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Review

Many different tumor types have polyclonal tumor origin: Evidence and implications

Barbara L. Parsons *

Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, HFT-120, 3900 NCTR Road, USFDA, Jefferson, AR 72079, United States

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ABSTRACT

Few ideas have gained such strong acceptance in the scientific community as the monoclonal origin of tumors; the idea that tumors start with a single mutated cell (or a single clone of cells) that go on to accumulate additional mutations as a tumor develops. The certainty with which this concept is held by the scientific community reflects the length of time it has been unchallenged and the experimental difficulty in obtaining direct evidence to the contrary. Yet, recent findings regarding X chromosome inactivation patch size indicate that the X-linked marker data previously interpreted as evidence of monoclonal tumor origin is actually more consistent with polyclonal tumor origin, a situation where two or more cells or clones of cells interact to initiate a tumor. Although most tumors show homotypy for X-linked markers (as expected given the bias conferred by X chromosome inactivation patch size), the literature contains numerous examples of tumors with X-linked marker heterotypy, examples of which encompass 24 different tumor types. Chimeric models have yielded direct unequivocal demonstrations of polyclonality in rodent and human tumors. Also, mutational data are consistent with polyclonal tumor origin. Methods that analyze levels of tumor-associated oncogene and tumor suppressor gene mutations demonstrate that initiated cells are much more common in normal tissues than previously realized. Also, while tumors have higher levels of mutation than normal tissues, oncogenic mutations frequently are present as subpopulations within tumors, rather than as the pure mutant populations expected to develop from a single initiated cell. Understanding the mutational basis of tumor etiology has important practical significance for assessing cancer risk, as well as in modeling and treating cancer. Therefore, the scientific community needs to re-examine this issue and consider the implications of polyclonal origin for, perhaps, a majority of tumors, encompassing many different tumor types.

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* Tel.: +1 870 543 7946; fax: +1 870 543 7393.
E-mail address: barbara.parsons@fda.hhs.gov.

Abbreviations: ACB-PCR, allele-specific competitive blocker polymerase chain reaction; ACF, aberrant crypt foci; APC, *adenomatous polyposis coli*; CML, chronic myelocytic leukemia; DGGE, denaturing gradient gel electrophoresis; FAP, Familial Adenomatous Polyposis; G-6-PD, glucose-6-phosphate dehydrogenase; HPRT, *hypoxanthine-guanine phosophoribosyltransferase*; *HUMARA*, human androgen receptor gene; Ig, immunoglobulin; MF, mutant fraction; RFLP, restriction fragment length polymorphism.

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1. Introduction

Monoclonal tumor origin refers to the idea that all cells within a tumor can be traced back to a single progenitor cell (Fig. 1A). The monoclonal origin of cancer is commonly accepted as fact. This tenet of cancer biology has become so entrenched within the scientific community that the data upon which this conclusion is based is no longer presented in the textbooks used in medical and graduate education. Many scientists are unaware that the data that led to the acceptance of the monoclonal origin of tumors have been re-interpreted in a manner that nullifies the original conclusions. Furthermore, positive evidence of tumor polyclonality has been accumulating. Polyclonal tumor origin refers to the idea that two or more different progenitor cells or clones of cells cooperate in the genesis of a tumor (Fig. 1B). While the acceptance of polyclonal tumor origin has been expressed in at least two editorials and one review on bladder cancer [1–3], the data supporting this mechanism have not been systematically collected and scrutinized for the purpose of refuting the generalized acceptance of monoclonal tumor origin. Thus, the main focus of this review is to re-evaluate the data from which the nature of the very earliest stage of tumor development (tumor origin) can be inferred. While it is understood that not all tumors have to be either monoclonal or polyclonal, both types of tumor origin may exist, any single tumor has to have been derived from either a single cell lineage (one cell or a clone derived from one cell) or from two or more cell lineages (two or more cells or two or more clones of cells). Distinguishing whether tumors arise from a single mutated cell or single clone of cells (monoclonal) or from two or more cells or clones of cells (polyclonal) is at the core of this review. Furthermore, this review is intended to argue that the monoclonal origin of tumors is an idea held with a certainty not supported by the literature, to point out how the categorical acceptance of this idea may be impeding progress in cancer research, and to stimulate investigation into the earliest events in tumor development, including the potential interaction between multiple mutant clones. This review does not deal with the large literature characterizing the relationship between multiple synchronous tumors, where genetic markers are investigated in order to distinguish the spread of tumor cells through a tissue from the development of multiple independent tumors from a field of cells with underlying genetic lesions (field cancerization).

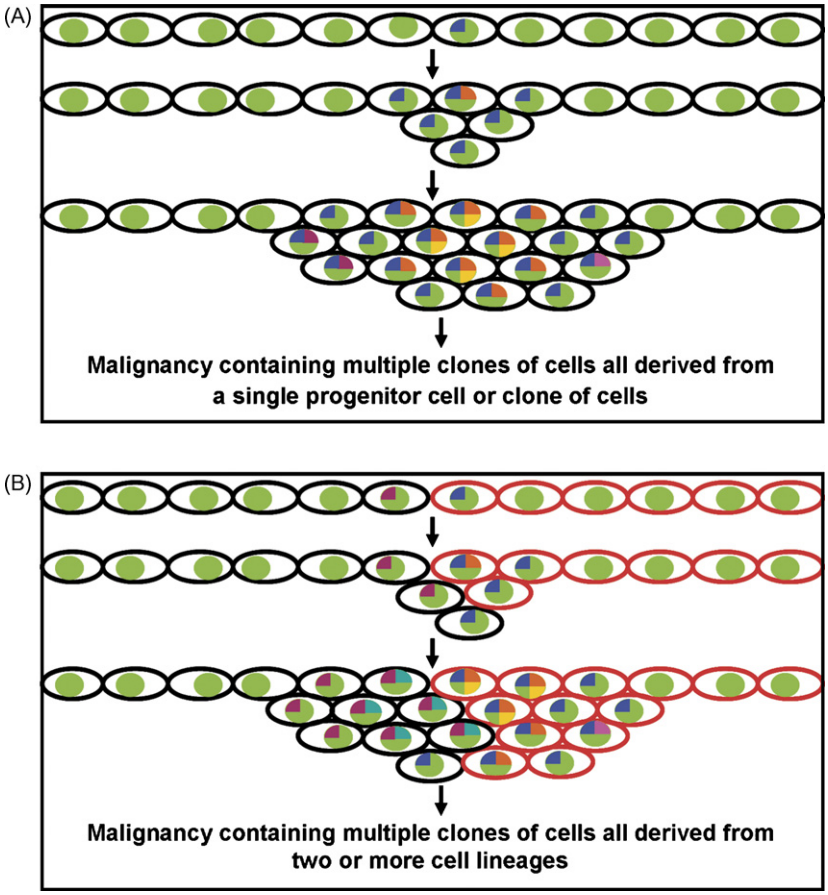

Fig. 1. Schematic representation of the initial events in carcinogenesis. Monoclonal (A) and polyclonal (B) tumor origins are depicted. The outline of the "cells" is colored (red or black) to represent a cell lineage marker independent of the carcinogenic process, like X-linked glucose-6-phosphate dehydrogenase activity or methylation at an X-linked locus. Nuclei are depicted as green circles. Genetic lesions are denoted by the different colored inserts in the nuclei. Theoretically, genetic lesions could include epigenetic changes, as well as different types of mutations (point mutations, insertions, deletions, translocations, etc.). According to monoclonal tumor origin (A), all the cells of the nascent tumor are derived from a single cell carrying the initial genetic lesions (indicated by the blue sector). Accumulation of additional mutations in this cell (or the daughters of this cell) follows, producing a clone of cells with multiple mutations whose propagation is favored (cells with blue, orange, and yellow sectors). Such multiply mutated cells are thought to develop into a malignancy, but tumors are heterogeneous because clones of cells containing different subsets of these genetic lesions, as well as additional genetic lesions (*e.g.*, pink sector), may also be present. According to polyclonal tumor origin (B), genetic lesions in two or more cells or clones of cells (indicated by blue and maroon sectors) interact to begin tumor development. Aside from this critical difference in the number of cells lineages involved in tumor initiation, concepts regarding the types of the genetic lesions, the need for the accumulation of additional mutations, and subsequent clonal selection are equally applicable to both schemes (monoclonal and polyclonal tumor origin).

2. Current mutation theory of carcinogenesis

The role of mutation in the etiology of cancer has been the subject of extensive investigation. Before considering the data on the clonal origins of tumors, it is useful to summarize the broad conclusions of these investigations because the clonal origins of tumors must be reconciled with the observations that are the basis of current mutation theory. Cancer has been recognized as a genetic disease from the time a cause and effect relationship between mutation and carcinogenesis was gleaned from studies of oncogenic viruses, the epidemiological association between chemical exposures and cancer, and Bruce Ames' seminal observation that carcinogens are mutagens [4,5]. It is accepted that the somatic mutations that cause cancer are rare events, with frequently cited values of $\sim 10^{-7}$ mutations/gene/generation or $\sim 10^{-10}$ mutations/basepair/cell generation [6]. It is generally accepted that tumors accumulate mutations during tumor development and that the eventual clonal outgrowth is derived through a process of clonal evolution [7]. In their landmark paper "*A genetic model of colorectal tumorigenesis*," Fearson and Vogelstein [8] presented the idea that accumulation of mutations results in the progression of pre-neoplastic lesions along the path to carcinogenesis. Fearson and Vogelstein described the most common order of mutation accumulation in colon cancer, but stressed that the total number of mutations was more important than the order, which may vary from tumor to tumor. Definitive evidence that mutant populations expand and acquire additional mutations comes from the analysis of mutant lineages from different areas of tumors [9,10].

Tumors have been shown to carry large numbers of different mutations [11]. Because somatic mutations are viewed as rare events, it has been concluded that the large numbers of mutations in tumors could not develop from a single mutant cell unless the tumor acquired a mutator phenotype during tumor development [6,11]. In support of the mutator phenotype theory, mutations in a variety of genes, including DNA polymerase, DNA repair, and cell cycle regulating genes produce higher frequencies of mutation and speed the carcinogenic process [12]. Familial cancer syndromes have been shown to result from germline acquisition of oncogene or tumor suppressor gene mutation [13,14]. Tumor development is more common and frequent in individuals carrying such germline mutations, presumably because the first step toward tumor development is already present in every cell of the individual.

While somatic mutation is clearly an integral part of carcinogenesis, it is generally acknowledged that not all of the signals or causes of tumorigenesis are mutational. For many years, it has been recognized that inflammation, immune suppression, and hormones have significant roles in carcinogenesis. In addition, evidence has accumulated regarding the role of epigenetic changes in tumor development. For example, specific regions of tumor DNA may be hypermethylated or hypomethylated compared to normal DNA [15]. Changes in the expression of non-coding RNAs and microRNAs also have been shown to play a role in tumorigenesis [16]. Therefore, the term "initiation" will be used hereafter to refer to the acquisition of any genetic or epigenetic change in a cell (including those induced experimentally by carcinogen exposure), which increases the risk that the cell will contribute to the development of a tumor.

In 2000, Hanahan and Weinberg [17] published a seminal paper "Hallmarks of Cancer," in which they identified the phenotypic characteristics or capabilities that must be acquired for a lesion to develop into a cancer, specifically self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evading apoptosis, sustained angiogenesis, tissue invasion, and metastasis. Several of these acquired characteristics can be conferred by mutation in a number of different genes, but tumorigenesis may also involve acquired characteristics conferred by non-mutational mechanisms. Recently, the hypothesis that tumors are initiated and maintained by a population of tumor stem cells has begun to be investigated [18,19].

The concepts mentioned above constitute current theory regarding the role of mutation in carcinogenesis. It is important to realize that none of these concepts requires *a priori* that tumors develop via a monoclonal as opposed to a polyclonal mode. Therefore, the nature of the initial events in carcinogenesis (hereafter referred to as "tumor initiation"), whether they occur in a single cell, two cells, or a group of cells must be established experimentally and cannot be inferred based upon simple acceptance of current mutation theory.

3. Strength and basis of the scientific acceptance of tumor monoclonality

The medical text, *Robbins & Cotran Pathological Basis of Disease* [20], describes the molecular basis of cancer by saying "a tumor is formed by the clonal expansion of a single precursor cell that has incurred the genetic damage (*i.e.*, tumors are monoclonal)." The Holland-Frei medical textbook *Cancer Medicine 7th edition* [21] includes a slightly more equivocal statement, "the genetic clonality observed in most cancers suggests they arise from a single neoplastic cell that has undergone multiple rounds of mutation, proliferation, and selection." A diagram of multistage carcinogenesis appears as an educational tool for teachers on the National Cancer Institute website, along with the text "cancer cells are indigenous cells—abnormal cells that arise from the body's normal tissues. Furthermore, virtually all malignant tumors are monoclonal in origin, that is, derived from a single ancestral cell that somehow underwent conversion from a normal to a cancerous state" (http://science.education.nih.gov/supplements/nih1/cancer/guide/understanding1.htm). Another National Cancer Institute website (http://www.cancer.gov/cancertopics/understandingcancer/genetesting/Slide26) states "Cancer usually arises in a single cell. The cell's progress from normal to malignant to metastatic appears to involve a series of distinct changes in the tumor and its immediate environment, and each is influenced by a different set of genes." Such statements may be dismissed as oversimplified descriptions of tumor etiology. But, in fact, the most commonly cited models of carcinogenesis (the multistage, multistep, or initiation/promotion/progression models of carcinogenesis) are based on the tenet of monoclonal tumor origin. These models simply include the idea that initiated cells may proliferate before a second genetic lesion occurs [22–24]. Thus, assertions of monoclonal tumor origin are ubiquitous in the scientific literature and now appear without discussion of the primary scientific literature used to reach the conclusion that tumors are monoclonal in origin. Consequently, an essential first step in re-evaluating the literature regarding the clonal origins of tumors is a description of the original literature used to conclude tumors are monoclonal in origin.

Most of the early evidence supporting monoclonal tumor origin came from study of X chromosome-linked markers. In the 1960s, it was determined that random X chromosome inactivation (lyonization) occurs in every cell at a point early in development, causing each somatic cell from heterozygous females to carry only one functional X-linked marker [25]. It was concluded that analysis of X chromosome markers could be used in heterozygotes to obtain evidence for unicellular or multi-cellular tumor origin, based upon the expectation that monoclonal tumor origin would produce tumors expressing only one allele of an X-linked gene, while polyclonal tumor origin would produce tumors expressing both

alleles [26–28]. This approach for determining tumor clonality was enthusiastically investigated and universally accepted at the time. The data generated from these studies are treated in the literature as the strongest evidence of monoclonal tumor origin; however, two significant facts regarding these early investigations are seldom communicated. First, the rigor with which this approach needs to be applied in order to prove tumors are monoclonal in origin was initially expressed as a mathematical argument, yet subsequent articles were silent regarding this important methodological caveat. Second, the fact that a percentage of the tumors analyzed in these early studies showed evidence of polyclonal tumor origin is seldom acknowledged.

In 1965, Linder and Gartler [26] were among the first investigators to use X-linked markers to investigate the clonal origin of tumors. They examined electrophoretic variants of the X chromosome-linked glucose-6-phospate dehydrogenase (G-6-PD) enzyme and reported that 27 leiomyomas from five heterozygous women contained only one electrophoretic variant while the surrounding myometrium contained two different variants. The authors went on to describe the appropriate interpretation of their results in this classic paper, including the significance of G-6-PD allele inactivation patch size. Patch size refers to the number of contiguous cells in a normal tissue with the same X chromosome inactivated. Patch size is determined by the developmental process of lyonization, the initial X chromosome inactivation that is subsequently maintained within a lineage of cells. Linder and Gartler concluded that although their data (the detection of 27/27 monotypic tumors) "are insufficient to exclude a two-cell origin of these tumors, and therefore statistically prove single cell origin, they do indicate that the tumors arise from a small number of cells." This report was followed by additional examples of tumors expressing a single X-linked marker. In 1967, Fialkow et al. [29] reported that the erythrocytes and granulocytes of three patients with chronic myelocytic leukemia (CML) were monotypic for the X chromosome-linked G-6-PD enzyme, while skin cells from these patients were heterotypic. Also, a 1967 case report by Beutler et al. [30] examined G-6-PD variants in normal and malignant tissues of two women heterozygous for G-6-PD and concluded that lymphocytic tumor masses of a chronic lymphocytic leukemia patient had a unicentric origin.

Early X chromosome-linked marker inactivation studies also produced evidence of heterotypy within tumors. Beutler et al. [30] demonstrated the primary tumor of a colon cancer patient had a multicentric origin, while the liver metastases had a unicentric origin. In 1971, Smith et al. [31] examined electrophoretic variants of G-6-PD in cervical neoplasia. They reported finding five patients with unicellular origin but two patients with multicellular origin. In 1971, Fialkow et al. [32] published a paper entitled *Multiple cell origin of hereditary neurofibromas* in which they characterized electrophoretic variants of G-6-PD in 14 neurofibromas from two G-6-PD heterozygous hereditary neurofibromatosis patients. All 14 tumors carried both variants. In this remarkable study, Fialkow et al. [32] used a mathematical approach (probability that all 14 tumors would carry both variants and information about patch size) to estimate the number of cells initiating the neurofibroma as ~150. They also postulated that a tumor might arise from hundreds of cells "if the oncogenic mechanism initially altered only a single cell and if this alteration subsequently influenced the pattern of growth in neighboring cells." In 1983, Hsu et al. [33] examined electrophoretic variants of G-6-PD in colonic polyps from three patients with Gardner's syndrome [considered a subcategory of Familial Adenomatous Polyposis (FAP) disease] and concluded they were multiclonal in origin.

Despite the conflicting reports on tumor clonality cited above, several significant papers of the 1970s and 1980s strongly asserted that the literature supported monoclonal tumor origin. Nowell [7] indicated in his 1976 paper, *The clonal evolution of tumor cell populations*, that there was "wide recognition that most neoplasms have a unicellular origin and clonal growth pattern" based on X-linked marker data. The idea that tumors are monoclonal in origin also was supported by Knudson [13]. In his landmark paper, *Mutation and human cancer* [13], Knudson catalogued genetic susceptibility to cancer (familial cancer syndromes) and the underlying genetic abnormalities associated with susceptibility. Knudson discussed cancer models involving two or more stages, the parameters of such models, and the "genetic consequences" of cancer models. Knudson stated "a major consequence of any somatic mutation hypothesis is that individual tumors should be derived from single cells." He cited lineage analysis and karyotypic changes in tumors; specifically the detection of a single X-linked G-6-PD marker in uterine leiomyomata (the work of by Linder and Gartler [26]) and the detection of a single X-linked G-6-PD marker in cells carrying the Philadelphia chromosome as evidence "that tumors often, if not always, naturally arise from a single cell [34]."

In the 1980s, the acceptance of monoclonal tumor origin was significantly strengthened by publication of two studies that examined the clonal origin of human tumors using restriction fragment length polymorphism (RFLP) analysis of X-linked genes, rather than electrophoretic variants of proteins. In 1985, Vogelstein et al. [35] analyzed the *hypoxanthine-guanine phopshoribosyltransferase* gene (*HPRT*) using two different restriction enzymes; one that would distinguish between the parental alleles and one that would digest the active, unmethylated copy but the not the inactive, methylated copy. Using this approach, they showed that the neoplastic cells from three individuals with acute myelocytic leukemia, along with a single Wilms' tumor and a leiomyoma were monotypic [35].

A second RFLP analysis of colorectal tumors was reported by Fearon et al. [36] in 1987. In this article it was argued that allele determination by RFLP would be more conclusive than a method based on electrophoretic variants of proteins because the potential contamination by non-tumor cell DNA would be directly proportional to the percentage of normal cell contamination observed histologically, whereas electrophoretic variants of G-6-PD might be expressed to different degrees in normal and neoplastic cells (suggesting minimal contamination by normal cells could cause a disproportionally large contribution to the electrophoretic variant profile). These publications appear to have greatly influenced the clonality debate and the methylation sensitive RFLP approach became the standard method for analyzing clonality based on X-linked markers. Like Nowell [7] and Knudson [13], Vogelstein et al. [35] emphatically accepted monoclonal tumor origin and rejected the possibility of polyclonal tumor origin, saying "a multiclonal tumor is logically inconsistent with the premise that a rare genetic event (for example, DNA mutation or chromosomal rearrangement) was responsible for formation of the tumor. Indeed, the assumption that tumors are monoclonal is a cornerstone of somatic mutation theories of carcinogenesis (citing Knudson [13])."

4. Failure to detect marker heterogeneity is not evidence of monoclonal tumor origin

Two important studies by Novelli et al. [37,38] significantly altered the interpretation of studies that analyzed X-linked markers. In 1996, Novelli et al. [37], using *in situ* hybridization with Y chromosome probes, examined the small and large intestinal mucosa of a phenotypically male FAP patient who was also an XO/XY chimera. The patient's colon contained thousands of tubular adenomas ranging in size from unicryptal to 2.5 mm in

diameter, as is typical of FAP patients. Staining with a Y chromosome probe demonstrated that 246 (94%) of the dysplastic microadenomas (greater than monocryptal in size) were exclusively XY, 4 (2%) were exclusively XO, and 13 (5%) were mixtures of XO/XY crypts. This led the investigators to re-examine the concept originally described by Linder and Gartler [26], that the frequency and distribution of the marker alleles would influence the ability to detect polyclonality. After taking into account the overall frequencies of the XO and XY alleles, Novelli et al. [37] estimated that 76% of the adenomas above monocryptal in size were polyclonal in origin.

These initial observations, made possible by the fortuitous discovery of a FAP XO/XY chimera, were followed by a systematic investigation into X chromosome inactivation patch size and how patch size would influence the interpretation of X-linked markers studies. Novelli et al. [38] pointed out that X chromosome inactivation (mediated by methylation at CpG islands) occurs early in mammalian embryogenesis (day 16 in the human female). Thus, the progeny of a single progenitor are localized to a given area in the adult, creating patches of tissue with the same X chromosome inactivated. Novelli et al. [38] used *in situ* staining for G-6-PD to analyze patch size in human colon, breast, and thyroid tissue. Their study demonstrated that the terminal ductular/lobular units of breast tissue were generally monomorphic (*i.e.*, derived from a single progenitor). Consequently, even if more than one cell was involved in the development of breast cancer, analysis of X chromosome inactivation in breast tissue would be unlikely to produce the heteromorphic signal interpreted as evidence of polyclonality. With regard to the colonic mucosa, patch size again leads to a bias for detecting monoclonality in tumors. Novelli et al. [38] described a colonic "crypt pair phenotype index," the percentage of colonic crypts adjacent to another crypt distinguishable by X chromosome inactivation. Because the crypt pair index was only 8.2% in the colonic mucosa, Novelli et al. [38] concluded that to exclude the possibility that 10% of adenomas were polyclonal, 430 adenomas would need to be examined and found to be monotypic.

In a parallel study, Jovanovic et al. [39] employed PCR amplification of polymorphic human androgen receptor gene (*HUMARA*) alleles to study X chromosome inactivation patch size in the normal human thyroid gland. They also concluded that the large patch size (demonstrated by frequent detection of monoclonality in normal thyroid epithelium) creates a bias against detecting polyclonality in X chromosome inactivation tumor clonality studies of the thyroid.

5. Re-interpreting data on heterotypy of X-linked markers

X-linked clonality studies were last reviewed in 1976 [40]. Because it has now been definitively demonstrated that X chromosome inactivation patch size biases analyses of clonality using X-linked markers toward the detection of monoclonality, studies that find even a small percentage of polyclonal tumors should be interpreted as providing evidence of polyclonal tumor origin for a particular tumor type. Consequently, studies employing this approach have been reviewed and organized into two categories, those finding any evidence of polyclonality and those finding no evidence of polyclonality. Studies which produced evidence of X chromosome heterotypy are listed in Table 1. This table lists the lesion or tumor type that was analyzed, the method and marker that were used to examine clonality, and indicates the authors' interpretation of their results. This table includes four different types of studies; (1) studies where the authors interpreted their data as evidence of polyclonal tumor origin (designated in Table 1 by P), (2) studies where the authors concluded some lesions or tumors where monoclonal in origin and some lesions or tumors were polyclonal in origin (designated by P & M), (3) studies that detected some evidence of heterotypic lesions or tumors and concluded the monoclonal lesions were contaminated with normal cells (designated by M/C), and (4) studies that include evidence of heterotypic lesions or tumors interpreted as evidence of monoclonal tumor origin (designated by M). The fact that a number of authors have found evidence of polyclonality yet dismissed their own results (attributing them to potential methodological errors) has driven continued acceptance of monoclonal tumor origin despite a significant body of evidence to the contrary. Heim et al. [1] noted the tendency to dismiss evidence of polyclonality [41] in deference to the general acceptance of monoclonal tumor origin, even though according to Heim et al. "there already exists a substantial body of evidence from cytogenetic studies of breast cancer indicating that many of them are polyclonal." The notation regarding the authors' conclusion (see Table 1) provides an indication of the frequency with which this has occurred.

With the aid of hindsight and knowledge of the technical improvements inevitable over time, a review of the X-linked marker literature reveals that X chromosome inactivation studies based on restriction enzyme digestion using methylation sensitive enzymes were subject to a number of technical pitfalls, as well as biases in interpretation [42–46]. An example of a study included in Table 1 in which the authors concluded monoclonality is provided by Siu et al. [47]. Their study used methylation sensitive restriction enzyme digestion followed by PCR of the *HUMARA* gene to evaluate the clonality of colonic aberrant crypt foci (ACF). They compared the relative intensities of the two *HUMARA* alleles in normal tissue to the relative intensity of the two *HUMARA* alleles in ACF. They determined a ratio of parental alleles in the PCR products amplified from the cut and uncut ACF DNA and interpreted their results as evidence of monoclonality, even though both alleles were detected in 9/11 (81.8%) ACF. Remarkably, several papers express the idea that if a lesion is polyclonal, then the ratio of the two alleles following methylation sensitive restriction digestion should be one. This concept was used to justify data analysis methods where the ratio of alleles in the non-methylation-discriminating situation is compared to the ratio of alleles in the methylation-discriminating situation to calculate the clonality ratio or allelic cleavage ratio. Using these data analysis methods, approximately two-fold changes in allele ratios are reported as evidence of monoclonality (clonality values of 0.2–0.4 or an allelic cleavage ratio of 2) [47–50]. Because polyclonality does not *per se* require equal contributions from only two clones, such studies are included in Table 1 as evidence of polyclonality, with the authors' conclusions indicated by "M."

Tumors include blood vessels and infiltrating lymphocytes that can potentially contribute to the X-linked marker heterotypy observed in tumors. To know whether non-tumor cell contamination can explain the heterotypy observed in tumors, one would need to know precisely: (a) how much contamination with stroma, endothelial cells, and lymphocytes was present in each tumor, and (b) what was the ratio of the two lineage markers in each tumor. Neither of these things can be known precisely for all the tumors included in this review. Levels of non-tumor cell contamination are likely to vary between different tumor types, tumors at different stages, and between individual tumors of the same type and stage. Much of the primary data included in Tables 1 and 2 was not presented in a form that could be visually evaluated. When visual evaluation was possible, however, tumors showing minor levels of heterotypy (potentially due to contamination by non-tumor cell types) was not taken as evidence of polyclonality. Given their small size, the pre-neoplastic lesions listed in Table 1 are unlikely to have significant non-tumor cell contamination. A bias in the direction of

Table 1
X chromosome inactivation studies with evidence of polyclonality[a]

Lesion or tumor type[b]	Method and X-linked marker examined[c]	Number of heterotypic lesions or tumors/number of informative lesions or tumors analyzed (percentage)	Authors' conclusion[d]	Reference
Pre-neoplastic lesions				
Atypical endometrial hyperplasia	RFLP/methylation in *HUMARA*	NRQ[e]/4	M	[126]
Stomach metaplastic gland	RFLP/methylation in *HUMARA*	41/86 (47.7%)	P & M	[127]
			P	[128]
Colonic aberrant crypt foci	RFLP/methylation in *HUMARA*	9/11 (81.8%)	M	[47]
		10/10 (100%)	P	[46]
Cervical precancer	RFLP/methylation in *HUMARA*	5/27 (18.5%)	P	[58]
Endometrial hyperplasia				
Without atypia	RFLP/methylation in *HUMARA*	2/15 (13.3%)	M	[59]
With atypia		9/21 (42.9%)		
Adenomas				
Neurofibroma	Protein variants, G-6-PD	14/14 (100%)	P	[32]
	Protein variants, G-6-PD	1/1 (100%)	P	[62]
	RFLP/methylation in *HUMARA*	3/7 (42.8%)	P or M/C	[129]
	RFLP/methylation in *HUMARA*	11/13 (84.6%)	P	[130]
Parathyroid adenoma	Protein variants, G-6-PD	12/12 (100%)	P	[60]
Fibroma	Protein variants, G-6-PD	1/1 (100%	P	[62]
Colon polyp	Protein variants, G-6-PD	2/2 (100%)	P	[33]
Schwannoma	RFLP/methylation in *HPRT*	1/8 (12.5%)	M/C	[131]
Pituitary adenoma	RFLP/methylation in *PGK* & *HPRT*	4/16 (25.0%)	P & M	[132]
Meningioma	RFLP/methylation in *HUMARA*	6/13 (46.2%)	P & M	[133]
	RFLP/methylation in M27β	6/17 (35.3%)	P & M	[134]
Head and neck tumor	RFLP/methylation in *HUMARA*	4/4 (100%)	M	[135]
Kaposi's sarcoma	RFLP/methylation in *HUMARA*	NRQ/28	M	[136]
Extra-ovarian papillary serous tumor	RFLP/methylation in *HUMARA*	NRQ/73	M	[137]
Bladder tumor	RFLP/methylation in *HUMARA*	16/32 (50.0%)	P	[138]
		27/45 (60.0%)	P & M	[138]
Oligoastrocytomas/glial tumor	RFLP/methylation in *HUMARA*	2/11 (18.2%)	P & M	[139]
Breast				
Fibroadenoma	RFLP/methylation in *HUMARA*	NRQ/19	P	[48]
Phyllodes tumor		5/7 (71.0%)	P	
Follicular thyroid adenoma	RFLP/methylation in *HUMARA*	NRQ/10	M	[49]
Ovarian serous cystadenomas	RFLP/methylation in *HUMARA*	25/29 (86.2%)	P	[140]
Carcinomas				
Colon carcinoma	Protein variants, G-6-PD	1/1 (100%)	P	[30]
Cervical carcinoma	Protein variants, G-6-PD	2/5 (40.0%)	P & M	[31]
Breast intraductal carcinoma	Cytophotometric measurement of nuclei	14/35 (40.0%)	P	[141]
	RFLP/methylation in *HUMARA*	4/12 (33.3%)	P & M	[41]
Ovarian carcinoma	RFLP/methylation in *PGK*	2/5 (40.0%)	M	[142]
Endometrial carcinoma	RFLP/methylation in *HUMARA*	NRQ/12	M	[126]
Basal cell carcinoma	RFLP/methylation in *HUMARA*	NRQ/5	M	[143]
	RFLP/methylation in *HUMARA*	10/25	M	[50]
	RFLP/methylation in *HUMARA*	2/8	P & M	[144]
Thyroid medullary carcinoma	RFLP/methylation in *HUMARA*	10/11 (90.9%)	P	[145]
Cervical squamous carcinoma	RFLP/methylation in *HUMARA*	1/2 (50.0%)	P	[146]
Bladder carcinoma	RFLP/methylation in *HUMARA*	9/11 (81.8%)	P	[147]
Non-medullary thyroid tumors				
Papillary thyroid carcinoma	RFLP/methylation in *HUMARA*	NRQ/23	M	[49]
Follicular thyroid carcinoma		NRQ/8		

[a] Studies involving known hereditary cancer syndromes are not included.
[b] Tumors without classification as benign or malignant were placed in the adenoma section of the table.
[c] RFLP analyses rely upon digestion with methylation-sensitive restriction enzymes but otherwise encompass a variety of technical variations, including Southern blotting and PCR amplification of particular polymorphic sequences.
[d] Authors' conclusions are summarized as: P, study data provide evidence for polyclonal tumor formation; M, study data provide evidence for monoclonal tumor formation; P & M, study data provide evidence for both monoclonal and polyclonal tumor formation: M/C, monoclonality concluded because heterogeneity detected ascribed to contamination with normal tissue.
[e] NRQ, study includes evidence of heterotypy that is not readily quantifiable in terms of all reported tumors.

Table 2
Studies reporting detection of a single X chromosome-linked marker in pre-neoplastic lesions or tumors[a]

Lesion or tumor type[b]	Method and x-linked marker examined	Number of lesions or tumors analyzed	Reference
Pre-neoplastic lesions			
Lung atypical adenomatous hyperplasia	RFLP/methylation in *HPRT*	10	[117]
Adenomas			
Leiomyoma	Protein variants, G-6-PD	27	[26]
Meningioma	RFLP/methylation in *HPRT*	9	[131]
Pituitary adenoma	RFLP/methylation in *PGK* & *HPRT*	6	[148]
Parotid gland pleomorphic adenoma	RFLP/methylation in *HUMARA*	5	[149]
Carcinoma			
Chronic myelocytic leukemia	Protein variants, G-6-PD	2	[34]
		3	[34]
		1	[53]
Acute myeloblastic leukemia	Protein variants, G-6-PD	1	[150]
Neuorfibrosarcoma	Protein variants, G-6-PD	1	[62]
Bladder cancer	RFLP/methylation in *PGK* and *HPRT*	10	[151]
Endometrial carcinoma	RFLP/methylation in PGK	5[a]	[118]
bronchioloalveolar carcinoma	RFLP/methylation in *HPRT*	7	[117]

[a] Polymorphic lesions detected but concluded to be hyperplastic, not neoplastic.
[b] Studies involving known hereditary cancer syndromes are not included.

dismissing heterotypy as contamination with non-tumor cell types has occurred. In a review of methods to assess tumor clonality, Wainscoat and Fey [28] write "a few exceptional cases of malignant tumors with double enzyme phenotypes including colonic carcinomas, breast cancers, and a hepatoma have been reported. These rare cases may be explained by admixture of normal cells in the tumor sample analyzed." But, by examining histology in conjunction with cell lineage markers, colon and breast cancers were shown to be polyclonal [37,51,52]. The X-linked marker analysis also showed they were polyclonal, yet this evidence was dismissed as contamination with normal cells. So, while contamination with normal cells is unlikely to account for the majority of the polyclonal tumors described in Table 1, the possibility that the appearance of polyclonality in some fraction of the tumors listed in Table 1 is due to contamination with non-tumor cell types cannot be excluded.

Table 1 shows that there is evidence for polyclonal tumor origin for 22 different human tumors (basal cell carcinoma, bladder tumor, breast fibroadenoma, breast intraductal carcinoma, cervical carcinoma, colon carcinoma, endometrial tumor, fibroma, follicular thyroid adenoma/carcinoma, head and neck tumor, Kaposi's sarcoma, meningioma, neurofibroma, oligoastrocytomas/glial tumor, ovarian tumor, papillary thyroid carcinoma, parathyroid adenoma, phyllodes tumor, pituitary adenoma, schwanoma, and thyroid medullary carcinoma). The human tissues shown to produce polyclonal tumors using this approach include: bladder, blood, brain, breast, cervix, colon, endometrium, head and neck, nerve tissue, parathyroid gland, pituitary gland, skin, stomach, and thyroid gland.

Studies of X chromosome inactivation that produced no evidence of polyclonality are listed in Table 2. The 13 studies listed in Table 2 detected no evidence of X-linked heterotypy in eight different tissues/organs. However, six of these tumor types or their benign precursors also are listed in Table 1, meaning there are only two tissue/organs (lung and parotid gland) for which there is currently no X-linked marker evidence of polyclonal tumor origin.

6. Other evidence of monoclonal tumor origin

The bias toward detecting monotypic X chromosome inactivation due to patch size suggests that a conclusion of monoclonal tumor origin should rely more heavily upon other types of data. The next most commonly cited data used to conclude tumors are monoclonal in origin are the detection of a particular chromosome

Table 3
Karyotypic analyses used to investigate clonality

Lesion or tumor type	Method and marker(s) examined	Number of heterotypic lesions or tumors detected per number of informative lesions or tumors analyzed (percentage)	Reference
B-cell lymphoma	Cell surface immunoglobulin	3/3 (100%)	[152]
Breast carcinoma	Aneuploidy by flow cytometry	10/104 (9.6%)	[153]
Ovarian carcinoma		5/77 (6.5%)	
Breast tumors	Aneuploidy by flow cytometry	14/74 (18.9%)	[154]
Breast epithelial hyperplasia	Cytogenetic analysis by FISH	1/7 (14.3%)	[155]
Breast papillomas		1/3 (33.3%)	
Breast fibroadenomas		4/5 (80.0%)	
Colonic adenoma	Immunostaining for Apc-c	NRQ/26	[156]
Cervical squamous carcinoma	LOH at chromosome 3p	8/14 (57.1%)	[146]
Oligoastrocytomas/glial tumors	Microsattelite analysis w/PCR	2/11 (18.2%)	[139]
Prostate phyllodes tumor	LOH at 12 micosattelites	6/6 (100%)	[157]
Esophageal carcinosarcoma	LOH at 25 microsatellites	0/6 (0%)	[9]
Metaplastic carcinoma of the breast	Sequencing the *p53* gene	0/14 (0%)	[158]

NRQ, study includes evidence of heterotypy that is not readily quantifiable in terms of all reported tumors.

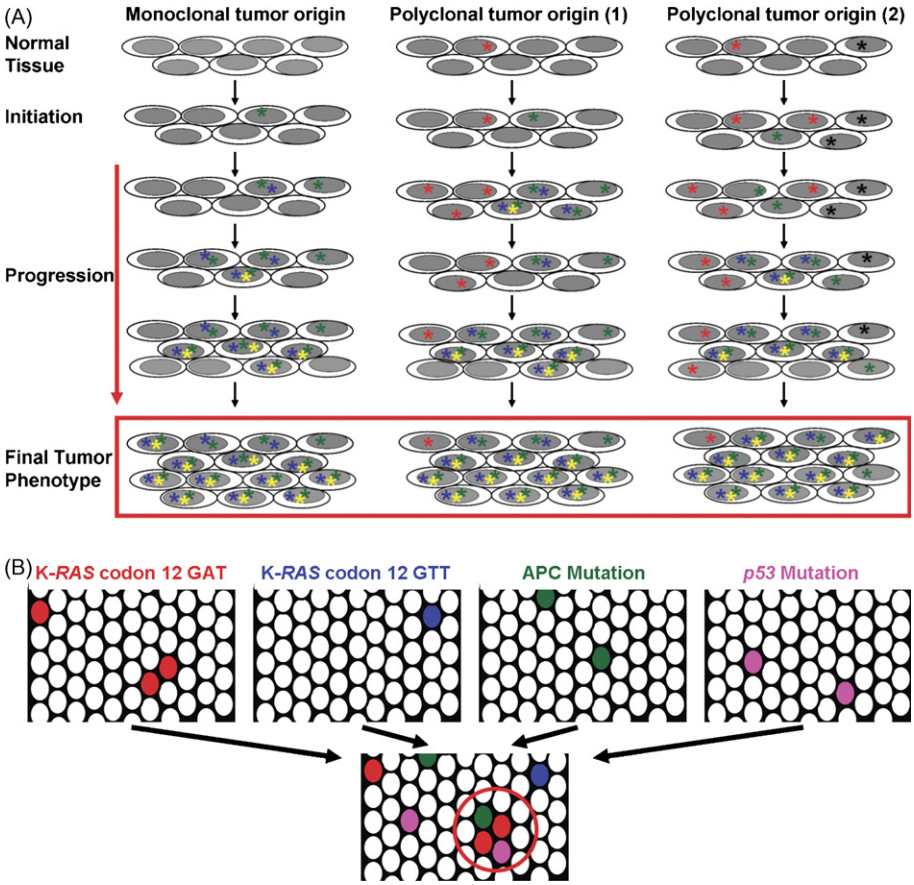

Fig. 2. Hypothetical accumulation of mutation during tumorigenesis. (A) Three different scenarios for mutation accumulation are shown, one representative of monoclonal tumor origin and two different versions of polyclonal tumor origin. Different color asterisks represent different somatic mutations. The illustration depicts pre-existing mutation (red and black in polyclonal scenarios) and initiation (green). During progression, additional mutation (blue) occurs in initiated cells (monoclonal and polyclonal scenario 1) or progression proceeds first by cooperative interaction among complementing mutant clones (polyclonal scenario 2). The figure also illustrates that: (1) with polyclonal tumor origin, initial mutation may not persist in the final tumor outgrowth (red and black), (2) monoclonal and polyclonal tumor origin can result in nearly identical and apparently homogeneous clonal tumor outgrowth (blue, green and yellow), (3) markers present at low frequency in the final tumor outgrowth may not be detected (red), and (4) detection of a prevalent genetic lesion in the final tumor outgrowth does not mean, *a priori*, that it was an initiating lesion (yellow). (B) A potential approach for the spatial modeling of polyclonal colon tumor development is depicted. Each oval represents an individual colonic crypt; shading indicates crypts carrying a particular mutation. It is envisioned that the frequency and size distribution of mutant clones could be used to estimate the probability of co-localization of complementing mutant clones, which in turn would relate to the frequency of tumor development.

aberration or genetic lesion throughout the tumor. The best known example of this is provided by the presence of the Philadelphia chromosome in patients with chronic myelocytic leukemia (CML). The Philadelphia chromosome is typically found in 80–100% of dividing marrow cells in CML patients [29,53–56]. Several investigators argued that the prevalence of the Philadelphia chromosome in CML is evidence of monoclonal tumor origin [57]. However, Fialkow [40] concluded "it was not possible to rule out the possibility that the abnormal leukocytes were derived from many cells" and that "the conclusion that CML has unicellular origin applies only to the stage of the disease at which it is clinically apparent. Conceivably, a number of cells might be affected at an earlier phase, but by the time CML becomes overt, one clone has evolved." In 1976, Fialkow [40] also reviewed analyses of tumor clonality based on immunoglobulin (Ig) markers. Studies of Burkitt-type leukemia, hairy cell leukemia, chronic lymphosarcoma leukemia, and non-hodgkin lymphoma only found monotypy for an Ig marker, while studies of Burkitt's lymphoma, chronic lymphocytic leukemia, Waldenstrom's macroglobulinemia, and multiple myeloma found at least some evidence of Ig heterotypy [40].

Many studies have concluded monoclonal tumor origin based on the prevalence of a particular chromosome aberration or genetic lesion throughout the tumor. Examples of such tumor marker studies are given in Table 3, which also includes studies that observed heterotypy in tumor markers. Table 3 does not, by any means, provide an exhaustive list of such studies. A complete review was not undertaken because in the author's opinion such studies do not provided conclusive evidence of either monoclonal or polyclonal tumor origin.

While many analyses of tumor markers report monotypy, there are a number of scenarios whereby this type of data can lead to an incorrect conclusion of monoclonal tumor origin. A polyclonal tumor might appear to be monoclonal if one clone has a higher proliferation rate than another clone initially involved in tumorigenesis. The clone with the higher proliferation rate (possibly due to the acquisition of additional genetic damage) will become the predominant clone in the tissue examined. Also, failure to detect cells lacking the genetic lesion or chromosome aberration present in the majority of the tumor mass does not prove that all cells in the tumor carry that genetic lesion or chromosome aberration. Technically, the inability to detect tumor cells without the genetic lesion in question only proves that the number of such cells present is below that detectable by the method employed. In fact, small polymorphic populations of cells within tumors are a common finding, one that is frequently

dismissed as contamination with normal cells or tumor stroma (see Table 1 study conclusions designation M/C).

Because initial heterotypy may be lost during tumor progression, the clonality of a developed tumor cannot be analyzed as a surrogate for the earliest events in carcinogenesis. A number of studies have shown polyclonality of a marker in a "precancer" but detection of only a single marker in the developed malignancy [48,58–62]. The idea that both initially monoclonal and initially polyclonal lesions could eventually give rise to "monoclonal appearing tumors" is illustrated in Fig. 2A, where the final tumor phenotype will appear the same for tumors with monoclonal and polyclonal mutational origins, at least when tumors are characterized by the most commonly used methods. Two different examples of polyclonal tumor origin are drawn in Fig. 2A. The fact that innumerable scenarios for both monoclonal and polyclonal tumor origin could be drawn, which would produce the same final tumor outcome, emphasizes the weakness of using the prevalence of a genetic lesion in the developed tumor to make conclusions regarding tumor origin. Expressed another way, it is circular reasoning to assume that detection of one genetic change in all the cells of a tumor indicates that the tumor was monoclonal in origin because, theoretically, this approach will only be valid under circumstances of monoclonal tumor origin. Also, it should be pointed out that in these types of genetic marker studies, it can never be proven that the marker being examined was actually an initiating lesion. For the same reason, detection of heterotypy for a particular genetic marker (unrelated to cell lineage) also does not prove polyclonal tumor origin.

7. Direct evidence of polyclonal tumors

Given the difficulties interpreting reports of tumor monoclonality, positive evidence of tumor polyclonality should weigh significantly in the debate regarding the clonal origin of tumors. Merritt et al. [51] developed a mouse model system analogous to the XO/XY chimeric FAP patient studied by Novelli et al. [37]. In the mouse intestine, the cells of a single crypt have been reported to have the same genotype (*i.e.*, they are monoclonal). The explanation for this is that all the cells in an individual crypt are derived from a small number of stem cells that all have the same progenitor [63]. Polyclonality in this system, then, means that tumors develop from the cells in two or more crypts. To investigate the clonal origins of mouse intestinal tumors, embryos heterozygous for the *ROSA26* marker allele and heterozygous for the Apc^{Min} allele were aggregated with embryos wild-type for the *ROSA26* marker allele and heterozygous for the Apc^{Min} allele [51]. The mice generated were heterozygous for the *Min* allele and had the high frequency of intestinal tumors characteristic of the *Min*/+ genotype. The presence or absence of the *ROSA26* marker allele in the intestinal epithelium and intestinal tumors of these mice was visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of whole mount tissue sections. Analysis of the intestinal tissue of two different chimeras showed the wild-type allele was 8.2- or 9.6-times more abundant than the *Rosa26* allele. Examination of whole mounts of 260 adenomas indicated that 229 (88%) appeared all white, 13 (5%) appeared all blue, and 18 (7%) were mixtures of white and blue. Sectioning and staining identified additional adenomas containing mixtures of blue and white crypts (7–10% of the adenomas examined) [51].

Based on X chromosome inactivation patch size and the relative frequencies with which the different X chromosomes were inactivated, the number of observed X-linked heterotypic tumors (blue and white) was extrapolated to the number that would be expected if there was not a bias against their detection. Using this approach, the authors estimated that 79% of the adenomas that developed in this chimeric mouse model were actually polyclonal. In addition, Merritt et al. [51] showed that the wild-type *Apc* allele had been lost in all of the lineages of the polyclonal tumors and proposed several mechanistic models to explain this result: (1) the region of polyclonal tumor formation is characterized by high levels of initiation, (2) the dysplastic growth of one *Apc*-negative clone causes subsequent conversion of the second clone, and/or (3) one *Apc*-negative clone enhances the growth and/or survival of a second clone.

A criticism of this work was that the intestines of the *Min* mice developed so many adenomas that the apparent polyclonality might result from the collision or conjoining of independently derived blue and white adenomas. In order to address this concern that mixed tumors were the result of collision between monoclonal adenomas, Thliveris et al. [64] generated similar chimeric mice that were also homozygous for a tumor resistance gene, *Mom1*. Using this modified system, the number of intestinal tumors was reduced 8-fold (on average 16 tumors/mouse intestine), yet 22% of the adenomas were comprised of both of blue and white crypts, a result consistent with polyclonal tumor origin. Statistical analysis of these data concluded that random collision of monoclonal adenomas was not a plausible explanation for the observed polyclonality [65]. Thliveris et al. [64] went on to analyze the spatial distribution of blue and white crypts in the intestinal mucosa and to develop a mathematical model of neighboring crypt interactions. From their analysis, they concluded that short-range interactions between clones across a distance of one to two crypt diameters would best explain the observed frequencies of blue, white and mixed crypts observed in the intestinal adenomas. The work of Merritt et al. [51] and Thliveris et al. [64] provides direct and compelling evidence that the intestinal tumors of *Min* mice are polyclonal in origin.

Winton et al. [66] also used chimeric mice to investigate the clonality of mouse skin lesions. They used embryo aggregation to generate mice chimeric for immunohistochemical markers ($H2^k$ and $H2^b$ alleles). Using this genetic model, they showed that the earliest recognizable mouse skin lesions induced using an initiation/promotion protocol are polyclonal [66]. Depending on the initiation/promotion treatment used, they found that between 16.7% and 24% of the epidermal focal hyperplasias they characterized were heterotypic.

Kisseberth and Sandgren [52] used a chimeric mouse model to investigate the clonality of mouse mammary lesions. They engineered chimeric mammary glands into a mammary tumor-sensitive mouse strain, by surgical excision of normal mammary gland tissue and implantation of two cell lineages expressing distinguishable cell markers. Using this genetic model, Kisseberth and Sandgren [52] demonstrated that the pre-neoplastic lesions (hyperplastic alveolar nodules) that developed from the chimeric mouse mammary glands were polyclonal, mirroring the polyclonal architecture of the tissue. This result, they argue, suggests that a non-cell autonomous local tissue microenvironment influences a group of cells rather a single cell during clonal outgrowth.

Additional studies produced evidence of polyclonal tumor origin by employing genetic markers that could distinguish cell lineage. Reddy and Fialkow [67] induced mouse fibrosarcomas with 3-methylcholanthrene and showed that 36/38 tumor sections contained two different alleles of the X-linked phosphoglycerate kinase gene [67]. Clayton and Farrell [68] examined surgically removed primary pituitary tumors and samples from where the tumors regrew. They examined heterotypy at the X chromosome-linked *HUMARA* gene, as well as loss of heterozygosity at a number of microsatellite markers and concluded their data support the existence of at least two clones in the primary tumor [68]. Two B-cell clones with distinguishable IgH VDJ rearrangements were

observed in the B-cell neoplasia, Waldenstrom's macroglobulineamia [69]. Three cases of B-cell lymphoma were composed of two different B-cell clones distinguishable by the re-arrangement of their immunoglobulin genes [70]. Through lineage analysis of immunoglobulin genes, evidence of polyclonal tumor origin was obtained for three additional tumor types (Waldenstrom's macroglobulineamia, B-cell lymphoma, and multiple myeloma), for a total of 24 different tumor types with evidence of polyclonal tumor origin.

In addition to the specific studies mentioned above, additional support for polyclonal tumor origin comes from the nature of tumors themselves. It is generally accepted that the interaction between tumor cells and the surrounding normal tissue is part of the carcinogenic process. Indeed, according to a review by Bissell and Radisky [71], tumorigenesis begins with a perturbation of intercellular communication, leads to a disruption of normal tissue or organ organization, which can then cause proliferation of pre-existing epithelial cells with neoplastic potential or the creation of such cells. Thus, one might argue that studies demonstrating a role of stroma in tumorigenesis provide *de facto* evidence of polyclonal tumor origin.

In summary, a considerable amount of evidence from X chromosome inactivation studies supports polyclonal tumor origin. The bias against detection of heterotypy due to X chromosome inactivation patch size means that failure to detect heterotypy using X-linked markers should be interpreted cautiously. Results obtained using other genetic markers, which cannot distinguish cell lineage, are generally equivocal because it cannot be assumed that the marker in question was involved in tumor initiation. On the other hand, studies performed using chimeric mice provide direct evidence of polyclonal tumor origin. This work demonstrates that when a model system is used that can reduce the bias against detecting polyclonality, polyclonality is detected.

8. Frequency of tumor-associated mutation in tissues and tumors is consistent with polyclonal tumor origin

According to monoclonal tumor origin theory, mutation occurs in one cell, this cell may proliferate, and then the next rare event occurs in one of the cells already carrying the first mutation. According to polyclonal tumor origin, two independent events occur in different cells that happen to be in relatively close proximity to each other, leading to polyclonal tumor origin. The low frequency of spontaneous somatic mutation has been cited as a reason to expect that tumors are monoclonal in origin [13,35,40,53]. For example, Fialkow et al. [60] states that "unicellular origin would be compatible with a rare oncogenic event, whereas multicellular origin might be seen with hyperplasia." Although the idea that multicellular origin is inconsistent with a rare event is quite apparent in the early literature, the reason why this would be so is not specifically enunciated. However, it may be surmised that the accumulated data on the frequency of spontaneous mutation led investigators to conclude that two rare independent events in close proximity was less likely to occur than the clonal proliferation of a single initiated cell leading to a second mutation. Thus, information on the frequencies of the initial events in carcinogenesis is relevant to a discussion of the respective probabilities of monoclonal and polyclonal tumor origin. For this reason, it is important to review the commonly cited literature regarding spontaneous mutation rate and spontaneous mutant frequency, as well as more recent findings obtained using DNA-based measurement of oncogene and tumor suppressor gene mutations.

Spontaneous mutation rate has been measured *in vitro* in cell cultures. A spontaneous mutation frequency of 1.2×10^{-7} per locus per cell generation was estimated based on changes in the electrophoretic mobility of proteins in cultured TK-6 cells [72]. Most studies addressing spontaneous mutation rate in cell cultures, however, rely on the use of a neutral reporter genes, which confer selectable mutant phenotypes. For example, Oller et al. [73] used 6-thioguanine to select for *HPRT* mutants and found a spontaneous mutation rate of 1.5×10^{-7} mutants/cell $\times$ days in cultures of AHH1 human lymphoblastoid cells. The *Pig-A* gene can be used as a reporter because mutation in this gene results in the loss of a set of cell surface proteins, with mutant cells quantifiable through the use of antibody binding and cell sorting [74]. Using the *Pig-A* system, B-lymphoblastoid cell lines were found to have spontaneous mutation rates of $2.4–29.6 \times 10^{-7}$ per locus per generation. Even lower rates of spontaneous mutation ($0.8–1.0 \times 10^{-7}$ per locus per cell per generation) were observed in the *adenine phosphoribosyltransferase* gene of embryonic stem cells using 2-fluoroadenine selection of mutants [73]. It should be noted that these are per locus mutation rates and it is expected that the per basepair mutation rates would be significantly lower depending on the number of mutable sites within the target locus. Only one study addresses the question of *in vivo* spontaneous mutation rate. Denver et al. [75] used direct sequencing of four megabases of *C. elegans* nuclear DNA from a set of mutation-accumulation lines (following generations of single progeny descent). By sequencing the same set of loci throughout the germline lineage, they determined a mutation rate of $\sim 2 \times 10^{-8}$ per basepair per generation.

While measurements of mutation rate are rare, there are numerous reports regarding the measurement of mutant frequency, the ratio of mutant to wild-type cells within a given population of cells. Again, this type of data is usually collected using *in vitro* selection of mutants within particular reporter genes. However, this approach can and has been used to characterize the spontaneous mutant frequency produced *in vivo*. The spontaneous mutant frequencies found in a variety of tissues, based on measurements in the *hprt*, *lacI*, *supF*, *cII*, *gpt*, ϕX174 *am3*, and *rpsL* loci have been reviewed [76]. Per locus, spontaneous mutant frequencies ranged between 2×10^{-7} and 25×10^{-5}. When these frequencies are divided by the number of known mutable sites within each target, the spontaneous mutant frequency per basepair can be estimated. Using this type of analysis, it was estimated that the mutable sites within these neutral reporter loci had spontaneous mutant frequencies of 10^{-8} to 10^{-7} per basepair [76]. These reporter gene assays have been very productive in understanding chemical carcinogenesis. Part of the rationale for using them is that mutations in these reporter gene targets can be used as surrogates for the mutations in oncogenes and tumor suppressor genes that initiate carcinogenesis. Taken together, the data on spontaneous mutation rate and spontaneous mutant frequency has led to the perception that the frequency of oncogene and tumor suppressor gene mutants (initiated cells) is extremely low.

A somewhat different picture emerges when the frequencies of specific oncogene and tumor suppressor gene mutations are examined directly. A number of DNA-based methods to quantify rare point mutations have been developed, including allele-specific competitive blocker-PCR (ACB-PCR) [77], the restriction site mutation (RSM) assay [78], mutant allele-specific amplification-PCR (MASA-PCR) [79], denaturing gradient gel electrophoresis (DGGE) [80], mutant allele enrichment (MAE) with DGGE [81], the needle-in-a-haystack mutation assay [82], and the mismatch amplification mutation assay (MAMA) [83].

Using these methods, oncogene and tumor suppressor gene mutations have been detected within a number of normal-appearing human tissues. With a sensitivity of $\leq 10^{-5}$, K-*RAS*

mutation was found in histologically normal colonic mucosa samples from patients with colon cancer [79,84–87]. With a sensitivity of $\leq 10^{-5}$, *p53* mutations were found in 257 of 463 human tracheal–bronchial epithelial samples [88]. With a sensitivity of $\sim 10^{-4}$, *p53* mutations were detected in nondysplatic esophageal tissue from individuals with Barrett's esophagus [89]. *p53* mutation has been found in normal human breast tissue [90] and in keratinocytes of normal human skin [91]. K-*RAS* mutations have been found in the non-neoplastic ductal lesions of patients with chronic pancreatitis [92]. Also, analysis of peripheral blood leukocytes from 19 normal individuals identified 17 independent mutations in the 66 different basepair targets examined within the H-*RAS*, N-*RAS* and *p53* genes [82].

These same methodologies have been applied to normal-appearing rodent tissues, and detectable levels of oncogene and tumor suppressor gene mutations were observed. In an elegant study, Cha et al. [93] showed that 70% of untreated rats carried H-*Ras* mutation detectable at a frequency of 10^{-5} and that *N*-nitroso-*N*-methylurea-induced rat mammary tumors arise from these pre-existing H-*Ras* mutant cells. Using ACB-PCR with a sensitivity of 10^{-5}, measurable levels of spontaneous oncogene and tumor suppressor gene mutations have been observed in a number of rodent organs, H-*Ras* mutation in mouse liver [94], K-*Ras* mutation in rat liver [95], *p53* mutation in mouse skin [96], K-*Ras* mutation in rat colon [97], and K-*Ras* mutation in mouse lung [98].

The detection/quantitation of oncogene and tumor suppressor gene mutations in normal-appearing tissues at frequencies at or above 10^{-5} indicates that tumor-associated mutations occur much more frequently than mutants in neutral reporter gene targets. This likely reflects the ability of these mutations to confer a selective advantage. While 10^{-5} is a low frequency, when one takes into account the number of cells in an organ, it becomes clear that oncogene and tumor suppressor gene mutations are not "rare." To illustrate, the human colon is estimated to contain 10^8 crypts, with $\sim$112 cells per crypt, for an estimated total of $\sim 1.12 \times 10^{10}$ crypt cells per colon [99,100]. By extrapolation, a mutation present at a frequency of 10^{-5} implies the presence of 100,000 mutants within the colon. Given such numbers, carcinogenesis initiated through the co-occurrence of cooperating oncogene and tumor suppressor gene mutant clones is a viable theory, one consistent with the stochastic nature of cancer.

Polyclonal tumor origin is expected to produce tumors in which oncogene and tumor suppressor gene mutations are present as subpopulations within the lesion/tumor cell population, whereas monoclonality predicts that a single molecular lesion (the initiating lesion) is present in every tumor cell. Therefore, the prevalence of oncogene and tumor suppressor gene mutations within developed tumors may provide some insight into the clonal origins of tumors. Evidence that oncogene and tumor suppressor gene mutations are frequently present as subpopulations within tumors comes from published DNA sequencing traces showing >50% wild-type allele. In addition, methods more sensitive than DNA sequencing find higher frequencies of mutation positive tumors [101–104]. Keohavong et al. [105] isolated pure tumor cell populations from three ductal carcinomas *in situ* and with PCR/SSCP showed that between 25% and 35% of cells in the samples were H-*Ras* mutant. But as previously discussed, identification of mutant subpopulations within any particular tumor does not prove polyclonality because, as with other tumor markers, it cannot be concluded that the mutation in question was an initiating mutation. However, in some cases, sets of tumors have been analyzed systematically for the levels of particular oncogene and tumor suppressor gene mutations. K-*Ras* mutant subpopulations in colon tumors have been estimated by RFLP-PCR and ACB-PCR [86,87]. H-*Ras* mutants were reported to comprise 14–47% of cells in PAH-induced mouse skin papillomas [106]. Because of its frequent and early occurrence in UV-B and SSL-induced skin tumors, it has been suggested that *p53* mutation may initiate mouse skin tumorigenesis [107–109]. Using ACB-PCR, a particular *p53* mutation was shown to occur as a subpopulation in 24/24 simulated solar light-induced mouse skin tumors at levels corresponding to between 1 in 5 and 1 in 218 cells (on average, 1 in 15 cells) [108]. Thus, while its presence as a subpopulation in every SSL-induced tumor argues this *p53* mutation is important for tumorigenesis, the levels of this mutation are not consistent with this being an initiating lesion in mouse skin tumors of monoclonal origin. The same type of argument can be made regarding the occurrence of K-*RAS* mutations in human colon tumors [87]. These findings suggest that either the typical analysis of tumor mutation is being over-interpreted in terms of surmising that detected mutations are the initiating events in particular tumors, or that these tumors are polyclonal in origin (as is supported by X-linked marker studies of skin and colon).

In a similar vein, it is worth noting that some of the mutational data on colon tumor development has never fit the well-known Fearon–Vogelstein model of sequential mutation accumulation during clonal evolution from a single cell [8]. The genes/lesions of the "Vogelgram" were ordered based on the prevalence with which they are detected at the different stages of tumorigenesis, with the *adenomatous polyposis coli* (*APC*) gene appearing first because it is found in the highest percentage of tumors, followed by K-*RAS* and *p53*. While lesions in these genes are, indeed, among the most commonly reported lesions in colon tumors, lesions in all three are seldom found within the same tumor [110,111]. In addition, K-*Ras* mutation is more prevalent than *APC* mutation in aberrant crypt foci (the presumptive pre-neoplastic lesion), while *APC* mutation is more prevalent than K-*RAS* mutation in adenomas or adenocarcinomas [112–115]. Also, when ACB-PCR was used to quantify levels of K-*RAS* mutation in adenomas and adenocarcinomas, significantly higher levels of the K-*RAS* codon 12 GTT mutation were found in adenomas as compared with adenocarcinomas [87]. These findings are difficult to reconcile with the sequential accumulation of mutation during the clonal expansion of a single mutant clone. On the other hand, these data are consistent with the hypothesis that different clones of mutant cells cooperate in initiating a tumor, with subsequent changes in the proportions of the different mutant clones occurring during the clonal evolution of the tumor.

9. Conclusions

From the evidence described above, it can be concluded with certainty that some tumors are polyclonal in origin. In fact, there is evidence that at least 24 different human tumors can have polyclonal tumor origin, including tumors of the bladder, blood, brain, breast, cervix, colon, endometrium, head and neck, lymph, nerve tissue, parathyroid gland, pituitary gland, prostate, skin, stomach, and thyroid gland. The fact that considerable evidence of polyclonality has been amassed (see Table 1), despite the bias against detecting polyclonality, suggests that a significant portion of tumors of the types mentioned above may be polyclonal. Existing data cannot exclude the possibility, however, that some tumors of these tumor types are monoclonal in origin. Future reports on tumor heterogeneity need to convey clearly that failure to detect polyclonality should not be interpreted as definitive evidence of monoclonality. More importantly, the broadly held belief that the currently available data support monoclonal tumor origin needs to be corrected.

It is important to recognize that polyclonal tumor origin is consistent with the observations that form the basis of the current mutation theory of cancer. Polyclonal tumor origin is consistent

with the observations that there are large numbers of mutations and genetic lesions within tumors, that clonal evolution of mutated cells occurs during tumor development, and that this clonal evolution may be driven by the acquisition of mutator phenotypes. Polyclonal tumor origin is consistent with the Hanahan–Weinberg theory that tumors progress as they acquire a set of phenotypic characteristics. And, polyclonal tumor origin is consistent with the recent observation that tumor stem cells make up only a fraction of the tumor cell population [116]. Indeed, many of these observations are more easily explained by polyclonal than monoclonal tumor development.

10. Implications of polyclonal tumor origin

Given that fully developed tumors frequently appear to be composed of a single predominant clone, it may seem like the discussion of whether tumors originate from one, two, or more cells is a semantic argument. Why would it matter whether tumors start from one, two, or three cells, if they eventually evolve into a more or less clonal population? In fact, the clonal origin of tumor development is important for four reasons: for developing an accurate scientific understanding of the initial events in carcinogenesis, for improving cancer risk assessment, for developing accurate mathematical models of tumor development, and for more accurate insight regarding the merits of different therapeutic approaches.

It is critical that the scientific community, physicians as well as researchers, have an accurate understanding regarding what is and is not known about the initial events in carcinogenesis. Pathologists, for example, are trained that clonality is a characteristic of neoplasia. Based on the strong acceptance and promulgation among the medical community that tumors are monoclonal in origin, pathologists have used clonality to conclude heterotypic lesions are "reactive" and not precancerous [117,118]. Yet, heterotypic hyperplastic lesions can be precancerous lesions [48,52,58–62]. An accurate evaluation of the neoplastic potential of polyclonal, hyperplastic lesions, therefore, has direct implications for patient care, particularly as molecular methods for pathological evaluation continue to develop.

An accurate understanding regarding the weight of evidence of polyclonal tumor formation is also important in terms of weighing research priorities and avenues of investigation. Polyclonal tumor origin clearly occurs in a larger proportion of tumors than previously realized; in fact, the data reviewed here are consistent with the idea that a majority of colon tumors may be polyclonal in origin [37,51]. Therefore, a greater research emphasis should be placed on understanding the molecular mechanisms underlying cell-to-cell interactions between complementing mutant cells or clones of cells [24]. Considerable evidence establishes that stroma influences tumor development to a significant extent. The ability of adherens, gap junctions, tight junctions, and desmosomes to influence carcinogenesis has been investigated for many years [71]. Such investigations need to proceed; however, more experimental strategies and additional informative models (like the chimeric models) are needed to investigate the potential involvement of multiple complementing cell clones in initiating a nascent tumor. Definitive studies on the genesis of tumors will require concurrent analysis of the genetic underpinnings of transformation (mutations and epigenetic changes), the effects on gene expression and signaling, and the roles of cell-to-cell interactions and changes in tissue architecture. Mechanistic studies are needed to identify which types of mutant cells can complement each other and to determine how the stromal compartment of a tumor participates in the earliest polyclonal lesion. Do the cells in the interacting stromal compartment carry some genetic or epigenetic lesion that contributes to tumor development or are these cells really "wild-type"? Until broader acceptance of polyclonal tumor origin is achieved, these types of questions will remain unasked and certainly unanswered.

A mechanistically correct understanding of the clonal origin of tumors is critical for accurately modeling the carcinogenic process, an activity with important practical implications for cancer risk assessment. Acceptance of the idea that tumors are the consequence of a mutational event in a single "initiated" cell (once the initiated cell receives one or more additional "hits" to become a tumor cell) has led to acceptance of the idea that carcinogens act through a single mechanism. For most chemicals this is probably an oversimplification. If one accepts that it is actually two or more cells or clones of cells with complementing genetic or epigenetic lesions that cooperate during the initial tumorigenesis, it becomes apparent that carcinogens may not act through a single mechanism. For example, chemicals that cause toxicity and compensatory cell proliferation could cause an underlying amplification of pre-existing mutation at the same time they have a direct genotoxic effect. Based on the idea of polyclonal tumor origin, the tumor response might not be directly attributable to either the cell proliferative effect or the genotoxicity, but rather their interaction. The risk assessment community currently employs linear low-dose extrapolation for genotoxic carcinogens but may employ a threshold-based approach for non-genotoxic carcinogens. This also was based on the idea that genotoxins are more of a concern because, in theory, every initiated cell has the potential to become a cancer. This distinction needs to be revisited in the face of the fact that initiating mutations in tumor-associated genes pre-exist in tissues and may participate in polyclonal tumor origin. Any chemical effect that causes even a transient increase in cell division has the potential to increase the number of tumor-associated mutant cells and, thereby, increase the probability that these cells could interact with clones carrying complementing mutations or epigenetic changes.

The presumption of monoclonal tumor origin also provided the mechanistic foundation for a generation of modelers who started with tumor incidence data and worked backwards, selecting particular mutation frequencies and birth and death rates of transformed cells, to calculate the number of mutational events or "hits" likely to be required for carcinogenesis [22,119,120]. Modeled mutation rates were constrained to be low enough that the tumor mass could be generated by a sequence of cell divisions following one initial mutation, an artificial constraint which might result in a downward bias in human risk estimates. These types of mathematical models of tumorigenesis provided a satisfying theoretical framework for scientists to conceptualize monoclonal tumor origin and subsequent tumor development. But what practical advances have been achieved using these approaches? Because these modeling approaches require information on the rate of mutation in oncogenes and tumor suppressor genes, information that has never been acquired experimentally, the practical utility of these approaches in predicting tumor development (or risk of tumor development) has been quite limited.

The evidence supporting polyclonal tumor origin suggests that a different approach to the mathematical modeling of carcinogenesis may be possible. In a tumor of polyclonal origin, one or more cells or groups of cells in close proximity, each carrying one of the phenotypic characteristics of tumors, may interact to initiate tumorigenesis [64]. This scenario suggests that it may be possible to model tumor development in a spatial manner (see Fig. 2B). Although mutations rates for tumor-initiating events have not been measured experimentally, absolute levels of "initiating" mutations can and have been measured in normal epithelium, as well as in tumor tissue. This, therefore, raises the possibility that

polyclonal tumor origin could be modeled based on direct measurements of the frequency and clonal distribution of various tumor-initiating events. According to this modeling approach, the exponential increase in tumor incidence with age relates to the probability that two or more complementing clones come within a certain proximity of each other, a probability that would increase with time. Recognition that many different tumor types may have polyclonal tumor origin, therefore, provides new opportunities for mathematical modeling of carcinogenesis, suggesting approaches that have the potential to produce biologically realistic models of tumor development.

Polyclonal tumor origin also has therapeutic implications. The medical and scientific community is currently exploring opportunities for personalized medicine. This includes selecting particular chemotherapeutic approaches based on the identification of particular molecular lesions within a patient's cancer. K-*RAS* mutation was shown to cause tumors of epithelial origin that are resistant to radiation therapy, yet K-*RAS* mutant tumors can be converted to a radiosensitive phenotype if treated with a particular inhibitor [121–123]. *p53* mutation confers sensitivity to cisplatin-induced apoptosis [124]. Detection of K-*RAS* mutation in colorectal cancer is associated with failure to respond to Cetuximab therapy [125]. Apparently, because K-RAS is a downstream effector of EGFR signaling, EGFR receptor inhibitors have no therapeutic benefit for tumors carrying an activating K-*RAS* mutation.

Monoclonal tumor origin predicts that an initiating lesion will be present in all the neoplastic cells of a tumor and, as such, would be an ideal chemotherapeutic target. Polyclonal tumor origin, however, raises the possibility that small undetected populations of distinct tumor cells are present among the seemingly monoclonal tumor. Such subclones might respond quite differently to the chemotherapeutic directed against the bulk of the tumor. This implies that molecular targets may need to be sampled in multiple areas of a tumor using a sensitive approach for mutation detection before selecting a particular therapy [3]. Initial response to therapy followed by tumor reoccurrence may result through clonal outgrowth of an untargeted tumor subpopulation. Therefore, the probability that a particular tumor type is polyclonal in origin needs to be taken into account when selecting a particular chemotherapeutic strategy. If polyclonality is, indeed, more common than monoclonality, then combination therapies may be more appropriate, in general, than monotherapies.

In summary, unequivocal and unjustifiable adherence to the conceptual model that tumors are monoclonal in origin has outlived its usefulness. The best available data cannot exclude the possibility that some tumor types may be monoclonal in origin; however, there is considerable evidence that many tumor types are polyclonal in origin. It is the intention of this review to provide a framework for reconsidering and re-evaluating the strengths and weaknesses of the different types of data, so that consensus can be reached regarding the clonal origins of specific tumor types. Increasing awareness that many tumors are polyclonal in origin may stimulate productive new experimental strategies to investigate the earliest stages of tumor development.

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Conflict of interest

None.

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