The clonal origin and clonal evolution of epithelial tumours

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Summary. While the origin of tumours, whether from one cell or many, has been a source of fascination for experimental oncologists for some time, in recent years there has been a veritable explosion of information about the clonal architecture of tumours and their antecedents, stimulated, in the main, by the ready accessibility of new molecular techniques. While most of these new results have apparently confirmed the monoclonal origin of human epithelial (and other) tumours, there are a significant number of studies in which this conclusion just cannot be made. Moreover, analysis of many articles show that the potential impact of such considerations as *patch size* and *clonal evolution* on determinations of clonality have largely been ignored, with the result that a number of these studies are confounded. However, the clonal architecture of preneoplastic lesions provide some interesting insights – many lesions which might have been hitherto regarded as hyperplasias are apparently clonal in derivation. If this is indeed true, it calls into some question our hopeful corollary that a monoclonal origin presages a neoplastic habitus. Finally, it is clear, for many reasons, that methods of analysis which involve the disaggregation of tissues, albeit microdissected, are far from ideal and we should be putting more effort into techniques where the clonal architecture of normal tissues, preneoplastic and preinvasive lesions and their derivative tumours can be directly visualized *in situ*.

Keywords: tumours; clonality; clonal evolution; carcinogenesis

One of the shibboleths which has arisen in modern tumour biology, possibly stimulated by the rarity of malignant transformation *in vivo*, has been that neoplastic proliferations, perforce, take origin in a single cell**.** Our ideas about the nature of the neoplastic process are heavily predicated by our concepts of its origin: it is widely accepted that tumours arise from a mutation or series of mutations in this single cell. This view has been largely influenced by numerous observations indicating that not only are chemically induced neoplasms in rodents clonal, but that the preneoplastic lesions from which they arise are also clonal in origin (Iannaccone *et al*. 1987). Thus, using the paradigm of colorectal cancer, Fearon (1994) has remarked 'most interesting are those

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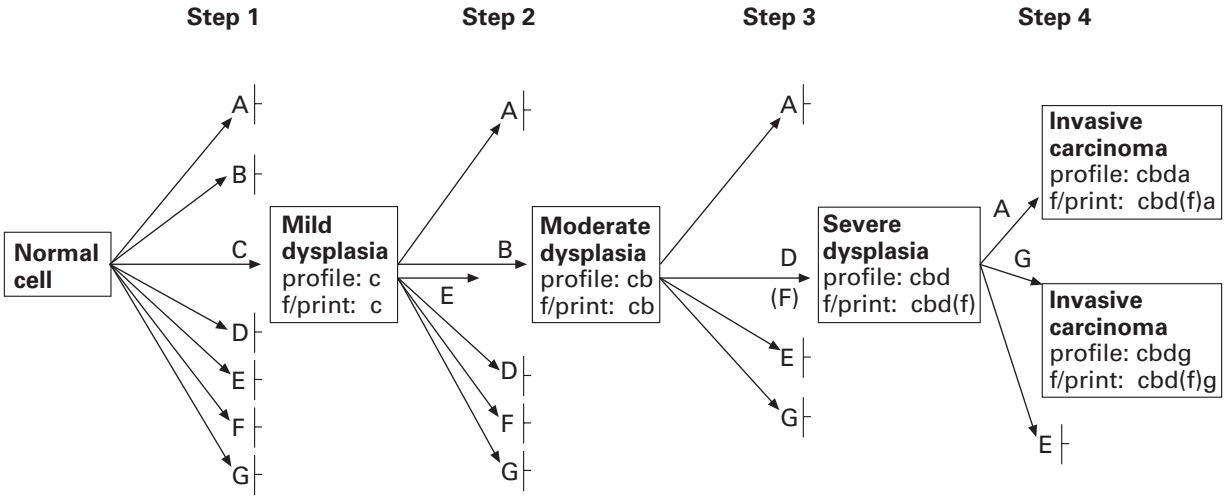

Figure 1. A diagrammatic representation of the process of mutation and selection, starting in a single cell and progressing through a sequence of steps through dysplasia to carcinoma. While based on the well-known Vogelstein model for colorectal cancer, the diagram is generally representative of neoplastic progression in a variety of epithelial tissues. The diagram represents genetic pathways in the development of a tumour, where A, B, C, D, E, F and G indicate the loci of mutations which, when selected, give survival advantage to the cells. Profile, mutationprofile; f/print, mutation fingerprint; |– not selected (from Ilyas & Tomlinson 1996, with permission).

mutations which arise in early tumours which are not found in the germ-line; these mutations are responsible for the clonal development of the adenoma'. The model of development of colorectal carcinoma subscribed to by many (Figure 1) involves a series of sequential mutations in single cells, which leads to the evolution of the definitive malignant phenotype.

So, largely accepted theories for carcinogenesis maintain, explicitly or implicitly, that tumours are derived from a single mutated cell and it's progeny. Neoplastic cells would then be capable of growth under what would otherwise be limiting conditions, and some accrue genetic changes that allow them to outcompete their neighbouring cells to eventually become the predominant subclone (Kern 1993). Tumours would then be the result of clonal expansion of such unusual cells surviving transformation (Chang 1982): indeed, much earlier work by Fialkow and colleagues (reviewed by Fialkow 1976), using electrophoretic studies of X-linked glucose-6-phosphate variants in homogenized tissues from heterozygous women, has shown monoclonality in a wide range of human tumours. The possible exceptions are hereditary tumours such as familial neurofibromas (Fialkow *et al*. 1971).

There is also the interesting corollary of this belief: if a lesion can be shown to be the result of a clonal proliferation, then, *ipso facto*, it is neoplastic, and this concept also seems widely accepted (e.g. Noguchi *et al*. 1992; Nilbert *et al*. 1995; Vogrincic *et al*. 1997; Niho *et al*. 1998; Walsh *et al*. 1998). In hyperplasias, where multiple cells respond to a stimulus and therefore we might anticipated a polyclonal expansion (Walsh *et al*. 1998), we shall see that hyperplastic lesions such as regenerative nodules in the liver, thyroid nodules in hyperplastic goitre and atheromatous plaques in coronary arteries, unlikely candidates for 'neoplastic' habitus, are also regarded by many as clonal proliferations.

Until comparatively recently, any departure from this concept (Alexander 1985; Rubin 1985) was indeed regarded as iconoclastic. But as Teixeira *et al*. (1994) have noted, support for the general conclusion that tumours are clonal in the human situation, at least from the cytogenetic viewpoint, comes from the study of mesenchymal and haematological neoplasms. The increasing karyotypic evidence of polyclonality in carcinomas might indicate a fundamental difference in this respect between epithelial tumours and other neoplasms. In fact, evidence is now accruing, at least for a subset of epithelial tumours, that by no means all of these lesions are clonal, with the result that we probably have to come to terms with the concept that tumourigenesis in epithelial systems demands the co-operation of several distinct clones, and somehow account for this in any global theory of carcinogenesis.

Methods for investigating the clonal composition of tumours

Because this is such an important baseline in our

attempts to understand malignant transformation in molecular terms, it is always worthwhile reminding ourselves of the experimental basis of this proposal. Why do we believe that epithelial tumours, such as the early adenoma in the colonic mucosa, are clonal proliferations? The earlier literature has been reviewed by several authors (Fialkow 1976; Iannaccone *et al*. 1987), but here we should note that the main methods which have been used for the analysis of clonality in human epithelial tumours have been based on X-chromosome inactivation analysis in females and the detection of somatic mutations. Studies of viral integration, for example by Southern blotting in EBV-associated tumours (Wainscoat & Fey 1990; Fukuyama *et al*. 1994; Leung *et al*. 1995; McClain *et al*. 1995; Shin *et al*. 1996) or in hepatitis B or C-associated liver tumours (Hsu *et al*. 1991) are also useful; Jiang & Yao (1996) record the excellent agreement between X-inactivation and EBV integration in nasopharyngeal carcinoma. To date, attempts to find methods of assessing the clonal architecture of male tissues and tumours in the human have proved unsuccessful (Zvejnieks *et al*. 1998).

Classically, in X-chromosome inactivation studies the haplotypes of glucose-6-phosphate dehydrogenase (G6PD) have been exploited (Fialkow 1973), replaced more recently by methods based on restriction length polymorphisms of X chromosome-linked genes such as glycerophosphate kinase (PGK), the androgen receptor gene (HUMARA) (Vogelstein *et al*. 1987), hypoxanthine phosphoribosyltransferase (HPRT) (Sternlicht *et al*. 1994), the M27β probe for DXS285 (Fraser *et al*. 1989; Li *et al*. 1993), and p55 together with glucose-6-phosphate dehydrogenase (Liu *et al*. 1997). Such methods are based on the principle that, early in embryogenesis, genes on either of the two X chromosomes are randomly inactivated by methylation of cytosine residues within promoter regions (Lyon 1972); once methylated, such CpG islands are functionally and heritably inactive (Jones 1996) and it is widely believed that this inactivation is stable, even during the neoplastic change. Thus in approximately half of the cells the paternal X chromosome is active, and in the rest, the X chromosome from the mother is active. Consequently the pattern of fragments produced by DNA digestion with a methylation-sensitive enzyme such as SnaB1 and a further endonuclease corresponding to a restriction fragment length polymorphism, in for example PGK – BstCX1 – can be used to investigate the apparent clonality of any tissue specimen. The percentage of informative cases in women using these markers are reported to vary from 45% with PGK and HUMARA (Vogelstein *et al*. 1987), to over 90% with M27β/DXS255 (Fey *et al*. 1992).

The methylation pattern of DNA can be abnormal in malignancy, with both increases and reductions in methylation (Jones & Buckley 1990), and the possibility exists that X-chromosome inactivation may not be valid as an indicator of clonality because of these abnormalities in DNA methylation (Jacobs *et al*. 1992). In fact, a cell is not distinguished by a specific sequence of methylated or nonmethylated sites: the methylation of different cell types is distinguished by the proportion of cells methylated, i.e. the overall methylation frequency, for each site in the population (Silva *et al*. 1993).

Moreover, it is possible that X-inactivation might be nonrandom, either constitutive (Tihy *et al*. 1994) or cell-type specific (Wengler *et al*. 1993). Some studies in normal haemopoietic and lymphoid tissue have shown skewed X-inactivation, possibly favouring the paternal or the maternal X-chromosome (Mutter & Boynton 1995), which could presage nonrandom X-chromosome inactivation patterns. While some studies claim that extremely unbalanced inactivation of the X chromosome is an uncommon phenomenon (Racchi *et al*. 1998), others report skewed inactivation in some 23% of women with HPRT and PGK, and 22–33% with M27β (Fey *et al*. 1992; Gale *et al*. 1992), present in peripheral blood leucocytes (Boyd & Fraser 1990; Fey *et al*. 1992) and in bone marrow and skin (Lo Coco *et al*. 1993; Tsuge *et al*. 1993), suggesting tissue-specificity, and possibly related to the number of stem cells in the tissue at the time of X chromosome inactivation. If this number is small, it will result in skewing, with increased probability with diminishing stem cell pool size (Fialkow 1973). In some embryonic tumours such as retinoblastoma and Wilm's tumour, loss of heterozygosity (LOH) on 13q and 11p, respectively, show the preferential loss of maternal and paternal alleles (Drya *et al*. 1989; Pal *et al*. 1990), also seen in sporadic osteosarcoma (Toguchida *et al*. 1989).

Finding the same mutation in key genes, such as k-*ras* (Dijkhuizen *et al*. 1997) or p53 (Franklin *et al*. 1997) in multiple tissue samples from the same tumour, or from apparently unconnected tumours, has also been proposed as indicating a clonal origin, notwithstanding several perceived problems, notably the possibility of the contemporaneous occurrence of the same mutation in separate precursor cells by a carcinogen – aflatoxin causes specific p53 mutations in hepatocellular carcinoma (Bressac *et al*. 1991; Hollstein *et al*. 1991; Hsu *et al*. 1991; Ozturk 1991). We should also note that DNA fingerprinting has also been proposed as a method for assessing clonal status (Fey *et al*. 1988).

Demonstration of heterogeneity of microsatellite instabilities, for example in multiple gastric carcinomas (Nakashima *et al*. 1995) has been used as a marker for

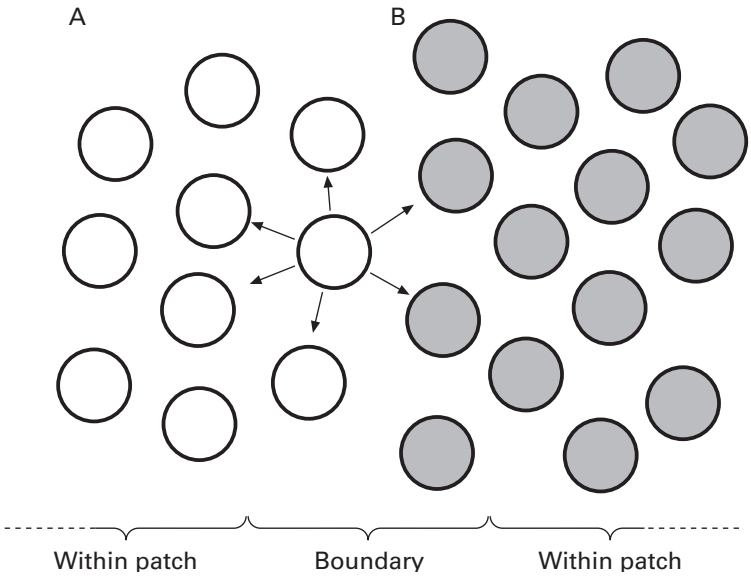

Figure 2. Showing the influence of patch size on the determination of clonality, here in colorectal epithelium. using X-linked markers; tumours arising within patches will perforce be clonal, and only lesions arising at the margins of patches (i.e. between A and B) will be detectable as a polyclonal proliferation. The concept can of course be extrapolated to any epithelial sheet, such as the epidermis or urothelium (from Schmidt & Mead 1990, with permission).

polyclonality, but if genome alterations continue to occur at microsatellite loci, with resulting genetic diversity within the same clone, microsatellite instability will not be an appropriate molecular marker of clonality. Others have looked to the presence of cytogenetically unrelated abnormal clones demonstrated by karyotypic analysis as evidence of polyclonality, but the existence of such clones might be due to chromosomal rearrangement in non-neoplastic epithelial or stromal cells. For example, it is now recognized that cytogenetically abnormal clones are present in apparently non-neoplastic breast lesions such as fibrocystic disease (from which breast carcinoma probably arises)(Petersson *et al.* 1994; Dietrich *et al.* 1995). Mutated clones have been reported even in histologically normal breast epithelium (Larson *et al.* 1998), several at potential tumour-suppressor gene sites, indicating that genetic abnormalities accumulate before pathological changes can be detected. There also exists the possibility, however unlikely, that the disparate clones could arise from a single transformed 'mother cell' (Teixeira *et al.* 1995). Or of course, such clones could indeed presage a polyclonal proliferation; indeed, because such karyotypic analysis is perforce carried out *in vitro*, there are reasons why karyotypic heterogeneity maybe underestimated in human tumours (Teixeira *et al.* 1995).

We should note that these observations, in the main, involve the biochemical or molecular examination of homogenized tissues, and in human epithelial tissues, there have been few opportunities indeed to directly observe the clonal development of very early tumours. But why should direct observation be so important? This importance is well illustrated for techniques using X-chromosome inactivation by the thoughtful article from Schmidt & Mead (1990), which introduces the concept of patch size into the argument. It is usual to define a clone as a family of cells which derive from a common progenitor, sometimes adding the caveat, for normal tissues, that these remain contiguous throughout the growth of the embryo (Nesbitt 1974). This definition thus refers to cell lineage. A patch, on the other hand, is a group of cells which share a common genotype, contiguous at the moment of consideration (Nesbitt 1974). Thus clone size and patch size are not strictly equivalent, since multiple clones of the same genotype could contribute to a single patch; similarly, a single clone could be anatomically separated into different patches.

Thus in Figure 2, which again uses the paradigm of the colon, an adenoma arizing from the centre of a patch will, by definition, be of clonal origin when assessed by the pattern of X inactivation. The only chance of detecting a polyclonal, or pleoclonal (Woodruff 1988) proliferation would be when such a lesion arises from the margin of a patch boundary. This is seen in patch boundaries in normal mouse epidermis, where hair follicles appear polyclonal, but of course are monophenotypic within patches (Winton *et al.* 1989). At X-inactivation, at about the time of implantation, the pool of stem cells is quite small, approximately 15 cells for the skin (Fialkow 1973). Skewing towards one parental allele is thus very possible, and indeed this is seen in human skin specimens (Tate *et al.* 1997), potentially indicating a large patch size. Such polyclonal tumours will therefore be commonest at patch boundaries, the opportunity for this occurring being dependent on the size of the patch (Schmidt &

Mead 1990), and thus the incidence of polyclonal tumours might indeed be small in any one analysis. And if rare polyclonal lesions are found, then the explanation that the lesion consists of more than one single tumour, each of clonal origin, that have mixed or collided, is often proffered (Farber 1997). But the rarity of such lesions would indeed be expected, it as seems likely, X-linked patches are quite large (Schmidt & Mead 1990; Chung *et al*. 1998).

Interestingly, the concept of patch size in respect of these arguments is rarely mentioned (Thomas *et al*. 1988), and, even when adjacent normal tissue is used as a control, and found to be polyclonal, the sample size, a critical factor, is rarely mentioned (e.g. Sawada *et al*. 1994; Mutter *et al*. 1995; Niho *et al*. 1998; Walsh *et al*. 1998). Indeed the finding of polyclonality in normal tissues, such as the normal liver adjacent to carcinomas, without mention of size, has been regarded as a validation of the PGK (Kawai *et al*. 1995), HUMARA (Mutter *et al*. 1995) or HPRT technology (Barsky *et al*. 1994), and even taken as evidence that the patch size is small (Apel *et al*. 1995). A recent paper by Chung *et al*. (1998) has brought this issue sharply into focus: a long-term belief has been that smooth muscle cells in atheromatous plaques are monoclonal (Benditt & Benditt 1973; Pearson *et al*. 1978, 1979, 1987; Murry *et al*. 1997) with the clonality in this case dependent upon the smooth muscle component of the plaque (Murry *et al*. 1997), and such plaques have even been regarded as some sort of benign neoplasm. This proposal has been based on X-chromosome inactivation studies with glucose-6-phosphate dehydrogenase and HUMARA analysis (Murry *et al*. 1997), supported by karyotypic abnormalities reflecting monoclonality (Casalone *et al*. 1991) and mutations in a microsatellite in the TGFB1 receptor gene (McCaffrey *et al*. 1997). Clonal proliferation is thus explained by a somatic mutation, induced perhaps by genotoxic chemicals, or an infection, giving a hit in a single cell, which then develops into an atheromatous plaque. Alternatively, since smooth muscle cells are now known to consist of multiple cell lineages (Lemire *et al*. 1994) a rare progenitor cell could be selected and clonally expanded to form the plaque (Schwartz & Murry 1998).

While plaques are indeed monoclonal based on such analyses, Murry *et al*. (1997) reported skewed X-chromosome inactivation patterns in normal media and intima, and Chung *et al*. (1998) found patch sizes in aortic media and intima exceeding 4 mm in diameter. Although plaques may indeed arise through a mutagenic event, X-chromosome inactivation analysis cannot be relied on to distinguish between a monoclonal and a polyclonal origin for such atheromatous plaques, because of the very large patch size. This underlines the significance of Schmidt and Mead's proposals, shown in Figure 2, and the importance of knowledge of the patch size. It will be interesting to see how many other investigations of clonality, which ignore or side-step this constraint, will be similarly confounded. Sometimes the *clone size* is preferable – thus relating the number of apparently mixed epidermal tumours in chimaeric mice (6/50) to the epidermal clone size (600 cells) allowed the conclusion that each tumour arises from 8 or fewer cells – the target size (Iannaccone 1980) (but see below for further *caveats*).

The study of multiple lesions in the same patient does provide a way of circumventing the problem of patch size, especially if more than one clonal marker is used (Sidransky *et al*. 1992a). Thus the probability that all the tumours examined would have the same X-chromosome inactivation pattern is 0.5^{n-1}, where n equals the tumour number. If say, allele loss on 9q, an independent event, was also sought, then the probability that the same pattern in each tumour is due to chance is $(0.5^{n-1})(0.5^{n-1})$, and so on for each independent marker used.

On the other hand, when methods based on the analysis of homogenized tissues have given rise to results suggesting polyclonality, this is often attributed to contamination with underlying stromal cells of different clonal derivation. Even so, some investigators insist on normal tissue contamination, even when the incidence of polyclonal tumours amounts to 40% of the total (Walsh *et al*. 1998). Having said this, it is clear that even the fibroblasts or myofibroblasts very closely applied to the epithelium in the colonic crypt are of different clonal derivation (Figure 3a). Moreover, there may be invading inflammatory cells (Figure 3b), which, if numerous enough, can give rise to disparate results in clonality analyses involving PCR techniques (Shroyer & Gudlaugsson 1994; Kawai *et al*. 1995). Figure 3(c) shows how normal tissue can be trapped and enclosed by surrounding neoplastic tissue, and again give discordant results unless microdissection procedures are stringent. The problem of poor sampling may give a false impression of monoclonality in a polyclonal tumour (Williams & Wynford-Thomas 1994; Fey & Tobler 1996; Piao *et al*. 1997). Enomoto *et al*. (1994) using the HUMARA method, has estimated that the bands generated by mixing monoclonal tumour tissue with peripheral blood leucocytes are distinguishable in the proportion 8 : 2, indicating that clonality can be assessed when clonally derived cells comprise 20% or more of the population; Krohn *et al*. (1998) quoted 40% and Noguchi *et al*. (1992) 50%.

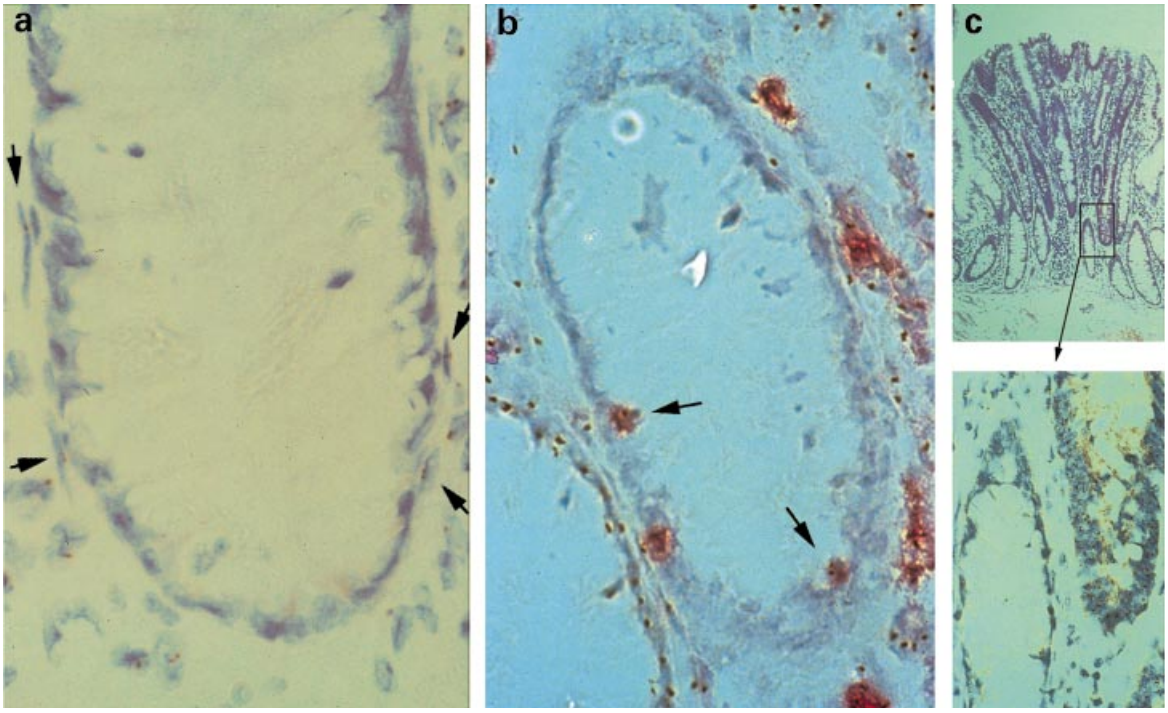

Figure 3. The problems of using tissue disaggregation techniques or less than stringent microdissection techniques in the determination of tissue clonality: (a) human colon from an XO/XY mosaic, with NISH demonstration of nuclei containing the Y chromosome. Myofibroblasts which are very closely applied to the epithelium show a different clonal origin; (b) white blood cells, shown red by immunohistochemistry with an antibody against leukocyte common antigen, are present between crypt epithelial cells, and are the source of Y-positive nuclei in an otherwise negative crypt, and (c) a tubular adenoma from the same individual showing a normal, negative crypt in a Y-chromosome positive adenoma, showing how easy it is to include normal tissue in a neoplastic lesion (from Novelli *et al.* 1996, with permission).

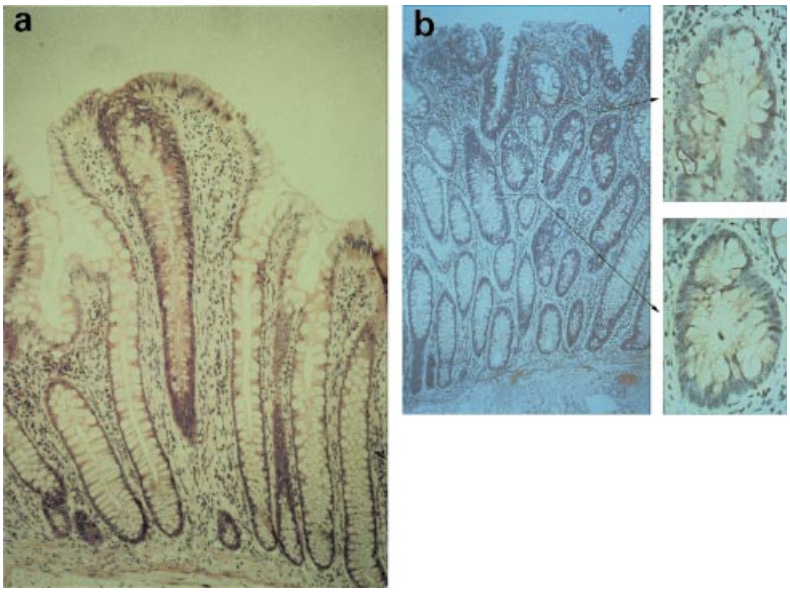

Figure 4. (a) A monocryptal adenoma from an XO/XY individual with FAP. These lesions are always monophenotypic, and (b) a microadenoma from the same individual, showing adenomatous crypts of different clonal derivation (from Novelli *et al.* 1996, with permission).

In animals, extensive use of aggregation chimeras, or tetraparental allophenic mice has been made to explore both the clonal architecture of epithelial tissues and their derivative tumours, but it was not until recently that similar advantage was taken of human mosaics to investigate the clonal architecture of human tissues and their tumours (Novelli *et al*. 1996).

A further *caveat* of particular relevance to clonal mutation analysis is the point, during the life history of the development of the tumour, at which it is examined. The analysis of mature lesions is inappropriate, since tumours of polyclonal origin may become clonal because of clonal evolution (Nowell 1976, 1986). All clones, except one, are eliminated or reduced to the point of being undetectable, apparently seen in chemically induced mouse fibroasarcomas, initially polyclonal, which evolve to a clonally derived population with time (Woodruff *et al*. 1982), because of the later selection of a dominant clone (Winton *et al*. 1989). Thus polyclonality gives rise to 'pseudomonoclonality' (Teixeira *et al*. 1994), when selection forces in the tumour change, as for example after invasion has occurred (Heim *et al*. 1989). This is possibly exemplified in colorectal adenomas, where heterogeneity of k-*ras* mutations is seen – which heterogeneity is lost after the lesion has evolved to become an invasive carcinoma (Shibata *et al*. 1993). Conversely, genetic heterogeneity in a tumour does not necessarily indicate a polyclonal origin, since genetic instability is a major feature of malignant tissues and many new clones may arise during tumour development (Nowell 1976, 1986). We return to this issue at the end of this article.

The clonal architecture of normal tissues

Most authors have drawn a direct comparison between genotype/phenotype determination and clonality: while apparently reasonable, this begs the patch size question, and some have used the terms monophenotypic and polyphenotypic (Thomas *et al*. 1988) or homotypic and heterotypic (Nomura *et al*. 1996) to avoid this problem. Thomas *et al*. (1988) cogently point out that a monophenotypic tumour arising in the colon, where intestinal crypts are themselves clonal populations (*vide infra*), can only be described as monocryptal and not monoclonal in derivation. Conversely, in the thyroid, where follicles are mainly (in the mouse) polyclonal, a monophenotypic tumour is much more likely to be monoclonal.

Investigations of clonality in many normal tissues has been aided by the use of tetraparental allophenic or chimaeric mice, coupled with suitable strain-specific markers to distinguish the chimaeric tissues *in situ*. Thus in the neonatal mouse, intestinal crypts are polyclonal, but, through a process known as 'purification', crypts become monoclonal by two weeks of age (Schmidt *et al*. 1988) and remain so throughout adult life (Ponder *et al*. 1985; Tatematsu *et al*. 1996b). This is similar to the retinal epithelium, where a random arrangement is seen in the embryo, but a pattern of small clones is present in the adult (West 1976a). In X-chromosome mosaics, adult mouse thyroid follicles are polyclonal (Thomas *et al*. 1988). The liver in the adult mouse chimaera apparently contains $>10^6$ small clones, indicating that considerable mixing occurs during development (West 1976b), although direct observations in rat chimaeras shows many large patches, though some small ones (<50 cells) are seen. Moreover, patch size does not follow anatomical boundaries such a lobules, unlike the intestinal crypt or adrenal cortex, which show clear evidence of clonal development (Weinberg *et al*. 1985). In chimaeric mouse epidermis, the average clone size was thought to be 600 cells, and the patch size approximately 15000 cells (Iannaccone 1980); in X-chromosome mosaics, the patch size was approximately 6000 cells (Deamant & Iannoconne 1987), and up to 3000 cells in human scalp (Gartler *et al*. 1971). At least in the mouse epidermis, this concept of fine-grained patchiness, based on the biochemical analysis of small fragments, is not seen when chimaeric epidermis is visualized *in situ*, when nonhomogeneous transversely orientated stripes are seen (Schmidt *et al*. 1990).

There is little data concerning the patch size in human tissues, even where the pattern of X-inactivation is concerned, but we have seen above that in human arteries, the smooth muscle cells are a diverse population composed of multiple lineages, and growth is by expanding smooth muscle cell clones, with little or no mixing of adjacent clones (Chung *et al*. 1998); the evidence indicates a very large patch size. In the human breast, earlier findings suggested that normal breast tissue was polyclonal in origin, with random distribution of X-chromosome inactivation (Noguchi *et al*. 1992). However, microdissection of normal breast epithelium shows that epithelial patch size is quite large, exceeding $0.45\,mm^2$, and that the larger terminal ductulo-lobular units represent the progeny of a single precursor or stem cell (Deng *et al*. 1996; Tsai *et al*. 1996). In the *mouse* mammary gland, where ducts and acini are apparently polyclonal, while a large patch size is seen due to coherent clonal growth during development (Thomas *et al*. 1988), there appears to be three multipotent but distinct epithelial stem cells in clonally derived mammary epithelial cell populations. When serially passaged, one stem cell could readily produce an entire mammary gland (Kordon & Smith 1998). Since these stem cells can be

mutated during organogenesis, and these mutations conserved in all progeny, such self-renewing, organ-specific stem cells may represent the major potential risk for carcinogenesis in mammary epithelium**.** As we shall see, this is an important point, since apparently normal breast can contain genetic abnormalities at potentially important sites (Larson *et al.* 1998). These observations have implications for claims that human breast cancers, and their precursors, are clonal proliferations (*vide infra*).

Further evidence for a large X-linked human patch size comes from the bladder epithelium – 1 cm^2 (Tsai *et al.* 1995) hair follicles −0.2–1.0 cm^2 (Gartler *et al.* 1971) and gastric epithelium – 1.0 cm^2 (Linder & Gartler 1965a), but other tissues, such as myometrium, may have a smaller patch size, probably less than 0.3 cm^2 (Linder & Gartler 1965b). The mature human ovarian follicle is composed of oligoclonally derived – not more than three – groups of granulosa cell progenitors (Deerlin *et al.* 1997).

Thompson *et al.* (1990) were the first to show that gastric glands are also clonal populations, using XO/XY mice and localizing the male nuclei with a Y chromosome-specific probe, and also to show that neuroendocrine cells shared the same clonal derivation as the glands. In the human stomach, the situation may be more complex than this: antral gastric glands are clonal, but in the body glands the foveolae and bases of the glands appear homotypic, whereas the isthmus and neck, the area which contains the gastric stem cell population, are heterotypic on HUMARA analysis (Nomura *et al.* 1996). These glands may be of polyclonal structure, possibly reflecting complex stem cell organization, unless differences in the timing of methylation are important, possibly occurring after the formation of gastric gland primordia in man (Nomura *et al.* 1996): the methylation of the inactive X chromosome is complete at 8 days in the foregut of the mouse (Tan *et al.* 1993). Additionally, when mice in which multiple copies of the *lacZ* gene were placed in tandem close to the *hprt* locus on the X-chromosome, Lyonization gives gastric glands of different phenotype for analysis (Nomura *et al.* 1998a). At birth, 75% of glands in the pylorus and 85% in the body are of mixed phenotype, but over the first six weeks of life most glands undergo monoclonal conversion to become monophenotypic. However, even in adult life there is a small fraction, approximately 5–10%, which remain mixed, and given that some monophenotypic glands can also be polyclonal, the total of polyclonal crypts might be as many as 15–26% in the body and 10–15% in the pyloric mucosa.

In the normal human intestine, investigation has been limited by the lack of suitable markers of clonality, although in adults, Fuller *et al.* (1990) have shown crypt-restricted loss of mucin O-acylation. This may be due to somatic mutation at the O-acylation transferase (OAT) locus and suggests that human crypts may be clonally derived – indeed Endo *et al.* (1995), using the HUMARA assay, have shown that individual, microdissected human colonic crypts are homotypic and clonal in origin. In human XO/XY mosaics, it is possible to differentiate between XO and XY cells using *in situ* hybridization with *Y* chromosome-specific probes (Novelli *et al.* 1996). In such an individual, intestinal crypts were composed almost exclusively of either XY or XO cells, including the crypt neuroendocrine cells. Thus, intestinal crypts are clonal populations, each derived ultimately from a single stem cell; neuroendocrine cells share this derivation, thus conclusively disproving Pearse's hypothesis of the neuroectodermal derivation of such cells in humans, as part of the global APUD cell theory (Pearse 1987). The patches of XO crypts were irregular in shape and patch size varied widely (mean 11.25 crypts, range 1–14 crypts).

There have been several statements about the polyclonal structure of human endometrium (cf. Mutter *et al.* 1995), without further refinement, but it is of interest that the clonal architecture of the colonic crypt is also reflected on the endometrium, where microdissection studies show that approximately 80% of glands are clonal in origin (Jiang *et al.* 1996).

The clonal architecture of non-neoplastic, preneoplastic and preinvasive proliferations

Non-neoplastic and preneoplastic lesions

We now move on to a consideration of the lesions which are thought to precede neoplastic development. Many tumours arise from preneoplastic lesions, not themselves neoplastic, which were previously regarded as hyperplasias, involving changes in many cells and therefore by definition polyclonal. However, it is becoming increasingly clear that such lesions often represent clonal proliferations. Thus in gastric mucosa three separate hyperplastic polyps of the fundus in a single patient which harboured foci of dysplasia showed the same k-*ras* codon 12 point mutation – glycine:aspartate, present in both hyperplastic and dysplastic areas (Dijkhuizen *et al.* 1997). Chance was discounted, as was a specific genetic lesion, and the explanation preferred was that the progeny of a single transformed cell has spread through the mucosa. It is difficult to envision how such spread could occur, although the unlikely possibility of surface spreading and surface implantation was

considered possible. It is of course possible that a single cell could populate a gastric gland, which could then spread through the mucosa by gland fission (Garcia *et al*. 1999).

In the female genital tract, endometrioid carcinomas are considered to arise from foci of endometriosis (Jimbo *et al*. 1997). Recent studies of informative individuals has shown the majority of such endometriotic foci to be clonal (Nilbert *et al*. 1995; Jiang *et al*. 1996; Jimbo *et al*. 1997; Tamura *et al*. 1998). Endometriosis arises either from implantation of shed endometrial cells, or from metaplasia of the pelvic peritoneum: if patch size considerations can be excluded, this means from a single endometrial or mesothelial cell. It is clear that these investigations have concentrated on the epithelial component of the lesion, although the formal definition does include the presence of glands and stroma, and the clonal relationships of the two tissues would be of interest. It is singular that Nilbert *et al*. (1995) and Jiang *et al*. (1996) ignored the 20–40% of cases which were polyclonal (Schmidt & Mead 1990) and also that Jiang *et al*. (1996) showed that 80% of normal endometrial glands are clonally derived. The patch size in the endometrium thus extends to the size of the gland (many hundreds of cells), and thus if implantation is the mechanism, this certainly increases the chance of an endometriotic focus being polyclonal (Thomas *et al*. 1988). Moreover, endometriotic foci can show aneuploidy (Ballouk *et al*. 1994) and loss of heterozygosity at candidate tumour suppressor loci in 9p, 11q and 22q (Jiang *et al*. 1996). Thus it is not entirely surprising that the derivative tumours, the endometrioid carcinomas, are clonally derived (Shroyer & Gudlaugsson 1994): they evidently arise from a monoclonal proliferation that itself can show genetic defects. These findings led Nilbert *et al*. (1995) to label endometriotic cysts as neoplasms, and while we might disagree with this proposal, there is a view that some of these lesions could well be endometrioid cystadenomas (Fox 1993). In the endometrium itself, carcinomas arise from areas of atypical hyperplasia (Tavassoli & Kraus 1978); such hyperplasias appear clonal (Sawada *et al*. 1994; Mutter *et al*. 1995; Esteller *et al*. 1997).

Interestingly, the preneoplastic lesions from which breast cancers develop – proliferative breast disease and similar lesions – show cytogenetic abnormalities suggesting the presence of multiple clones (Petersson *et al*. 1994; Noguchi *et al*. 1994b; Diettrich *et al*. 1995; Lakhani *et al*. 1995, 1996; Petersson *et al*. 1996; Chaquai *et al*. 1997; Kasami *et al*. 1997). X-chromosome inactivation studies shows that atypical duct hyperplasias and intraduct papillomas appear clonal proliferations (Noguchi *et al*. 1994c), as do detection of monoclonal microsatellite alterations (Rosenberg *et al*. 1997) and consequently there is no reason not to believe that cytogenetic alterations have not already occurred at this stage, remembering the *caveat* about the patch size in normal breast, and noting a further report which claims that intraduct papillomas are polyclonal (Malamou-Mitsu *et al*. 1996).

Naevocellular or melanocytic naevi (thought to be, in some cases the precursor of malignant melanomas), are either congenital or acquired, and indeed studies with HUMARA only show that some 80% of acquired naevi are apparently clonal lesions (Robinson *et al*. 1998). However, in other work, also using X-chromosome inactivation analysis but here combining HUMARA and PGK, such naevi were polyclonal; malignant melanomas were clonal (Harada *et al*. 1997). If melanocytic naevi are indeed polyclonal proliferations. it might imply, as is widely held that such naevi are hamartomas – an abnormal proliferation of cells in an organ or tissue where these cell types would normally be found – here melanocytes. However, hamartomas in tuberous sclerosis, where multiple cell types are seen, show clonal 9q34 or 16p13.3 LOH and clonal X-inactivation patterns (Green *et al*. 1996), while pulmonary chondroid hamartomas also contain clonal cytogenetic abnormalities only in the chondroid component (Fletcher *et al*. 1993).

Extremely relevant to the concept of field change is the growing recognition that mutations in important genes such as p53 are found in squamous epithelium preceding any dysplastic change (Ahomadegbe *et al*. 1995; Brennan *et al*. 1995; Gallo & Bianchi 1995; Nakanishi *et al*. 1995; Chung *et al*. 1996; Mandard *et al*. 1997; Ogden & Hall 1997). Such mutations are seen even in normal mucosa (Nees *et al*. 1993; Brennan *et al*. 1995) and in sun-exposed normal epidermis (Ponten *et al*. 1997). In this respect, it is important to distinguish between field change adjacent to a tumour and in the absence of tumour. Clones of keratinocytes with immunoreactive p53 and p53 mutations (exons 5–8) are seen in sun-exposed but otherwise normal skin (Jonason *et al*. 1996; Ren *et al*. 1996; 1997a,b; Ponten *et al*. 1997), and in morphologically normal mucosa from individuals with upper aerodigestive tract tumours (Nakanishi *et al*. 1995; Mandard *et al*. 1997). Moreoever, microsatellite instability has been found in normal mucosa from patients with ulcerative colitis (Brentnall *et al*. 1996), reflecting the increased risk of malignancy in these patients, since microallelotyping shows no allelic loss in transitional mucosa adjacent to colorectal neoplasms (Boland *et al*. 1995). However, such losses have been reported in normal breast tissue (Larson *et al*. 1998).

There is good evidence from rat chimaeras that the

preneoplastic nodules which give rise to hepatocellular carcinomas are clonal (Weinberg & Iannoccone 1988). Such experimental mouse hepatic tumours are also monoclonal, as assessed by the X-linked tissue-specific gene ornithine carbomyl transferase (Howell *et al*. 1985; Tsuji *et al*. 1988), In the human liver however, opinion is sharply divided concerning the clonality of lesions often regarded as preneoplastic: several studies have shown that while lesions such as benign adenomatous hyperplasia and focal nodular hyperplasia are polyclonal, hepatic adenomas and small (< 25 mm) hepatocellular carcinomas are clonal (Kawai *et al*. 1995; Paradis *et al*. 1997b). But Tsuda *et al*. (1988), examining the integration patterns of hepatitis B virus, claimed that atypical adenomatous hyperplasia was clonal, and similarly, Gaffey *et al*. (1996) considered that most focal nodular hyperplasias also appear clonal, as do genuine hepatic adenomas. Hepatocellular carcinoma is frequently multifocal (Sheu 1997) and whether these arise from a single clone or independently is controversial: Esumi *et al*. (1986, 1989) and Govindarajan *et al*. (1988) proposed a clonal origin for multiple tumours, but most authors agree that an independent origin is more likely (Aoki & Robinson 1989; Chen *et al*. 1989; Shimoda *et al*. 1990; Hsu *et al*. 1991; Ding *et al*. 1992; Tsuda *et al*. 1992; Sheu *et al*. 1993), including recurrences (Chen *et al*. 1989). But Yamamoto *et al*. (1999) have proposed that the finding of monoclonality in multiple hepatitis B-associated hepatocellular carcinomas indicates intrahepatic metastases from a primary tumour, while a polyclonal pattern suggests independent multicentric origins.

Thus the strategy of tumour development in the liver consists of two major sequences: (a) after induced liver damage, clonal selection occurs during regeneration (Wada *et al*. 1990). leading to the genesis of persistent benign focal proliferations, shown to be clonal nodules in the rat (Weinberg & Iannoconne 1988) and (b) the development of clonal hepatocellular cancer from one or more such nodules (Farber 1984, 1990; Weinberg *et al*. 1987; Aihara *et al*. 1994; Kawai *et al*. 1995) – in animals, such induced hepatocellular carcinomas are mainly clonal (Howell *et al*. 1985; Lee *et al*. 1991; Tsunashima *et al*. 1996). Thus in man there is substantial evidence from hepatitis B (Aoki & Robinson 1989; Yasui *et al*. 1992) and hepatitis C (Aihara *et al*. 1994) virus integration that between 0.5 and 43% of regenerative nodules in cirrhosis are monoclonal. This has recently been confirmed using X-inactivation analysis (Paradis *et al*. 1997a), where 54% of macronodules in cirrhotic livers were monoclonal: neither the size of the nodule, its histology or the aetiology of the process were correlated with its clonal status. This underlines the finding of loss of heterozygosity for the mannose-6-phosphate/IGF-II receptor locus in histologically normal nodules adjacent to hepatocellular carcinomas, again suggesting a clonal habitus (Yamada *et al*. 1997). This might suggest that large regenerative liver nodules showing a polyclonal pattern evolve into a clonal population, developing into hepatocellular carcinomas which are also clonal by X-inactivation, and differ from the nodules by 18q loss (Sakamoto *et al*. 1989; Kawai *et al*. 1995; Piao *et al*. 1997).

Pre-invasive lesions

It is usually held that the field cancerization hypothesis (Slaughter *et al*. 1953) predicts that multiple cells form independent epithelial tumours. Carcinogenic exposure affects multiple cells in the field, and second primary or synchronous tumours arise from independent genetic events. This concept originated from the observation that 11% of individuals with oral cancer had multiple upper aerodigestive tract tumours, and the finding of multiple invasive foci associated with overlying areas of *in situ* squamous carcinoma in such lesions. Indeed there is genetic evidence for such an independent origin: in the upper aerodigestive tract, multiple synchronous squamous tumours appear independent and multicentric in origin (Chung *et al*. 1993; Yang *et al*. 1995). This search for commonality in genetic lesions in multiple preinvasive or invasive lesions in the same patient has been a popular pursuit in those interested in trying to verify or falsify the field cancerization hypothesis. However, it has been proposed that the concept of clonal origin and expansion is problematic in organs where several metachronous tumours appear (Sidransky *et al*. 1992a), and a synchronous or second primary tumour may reflect recurrence or indeed lateral spread from a single tumour. Thus, although the field cancerization hypothesis would predict a multicentric, polyclonal origin, if indeed such tumours could be shown to be clonal, then lateral migration from the original clone would be a distinct possibility.

In fact, conflicting evidence for the nature of field cancerization comes from the study of tumours of the upper aerodigestive tract and adjacent mucosa (Bedi *et al*. 1996). In laryngeal and pharyngeal tumours, multiple samples taken at tumour-distant sites show different and independent mutations in the p53 gene, assessed by SSCP and direct sequencing (Chung *et al*. 1993; Yang *et al*. 1995), favouring a discontinuous, multifocal and polyclonal process of field cancerization, rather than migration of premalignant basal keratinocytes giving a clonal development of multiple primary, secondary or recurrent tumours (Gusterson *et al*. 1991). That this is not

the whole story is shown by an exhaustive karyotypic and FISH analysis of two synchronous but anatomically distinct oral squamous carcinomas, and their recurrences, which all contained a Y chromosome rearrangement, acting as a unique marker (Worsham *et al*. 1995). These lesions also contained the same aneuploid patterns in a number of chromosomes, indicating that the synchronous primary and recurrent tumours were of clonal origin.

But Sozzi *et al*. (1995) examining 3p deletions, p53 and k-*ras* mutations, also concluded that preneoplastic lesions, as well as the ensuing tumours of the bronchus, were genetically distinguishable and therefore independent in origin. In the stomach, discordant mutation patterns of *APC*, *MCC* and p53 were found in 12 out of 13 cases of multiple gastric carcinomas (Kang *et al*. 1997), again in accord with a multicentric origin, also seen in multiple colorectal tumours (Fearon *et al*. 1987), parathyroid adenomas (Arnold *et al*. 1988) and in separate prostatic intraepithelial neoplastic lesions, which show different clonal patterns of allele loss at 8p12–21, suggesting an independent origin (Emmert Buch *et al*. 1995; Bostwick *et al*. 1998).

However, while the field hypothesis has also been raised to account for multifocal ovarian and urothelial cancers (e.g. Mills *et al*. 1988), multiple synchronous carcinomas in the bladder and other pelvic organs apparently show a common clonal origin – X-chromosome inactivation and allele loss at 9q and 17p are identical, as are c-erB2 and p53 mutations in multiple synchronous urothelial tumours (Lunec *et al*. 1992). Diffuse carcinomas of the stomach, often apparently arizing multifocally and spreading diffusely through the gastric wall, are predominantly polyclonal (Bamba *et al*. 1998). In such a tissue as the peritoneum, where implantation is commonly seen, it is not possible to say whether the lesion spreads from a single site or started contemporaneously at multiple sites. The presence of multiple sites of occurrence of sporadic ovarian cancer on the ovarian surface and pelvic peritoneum has been advanced as a possible example of field cancerization change, although in fact most cases are clonal, as assessed by LOH, p53 mutations and X-chromosome inactivation analysis in the same cases (Jacobs *et al*. 1992; Li *et al*. 1993), or in a few cases, of dual primary origin. Multiple serous adenocarcinomas in the ovaries, peritoneum and endometrium showed the same p53 mutation (Mok *et al*. 1992; Kupryjanezyk *et al*. 1996) and clonal cytogenetic abnormalities (Pejovic *et al*. 1991), findings also confirmed by X-chromosome inactivation (Sawada *et al*. 1994).

In multifocal breast carcinomas, an increasingly common finding (Dawson 1993), karyotypically identical clones were detected (Teixeira *et al*. 1994; Pandis *et al*. 1995), indicating intramammary spread from a single carcinoma either by focal lymphatic spread or by intraductal spread. However, intra- and inter-focal karyotypic heterogeneity was also found, some of which were related clones, possibly evidence of clonal evolution from a single mutated cell, but other clones were unrelated. This finding was interpreted by Teixeira *et al*. (1994) as indicating a polyclonal derivation for at least a subset of breast carcinomas, and that clonal synergy might be necessary for tumour progression. But in rats, multiple methyl-nitrosourea-induced mammary carcinomas emerge from independently initiated cells and are each of clonal origin (Lu *et al*. 1998). However, in phyllodes tumours, widely separated deposits show the same monoclonal stromal component (Noguchi *et al*. 1993).

But if lesions some distance away from each other are clonal, how can this be explained? Of course there is always the possibility, always very difficult to exclude, that the disease process which causes the tumour to develop has a characteristic genetic fingerprint, which is seen in all examples of the tumour. The other possibility is that of a common mutated progenitor cell, and the spread of this mutant clone in some way, throughout the epithelium at an early stage in tumour development, How can this occur? An interesting link between the clonal origin of cancer and the field cancerization hypothesis is provided by the work of Franklin *et al*. (1997): examining p53 mutations in the nonmalignant but dysplastic tracheobronchial mucosa of a smoker (Sundaresan *et al*. 1992; Sozzi *et al*. 1995) by SSCP and direct sequencing, they found an G:C to T:A transversion in codon 245 in 7/10 sites in both lungs. The results were interpreted in terms of the expression and dispersion of a single mutant progenitor bronchial epithelial cell clone throughout the airways, a ‘novel mechanism for field cancerization’.

What are we to make of these disparate p53 studies? Clonal p53 mutations could of course be explained by the same carcinogen leaving a fingerprint mutation, and polyclonal p53 mutations by the mutation being a late event in aerodigestive tract tumourigenesis with migration of tumour cells before the development of the p53 mutations. But Sundaresan *et al*. (1992) and Chung *et al*. (1993) have argued that p53 mutations are early events in upper aerodigestive tract carcinogenesis, prior to the development of invasive lesions (cf. Sozzi *et al*. 1995), being found in premalignant lesions of the head and neck, lung and oesophagus. p53 mutations apparently do not show an increased incidence with cancer progression (Dolcetti *et al*. 1992), but do show clonal fidelity in a variety of tumours (e.g. Sameshima

et al. 1992; Sidransky *et al*. 1992b). Against this Boyle *et al*. (1993) report that p53 mutations increase with progression of head and neck cancer. Importantly, Chung *et al*. (1995) show that allele loss on 3p precedes p53 mutation in squamous metaplastic, dysplastic bronchial mucosa. Thus an early event, prior to p53 mutation, might establish a mutated clone, which migrates laterally, possibly aided by a mutation in a cell adhesion molecule (Worsham *et al*. 1995). In multiple bladder cancer, all tumours were found to have lost the same 9q allele as an early event, possibly a growth control or adhesion molecule, enabling cells to repopulate the urothelium by lateral migration or mucosal seeding (Sidransky *et al*. 1992a). Moreover, in most discontinuous foci of CIN3 in the cervix, individual lesions show the same X-chromosome inactivation pattern, suggesting intraepithelial spread (Enomoto *et al*. 1997). In the skin, it is not uncommon to see migration of morphologically abnormal cells laterally from a lesion such as Bowen's disease – the so-called Borst phenomenon (Pinkus & Mehregan 1986). But the multifocality of the bronchioloalveolar lung carcinoma, long thought to be due to intra-alveolar spread (Liebow 1960) has been shown by X-chromosome inactivation to be multiclonal, arguing for multiple independent tumours (Barsky *et al*. 1994). In fact, atypical adenomatous hyperplasias of the lung, postulated as a possible precursor lesion for bronchioloalveolar carcinoma, are individually monoclonal proliferations, and when multiple, also appear to arise from separate events (Niho *et al*. 1999). Multiple uterine leiomyomas also appear to arise independently, though individually clonal in origin (Mashal *et al*. 1994; Hashimoto *et al*. 1995). But the multiple lesions of disseminated peritoneal leiomyomatosis appear to derive from a clonal, unicentric origin (Quade *et al*. 1997).

Interestingly, it is claimed that the multiple deposits of Kaposi sarcoma, a widely disseminated malignancy, appear clonal in any one patient (Rabkin *et al*. 1995, 1997), interpreted as indicating a clonal origin and wide intravascular dissemination; other interpretations include an initial vascular hyperplasia, with later clonal evolution (Delabesse *et al*. 1997; Gill *et al*. 1997, 1998). Diffusely infiltrating gliomas are also clonal (Kattar *et al*. 1997), and multiple discrete meningiomas share clonal neurofibromatosis 2 (NF2) mutations (Stangl *et al*. 1997); most individual meningiomas appear clonally derived (Zhu *et al*. 1995). In the urothelium, identical point mutations in multiple synchronous and metachronous carcinomas were interpreted as being due to intraluminal seeding rather than intraepithelial spread (Habuchi *et al*. 1993). Additionally, in situations where migration might be considered unlikely (cf. pelvic peritoneum with multiple ovarian tumours), somatic genetic mosaicism has been proposed, where postzygotic mutations in the embryo are present at multiple sites – and possibly multiple organ systems (Kupryjanezyk *et al*. 1996).

Nodules, and nodular hyperplasia, in endocrine organs such as the thyroid, parathyroid, pancreas and adrenal glands present a special problem in clonality studies. In several endocrine organs, such as the thyroid and the parathyroid, the distinction between a nodule and a neoplasm is difficult to make on purely histological grounds. However, we have seen that, in mice, the majority of thyroid follicles are polyclonal in origin (Thomas *et al*. 1989). In humans, sporadic, multinodular goitre contain nodules which are generally regarded as being hyperplastic; consequently, we might expect that these lesions would be predominantly polyclonal (Harrer *et al*. 1998a,b), but there is substantial X-chromosome inactivation evidence that many of these nodules comprise clonal populations (Hicks *et al*. 1990; Namba *et al*. 1990; Aeschimann *et al*. 1992; Apel *et al*. 1995; Chung *et al*. 1999). However, it does appear that some nodules, including hyperfunctioning or toxic nodules, can be polyclonal (Namba *et al*. 1991). In these lesions, the presence of a TSH receptor mutation may be important (Krohn *et al*. 1998): 10/11 cases showed monoclonality on HUMARA analysis, but only 6/12 toxic nodules with no TSH receptor mutation are clonal, raising the possibility that during thyroid hyperplasia a cell with a mutation at this locus leads to the initiation of autonomous growth. In recurrent goitres, nodules are predominantly polyclonal (Harrer *et al*. 1998a). In several studies of clonality in multinodular goitre, the proportion of clonal nodules varies, and it is clear that clonal and polyclonal nodules can coexist in the same gland (Namba *et al*. 1990; Bamburger *et al*. 1993; Kim *et al*. 1998), with no apparent correlation with morphology, although clonal nodules might be larger (Kim *et al*. 1998). Multiple nodules in the same patient are mostly clonal, with activation of the same allele, possibly indicating intraglandular spread by follicular budding, although clonal nodules with different X-chromosome inactivation patterns can be seen in the same multinodular gland (Kopp *et al*. 1994). This could mean a different pathogenetic mechanism for clonal and polyclonal nodules, or indeed evolution of clonal from polyclonal nodules (Kopp *et al*. 1994), as generally proposed by Woodruff *et al*. (1982) and Alexander (1985). In animals, goitrogen-induced 'monomorphic adenomas' are clonal, but 'nodules' are polyclonal in X-chromosome mosaics (Thomas *et al*. 1989). In man, most follicular, papillary and anaplastic carcinomas are clonal (Fialkow 1976; Hicks *et al*. 1990; Namba *et al*. 1990; Fey *et al*. 1992). But while earlier glucose-6-phosphate

dehydrogenase allelotype studies showed a clonal origin of medullary carcinoma in MEN2 (Baylin *et al*. 1976, 1978), later X-chromosome inactivation studies have shown that such tumours, all of which showed mutations in exon 11 of *ret*, were polyclonal, whereas a case with no family history was monoclonal (Ferraris *et al*. 1997).

A similar pattern appears to exist where adrenocortical hyperplasias and neoplasms are concerned: ACTH-dependent hyperplasia with associated Cushing's syndrome shows a polyclonal pattern, while adenomas and carcinomas are clonal (Beuschlein *et al*. 1994). Glands in ACTH-independent adrenocortical hyperplasia contain polyclonal nodules, but a larger monoclonal nodule can emerge, suggesting again clonal evolution.

Parathyroid adenomas are monoclonal (Arnold *et al*. 1988; Noguchi *et al*. 1997), as indicated by X-chromosome inactivation and the identification of clonal abnormalities of the parathyroid hormone gene. This is against previous beliefs, based on studies with G6PD allelotypes (Fialkow *et al*. 1977; Jackson *et al*. 1982). In MEN 1, where all four parathyroids are enlarged and appear hyperplastic, allelic loss on 11q indicates that proliferation is clonal (Friedman *et al*. 1989), although when LOH on 11q was combined with X-chromosome inactivation studies, the parathyroid lesions in MEN1 were shown to be polyclonal, suggesting that multiple neoplastic clones grow, coalesce and replace the parathyroid gland (Lubensky *et al*. 1996). On the other hand, parathyroid hyperplasias, which either occur as a primary phenomenon or secondary to such conditions as chronic renal failure, were previously regarded as polyclonal proliferations (Arnold *et al*. 1988), but X-inactivation and allelic loss of 11q shows that 38% of primary parathyroid hyperplasias and 64% of hyperplasias secondary to renal failure are clonal, interpreted as neoplastic evolution from a pre-existing polyclonal hyperplasia (Falchetti *et al*. 1993; Arnold *et al*. 1995; Shan *et al*. 1997). However, Tominaga *et al*. (1996) reported that diffuse parathyroid hyperplasias in uraemia were polyclonal, but the individual nodules in nodular hyperplasia were clonal, indeed showing different clonal patterns of X-inactivation in the same gland, once more favouring the hypothesis that monoclonal proliferations evolve from hyperplasias. Parathyroid grafts, all taken from nodular glands, retained clonality. These findings favour the view that hyperplasia begins in endocrine glands as a polyclonal process, but then becomes a clonal hyperplasia, and the borderline between this phase and that of a benign clonal neoplasm is difficult to delineate. In benign pancreatic endocrine neoplasms, some 50% of lesions appeared polyclonal, and 60% of malignant tumours were shown to be clonal in derivation; these results were again interpreted in terms of such tumours being poly/oligoclonal neoplastic lesions which are eventually outgrown by a single, more aggressive clone with invasive and metastatic potential (Perren *et al*. 1998).

Invasive carcinomas of the cervix, which are clonal in origin (Enomoto *et al*. 1994; Park *et al*. 1996), are considered to originate in areas of cervical intraepithelial neoplasia (CIN). Severe dysplasia or CIN3 is also a clonal proliferation, although lesser degrees of dysplasia (CIN2) appear more commonly polyclonal in X-inactivation studies (Park *et al*. 1995, 1996; Enomoto *et al*. 1994), with a strong correlation between HPV subtype and clonality. In this respect, some vulvar hyperplasias, considered to be preneoplastic lesions in this tissue, appear clonal: the derivative VIN and invasive carcinomas are also clonal proliferations (Tate *et al*. 1997), but in a smaller series, Kim *et al*. (1996) showed that, while invasive carcinomas are indeed clonal, adjacent squamous hyperplasias were polyclonal and did not show p53 mutations in instances where p53 in the tumour was mutated. While VIN itself was not apparently examined, Kim *et al*. were doubtful about the provenance of VIN as a preneoplastic lesion in a proportion of cases. The squamous hyperplasias associated with HPV-negative carcinomas may also not be direct precursor lesions, based on clonal analysis (Young-Tak *et al*. 1996). In nasophayngeal carcinoma (NPC), a combination of X-inactivation studies (PGK), X-linked RPLPs, and EBV integration shows that carcinomas were mainly clonal (Jiang & Yao 1996). However, hyperplastic epithelia, and early atypical hyperplastic epithelia are polyclonal, and clonality emerges only at the moderate/severe dysplastic stage; only NPCs were EBV-positive. At this stage, i.e. severe atypia, p53 (Sheu *et al*. 1995) and bcl-2 (Lu *et al*. 1993) mutations appear, and clonality is established.

A relationship between intestinal metaplasia and carcinoma of the stomach has long been suspected, possibly as a direct precancerous lesion, as an epiphenomon caused by the same mutation(s) in tumourigenesis, or possibly the sharing of a carcinogenic milieu. Since intestinal crypts are clonal in both animals (Ponder *et al*. 1985) and man (Endo *et al*. 1995; Novelli *et al*. 1996), it would be anticipated that intestinal metaplastic crypts would also be clonal (Slack 1985). They are not, even in human pyloric mucosa, where HUMARA analysis indicates a clonal origin for gastric glands (Nomura *et al*. 1996). About 50% of intestinal metaplastic glands are heterotypic or polyclonal (Nomura *et al*. 1998b); if we can disregard stromal contamination or white cell insudation, or X chromosome inactivation not being determined in stem cells until after the formation of gastric glands, then intestinal metaplastic glands are polyclonal and contain

multiple, independent, clonally derived cell lineages. If body-type glands are indeed polyclonal (Nomura *et al.* 1996), then a metaplastic change in such glands could well be polyclonal, but in the antrum, where gastric glands are clonal in origin? Such polyclonal crypts were explained by the presence of dormant stem cells, while the other possibility entertained, of gastric and intestinal cells in a single gland, which does occur (Silva *et al.* 1990), would not explain the phenomenon, since, in the antrum, both lineages would be of the same clonal origin. In addition, 11/13 metaplastic areas, each 6 mm in diameter, contained glands of different clonal derivation, suggesting that clonal expansion, at least in intestinal metaplasia, does not occur by the gland fission process proposed for the colonic mucosa (Garcia *et al.* 1999). But several groups have reported mutations in oncogenes and tumour suppressor genes in intestinal metaplasia including evidence for clonal expansion of cells showing the same p53 mutation (Ochiai *et al.* 1996). It is possible that the process of field cancerization in the gastric mucosa is in fact intestinal metaplasia.

Carcinoma in Barrett's oesophagus arises from dysplastic changes in metaplastic epithelium that replaces the stratified squamous epithelium of the lower oesophagus. While the nature of this metaplastic change is disputed – whether it is a unique, specific change or related to intestinal metaplasia – the nondysplastic metaplasia adjacent to carcinomas is clonal on X-inactivation analysis (HUMARA), and also shows LOH for APC, changes also seen in the dysplastic and neoplastic tissues of Barrett's oesophagus (Zhuang *et al.* 1996). In the context of a field change, it is not yet clear if the *APC* change is clonal, but microsatellite analysis shows allelic imbalance not only on 5q but at multiple other sites, in both premalignant and malignant Barrett's epithelium, supporting the concept of clonal expansion from metaplasia through dysplasia to carcinoma (Gleeson *et al.* 1998).

In conclusion, it is true to say that there are preneoplastic changes which appear to be clonal but may not be neoplastic and that clonality in many instances is derived quite early on, before the emergence of severe dysplastic changes.

Clonality in established tumours – conflicting evidence

Tumours are clonal in origin

Earlier reviews (Iannaccone *et al.* 1987) of experimental models of chemically induced neoplasms in mouse chimaeras showed that benign and malignant neoplasms, as well as precursors of these neoplasms, arise from the clonal expansion of a single transformed cell, and very few studies demur (Woodruff 1988). But we should note here the constraints of Williams & Wynford-Thomas (1994), who point to the considerable variation in patch size in these models and the possible differences in susceptibility to carcinogens between the two strains; possibly models where mice are heterozygous for a defective X-linked gene (Howell *et al.* 1985) or X-linked PGK polymorphisms (Tsunashima *et al.* 1996) are more reliable. More recently, Tatematsu *et al.* (1996a), using C3H/Balb/c chimaeras and a C3H strain-specific antigen (CSA) claim that the preneoplastic hyperplastic changes in the squamous forestomach of chimeric mice treated with methylnitrosourea (MNU) or diethylnitrosamine are polyclonal aggregates from which monoclonal squamous cell carcinomas are derived. Polyclonal carcinomas, which occurred at low incidence (2/62) during progression, were dismissed as being due to two or more lesions coalescing. The patch size is considerable, not measured, but up to 5 rete pegs in diameter (see Figure 2). But in focal epidermal hyperplasias initiated with DMBA or acetone in CBA/C57Bl chimaeric mice, and promoted with TPA or DMBA, some 20% were polyphenotypic, using anti-Hsk and H2b antibodies as discriminating markers (Winton *et al.* 1989). Of the early papillomas which arose from these preneoplastic lesions, some 25% appeared polyclonal: this would be a minimum value, recalling the *caveat* about patch margins and patch size, which was about 100 basal cells in diameter.

Using the same marker, Tatematsu *et al.* (1994) also showed that experimentally induced adenomatous hyperplasias and carcinomas in the mouse stomach were apparently clonal proliferations, although later, clones emerged in the carcinomas which displayed different differentiation features. Furthermore, Tatematsu *et al.* (1996b) found only one polyclonal adenoma in 91 adenomas and 12 polyclonal adenocarcinomas in a total of 119 colon tumours induced by dimethylhydrazine in chimaeric mice, results interpreted in terms of a clonal origin of colonic adenomas: the mixed tumours were all explained by coalescence. Previous results with azoxymethane (Ponder & Wilkinson 1986) indicated that small, carcinogen-induced dysplastic foci in chimaeric mice were monoclonal, although 5/17 larger lesions were polyclonal, explained as collision tumours. These observations led Farber (1997) to conclude that individual cancers are derived initially or early on from single cells showing monoclonal growth.

In the human, there are many articles claiming a clonal origin for tumours (see review by Fialkow 1976). In more

recent years, X-linked restriction fragment length polymorphisms showed 50 human colorectal tumours, adenomas and carcinomas, of both familial and sporadic type to be monoclonal (Vogelstein *et al*. 1985, 1987; Fearon *et al*. 1987). However, because the tumours were of sufficient size to allow microdissection, these results are consistent either with a true clonal origin, or a polyclonal proliferation with later outgrowth of a dominant tumour clone. The ability to use X-linked polymorphic markers for the HUMARA or PGK genes in microdissected, paraffin-embedded tissues has again shown apparent clonality in many tumours and tumour-like lesions: sclerosing haemangioma of the lung (Niho *et al*. 1998), with both pale and cuboidal cells sharing the same clonal origin; most gynaecological cancers – cervix, endometrium, fallopian tube and ovary appear clonal (Jacobs *et al*. 1992; Enomoto *et al*. 1994), including pseudomyxoma peritonei, which however, appears usually associated with a mucinous adenoma of the appendix rather than the ovary (Szych *et al*. 1999). Leiomyomas of the uterus (Nilbert & Strombeck 1992; Mashal *et al*. 1994; Hashimoto *et al*. 1995) and Wilm's tumour (Vogelstein *et al*. 1987; Wilimas *et al*. 1991) also appear to be clonal proliferations. Most pituitary adenomas are clonal in X-inactivation studies (Alexander *et al*. 1990; Herman *et al*. 1990), and gastric carcinomas are also reported as clonal (Cho *et al*. 1994). Immature teratomas are clonal by X-inactivation analysis (Sinnock *et al*. 1996), while malignant teratoma in men contains the i(12p) clonal marker in all derived malignant lineages (Motzer *et al*. 1998); combined seminomatous/nonseminomatous germ cell tumours in males are also clonal (Gillis *et al*. 1994). Basal cell carcinomas are clonal by X-inactivation (Walsh *et al*. 1996, 1998) and by the demonstration of a 'founder' p53 mutation in multiple samples from the same tumour (Ponten *et al*. 1997).

Mixed tumours, which consist of both epithelial and stromal components, have for some time generated considerable debate about their histogenesis. Carcinosarcomas pose a special and interesting problem in clonality (Wick & Swanson 1993). The available evidence, from commonality of p53 protein immunohistochemistry in both malignant lineages, would support the same clonal origin for both (Mayall *et al*. 1994), providing objections, such as the possible unreliability of immunohistochemistry in detecting mutated p53, or the presence of different mutations, or indeed no mutations at all being present, can be met (Williams & Wynford-Thomas 1994). However, cell disaggregation of carcinosarcomas, with consequent cell sorting and clonality determination by the X-linked HPRT gene also showed the same clonal origin for both lineages (Thompson *et al*. 1996). Further X-inactivation and cytogenetic studies on carcinosarcomas from multiple sites (Thompson *et al*. 1996), from the pancreas (Millis *et al*. 1994) and from the breast (Zhuang *et al*. 1997; Teixeira *et al*. 1998) confirm the same clonal origin for both components. An oesophageal carcinosarcoma showed the unusual production of granulocyte colony stimulating factor in both components, suggesting a common origin (Ota *et al*. 1998). Zhuang *et al*. (1997) noted co-ordinated LOH for int2 and NM23 in the epithelial and stromal components of a breast carcinosarcoma, and the finding of the same clonal pattern in the *in situ* epithelial component favours the view that the epithelial component contains the progenitor cell. Finally, we should note that mixed Mullerian tumours in the female genital tract show the same clonal p53 mutation in all components (Kounelis *et al*. 1998).

In other apparently mixed tumours, such as pulmonary chondromatous hamartomas, combined chromosome banding and immunohistochemistry on disaggregated tissues revealed that only the stroma possessed a clonal cytogenetic abnormality, and thus was possibly neoplastic (Fletcher *et al*. 1993). Paraganglionomas, a biphasic tumour which often shows a familial element and allelic imbalance on 11q, appear polyclonal on X-inactivation analysis (Devilee *et al*. 1994). We have seen that gliomas are themselves clonal (Kattar *et al*. 1997), and furthermore, biphasic gliomas are clonal (Kattar *et al*. 1997) as are gangliogliomas (Zhu *et al*. 1997).

Mixed tumours of the breast such as fibroadenomas, have presented special problems: several studies have shown karyotypic abnormalities (e.g. Calabrese *et al*. 1991; Dietrich *et al*. 1995), but opinion is divided as to where these occur – either in the epithelial (Dietrich *et al*. 1995) or stromal component (Belda *et al*. 1993; Fletcher *et al*. 1993). In the related phyllodes tumour, where the stromal component is cellular and can be atypical in appearance, the stroma and not the epithelium shows clonal expansion (Noguchi *et al*. 1993). The same PGK technique applied to fibroadenomas showed that both epithelium and stroma were polyclonal, which is really inconsistent with the claim that such lesions develop from a single lobule if patch size in the breast comprises the terminal ductal-lobular unit (Tsai *et al*. 1996). However, others (Dietrich *et al*. 1994; Petersson *et al*. 1997) have demonstrated deletion of 3p and gain of 1q in the epithelial portion of phyllodes tumours, and multiple cytogenetically abnormal and unrelated clones have been identified in breast fibroadenomas (Stephenson *et al*. 1992; Diettrich *et al*. 1995).

Tumours are polyclonal in origin

Not everyone has accepted uncritically the clonal origin of neoplasms, insisting instead that carcinogenesis requires the interactions of multiple cells (Rubin 1985; Alexander 1985), with outgrowth of a dominant clone during subsequent development. Others have pointed to the marked heterogeneity of tumour cell populations in regard to both their morphology and metastatic behaviour (Poste *et al*. 1981), notwithstanding the view that such phenotypic diversity could develop within a clonal population (Marsh *et al*. 1993). Indeed we have seen that some recent studies using aggregation chimeras support the concept of a polyclonal origin: the earliest morphologically recognizable stages of development of mouse skin papillomas induced by a chemical initiation-promotion regime are polyclonal, suggesting that interaction between cells of more than one clone is involved (Winton *et al*. 1989).

Mammary tumours and hyperplasias arising in mice appear to be clonal populations (Cardiff & Aguilar-Cordova 1988; Kordon *et al*. 1995; Kordon & Smith 1998), and we have seen above that the preneoplastic lesions in the breast and breast cancer itself in man also appear to be clonally derived (Noguchi *et al*. 1992). However, cytogenetic analysis of multifocal and bilateral breast carcinomas showed independent origins (Shibata *et al*. 1996a), and indeed unrelated clones, prompting the suggestion that breast carcinoma may be polyclonal (Teixeira *et al*. 1994; Pandis *et al*. 1995). It is surprising how often the pattern of chromosomal aberrations in breast cancer is nonrandom: in fact, cytogenetically unrelated clones are found in some 50% of breast cancers (Heim 1992; Pandis *et al*. 1993, 1995; Teixeira *et al*. 1994, 1995) and include all the three most common breast cancer deletions – del(1 : 16, i(1q) and del(3p). Most breast cancers are cytogenetically complex when this is related to anatomical site (Heim *et al*. 1997), and because proliferative and preneoplastic breast lesions also contain similar unrelated clones, it maybe that such clonal divergence is an early phenomenon (Heim *et al*. 1997).

Now, does the finding of multiple unrelated clones in breast cancer imply a polyclonal origin of the tumour? Well, Heim *et al*. (1997) and Teixeira *et al*. (1996) point to the result that when no histological evidence of tumour is visible, then no chromosomal abnormalities are present in mammary epithelia, but we have seen that histologically normal breast tissue can show LOH at potential tumour suppressor gene loci (Larson *et al*. 1998). Thus somatic cell mosaicism is present in normal breast and could, of course, be the origin of the independent clones by clonal divergence. Are the clones really unrelated? Heim *et al*. (1997) emphasize the evidence that breast cancers show a far higher incidence of cytogenetic polyclonality than haematological (Heim & Mittelman 1989) or mesenchymal neoplasms (Orndal *et al*. 1994). X-inactivation demonstrations of clonality (Noguchi *et al*. 1992) with skewing towards one X chromosome was regarded as being 'eminently compatible with multiple clones of various size', and clonal proliferation of cells as shown by X-inactivation could occur with different oncogene or tumour suppressor gene mutations, even in premalignant cells as part of field cancerization (Chung *et al*. 1993). Thus if X-inactivation is an early marker of clonal relationships, later genetic events may then show disparate patterns in a tumour(s) which are still clonally related (Fey & Tobler 1996). Heterogeneity in a marker (cf. clonal LOH demonstrated by microsatellite analysis) is no unequivocal demonstration of independent clonal origin, since such genetic heterogeneity could mark clonal relationships as subclones (Sidransky *et al*. 1992a; Fey & Tobler 1996). Thus molecular heterogeneity within and between lesions, as a result of clonal evolution, does not predict clonal independence.

However, an interesting development is the recent study of an acinic cell tumour, a low grade salivary carcinoma, which might provide at least a partial answer to this problem. Subcultures containing two cytogenetically independent clones were shown to have different X-chromosome inactivation patterns, showing that the clones must have originated from different cells (Jin *et al*. 1998): true demonstration of a polyclonal origin? But in gastrointestinal cancers showing a uniform X-inactivation pattern, LOH at 18q and clonal microsatellite mutations which differed markedly between defined areas would be expected on the basis of clonal evolution, urging a note of caution, at least where these markers are concerned (Nagel *et al*. 1995). Heterogeneity in c-k-*ras* mutations in multiple regions of the same tumour occurs in human adenomas in that substantial areas do not show mutated k-*ras*, but interestingly, this is not seen in carcinomas, which are uniformly positive, and also show mutated p53 (Shibata *et al*. 1993). Adenoma formation may include a stage in which multiple and genetically distinct neoplastic clones are present, while most carcinomas appear to have a homogeneous composition that results from the successful progression of one clone. This concept has been recently reinforced by the observation that clonal divergence and DNA aneuploidy seems to take place during the transition from adenoma to carcinoma (Tollenaar *et al*. 1997).

MMR deficient mice have been generated by targeted

gene disruption (De Wind *et al*. 1998) of the *Msh2* gene: introduction of the *Apc* ^{min} allele leads to the development of intestinal tumours with loss of the wild type *Msh2* allele in a significant fraction of the *Apc*+/^{min}/*Msh*+/− mice, and some areas of the same tumours showing loss of the *Msh*+/+allele but not of the *Apc*+ allele (De Wind *et al*. 1998). Other areas were Msh2 positive and showed LOH for *Apc*. These findings indicate a biclonal origin for these tumours, arguing against speculation (Jass *et al*. 1994), with loss of *Apc* and Msh2 in separate ancestor cells. Since only three tumours were found per animal, coalescence is an unlikely event, and suggests that RER-and RER + tumour areas develop interdependently and have a different clonal origin, rather than loss of MMR being a late event in the genesis of HNPCC. Again this biclonality was interpreted as showing a requirement for collaboration between independent tumour clones during carcinogenesis. In this respect, Palacios (1984) has interestingly hypothesized that bi-clonal interaction, analogous to that underlying embryogenesis, could be the origin of the earliest malignant phenotype.

These results have recently been confirmed (Merrit *et al*. 1997) using a model in which a chimaera was made from a *Min* mouse, which has an *Apc* mutation, and a *Min*/ROSA mouse, allowing the clonality of the resulting intestinal tumours to be assessed; 79% of adenomas were polyclonal. Moreover, there was evidence that the neoplastic proliferations lost the wild-type *Apc* allele in both lineages: both quantitative PCR for the *Min* region of *APC* and immunohistochemistry for the *APC* gene product showed that all cell lineages within the polyclonal adenomas had lost APC expression. This is in contrast to the findings of Bjerknes *et al*. (1997) who reported that adenomatous crypts from FAP patients contained a mixture of APC + and APC-cells. Thus in these mouse models, adenomas appear distinctly polyclonal, unlike chemically induced lesions.

In the patient reported by Novelli *et al*. (1996) the colon contained hundreds of adenomas, ranging in size from monocryptal adenomas to microadenomas of 2.5 mm in diameter (Figure 4a,b); no larger adenomatous polyps were seen. If an adenoma were of clonal origin, all dysplastic crypts within it would be expected to be entirely *XO* or entirely *XY*. NISH showed that monocryptal adenomas were entirely *XO* or *XY*, with no mixed pattern (Figure 4a); this is perhaps not surprising, given the clonal nature of normal crypts. However, many adenomas – 76% – were polyclonal. Isoenzyme studies of G6PD in black females have shown that colonic adenomas from three patients with Gardner syndrome were polyclonal (Hsu *et al*. 1983), while X-linked RFLPs showed that both spontaneous and familial adenomas were clonal (Fearon *et al*. 1987); the minimum size of the adenomas in this latter study was 3–4 mm, much larger than the very early lesions examined by Novelli *et al*. (1996), which might explain the discrepancy, through monoclonal conversion.

Novelli *et al*. (1996) and Merritt *et al*. (1997) have speculated on possible mechanisms of polyclonality in these lesions: 'field' effects may cause adenomas to cluster (nonrandom collision), a passive process involving fusion of two or more *APC*-negative clones early in tumour development. The high frequency of mixed adenomas found in both these studies is inconsistent with a random appearance of *APC*-negative clones in the mucosa, and suggests instead that some regions of the intestine have an increased potential for initiation. Multiple adenomatous clones may be required for (or strongly favour) early adenoma growth; or perhaps more likely, early adenomas may induce adenomatous growth in surrounding crypts (this may apply especially in FAP since all cells already have a single *APC* mutation and perhaps some derangement of APC function). These latter scenarios imply active co-operation between clones.

The importance of these results may have basic implications for more generalized models of tumorigenesis (Merritt *et al*. 1997). If in excess of one crypt must be involved to produce an adenoma, this would predict that not one but two hits would be required in FAP and four hits in sporadic adenomas. We should perhaps note that adenomas in FAP do not make their appearance until several years after birth, even in an environment where dietary carcinogens are common, and a two or three-hit model does not fit the age incidence for colorectal cancers (Moolgavkar & Luebeck 1992). This hypothesis would suggest that at least four hits were required. In respect of FAP, it is of note that desmoid tumours, which contain an APC mutation (Sen Gupta *et al*. 1993) are clonal (Lucas *et al*. 1997) as are desmoid fibromatoses (Li *et al*. 1995).

The concept of interactions between different neoplastic clones as a prerequisite for tumour development has been underlined by Muller *et al*. (1988), in an analysis of multiple preneoplastic or neoplastic lesions in transgenic mice, to assess the stepwise progression of carcinogenesis in mammary epithelium. Unlike the stochastic occurrence of solitary mammary tumours in transgenic mice bearing the MMTV/c-*myc* or the MMTV/v-Ha-*ras* oncogenes, transgenes uniformly expressing the MMTV/c-*neu* gene, an activated c-*neu* oncogene driven by a mouse mammary tumour virus (MMTV), develop mammary adenocarcinomas that involve the entire epithelium in each gland. These tumours arise synchronously and

are polyclonal in origin. In contrast, expression of c-*neu* transgene in the parotid gland or epididymis leads to benign, bilateral hypertrophy and hyperplasia which does not progress to full malignant transformation during the observation period. These results indicate that the combination of activated oncogene and tissue context are major determinants of malignant progression and that expression of the activated form of c-*neu* in the mammary epithelium has particularly deleterious consequences.

Clonal evolution of epithelial tumours and its relationship to determination of tumour clonality

The concept of sequential mutations arizing in tumour cells as neoplastic progression proceeds is now well-engrained in our consciousness (Vogelstein *et al*. 1987; Jen *et al*. 1994), This concept of clonal evolution (Nowell 1976, 1986) has profound implications for our perceptions of tumour clonality, both from the viewpoint of possible regression to monoclonality through clonal proliferative advantage (Woodruff *et al*. 1982) and for clonal divergence (Symmans *et al*. 1995). Thus single tumours studied during sequential recurrence, while retaining the changes seen *ab initio*, develop multiple karyotypic abnormal clones. We have already noted how this genetic heterogeneity does not predict clonal independence, and the coexistence of these clones of sequentially mutated cells (Figure 1) give insights into the mechanisms of tumour heterogeneity (Heppner 1984; Fey & Tobler 1996) particularly where the distribution of subclones is concerned. A corollary of this is that clonality assessment is constrained to the time of analysis, and the clonal composition may of course change in the course of clonal evolution (Van Elsas *et al*. 1995; Balaban *et al*. 1986; Orndal *et al*. 1993; Fey & Tobler 1996; Jin *et al*. 1998).

It is very clear that clonal evolution occurs in tumours as they become invasive and metastasize (Gotoh *et al*. 1998; Hovey *et al*. 1998; Pandis *et al*. 1998). At its simplest level, the incidence of aneuploid stem cells increase from 30% in small colorectal adenomas to 82% after invasion (Tollenaar *et al*. 1997), while larger adenomas also show the development of cytogenetically distinct subclones (Bomme *et al*. 1998). This probably reflects the distribution of p53 mutations in these lesions, i.e. in high grade dysplastic and invasive lesions, since immunohistochemically detectable p53 is correlated with an increased frequency of aneuploid stem lines (Carder *et al*. 1995), indicating that clonal p53 mutation precedes clonal divergence in colorectal tumour progression. Microsatellite LOH analysis for 5q, 17p and 18q also indicates a rapid clonal expansion at this stage (Boland *et al*. 1995): most early adenomas with mild dysplasia show LOH for 5q, reflecting *APC* allele loss, with a few showing 18q allele loss, but it is not until the development of high grade dysplasia that 17p allele loss, indicating p53 allele loss, was detected. This reflects abrupt waves of clonal expansion during adenoma development, with clonal homogeneity (for 5q in early adenomas, but clonal heterogeneity with widespread allelic instability in lesions with high grade dysplasia, Young *et al*. 1993). However, where the mutator phenotype is concerned, greater genetic diversity can be expected in older regions of the tumour (Shibata *et al*. 1994, 1996b; Shibata 1998). Rapid clonal expansion is thus associated with relatively homogeneous microsatellite alleles, whereas older lesions should show a higher number of alleles – while considerable diversity was seen in a colorectal carcinoma, the antecedent adenoma showed greatest variation.

Allele loss seen in the clonal ductal carcinoma *in situ* (DCIS) in the breast is shared by the derivative and clonal invasive and metastatic component (Noguchi *et al*. 1994b; Radford *et al*. 1995), strongly suggesting a clonal relationship between these lesions, but other changes, cf. LOH on 11p, can be restricted to the invasive component; such observations obviously should not be interpreted in terms of a polyclonal process.

While early mutations might be expected to be present in all alleles, mutations confined to subclones might therefore be restricted to particular geographical areas: implicit is the concept that regional (in the tumour that is) separation of subclones occurs (Ajiki *et al*. 1994), a situation which does not occur in prostatic carcinoma, where cytogenetic heterogeneity is a prominent feature but clonal cytogenetic aberrations are apparently confined to advanced prostatic carcinomas (Micale *et al*. 1992; Henke *et al*. 1994). Both the overall LOH rate and the mean fractional allele loss are lower in grade 1 compared with grade 2 and 3 areas in prostatic cancers, with increased frequency of loss of Rb, DCC, BBC1, and p53 in grade 2 and 3 areas, suggesting clonal development (Hugel & Wernert 1999). Using chromosome 1 probes and FISH, Alers *et al*. (1995) showed not only intraregional but intraglandular cytogenetic heterogeneity, reflecting prominent interspersion of these subclones.

Any one colorectal tumour (Carder *et al*. 1995) shows a clonal p53 mutation, and this may favour the emergence of aneuploid subclones in carcinomas. The overgrowth of low-grade astrocytomas by cells with acquired p53 mutations, already present in such low grade lesions, is associated with movement to a more aggressive glioblastoma habitus (Fults *et al*. 1992; Leenstra *et*

al. 1992; Sidransky *et al*. 1992b; Tada *et al*. 1996). The pattern of p53 mutations in any one advanced gastric carcinoma is also constant (Cho *et al*. 1994), although in basal cell carcinomas, subclones arise with a second or even a third p53 mutation (Ponten *et al*. 1997), explained again by clonal evolution rather than polyclonality, since all samples from the same tumour possessed a unique 'founder' mutation in p53. Clonal evolution in squamous carcinoma of the lung shows additional and different p53 mutations arising over a 9-month period (Chung *et al*. 1996), also seen in some colorectal carcinomas (Kuwabara *et al*. 1998). The distribution of k-*ras* mutations in colorectal adenomas is heterogeneous, but in invasive lesions becomes homogeneous, reflecting clonal evolution during growth (Shibata *et al*. 1993, 1994; Dix *et al*. 1995), but variation is also seen in k-*ras* mutations in adenomas and the resulting carcinoma (Ohmura & Hattori 1996). Thus a serious implication for clonal studies in tumours is the possibility of mutation in a marker gene, changing its nature or expression in a subclone, and indicating polyclonality in a clonally derived population, or indeed leading to apparent monoclonal regression, both seen in experimental (Griffiths *et al*. 1988) and human studies (Beutler *et al*. 1967; Williams & Wynford-Thomas 1994).

Conclusions

It is difficult to come to a firm general conclusion. While most evidence does favour the single cell origin of human tumours, many investigators have been less than critical in their experimental analysis, have ignored aspects of basic tissue architecture, and have often over-interpreted the data. On the other hand, protagonists of polyclonality have often ignored clonal evolution as a concept. The relationship between the clonal architecture of normal tissues, preneoplastic and preinvasive lesions, and their ensuing tumours is complex and difficult to analyse. This review underlines the need for the development of more general markers that can be used to visualize these relationships directly in space and time.

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