research has been based on established lines with high plating efficiency and endless capacity for proliferation. However, such results may not be trustworthy or possible to generalise from, because the cells do not possess normal genotypes and could easily have unidentified defects in DNA repair. Use of diploid lymphoblastoid lines may provide a solution to estimating background mutation rate in normal human cells [60], but the method has not yet been widely used.

4.4.1. Genetic instability in human cancer cells

The usual starting point has been analysis of cell populations from longstanding cancers where, as techniques have become increasingly sensitive, there have been striking instances of a multitude of alterations at all levels of genetic analysis.

Thanks to monumental work by Mitelman and collaborators [61,62], a catalogue of karyotypic abnormalities in human cancer exists, which abundantly shows how common various forms of heteroploidy are and how vast the heterogeneity found particularly in epithelial cancers of non-endocrine origin on a background of non-random development of karyotypic stem lines [62,63]. Mechanisms behind aneuploidisation which, according to old data may be preceded by tetraploidisation, are not well understood, but a host of possibilities have been supported, such as mutations in genes that control spindle formation and function of centromeres, leading to non-disjunction. Reduction of telomeric length has been suggested, since this would create unstable chromosomes [64–66]. Other possibilities include defunct cell-cycle checkpoints in either G_1 or G_2 or a disturbed stoichiometry among histones ([67]; see also Refs. [41,49]).

By use of two-dimensional electrophoretic analysis of DNA fragments obtained after digestion with restriction enzymes, an overall picture of deviations from the normal may be obtained. Cancer tissue has then shown a multitude of aberrantly sized fragments that can only be explained by presence of numerous mutations ([68–70]. Normal tissue, however, will show no significant deviations from a 'standard' human profile.

Taken at face value, these results suggest that the number of mutations, including structural chromosome changes, in many cancer cell populations could run into the hundreds or even thousands. There are caveats, however. Mitelman's group has interpreted its findings of a large number of 'clonal' karyotypes to indicate that epithelial cancers are of polyclonal origin [71–73]. In that case, analysis of large populations of cancer cells may reflect different sets of genetic lesions in parallel cancer lineages rather than genetic instability in each. It cannot, furthermore, be excluded that large global findings of a multitude of base changes in a population of cancer cells at least partly reflect heterogeneity caused by 'senescence' in neoplastic and/or normal cells known to be accompanied by aneuploidy [74]. Conclusions about genetic instability based solely on genetic heterogeneity in a large cancer cell population may be spurious. It would be important to find ways to specifically analyse stem cells known to be on the lineage path to precancer or cancer (Figs. 1 and 2) in order to settle this controversial issue.

Results from all types of cancer have not been equally clear-cut. Many leukaemias, lymphomas and sarcomas, particularly of high differentiation, do not show heteroploidy [62,63]. The same seems to be true for well differentiated endocrine neoplasms, such as carcinoids of the gut and thyroid carcinoma. There do not seem to be sufficient data on restriction fragment length polymorphism to permit conclusions about the overall numbers of point mutations in these subcategories of cancer.

4.4.2. Numerical estimates of mutation frequencies in human cells

Since no direct way of determination of mutation frequency *in vivo* is at hand, one has to rely on uncertain indirect estimates.

There are still large gaps in our understanding of DNA synthesis and repair, but an outline that agrees with most data for eukaryotic cells from higher animals including humans may be presented as a framework for discussion and hypotheses. It will no doubt be changed and refined in the future.

Cells resting in G_0 or moving forward in G_1 are subject to spontaneous base alterations, including depurination, hydrolytic deamination, oxidation and

methylation [125]. These are repaired predominantly by a base excision repair pathway, which is considered efficient enough to leave few if any base changes behind, even if speculations to the contrary have been voiced [23]. The rate of base changes will be strongly elevated by several insults, of which mutagenic chemicals, reactive oxygen radicals and UV light deserve most attention from a precarcinogenetic point of view. The base excision repair pathway is then aided by a nucleotide excision repair (NER) system that is particularly important for lesions that cause more serious distortion of DNA than for instance depurination, and a mismatch repair system (MMR) which takes care of larger lesions, mainly heteroduplexes. Cyclobutane pyrimidines and 6–4 pyrimidine–pyrimidones after UV require NER to be mended [75], as do bulky DNA adducts caused for instance by benzopyrene (in tobacco smoke) and cisplatin. Methylated bases, including the commonly occurring O^6-methylguanine induced by alkylating agents, may use MMR [76].

An inevitable drawback of NER is that it fails to distinguish between the correct DNA strand and the strand carrying the single base pair alteration that caused the mismatch. This will by chance in up to 50% of instances result in misincorporation in the correct strand and thus a permanent mutation instead of proper repair. NER in human cells is not completely understood. A 27–29 unit long oligonucleotide is excised, including the damaged bases, and replaced by the correct bases. It requires at least 17 polypeptides, including those that are mutated in the different types of xeroderma pigmentosum (XP).

NER is triggered partly by interrupted transcription, which explains preferential repair of the transcribed strand [77]. Even within transcribed genes, those parts not predestined to become hot spots are preferentially repaired [78].

MMR is under control of at least four different genes in the human. These are all of suppressor type, i.e. only after homozygous incapacity will microsatellite instability ensue [79].

G_0/G_1 will have to be sufficiently long to permit repair to be finished after a burst of base damage. *TP53* retards G_1 after primary DNA damage. This will prevent fixation of mutations arising from replication of mutated single-strand DNA [80].

It is not certainly known how many base pair changes a normal cell is burdened with at the end of G_1, but this number should be a reflection of the length of G_1. In untouched stem cells on lineages towards malignant transformation, with their slow rate of multiplication, this number may approach zero in view of the efficiency with which base, nucleotide and mismatch excision repair pathways are presumed to operate. If, however, the rate of damage is increased by orders of magnitude, for instance in UV exposed epidermis, numbers of changed base pairs become templates for DNA polymerases during S phase. Particularly if repair is defunct, this number becomes very large, as dramatically shown in XP, where even such a tiny fraction of the genome as a few exons of *TP53* showed abundant missense mutations [81].

During S phase, any remaining misincorporated base pairs or other nucleotide alteration is copied and transmitted to one daughter cell as a permanent mutation. Additional mutations are created because of imperfections in the DNA synthesis machinery centred around five DNA polymerases and a number of accessory proteins. The resultant of two opposite forces will determine the final number of misincorporated bases, which will show up as mutations in G_2 and subsequent cell generations. This number is believed to form the major determinant of the spontaneous mutation rate. During extension of the nucleotide chain, insertion of the correct base has an estimated likelihood of about 10^5 compared with misincorporation. If this was not counteracted, each round of DNA synthesis would generate 60 000 base pair changes. However, excision by 3′–5′ exonucleases will remove misincorporations and thus reduce error frequency. The efficiency of this proofreading is not accurately known, but it cannot be expected to eliminate all misincorporated bases, as suggested by the observed spontaneous mutation rate of about 10^{-7} in human B cells [60,82,83].

The second type of error introduced during S phase derives from the tendency to create mismatches, particularly of repeated nucleotide sequences, where so-called slippage will increase or decrease the number of repeats. This need not have any functional consequences because most repeats are found outside of genes and other functionally important parts of DNA. Those few repeats found in introns are, however, a potential risk because slippage here would easily create frameshift mutations with dire consequences. The importance of creation of repeats (microsatellites) is profoundly discussed elsewhere in this issue by Shibata. Mutations of MMR genes may create hundreds or even thousands of abnormal length repeats.

Not much is known about mutations and their repair in G_2. It seems reasonable to assume that the same mechanisms as in G_0/G_1 have a role also here.

Mitosis—the next phase of the cell cycle—is prone to mistakes, but the error rate has not been determined [84]. Endomitosis, non-disjunction, sister chromatid exchanges, other types of recombinations and deletions (LOH) have been observed in normal cells (see Ref. [85]). There is also suggestive evidence from DNA measurements of instances of unequal distribution of DNA at mitosis [86,87]. Since such gross effects will result from any chromosomal rearrangement, most mistakes can be expected to result in non-viable progeny. Certain abnormalities will, however, be stable and could at least theoretically become part of a precancerous genotype.

For unclear reasons, certain chromosome configurations are unstable in the sense that continuous creation of new karyotypes takes place from which there will often be a selection of stem lines with reduced heterogeneity [67]. Such instability is a hallmark particularly of the bulk of epithelial cancers, where it has been regarded as more important than accumulation of point mutations for creation and maintenance of the malignant phenotype [88].

Chromosomal instability has repeatedly been associated with progression of precancer to cancer. Direct observation of karyotypes suffers from a quantitative problem of collecting enough metaphases. This method also has to resort to short-term cultivation with built in problems of selection [37]. More extensive data have arisen from cytophotometrical measurements of nuclear DNA content, which in the most penetrating studies have been combined with time-lapse filming. For established cancer cell lines, rapid restoration of heteroploidy with large intercellular differences between individual cells could be established. It was not possible to isolate stable clones, heteroploidy being recreated as the cells multiplied [89]. Similar observations of precancer or dysplastic cells with their nuclear atypia could be rewarding.

It has not been possible to arrive at a firm estimate of the average number of changed base pairs and other genomic lesions that physiologically and inevitably are consequences of each cell cycle. This is in spite of rapidly increasing insight into molecular aspects of DNA damage and its repair. This number will almost certainly vary but, for any understanding of the road to precancer and cancer, it would be particularly valuable to have an estimate for stem cells on a lineage towards malignant transformation (Fig. 1).

It is well established that the diploid human genome has 6×10^9 bp. About 10% of these (6×10^8) are genes, i.e. they are transcribed. The total number of alleles has been variously estimated, and 2×10^5 will be used here. This gives $6\times10^8/2\times10^5=3\times10^3$ bp as an average size of an allele. Two-thirds of these may constitute introns, giving 1 kb as the average size of the translated exons per allele. Hence, $10^3\times2\times10^5=2\times10^8$ bp suffice to encode all exons. The remaining base pairs are partly repeats and partly perform functions as promoters, enhancers and so on. The number of base pairs that do not participate in any function is unknown, but they form an important platform for neutral mutations. As a basis for subsequent calculations 50×10^8 bp are assumed to serve no function. The result is that 60×10^8 bp are divided into three major classes: (a) exon coding 2×10^8; (b) other functions 8×10^8; and (c) no function 50×10^8. By definition, mutations in (c) are neutral, whereas mutations in (a) or (b) are neutral or of positive or negative selective value. All in (a) and (b) have phenotypic effects.

Imperfect DNA polymerase function seems to be the major conveyor of base pair errors to daughter genomes. The intricacies of the fidelity by which DNA synthesis is carried out are still to a large extent unknown. Estimates centre around an error rate of 10^{-5} [82], which may vary considerably depending on such factors as alternate use of different polymerases, imbalance within dNTP pools and availability of accessory proteins (for review, see Ref. [24]). Then $10^{-5}\times6\times10^9=60\,000$ bp would be permanently altered at each round of DNA synthesis. They are divided into three classes: exons 2000 bp, other functions 8000 bp, no function 50 000 bp. This is a maximum figure and, speculatively DNA repair could reduce it by two orders of magnitude, in which case 20, 80 and 500 bp, respectively, would be mutated.

Table 1 summarises forecasts of the mutational load. The minimum number of fixed bp changes corresponds to a mutation rate of 5×10^{-5} computed as functional changes per allele. This figure is about 100 times higher than Knudson's figure for neuroblastoma [9]. In view of the uncertainties in both the current and Knudson's estimates, this is not inexplicable. A substantial proportion of the 'mutations' in retinoblastoma are severe chromosome rearrangements ([16], see also Ref. [85]). These are expected to be less common than base pair misincorporations. The calculations in Table 1 do not take selective advantage into consideration, whereas the *RB* mutations, to have a visible phenotypic effect, necessarily have to permit clonal expansion after malignant transformation. The calculations in Table 1 may be way off in terms of guesses about efficiency of repair.

It is not improbable that the human genome, because of physiological and biochemically unavoidable imperfections in synthesis and repair of DNA, endures a high load of mutagenic base pair changes. Their prevalence in lineages leading to precancer and cancer will vary from case to case, depending on the selective value of the respective mutations. Lethal mutations would at any

Table 1
Estimates of average mutation rates for three categories of human DNA

Type of DNA	Altered bp[a]	No. mutations[b]	Mutation rate[c]
Exons	20–2000	10–1000	5×10^{-5}–5×10^{c}
Other functions	80–8000	80–8000	4×10^{-4}–0.4
Neutral	500–50 000		
Total	600–60 000	90–9000	

See text for explanation of categorization and enumeration of alleles and rationale behind calculation of minimal and maximal values.

[a] Computed per round of DNA synthesis.

[b] Only 50% of bp changes predicted to give a significant functional disturbance.

[c] Computed from $2\times100\,000$ alleles/human genome.

stage interrupt a lineage, but neutral base pair alterations would accumulate at each cell cycle. In one example shown in Fig. 2, 4000 rounds of DNA synthesis in epidermal stem cells could be expected to result in between 2×10^6 and 2×10^8 changed base pairs without any impact on the cell phenotype at the age of 55. The latter figure corresponds to 4% of all 'functionless' base pairs at risk.

If a heavy genetic load suggested in Table 1 is created per somatic cell, it comes as no surprise that, for instance, old skin shows many signs of profound pathology, such as focal hyperpigmentation, depigmentation, basal cell papillomas, senile lentigo, atrophy and some precancers (melanoma-*in-situ*, squamous cell dysplasia). This is particularly true after exposure to ultraviolet (UV) light, which may increase the number of mutations by one to several orders of magnitude.

That human cells are able to sustain heavy loads of mutations is suggested by findings in xeroderma pigmentosum (XP). In a case of XP, subgroup C, studied by Williams and colleagues [81], 29 different mutations were found in exons 5–8 of *TP53*. This corresponded to an amazing 17% of all possible UV characteristic Py–Py mutations of the non-transcribed strand. To become detectable, the mutations, virtually all missense, had to be accompanied by selective clonal cell expansion. They were also clustered at known hotspots. By extrapolation, and since there is no reason to assume any particular hypersensitivity to UV in the *TP53* gene, the conclusion is that each average allele must have suffered a number of mutations. The dynamics of this striking scenario is not understood, but the findings support that the human somatic genome is able to sustain a substantial number of point mutations.

4.4.3. Is a precancer lineage characterised by hypermutability?

This difficult question has never been approached directly. Indirect evidence is, however, provided by analysis of aneuploidy in precancer, mainly in colon, cervix and lung. The most extensive data come from cytometrical measurements of DNA content in adenoma, dysplasia and adenocarcinoma-*in-situ* of the colorectum. The overall picture has been rather consistent. Normal mucosa surrounding neoplastic lesions is diploid. A large majority of adenomas have near diploid profiles [90–92]. With increasing degree of dysplasia, the proportion of aneuploid nuclei increased [93–95]. Aneuploidy was more prevalent, together with villous rather than tubular morphology [96]. About 80% of invasive cancers were DNA aneuploid [97,98].

A sequence of changes has been proposed from an early start with development of nuclei with near diploid (particularly hypodiploid) DNA content, followed by tetraploidisation, followed by subtetraploid aneuploidy [99], where aneuploidisation roughly coincides with appearance of severe dysplasia. Individual departures from this scheme are considerable.

One attempt has been made to bring rough measurements of DNA content into cell biological context [100]. Computerised quantitative image analysis of nuclei in colorectal tumours confirmed the time-honoured cytopathological observation that nuclear 'atypia' increases with progression along the adenoma→invasive cancer axis. There was no correlation, however, between *RAS* mutations and allelic loss on 5q, 18q and 17p, either alone or in combination with nuclear texture (atypia). The important implication by this finding is that genetic alterations, generally considered 'hits' responsible for creation of the malignant phenotype [101–103], could not explain the individual characteristics of atypia, i.e. one diagnostic hallmark of malignancy. A dose–response relation was, however, suggested between certain atypical morphological features and number of genetic 'hits'.

In all essential respects, results from cervical and bronchial cancer are compatible with the scheme provided by colorectal cancer [104–106]. The same also seems to be true for experimentally induced skin cancer in the mouse [107].

The conclusion would be that there is no proof that genetic instability/hypermutability is required for the development of precancer. Many precancers, such as adenomas in endocrine glands or the gut, do not show the conspicuous genetic or chromosomal heterogeneity quoted as the major argument for genetic instability. Whether transition to invasive cancer is preceded or even caused by acquisition of genetic instability is unresolved, although this is suggested by development of heteroploidy and considerable alterations of restriction fragment lengths, at least in the majority of human cancer types. Careful analysis of clonally related precancers and cancers would stand a good chance of illuminating this dark but important corner of tumour biology. Lineages of non-transformed cells with comparable lengths to transformed ones have to be analysed genetically to provide a reliable estimate of mutability. This has to be done on a single-cell basis, and pooling of many lineages will randomly dilute any mutations only found in one lineage. Polymerase chain reaction analysis of single microdissected cells was recently introduced [108] for *TP53* and may be further developed to cover many potential sites of mutations.

4.4.4. Is precancer reversible?

Dysplasia and carcinoma-*in-situ* are characterised by cytological atypia. In at least a proportion of cases, genomic alterations have been noted, ranging from abnormal amounts of DNA via chromosome aberrations to defined mutations. Against such a background, it would not be unreasonable to assume that the

population of precancerous cells is 'transformed' to irreversible commitment to continued net increase, but there exists good evidence that a proportion of precancers are reversible. In the case of cervical cancer, the much higher incidence of carcinoma-*in-situ* than of invasive cancer cannot be explained unless one accepts that a proportion of the former will never appear as invasive cancers [109,110]. Statistical–epidemiological evidence has been supported by direct observation of regression without intervention [111,112]. The likelihood of spontaneous regression has been conservatively estimated at about 30% [113], but other estimates have landed at 80% [109].

The principal tumour biological significance of regression in cervical cancer can be questioned, mainly because unusual immunological responses could be expected in view of the common involvement of papillomavirus [114], but also in skin cancer there is evidence for spontaneous regression. About 25% of dysplasias disappeared during continuous observation [115]. Reversibility was also demonstrated in bronchial cell atypia [116].

The mechanism by which regression is instigated has hardly been studied apart from vague speculations about immunity and increased apoptosis [117,118]. Its cellular biology may, however, be very important to elucidate, particularly since an absolute difference may exist to invasive cancer, where spontaneous regression is extremely rare if it exists at all [119].

Existence of reversibility in precancer shows, furthermore, that early hits on a lineage to cancer can give phenotypic effects in the form of atypia but that these hits need to be followed by a decisive last hit to prevent regression and accomplish malignant transformation.

4.4.5. Can precancer be epigenetic?

Experimental carcinogenesis in rat liver has revealed a rather clear sequence of events [120]. The main difficulty has been to construct a system where non-specific influence of toxicity and reactive cell proliferation can be avoided or at least controlled. On a morphological level, it seems by now well established that the initiation–promotion path towards cancer after application of a variety of chemical carcinogens begins with multiple focal hepatocellular changes. These foci will then, after a phase of mitotic inhibition, rather synchronously begin to show increased rates of cell division. A few will later show cellular dysplasia and grow expansively as 'adenomas'. At some stage, a minority of these foci will transform into rapidly growing nodules considered *in-situ* cancers because of their high probability of further development into invasive hepatocellular cancer.

Application of the current dogma that cancer arises via sequential addition of somatic genetic changes and selection would explain this as a result of original mutations in a large number of stem cells, which then would acquire additional mutations until a few of them by chance had accumulated a set that caused malignant transformation and selection as hepatocellular carcinomas. Precancer would take up an intermediate position with rather few mutations which, however, would have distinct phenotypic consequences.

This view has been challenged. It has been observed that the earliest foci are characterised by metabolic alterations explicable by increased local exposure (or sensitivity) to insulin which, in turn, would lead to abnormal glycogen metabolism partly involving mitochondrial systems. Similar foci are produced in a variety of ways, including intrahepatic transplantation of Langerhans islets, hepatitis virus and chemicals, which cannot be expected to cause an identical initial causative mutation [121]. It has therefore been suggested that irreversible epigenetic mechanisms akin to forces driving embryonic development are active [122,123]. Attempts to search for *RAS* mutations and other oncogenes/suppressor genes have yielded only negative results in the early histochemically detected foci.

Epigenetic mechanisms are responsible for essentially irreversible well ordered embryonic development with appearance of 'new' phenotypes. The speculation is that similar mechanisms may, in reverse, by dedifferentiation, create foci of cells that now have acquired a low but definite likelihood of undergoing progression, possibly because unknown promoters are acting on a phenotype hypersensitive to malignant transformation. The challenge to the somatic genetic dogma of carcinogenesis has been given a general formulation [124].

5. Overview

The field of precancer has been, and still is, plagued by ambiguous terminology and confusion about definitions of precursors of invasive cancer. This section presents a cell and tumour biological approach based on the idea that precancer, in conformity with cancer, should be understood as a result of a chain of mutations (the term being taken in a wide sense and possibly mixed with irreversible epigenetic changes) affecting an uninterrupted cell lineage. The phenotypic consequences of this genotypic progression will vary from case to case and type to type of cancer. In some instances, exemplified by endocrine and neuroendocrine tumours, mutations en route to malignant transformation may change the phenotype predictably from hyperplasia to adenoma to carcinoma-*in-situ* to invasive cancer. The other extreme is exemplified by retinoblastoma, basal cell cancer and probably also some forms of lymphoma/leukaemia, where only the final mutation will lead to a phenotypic change in the form of cancer. The vast majority of precancers/cancers are intermediate. Some will develop via recognisable forms of dysplasia and/or

Table 2
Genetic determinants of malignant transformation

Rate of transforming mutations per round of DNA synthesis	This is a function of total rate of mutations (background + actively acquired). The frequency is increased by deficient repair, decreased fidelity of DNA synthesis and exposure to mutagens
No. of DNA rounds in the lineage which ends in transformation	This will vary between a few hundred and several thousands depending on the tissue
No. of lineages susceptible to transformation	This number reflects number of stem cells. It will vary widely within an individual depending on embryological development of the respective types of cells
Time of mutation	Mutations in a lineage possessing symmetrical stem cell divisions will affect more progeny than mutations in lineages with only asymmetric divisions
Effects of competing mutations	Mutations outside oncogenes and suppressor genes outnumber transforming mutations by a wide margin. Sublethal/lethal mutations may indirectly give a transforming lineage selective advantage
Phenotype of lineage	Certain cell types require fewer hits than others
Species derivation of target lineages	Human cells are more resistant to malignant transformation than murine cells after an equal number of externally inflicted mutations

Mutation = genetic change with significant impact on encoded protein. Transforming mutation = mutation, which in combination with other transforming mutations, causes transformation to precancer or cancer. Genes subject to transforming mutations are either oncogenes or suppressor genes.

in-situ cancer, others will appear without any traces of precursors. Current evidence indicates a stochastic process by which crucial mutations/chromosomal rearrangements are added to the genotype. These mutations are drawn from several hundred oncogenes/suppressor genes. The mutational spectrum and the specifically involved oncogenes/suppressor genes, together with the additional effect of mutations in non-oncogenic genes, will fashion a phenotype that may be unique. Inherent in such a unique genotype lineage is a possibility that all essential features determining subsequent clinical malignancy are laid down in the precancer and held in abeyance until the final hit that causes invasive growth. Such clandestine hits may determine degree of differentiation, amplification of oncogenes, capacity for invading vessels and so on. Other genotypes in precancer may not have a stable selective advantage, for example because telomere shortening is not stopped or self-renewal of stem cells is insufficient and will therefore die out and score as regressors. Determinants of malignant transformation are summarized in Table 2.

Genetic links have usually not been established between precancers and invasive cancers. Formal evidence for direct transition is thus generally lacking, but circumstantial morphological and epidemiological evidence supports the time-honoured notion that precancers develop into cancer via selection of fit genotypes. Elimination of precancer will prevent cancer only if it indeed can be proven that cancer develops from the same lineage as precancer.

Existence of hypermutability in precancer has never been substantiated except in colonic adenomas in the mismatch repair deficiency syndrome. If hypermutability (genetic instability) is functionally important it may be so only after malignant transformation, and then not as a regular or necessary trait.

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