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Cancer-causing karyotypes: chromosomal equilibria between destabilizing aneuploidy and stabilizing selection for oncogenic function

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Abstract The chromosomes of cancer cells are unstable, because of aneuploidy. Despite chromosomal instability, however, cancer karyotypes are individual and quasi-stable, as is evident especially from clonal chromosome copy numbers and marker chromosomes. This paradox would be resolved if the karyotypes in cancers represent chromosomal equilibria between destabilizing aneuploidy and stabilizing selection for oncogenic function. To test this hypothesis, we analyzed the initial and long-term karyotypes of seven clones of newly transformed human epithelial, mammary, and muscle cells. Approximately 1 in 100,000 such cells generates transformed clones at 2−3 months after introduction of retrovirus-activated cellular genes or the tumor virus SV40. These frequencies are too low for direct transformation, so we postulated that virus-activated genes initiate transformation indirectly, via specific karyotypes. Using multicolor fluorescence in situ hybridization with chromosome-specific DNA probes, we found individual clonal karyotypes that were stable for at least 34 cell generations—within limits, as follows. Depending on the karyotype, average clonal chromosome numbers were stable within ± 3%, and chromosome-specific copy numbers were stable in 70−100% cells. At any one time, however, relative to clonal means, per-cell chromosome numbers varied ±18% and chromosome-specific copy numbers varied ±1 in 0−30% of cells; unstable nonclonal markers were found within karyotype-specific quotas of <1% to 20% of the total chromosome number. For two clones, karyotypic ploidies also varied. With these rates of variation, the karyotypes of transformed clones would randomize in a few generations unless selection occurs. We conclude that individual aneuploid karyotypes initiate and maintain cancers, much like new species. These cancer-causing karyotypes are in flexible equilibrium between destabilizing aneuploidy and stabilizing selection for transforming function. Karyotypes as a whole, rather than specific mutations, explain the individuality, fluidity, and phenotypic complexity of cancers.

1. Introduction

1.1. The unstable chromosomes of cancers

The chromosomes of cancer cells are numerically and structurally unstable [1−5]. This chromosomal instability of cancer cells is proportional to the degree of aneuploidy or chromosomal imbalance, and is dominant in fusions with stable cells [6−9]. Based on this evidence, we have recently proposed that aneuploidy destabilizes the numbers and structures of chromosomes autocatalytically, because it unbalances teams of proteins that segregate, synthesize, and repair chromosomes [7−11].

Owing to the inherent chromosomal instability of aneuploidy, the progeny of the stem cells of clonal cancers typically evolve subclonal and nonclonal aneusomies over time, generating karyotypic diversity within tumors [2,3,8,9,12−16]. Accordingly, the karyotypes of cancers are heterogeneous, unlike those of normal tissues [10,17,18].

1.2. The paradox of the stable individual karyotypes of cancers

The karyotypes even of highly aneuploid cancers are individual [19] clonal, and thus stable over time, despite

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chromosomal instability [2,20–26]. The karyotypic stability of cancers is especially evident from the clonality of chromosome-specific copy numbers revealed by comparative genomic hybridization [27–29], and from clonal marker chromosomes [2,30]. Moreover, comparative genomic hybridizations and conventional cytogenetic analyses, tracing the karyotypes of individual cancers over multiple stages of carcinogenesis up to 17 years, have identified in primary lesions "karyotypes that were as complex as their paired relapses" [21,22,24,25,31–37] (quotation from Jin et al. [31]).

The individuality and basic stability of the cancer karyotypes is, however, a paradox, in view of the notorious instability of cancer chromosomes. Accordingly, an influential review by Wolman [20] notes that the karyotypes of cancers are "surprisingly stable" despite "karyotypic progression." "Contrary to expectation, … three distinct stages of breast cancer [had] highly similar … transcriptomes [reflecting the chromosomal imbalances of the karyotypes, see 4.1]"[38]. Likewise, it was noted with surprise that human clear cell sarcomas maintained the same karyotypes in serial transplantations in nude mice [39], and that rat tumors maintained the same karyotypes over years of serial transplantations in rats [40,41]. Similarly, it was described as an oddity that certain cancers, which are naturally transplanted by bites and sex among histocompatible dogs and Tasmanian devils, have maintained identical karyotypes over many such transplant generations [42–44].

Moreover, several researchers have pointed out that the karyotypes of cell lines derived from human cancers are "unexpectedly" [45], "relatively" [46], and "remarkably" [4,16,46] more stable than predicted from the chromosomal instability of cancer cells [17,46,48–51]. The HeLa cell line, which was derived from a human cervical cancer in 1951, is a primary example. The line has apparently maintained its individual karyotype with an average per cell chromosome number of ~78 and with line-specific chromosome copy numbers and marker chromosomes for >50 years in cell culture [47,52–54]. Further, Reshmi et al. [16] found that, even though the modal number is conserved in cancer cell lines, the chromosomes within are "not necessarily … the same."

Gusev et al. [17] termed this paradox "stability within instability," and Albertson et al. [4] commented in a recent review that "these cells do show substantial cell-to-cell variability but the average genotype is stable."

1.3. Hypothesis: selection for oncogenic function stabilizes the karyotypes of cancers

The apparent paradox of individual and stable karyotypes in the presence of unstable chromosomes would be resolved if cancers are generated and maintained by new individual karyotypes—much like new species [55]. Because the karyotypes of cancers are aneuploid and thus unstable, their stability would depend on constant karyotypic selection for oncogenic function. Already in 1969, Levan [43] considered cancer-specific karyotypes as an alternative to "invisible genetic changes" (i.e., mutations): "it would be reasonable to expect a priori that each tumor type would be characterized by one karyotype, just as … a species is characterized by its karyotype." In the same year, Foulds [19] included "cytogenetic and biochemical individuality" in the definition of cancers.

Because aneuploidy imparts a persistent risk on karyotypes of losing their identity by random gains and losses of chromosomes, the karyotypes of cancers must evidently be selected for quotas of alternative chromosomal variations that retain transforming function—if they are indeed cancer-causing genomes. This is particularly relevant for cancer genomes, because their phenotypes are variable and even include so-called immortality [9,18,56]. The risk of function loss by karyotype alterations is illustrated by the fact that fusion with normal cells, or the introduction of specific chromosomes, can obliterate the transformed phenotypes of cancer cells [57–63].

To test the karyotypic cancer theory, we have studied here the initial and long-term karyotypes of seven newly formed clones of transformed human epithelial, mammary, and muscle cells. Approximately 1 in 100,000 of such human cells generate clones of transformed cells, by 2–3 months after introduction of retrovirus-activated cellular genes or the tumor virus SV40 (simian virus 40) [15,64–67]. These frequencies are, however, too low for direct transformation by genes [15,66,68–70], so we postulated that these virus-activated genes would transform indirectly, by inducing the aneuploid oncogenic karyotypes predicted by our hypothesis. The known ability of such genes, particularly those of SV40, to destabilize chromosomes supports this assumption [15,40,71–76]. In addition, we have studied the karyotype of an SV40-transformed human mesothelial cell line [65].

We now describe the stability over time and per-cell variability of three karyotypic parameters of the seven newly transformed clones and the cell line: (a) clone-specific chromosome numbers, (b) clone-specific copy numbers of intact and clonal marker chromosomes, and (c) gains and losses of nonclonal marker chromosomes. These karyotypic parameters were determined by analyses of metaphase chromosomes hybridized in situ with chromosome-specific, color-coded DNA probes (m-FISH) [10,15]. The results indicate that each transformed clone contained an individual, quasi-stable karyotype.

2. Materials and methods

2.1. Tumorigenic Transformation of human cells with 6 retrovirus–activated genes and with SV40

Cultures of primary human mammary and muscle cells transduced with retrovirus-activated cell-derived genes and hence termed Ma6 and Mu6 cells, were kindly provided by Christopher M. Counter, who, with coworkers [66], constructed these genes by splicing genes for human telomerase, cyclin, cyclin kinase, p53, myc, and ras

proteins into murine retrovirus vectors. Cells carrying such virus-activated genes form clones of transformed tumorigenic cells at frequencies of ~1 in 100,000 within 2 to 3 months after transduction [15,66].

To transform normal human dermal fibroblasts (PromoCell, Heidelberg, Germany) with SV40, we incubated ~100,000 cells in a 5-cm culture dish at a multiplicity of infection close to 10 for 1−12 hours in serum-free RPMI 1640 medium at 37°C [65]. The cultures were then supplemented with 10% fetal calf serum and incubated until confluent. Confluent cultures were split fourfold once or twice and aliquot cultures were incubated without further subdivisions to allow focus formation. Approximately 1−10 foci of transformed three-dimensionally growing cells began to appear 2−3 months after infection of 100,000 cells. Such foci were picked out with micropipettes and propagated for cytogenetic analyses. A clonal line of mesothelial cells transformed with SV40 in its 62nd passage in vitro was kindly provided by Michele Carbone (see Bocchetta et al. [65]).

2.2. Cloning transformed cells in 0.4% agar gels

To obtain single-cell-derived clones of transformed human mammary and muscle cells, the Ma6 and Mu6 cells transduced with 6 retrovirus-activated genes were propagated in agar gel suspensions, to discriminate against growth of normal cells [77]. For this purpose, ~10^5 to ~10^6 Ma6 or Mu6 cells were suspended in 3 mL of 0.35−.4% agar (A9915; Sigma−Aldrich, St. Louis, MO) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 40°C. The suspension was layered on 2.5 mL of solidified 0.4% agar in the same medium in a 5-cm culture dish. On the next day, the cultures were overlaid with 1 mL of RPMI 1640 medium supplemented with 2.5% fetal calf serum and 2.5% calf serum. This over-layer was changed once or twice a week until colonies appeared in the agar suspension, 1−2 months later. These colonies were picked out with micropipettes and propagated for karyotype analyses.

2.3. Identification of metaphase chromosomes by m-FISH

At 1−2 days before karyotyping, cells were seeded at <50% confluency in RPMI 1640 medium containing 2.5% fetal calf serum and 2.5% calf serum. After ~75% confluency was reached, the medium was replaced with 3 mL of fresh culture medium per 5-cm culture dish. Cultures were then incubated either directly or after another day in culture with 0.15 μg Colcemid solution (KaryMax; GIBCO Invitrogen Co., Grand Island, NY) at 37°C for 1−4 hours. The medium was then collected, and the cells were washed with phosphate-buffered saline at pH 7, dissociated with 1 mL 0.5% trypsin−EDTA (GIBCO Invitrogen) for a few minutes at 37°C just until they detached from the dish, combined with the removed growth medium, and centrifuged for 6 minutes at 175 *g* at room temperature. The cells were then resuspended in 10 mL hypotonic KCl at 0.075 mol/L (Sigma Co., St Louis, MO) and incubated at 37°C for 16 minutes. Thereafter, the cells were cooled in ice-water for 3 minutes, and then 5% (v/v) of freshly prepared ice-cold fixative was added, as a mixture of 3 volumes of methanol and one volume of acetic acid [78,79]. This solution was then centrifuged for 6 minutes at 175 *g*. The cell pellet was resuspended in 0.3−0.5 mL supernatant and ~10 mL of ice-cold fixative was mixed in dropwise. The solution was incubated at room temperature for 15 minutes, then centrifuged again at 175 *g* as described and resuspended once more in fresh fixative. After 15 minutes at room temperature, the cells were again pelleted and resuspended in ~0.5 mL of the supernatant fixative.

Ten microliter aliquots were placed with a micropipette onto glass microscope slides held at an angle and immediately inspected under a phase contrast microscope for the presence of metaphase chromosomes. Slides with suitable metaphase chromosomes were then processed for m-FISH hybridization with chromosome-specific, color-coded DNA probes as described by the manufacturer (MetaSystems, Boston, MA; Altlussheim, Germany) and by us previously [10].

3. Results

3.1. Karyotypes of four clones of newly transformed human mammary cells

To test the hypothesis that aneuploid karyotypes consisting of unstable chromosomes are nonetheless sufficiently stable to initiate and maintain transformation, we first analyzed the karyotypes of four clones of newly transformed human mammary cells. These clones were prepared by incubating primary mammary cells (into which 6 retrovirus−activated genes had just been introduced) in soft (0.4%) agar gels, which discriminate against the growth of normal cells [66,77] (described in section 2.2.). After incubation for 1 to 2 months, ~1 in 100,000 of these Ma6 cells grew into colonies of transformed cells, confirming earlier results of Kendall et al. [66].

Cells prepared from such single-cell-derived clones grew at high rates in culture dishes. The karyotypes of the cells were analyzed by in situ hybridization of metaphase chromosomes with color-coded chromosome-specific DNA probes (described in section 2.3). As observed previously, a minority of these cells nevertheless died and detached from the culture dish, rendering the culture medium slimy (due to cellular DNA) [15], even while the majority of the cells continued to thrive.

3.1.1. Average chromosome numbers of Ma6 clones stable within ± 1.5% over many cell generations

A representative m-FISH karyotype of Ma6 clone 7 (C7) is shown in Figure 1 A. All four Ma6 clones (C1, C5, C7, and C10) had near-tetraploid stemlines (Fig. 1A).

The total per-cell chromosome numbers of 20 cells of each Ma6 clone were heterogeneous (Table 1). The chromosome numbers of Ma6 clone C1 ranged from 76 to 93 (with the exception of one outlier with 46 chromosomes), those of C5 ranged from 75 to 81, those of C7 from 72 to 84, and those of C10 from 76 to 83. The clonal averages were 81 for C1, 79 for C5, 80 for C7, and 79 for C10 (Table 1). Thus, the per-cell chromosome numbers of the four Ma6 clones varied within −16% to +15% around their clonal averages. We will show later in this article that the cell-to-cell variability of the chromosome numbers is not only the product of numeric instabilities of intact chromosomes and clonal marker chromosomes, but also of structural instabilities that generate and drop nonclonal marker chromosomes at relatively high rates.

To test our hypothesis that the karyotypes of cancers are quasi-stable because of selection for various transforming functions (i.e., oncogenic function), we first analyzed the stability of the average clonal chromosome numbers over two passages differing by ~10 cell generations. After 10

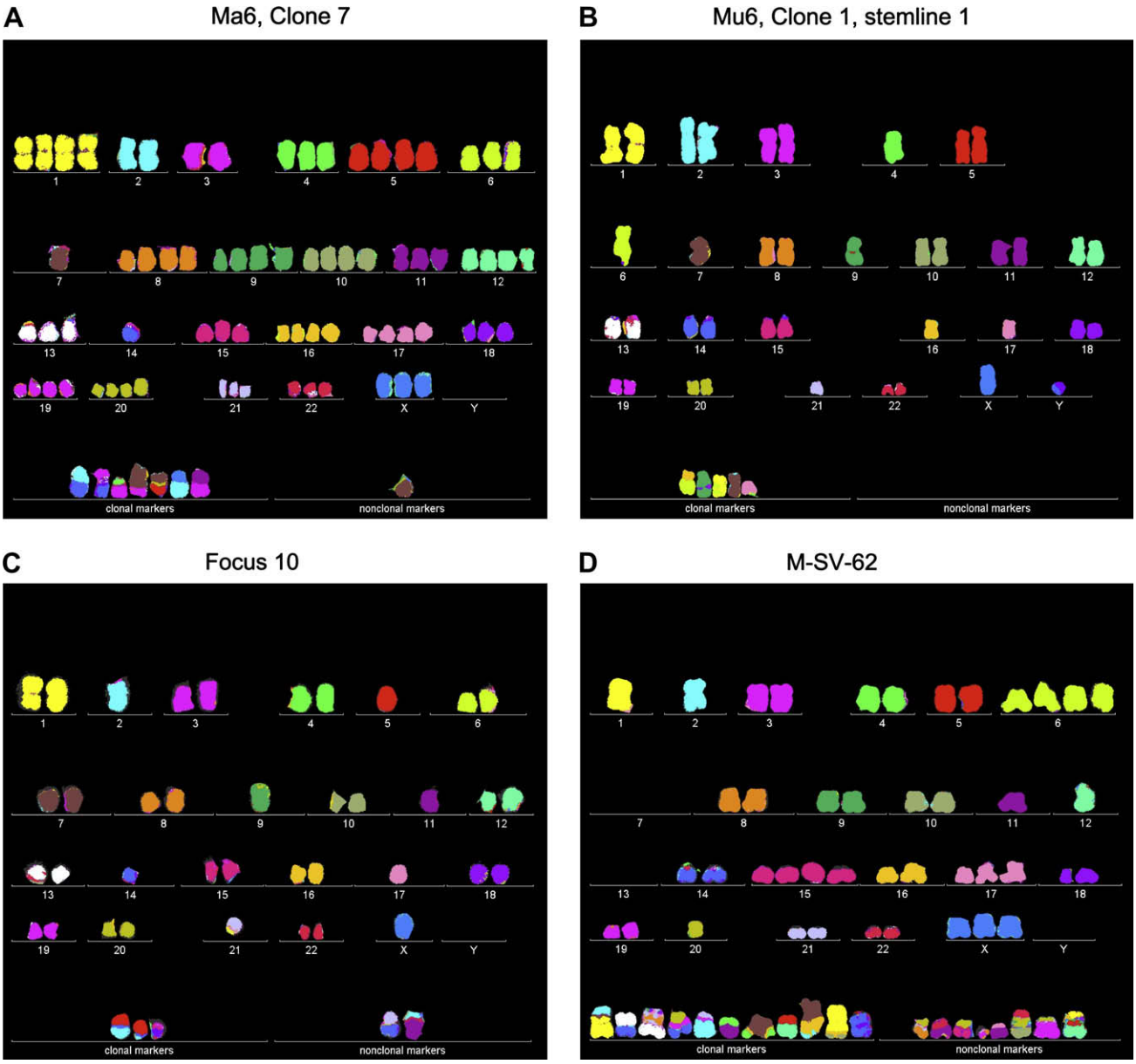

Fig. 1. Representative multicolor fluorescence in situ hybridization (m-FISH) karyograms for clones of newly transformed human primary cells and of a human cell line: (A) Karyogram from a clone of transformed human mammary cells, termed Ma6. (B) Karyogram from a clone of transformed human muscle cells, termed Mu6 (stemline 1). (C) Karyogram from a clone of transformed human dermal epithelial cells, termed Focus 10. (D) Karyogram from a line of transformed human mesothelial cells, termed M-SV-62. Metaphase chromosomes were hybridized in situ with chromosome-specific, color labeled DNA probes. Ma6 and Mu6 clones were obtained from cells transduced with 6 retrovirus-activated cell-derived genes. Focus 10 and the M-SV-62 line were obtained from cells infected with the SV40 tumor virus.

Table 1
Chromosome count for 20 metaphases of four Ma6 clones of transformed human mammary cells.

	Per-cell chromosome count																				
Clone and passage	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	Avg ± SD
Clone C1																					
p1	93	83	82	82	82	82	81	80	80	80	80	79	79	79	79	79	79	78	76	46[a]	81 ± 3
p2	93	83	83	83	83	82	82	82	82	82	82	81	81	80	80	79	79	78	75	68	81 ± 4
Clone C5																					
p1	81	81	81	81	81	80	80	80	80	80	79	79	79	79	78	78	78	78	76	75	79 ± 2
p2	85	81	80	80	80	79	79	79	79	79	79	79	79	78	78	78	77	75	74	68	78 ± 3
Clone C7																					
p1	84	83	83	83	83	83	83	82	82	82	81	81	81	81	78	77	77	74	73	72	80 ± 4
p2	84	83	81	81	81	81	80	80	80	80	80	79	78	78	78	76	75	72	72	71	79 ± 4
Clone C10																					
p1	83	82	82	82	81	81	81	80	80	80	80	78	78	78	78	77	77	76	76	76	79 ± 2
p2	84	82	82	81	81	81	81	81	81	81	81	81	81	80	80	80	78	78	77	76	80 ± 2

Abbreviations: Avg, average; C, clone; p, passage; SD, standard deviation; M, metaphase.
[a] Excluded from average.

cell generations, the per-cell chromosome numbers of the four clones at passage 2 were again heterogeneous, varying within nearly the same ranges around the same clonal averages as at passage 1; thus, the average chromosome numbers, differing by only 0 to 1, were stable within 1.5% over 10 cell generations (Table 1). Because the Ma6 clones consisted of ~10^7 cells by the time they were first karyotyped, their cells were already 24 generations ($10^7 \approx 2^{24}$) away from their stem cell at passage 1, or ~34 generations at passage 2—so they were probably stable for 34 cell generations.

Considering the population heterogeneity (i.e., that the cell-to-cell chromosome numbers fluctuated as much as −16% to +15% around clonal averages), the clonal averages of the chromosome numbers could have drifted far beyond the ±1.5% range during these 34 cell generations. For example, if the Ma6 C1 cell with the highest observed chromosome number, 93, had predominated in the generations after passage 1, the average chromosome number of the clone could have increased from 81 to 93—but this was not the case. It follows that some fitness selection, presumably for oncogenic or transforming functions, held the average per-cell chromosome numbers of each of the four clones close to that of their original stem cell for 34 cell generations.

3.1.2. Clonal copy numbers of intact and marker chromosomes

The copy numbers of most intact chromosomes and of seven highly clonal marker chromosomes were also clone-specific in 70–100% of the cells of each of the four Ma6 clones (Table 2). In Ma6 clone C5, for example, the copy number for chromosome 1 was 4 in 90–95% of cells; that of chromosome 14 was 1 in 75–95% of these cells, and that of the clonal marker chromosome der(3,14) was 1 in 90–100% of these cells. The subclonal marker chromosomes (Table 2) are described in the next section.

In the remaining 0–30% cells, the copy number of most intact chromosomes typically oscillated by ±1 above and below the clonal copy number, with the exception of a few outliers. (For an example, see the 20 metaphases of Ma6 clone C10 in Table 3.) The losses, however, outnumbered the gains by ~3:1 among intact chromosomes. Probably for the same reason, the highly clonal marker chromosomes were missing (most of them were present only as single copies) in 0–20% cells. The specific copy numbers of a few intact chromosomes (e.g., chromosomes 8 and 16) oscillated between adjacent clonal values—that is, with either 3 or 4 copies, at clonal percentages of 55/35 for chromosome 8 and 15/75 for chromosome 16.

The 70–100% clonality of the copy number of the intact chromosomes and of the seven highly clonal marker chromosomes could reflect either gradual deterioration of a stem cell karyotype, or alternatively, as proposed in our hypothesis (section 1.3), a dynamic equilibrium between chromosomal instability and selection for oncogenic karyotype.

To distinguish between these two possibilities, we again compared two passages of these clones differing by ~10 cell generations for the stability of the copy numbers of individual chromosomes. The copy numbers of the intact and clonal marker chromosomes of passage 2 were within 20% of those of passage 1, with the exception of a few outliers (Table 2). Thus, the clonal chromosome-specific copy numbers were stable within the stated limits for at least 10 cell generations, and probably for 34 generations (as noted in the previous section). This supports the idea of a dynamic chromosomal equilibrium with selection for transforming function counteracting the inherent instability of aneuploidy.

The stability test further revealed that eight subclonal markers, present in only a low percentage of cells per clone, were also passage-independent and thus stable (Table 2). Their absence in some passages of the same clone could reflect their presence in <1 per 20 cells. According to our hypothesis, such subclonal markers are stable acquisitions

Table 2
Percentage copy number of intact and clonal marker chromosomes in the four Ma6 clones at passages p1 and p2

		Copy number in 20 cells, %							
		Clone C1		Clone C5		Clone C7		Clone C10	
Chromosome	Copies, no.	p1	p2	p1	p2	p1	p2	p1	p2
1	4	80	95	100	90	90	85	95	90
2	2	90	90	100	80	95	100	95	80
3	2	75	85	65	40	70	80	70	85
4	3	85	75	100	85	90	100	90	100
5	4	90	95	75	70	90	75	85	80
6	3	70	85	90	100	95	80	85	100
7	2	75	85	90	95	95	90	65	95
8	3/4	55/35	70/30	15/85	55/35	15/75	20/80	35/65	75/15
9	4	85	80	90	75	95	90	75	95
10	4	75	95	70	30	85	65	70	100
11	3	70	90	95	95	80	95	85	85
12	4	90	90	95	95	75	85	80	90
13	4	65	85	40	35	70	65	65	65
14	1	65	90	75	95	95	85	85	85
15	3	80	100	85	90	90	90	95	90
16	3/4	15/75	25/65	15/80	50/50	15/85	60/35	35/50	30/55
17	4	90	95	95	90	95	90	90	85
18	3	80	80	75	90	80	85	90	95
19	4	80	65	75	80	60	75	75	70
20	4	85	80	85	90	75	95	80	80
21	3	80	95	60	85	75	75	85	90
22	3	75	85	75	90	90	90	75	95
X	3	80	90	75	60	85	60	85	80
der(2;14)	1/2	85/0	30/70	100/0	100/0	95/0	100/0	65/25	40/60
der(3;14)	1	90	95	100	100	95	100	100	90
der(3;4)	1	55	95	90	90	100	95	100	100
der(3;7)	1	80	95	85	100	95	100	80	95
der(5;7)	1	80	95	80	95	100	100	85	95
der(X;2)	1	85	80	100	80	100	100	95	85
der(3;11)	1	85	90	90	90	90	95	10	55
der(10;13)	1	—	—	55	60	—	—	—	—
der(8;19)	1	5	10	—	—	5	—	5	—
der(10;18)	1	—	—	5	5	—	—	—	—
der(3;10)	1	—	—	5	5	—	—	5	—
der(2;16)	1	—	—	—	—	—	—	5	5
der(1)	1	—	—	—	—	—	—	10	10
der(3)	1	5	—	5	5	20	—	20	20
der(2)	1	—	—	5	—	10	—	15	—

If specific copy numbers oscillated between adjacent clonal values, two values are reported, separated by a slash mark. For example, chromosome 8 had either 3 or 4 copies (3/4), at a clonal percentage of 55% or 35%, respectively (55/35).

Abbreviations: Ma6, transformed human mammary cells.

of stochastically generated marker chromosomes by the original clone (as discussed in the next section), which can generate new subclones or sidelines. The same is also typical of natural cancers [2,3,80].

Contrary to expectation, all four single cell-derived Ma6 agar-clones shared nearly the same average clonal chromosome numbers, shared similar copy numbers of intact and highly clonal marker chromosomes, and shared the same quotas for nonclonal marker chromosomes (see next section). This indicates that the four clones derived from a common stem cell and that this stem cell must have been the predominant agar-clonogenic cell in the original Ma6 line. A karyotype analysis of the original Ma6 line indeed revealed a candidate precursor with a near-tetraploid karyotype similar to that of the four Ma6 clones, including three of the seven highly clonal marker chromosomes shared by the four Ma6 clones (data not shown). Given a common precursor, it can be deduced that all four Ma6 clones studied were >68 generations apart from each other, because at passage 2 they were each ~34 generations away from their common precursor (see section 3.1.1). It follows that the average clonal chromosome numbers and chromosome-specific copy numbers of the four Ma6 clones were quasi-stable for 68 generations.

3.1.3. Nonclonal marker chromosomes

Any unstable nonclonal and subclonal passage-dependent markers of the four Ma6 clones were identified

Table 3
Copy number of intact and clonal marker chromosomes for 20 metaphases of Ma6 clone C10, passage 2

	Per-cell copy number																			
Chromosome	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
Total	n=84	n=82	n=82	n=81	n=81	n=81	n=81	n=81	n=81	n=81	n=81	n=81	n=81	n=80	n=80	n=80	n=78	n=78	n=77	n=76
1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	4
2	2	2	2	2	2	2	2	4	3	3	2	2	2	2	2	2	2	2	2	1
3	1	2	2	2	2	1	2	1	2	2	2	2	2	2	2	2	2	2	2	2
4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
5	3	4	4	3	4	3	4	4	4	4	4	4	4	4	4	3	4	4	4	4
6	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
7	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2
8	5	3	3	3	4	3	3	3	3	3	3	4	3	3	3	3	1	3	3	4
9	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
10	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
11	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	3	2	3
12	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	3	4	4	4
13	4	4	4	4	4	4	4	3	4	4	3	3	4	4	3	3	4	3	4	2
14	2	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	2
15	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3	2	3	3	3	3
16	4	4	3	4	3	4	4	2	4	4	5	3	4	3	4	3	3	2	4	4
17	4	4	4	4	4	4	4	4	3	3	4	4	4	4	4	4	4	4	4	2
18	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3
19	3	4	4	4	3	3	4	4	4	4	4	5	4	4	3	4	4	4	4	3
20	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	3	3	3	4	4
21	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	3
22	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3
X	3	3	3	3	3	2	3	3	2	2	3	3	3	3	3	3	3	3	2	3
der(2;14)	2	2	1	1	2	1	1	2	2	2	2	2	1	1	2	2	1	2	2	1
der(3;14)	1	1	1	1	1	2	1	—	1	1	1	1	1	1	1	1	1	1	1	1
der(3;4)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
der(3;7)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	—
der(5;7)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	—	1	1	1
der(X;2)	1	1	1	1	1	1	1	—	1	1	1	1	1	1	1	1	1	—	—	1
der(3;11)	1	—	—	1	1	1	1	1	—	—	—	—	—	—	—	1	1	1	—	1

Ma6 C10 is a clone of transformed human mammary cells.
Abbreviations: M, metaphase.

by comparisons of two passages differing by 10 cell generations. These comparisons revealed that the Ma6 cells contained on average 0.6 to 1.5 such markers, and a maximum of five (Table 4). The simplest explanation for the large numbers of nonclonal and passage-dependent, subclonal markers is again a dynamic, karyotype-specific equilibrium of gains and losses without stability selection (see previous section). In this equilibrium, the nonclonal and the passage-dependent subclonal markers would have half-lives of <10 generations and would thus be unstable.

Because these markers are not stable and appear in only a minority of transformed cells, we conclude that they are not relevant for oncogenic function. Nevertheless, such markers could yet become relevant for oncogenic function and thus become stable under new selective conditions, in what is called tumor progression [19] (see Discussion).

According to our hypothesis, the nonclonal and unstable subclonal markers are inevitable consequences of the inherent chromosomal instability of aneuploid karyotypes. The apparent limit of five such markers per Ma6 cell confirms the view (as noted in the Introduction) that there is a karyotype-specific quota above which these nonclonal markers would either corrupt the oncogenic function or the viability of the respective karyotypes.

In sum, these results support the idea that the Ma6 karyotypes are held to their clonal chromosome numbers, their chromosome-specific copy numbers, and their quotas of unstable nonclonal markers by selection for oncogenic function encoded by the karyotypes as a whole—despite surprisingly high frequencies of chromosomal instabilities. By way of example for chromosomal instability within karyotypic stability, of the 20 Ma6 clone C1 cells at passage 1, no 2 cells have identical karyotypes, even if nonclonal markers are excluded (Table 3). Of the four Ma6 clones analyzed, two contained a few cells per 20 with identical karyotypes: Ma6 clone C5 at passage 1 had three such cells, and Ma6 clone C10 at passage 2 had two identical karyotypes per 20 cells—excluding the nonclonal markers (data not shown).

3.2. Karyotypes of clones of newly transformed human muscle cells

Following the experimental outline developed here, we next tested two clones of newly transformed human muscle

Table 4
Nonclonal and passage-dependent subclonal marker chromosomes of the four Ma6 clones

Nonclonal and subclonal markers per metaphase, with average per clone and passage																
Clone C1				Clone C5				Clone C7				Clone C10			
p1		p2		p1		p2		p1		p2		p1		p2	
M1	der(4;5)	M1	t(1;2)	M1	der(21;18)	M1	der(1;5)	M1	del(6)	M1	der(1;5)	M1	der(3;16)	M1	der(5;21)
	der(8;17)		t(2;1)		del(6p)	M2	—	M2	—		der(1;2;4)	M2	der(8;14)	M2	der(X)
M2	del(13p)		der(2;8)	M2	der(3;7)	M3	—	M3	—		der(3;?)	M3	del(16)		der(8)[a]
	del(2p)[a]	M2	der(8)	M3	der(7;19)	M4	—	M4	—	M2	—		der(1;16)	M3	—
M3	der(3;15)	M3	—		der(13;14)	M5	—	M5	—	M3	—		der(3;21)	M4	der(2;3)
M4	der(3;4;7)	M4	—	M4	der(19;22)	M6	der(4;16)	M6	der(8;12)	M4	—	M4	del(9)		der(9;15)
M5	der(15;21)	M5	der(6;8)		der(11;22)	M7	—		der(1;17)	M5	der(5;13)		der(X;3)		der(16;20)[a]
M6	—	M6	—		der(?)	M8	der(2;15)	M7	—	M6	—		der(7;19)	M5	—
M7	—	M7	der(16)	M5	—	M9	der(5;9)	M8	—	M7	—	M5	—	M6	—
M8	del(9p)		der(8;16)[a]	M6	tas(13;15)		der(1;12)	M9	—	M8	—	M6	der(2;3)	M7	der(12)
	der(1;18)	M8	der(8;16)[a]		tas(13;14)	M10	der(8;13)[a]	M10	—	M9	der(1)		der(X;2;9)		der(3;12)[a]
M9	der(8;11)	M9	—	M7	der(7;20)		der(13;16)	M11	—	M10	der(3;19)	M7	—	M8	—
	der(2;13)	M10	—	M8	der(14;22)	M11	der(8)[a]	M12	—	M11	—	M8	dic(4;12)	M9	—
	der(4;8)	M11	der(3;8)		der(6)[a]	M12	der(8)[a]	M13	—	M12	der(2;8;16)		der(X;9)[a]	M10	der(3;12)[a]
	der(X;14)	M12	der(8;16)[a]		i(10p)	M13	der(2;3)	M14	der(19;21)	M13	der(X;6)	M9	—	M11	der(3;13)
	i(10p)	M13	der(19)	M9	—	M14	—		der(11;14)		der(X;16)	M10	der(X;9)[a]		der(7;13)
M10	del(16p)	M14	der(17;19)	M10	der(20;19)	M15	der(8;13)[a]	M15	der(16;17)	M14	—	M11	der(?)	M12	der(8;18)
	del(1p)[a]	M15	der(8;16)[a]		der(?)	M16	der(8)[a]	M16	der(16;12)	M15	der(7)	M12	—		der(5;16)
M11	—	M16	—	M11	—		der(2;10)		der(10;20)	M16	—	M13	der(13)		der(15;20)
M12	der(22;?)	M17	—	M12	der(10;11)		der(3;8;11)	M17	der(13;14)	M17	der(8;12)		der(15;21)		der(16;20)[a]
	del(1p)[a]	M18	—	M13	der(6)[a]		der(10)	M18	der(4)		der(14;20)	M14	der(5;12)	M13	der(16;20)[a]
M13	—	M19	der(9;12)	M14	der(5;12)		der(X;16)		der(X;12)	M18	—		der(8;13)	M14	der(3;21)
M14	—		der(19)	M15	der(21;22)	M17	der(16;20)	M19	der(20;21)	M19	—	M15	—	M15	—
M15	del(2p)[a]	M20	der(6;18)		der(13;18)		der(5)	M20	—	M20	der(16)	M16	—	M16	—
	der(10)			M16	—	M18	der(5;10)					M17	—	M17	der(1;3)
M16	—			M17	der(13;22)[a]	M19	der(16;21)					M18	der(12;17)	M18	der(5;8)
M17	der(7;13)			M18	—		der(5;16)					M19	der(6;13)		der(11;16)
	del(1p)[a]			M19	der(9;15)	M20	—						der(2;18)		der(2;12)
M18	der(2;3;7)			M20	der(13;22)[a]							M20	—		der(2;11)
M19	—													M19	der(5;14)
M20	der(8;14)													M20	der(9)
	der(13;17)														der(8)[a]
	der(1;2)														der(1;2;3)
															der(X;14)
	Avg ± SD		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**
	1.3 ± 1		0.8 ± 1		1.3 ± 1		1 ± 1		0.8 ± 1		0.6 ± 1		1.2 ± 1		1.5 ± 1

Abbreviations: Avg,average; p, passage; M, metaphase; Ma6, transformed human mammary cells; SD, standard deviation

[a] Shared by ⩾2 cells in the same passage.

cells, termed Mu6 C1 and C4, for their compatibility with the hypothesis that specific karyotypes generate and maintain cancers. The isolation of these clones by growth in agar gels was as described in section 2.2, and a preliminary analysis of the karyotype of Mu6 C1 was reported previously [15].

3.2.1. Average chromosome numbers of Mu6 clones stable within ± 3% over many cell generations

A representative karyotype of Mu6 clone C1 (stemline 1, passage 1) is shown in Figure 1B. Mu6 clone C1 contained two near-diploid stemlines (Table 5) with distinct chromosome-specific copy numbers (Table 6), and that Mu6 clone C4 contained only one near-diploid stemline. Further work is needed to determine, whether the two stemlines of C1 segregated as a single agar-clone by chance or because of some neoplastic cooperation.

Both stemlines of Mu6 clone C1 were associated with several ploidy variants, which were multimers of the near-diploid stemlines because the specific copy numbers of their chromosomes were approximate multimers of those of the diploid stemlines (Table 6). Thus, the ploidies of the Mu6 C1 stemlines were unstable, varying among near-diploid, near-tetraploid, and higher polyploids.

At passage 1, the per-cell chromosome numbers of the near-diploid Mu6 clone C1 stemline 1 ranged from 42 to 57, and the chromosome numbers of the near-tetraploid variant ranged from 83 to 98; for the near-diploid stemline 2 of C1, chromosome numbers ranged from 40 to 50; and the per-cell chromosome numbers of the near-diploid Mu6 clone C4 ranged from 38 to 49 (Table 5). The respective clonal averages were 44 (C1, stemline 1), 48 (C1, stemline 2), and 43 (C4). Thus, the per-cell chromosome numbers of the near-diploid stemlines varied within −20 to +14% around their clonal averages.

To estimate the stability of the average clonal chromosome numbers of the Mu6 clones, two passages of the clones were again compared, the passages differing by ~10 cell generations. The average chromosome numbers of the C1 stemline 1 was 44 at passage 1 and 45 at passage 2, and those of stemline 2 were 48 at passage 1 and 49 at passage 2, and those of C4 were 43 at passage 1 and 46 at passage 2 (Table 5). Thus, the average clonal chromosome numbers of the Mu6 clones were stable within ± 3% over 10 generations, although at any one time their per-cell chromosome numbers fluctuated within −20 to +14% around clonal averages.

As we have noted, the chromosome copy numbers of these clones were probably stable for 34 generations, because they were clonal when they were first tested at 24 clonal generations at passage 1 (see section 3.1.1 for Ma6 cells). Moreover, because the karyotypes of C1 stemline 1 and C4 were almost identical (Table 6), one can argue, as before, that the two lines had a common precursor and thus were numerically stable within ± 3% for ~68 generations.

3.2.2. Clonal copy numbers of intact and clonal marker chromosomes

The copy numbers of the intact and five highly clonal marker chromosomes of Mu6 clone C4 and of Mu6 clone C1 stemline 1 were the same in 80–100% of the cells, with few exceptions. The compositions of the five markers were also the same (Table 6). Thus the karyotypes of Mu6, C4, and C1 stemline were nearly identical. The copy numbers of the intact chromosomes and five highly clonal markers of Mu6 clone C1 stemline 2 were, however, line-specific, and thus unrelated to the other two (Table 6).

In the remaining 0–20% cells of each clone, the copy numbers of most intact chromosomes again oscillated ±1 around clonal copy numbers, but more typically below than above the clonal copy numbers, and single-copy marker were missing, as described for the Ma6 clones (see section 3.1.2 and Table 3).

A stability test over 10 cell generations showed that the chromosome-specific copy numbers of all three Mu6 clones were within 10% of each other, with the exception of a few outliers (Table 6). Thus, the chromosome-specific copy numbers of the two Mu6 clones were stable for at least 10 cell generations and probably for 34 generations, as explained for the Ma6 clones (see section 3.1.1).

The stability test also revealed two subclonal markers. One was present at a high percentage in Mu6 clone C4 at passage 2, but at a low percentage at passage 1; the other was also present at a high percentage in C4 at passage 2 and at low percentages in C1 stemline 1 at both passages 1 and 2 (Table 6). Note again that absence in one of two passages could mean presence in <1 per 20 cells (see section 3.1.2).

3.2.3. Nonclonal marker chromosomes

Any unstable nonclonal and subclonal passage-dependent markers of the Mu6 clones were identified by comparisons of two passages differing by 10 cell generations. These comparisons revealed that the near-diploid Mu6 cells contained on average 0.15 to 0.7 and a maximum of 4 such markers (Table 7). None of them was shared by different passages. The relatively wide differences of these averages seem to reflect rare stochastic episodes in which relatively high numbers of such markers (up to four) were generated simultaneously, as observed previously by Camps et al. [49]. These episodes may reflect the destabilization of the basic Mu6 karyotype following the stochastic acquisition or loss of a chromosome (see section 3.1.2, and also section 1.3).

The simplest explanation for the nonclonality or passage-dependence of these markers is again that they reflect a dynamic, karyotype-specific equilibrium of gains and losses without stability selection (see section 3.1.3). In this equilibrium, the Mu6 cells with nonclonal markers would have half-lives of less than 10 generations, and would thus be unstable. Because they were both unstable

Table 5
Chromosome count for 20 metaphases of two Mu6 clones of transformed human muscle cells

Clone and passage	Ploidy	Per-cell chromosome count																		Avg ± SD
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	
C1, stemline 1																						
p1	2*n* ±	57[a]	47	46	45	45	45	45	45	45	45	45	44	44	44	44	44	43	43	42	42	44 ± 1
p1	4*n* ±	98	91	91	91	90	90	90	85	83	—	—	—	—	—	—	—	—	—	—	—	90 ± 1
p1	8*n* ±	172	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
p2	2*n* ±	47	46	45	45	45	45	45	45	45	44	44	44	44	44	44	44	44	44	44	43	45 ± 1
p2	3*n* ±	67	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
p2	4*n* ±	85	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C1, stemline 2																						
p1	2*n* ±	50	50	50	50	49	49	49	49	49	49	49	49	49	49	48	48	48	47	47	40	48 ± 2
p2	2*n* ±	52	50	50	49	49	49	48	47	46	45	—	—	—	—	—	—	—	—	—	—	49 ± 2
p2	4*n* ±	95	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C4																						
p1	2*n* ±	49	46	45	45	45	45	44	44	44	44	43	43	43	43	43	43	42	40	40	38	43 ± 2
p2	2*n* ±	48	46	46	46	46	46	46	46	46	46	46	46	46	45	45	45	45	45	44	43	46 ± 1

Abbreviations: Avg, average; C, clone; p, passage; SD, standard deviation; 2*n* ±, near-diploid.
[a] Excluded from average.

and rare, we conclude that they are not relevant for oncogenic function.

The presence of such nonclonal marker chromosomes in our clones and in so-called clonal cancers is compatible with the view that cancer genomes made of inherently unstable, aneuploid karyotypes must have a quota of functionally permissible clonal variations to function as genomes of inherently unstable cancer cells. Karyotype-specific quotas, however, limit this flexibility to numbers that do not cancel the proposed oncogenic function of these karyotypes (see presentation of hypothesis, section 1.3).

In sum, the karyotypes of the three Mu6 clones were also stable, within narrow ranges, for up to 68 cell generations. This result again supports the idea that the clones originated with these or closely related karyotypes and that these karyotypes are maintained by selection for their oncogenic function, counteracting the inherent instability of aneuploidy.

3.3. Karyotype of a focus of human epidermal cells newly transformed with SV40

We tested the karyotype of a focus, also a clone, of transformed cells that appeared in a culture of primary human epithelial cells after infection with the tumor virus SV40 (as described in Materials and methods, section 2.1). Approximately 1 in 100,000 SV40-infected epithelial cells generated such foci by 2 to 3 months after infection, confirming the view that this virus is not sufficient for transformation (see section 1.3). Cultures derived from these foci grew very fast, whereas other cultures are reported to grow slowly or stop growing altogether [65,73,74]. This again suggests that the virus is not sufficient for neoplastic transformation. One of the fast-growing SV40 foci, identified as Focus 10, was chosen for karyotype analysis. Despite vigorous growth, the culture also desquamated dead cells which turned the growth medium slimy with the DNA of dead cells, as has been reported previously [72–74].

3.3.1. Average chromosome numbers of focus 10 were stable within ± 2.5% over many cell generations

A representative near-diploid karyotype of Focus 10 is shown in Figure 1C. Focus 10 contained several karyotypic sublines differing in their ploidies: one near-diploid, one near-tetraploid, and several hypertetraploid sublines. The polyploid lines were multimers of the near-diploid line, because the specific copy numbers of their chromosomes were approximate multimers of those of the near-diploid line (see further in the next section, with Table 9). Thus, the karyotypic ploidy of Focus 10 is unstable. This is similar to that of the near-diploid Mu6 clone C1, whereas the ploidies of the near-tetraploid Ma6 clones were stable.

The per-cell chromosome numbers of the near-diploid stemline of Focus 10 ranged from 42 to 57, averaging 45

Table 6
Copy number and percentage of intact and marker chromosomes in two Mu6 clones at passages p1 and p2, by ploidy

	Copy number in 20 cells, no. (%)										
	Clone C1, stemline 1						Clone C1, stemline 2			Clone C4	
	p1			p2			p1	p2	p2	p1	p2
Chromosome	2*n* ±	4*n* ±	8*n* ± [a]	2*n* ±	3*n* ± [a]	4*n* ± [a]	2*n* ± [b]	2*n* ± [b]	4*n* ± [a]	2*n* ± [c]	2*n* ± [c]
1	2 (95)	4 (88)	8 (5)	2 (80)	2 (5)	4 (5)	1 (100)	1 (90)	2 (5)	2 (90)	2 (95)
2	2 (90)	4 (100)	8 (5)	2 (95)	3 (5)	4 (5)	2 (100)	2 (100)	4 (5)	2 (100)	2 (100)
3	2 (95)	4 (100)	8 (5)	2 (90)	2 (5)	4 (5)	2 (95)	2 (90)	4 (5)	2 (90)	2 (100)
4	1 (100)	2 (100)	4 (5)	1 (80)	1 (5)	2 (5)	2 (90)	2 (100)	4 (5)	1 (90)	1 (100)
5	2 (95)	4 (63)	7 (5)	2 (100)	4 (5)	4 (5)	3 (90)	3 (60)	4 (5)	2 (90)	2 (100)
6	1 (95)	2 (63)	4 (5)	1 (100)	1 (5)	2 (5)	2 (95)	2 (80)	4 (5)	1 (90)	1 (100)
7	1 (100)	2 (88)	8 (5)	1 (95)	1 (5)	2 (5)	2 (90)	2 (80)	4 (5)	1 (85)	1 (90)
8	2 (95)	4 (88)	4 (5)	2 (95)	3 (5)	4 (5)	1 (95)	1 (90)	2 (5)	2 (100)	2 (100)
9	1 (95)	2 (100)	4 (5)	1 (95)	2 (5)	2 (5)	2 (100)	2 (100)	4 (5)	1 (100)	1 (100)
10	2 (90)	4 (88)	8 (5)	2 (100)	4 (5)	4 (5)	2 (85)	2 (90)	4 (5)	2 (90)	2 (100)
11	2 (95)	4 (88)	8 (5)	2 (100)	3 (5)	4 (5)	2 (95)	2 (90)	4 (5)	2 (95)	2 (100)
12	2 (100)	4 (100)	8 (5)	2 (100)	4 (5)	4 (5)	2 (90)	2 (100)	4 (5)	2 (90)	2 (95)
13	2 (90)	4 (75)	8 (5)	2 (80)	3 (5)	4 (5)	1 (100)	1 (100)	2 (5)	2 (95)	2 (100)
14	2 (100)	4 (88)	5 (5)	2 (100)	2 (5)	4 (5)	2 (95)	2 (70)	4 (5)	2 (95)	2 (90)
15	2 (85)	4 (75)	8 (5)	2 (100)	4 (5)	4 (5)	2 (95)	2 (90)	4 (5)	2 (95)	2 (90)
16	1 (95)	2 (75)	4 (5)	1 (95)	2 (5)	2 (5)	1 (100)	1 (100)	2 (5)	1 (95)	1 (100)
17	1 (95)	2 (75)	4 (5)	1 (85)	2 (5)	2 (5)	2 (90)	2 (90)	4 (5)	1 (75)	1 (95)
18	2 (85)	4 (63)	8 (5)	2 (50)	1 (5)	4 (5)	2 (90)	2 (100)	4 (5)	1/2 (80/20)[d]	1 (100)
19	2 (95)	4 (75)	8 (5)	2 (75)	3 (5)	4 (5)	2 (90)	2 (90)	2 (5)	2 (70)	2 (100)
20	2 (90)	4 (88)	8 (5)	2 (85)	3 (5)	4 (5)	2 (95)	2 (80)	4 (5)	2 (95)	2 (90)
21	2 (65)	2 (63)	4 (5)	2 (95)	3 (5)	2 (5)	2 (100)	2 (100)	4 (5)	2 (65)	2 (95)
22	2 (90)	4 (88)	8 (5)	2 (100)	3 (5)	4 (5)	2 (100)	2 (80)	4 (5)	2 (90)	2 (100)
X	1 (95)	2 (88)	4 (5)	1 (100)	1 (5)	2 (5)	1 (100)	1 (100)	2 (5)	1 (95)	1 (100)
Y	1 (95)	2 (88)	4 (5)	1 (85)	2 (5)	2 (5)	2100)	280)	4 (5)	1 (90)	1 (100)
der(6;16)	1 (95)	2 (88)	4 (5)	1 (100)	2 (5)	0 (5)	—	—	—	1 (95)	1 (95)
der(9;6;9)	1 (90)	2 (100)	4 (5)	1 (95)	2 (5)	2 (5)	—	—	—	1 (95)	1 (100)
del(6)	1 (90)	2 (75)	4 (5)	1 (100)	1 (5)	2 (5)	—	—	—	1 (80)	1 (100)
del(7)	1 (90)	2 (88)	4 (5)	1 (95)	2 (5)	2 (5)	—	—	—	1 (90)	1 (95)
der(17q)	1 (80)	2 (88)	4 (5)	1 (85)	1 (5)	2 (5)	1 (10)	1 (20)	—	1 (75)	1 (95)
der(18)	—	—	—	—	—	—	—	—	—	1 (20)	1 (95)
der(4)	1 (5)	—	—	1 (50)	—	—	—	—	—	—	1 (95)
der(X;16)	—	—	—	—	—	—	1 (100)	1 (100)	—	—	—
der(1;7;16)	—	—	—	—	—	—	1 (100)	1 (90)	—	—	—
der(1;12;14;16)	—	—	—	—	—	—	1 (100)	1 (100)	2 (5)	—	—
del(12q)	—	—	—	—	—	—	1 (70)	1 (70)	1 (5)	—	—
der(X;7)	—	—	—	—	—	—	1 (85)	1 (90)	2 (5)	—	—

Abbreviations: 2*n* ±, near diploid.
[a] Only 1 cell of the 20 exhibited this ploidy at this passage.
[b] Copy number was identical at both passages for this ploidy.
[c] Except for chromosome 16 and marker der(4), copy number was identical at both passages.
[d] The specific copy number oscillated between adjacent clonal values: 1 or 2 copies (1/2), at a clonal percentage of 80% or 20%, respectively (80/20).

chromosomes, and the numbers of the near-tetraploid line ranged from 67 to 90, averaging 81 (Table 8). Thus, the per-cell chromosome numbers of two related stemlines of Focus 10 varied within −16 to +28% around their clonal averages.

A test of the karyotypic stability over time once more showed stability: the average chromosome numbers of the near-diploid line of Focus 10 were stable within ± 2.5% over 10 cell generations, varying only between 45 at passage 1 and 43 at passage 2 (Table 8). The average chromosome number of the near-tetraploid lines was 81 at passage 1 and 76 at passage 2. Based on our argument (see section 3.1.1), the stability of the average chromosome numbers over 10 generations can be extrapolated again to 34 generations—the estimated age of the Focus 10 at passage 2.

Because at each point in time the per-cell chromosome numbers of Focus 10 varied within −16% to +28% around their clonal averages, the average chromosome number of Focus 10 could, if there were no stabilizing influences, spread far beyond 95% within a few cell generations. The stability of the average chromosome number thus again supports the view that neoplastic karyotypes are stabilized by selection for oncogenic function.

Note that the various karyotypic ploidies of Focus 10 converged (Table 8), going from a heterogeneous pattern at passage 1 to a less heterogeneous pattern at passage 2 during 10 generations—possibly also due to stability selection.

Table 7
Nonclonal and passage-dependent subclonal markers per metaphase of 20 Mu6 cells, with average for clone, passage, and ploidy

Clone C4				Clone C1, stemline 1										Clone C1, stemline 2					
p1 (2*n* ±)		p2 (2*n* ±)		p1 (2*n* ±)		p1 (4*n* ±)		p1 (8*n* ±)		p2 (2*n* ±)		p2 (3*n* ±)		p2 (4*n* ±)		p1 (2*n* ±)		p2 (2*n* ±)		p2 (4*n* ±)	
	NCM		NCM		NCM		NCM		NCM		NCM		NCM		NCM		NCM		NCM		NCM
M1	der(1)[a]	M1	der(15)[3]	M1	del(10)	M1	dic(10;14;19)	M1	dic(5;14)	M1	—	M1	—	M1	—	M1	—	M1	der(12;20)	M1	der(12;19)
M2	der(10)	M2	—		der(7)		der(7;15)	M2	—	M2	dic(1;7)	M2	—	M2	—	M2	del(10p)		del(7p)		der(12;14)
M3	der(1)[a]	M3	—	M2	—		der(14)	M3	—	M3	—	M3	—	M3	—	M3	der(10)		del(7q)		der(2)
M4	der(18)	M4	—	M3	—		del(10p)	M4	—	M4	—	M4	—	M4	—	M4	der(8;21)		der(14;20)	M2	—
M5	der(19)	M5	—	M4	—	M2	—	M5	—	M5	—	M5	—	M5	—	M5	—	M2	der(20;22)	M3	—
M6	der(6;10)	M6	—	M5	—	M3	der(21)	M6	—	M6	—	M6	—	M6	—	M6	—	M3	—	M4	—
M7	—	M7	—	M6	—	M4	—	M7	—	M7	—	M7	—	M7	—	M7	—	M4	dic(1;7)	M5	—
M8	der(1)[a]	M8	—	M7	—	M5	del(6p)	M8	—	M8	—	M8	—	M8	—	M8	der(10)	M5	—	M6	—
M9	der(11)	M9	—	M8	—		der(5;15)	M9	—	M9	—	M9	—	M9	—	M9	—	M6	—	M7	—
M10	—	M10	—	M9	—	M6	—	M10	—	M10	der(1;3)[a]	M10	—	M10	—	M10	—	M7	—	M8	—
M11	—	M11	—	M10	—	M7	der(5)	M11	—		der(1;19)[a]	M11	—	M11	—	M11	—	M8	—	M9	—
M12	—	M12	—	M11	—	M8	—	M12	—	M11	der(1;3)[a]	M12	—	M12	—	M12	der(4)	M9	—	M10	—
M13	—	M13	—	M12	—	M9	—	M13	—		der(1;19)[a]	M13	—	M13	—	M13	—	M10	der(5;6)	M11	—
M14	—	M14	—	M13	—	M10	—	M14	—	M12	der(1;3)[a]	M14	—	M14	—	M14	—	M11	—	M12	—
M15	—	M15	—	M14	—	M11	—	M15	—		der(1;19)[a]	M15	—	M15	—	M15	der(9)	M12	—	M13	—
M16	—	M16	—	M15	—	M12	—	M16	—	M13	der(1;3)[a]	M16	—	M16	—	M15	der(12;19)	M13	—	M14	—
M17	—	M17	—	M16	—	M13	—	M17	—		der(1;19)[a]	M17	—	M17	—	M16	—	M14	—	M15	—
M18	—	M18	—	M17	der(9;21)	M14	—	M18	—	M14	—	M18	—	M18	—	M17	—	M15	—	M16	—
M19	—	M19	—	M18	—	M15	—	M19	—	M15	—	M19	—	M19	—	M18	—	M16	—	M17	—
M20	—	M20	—	M19	—	M16	—	M20	—	M16	der(8;17)[a]	M20	—	M20	—	M19	—	M17	—	M18	—
				M20	—	M17	—				der(20p)					M20	der(3)	M18	—	M19	—
						M18	—			M19	der(8;17)[a]						der(?)	M19	—	M20	—
						M19	—			M20	—							M20	—		
						M20	—														
	Avg ± SD		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**		**Avg ± SD**
	0.55 ± 0.9		0.15 ± 0.2		0.15 ± 0.5		1 ± 1.4		1		0.6 ± 0.9		0		0		0.45 ± 0.7		0.7 ± 1		3

Abbreviations: Avg, average; M, metaphase; NCM, nonclonal marker chromosome; p, passage; SD, standard deviation.

[a] Shared by ≥2 cells in the same passage.

Table 8
Chromosome count for 14 metaphases of a focus of SV40-infected human dermal fibroblasts (Focus 10)

Passage and ploidy	Per-cell chromosome count														
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Avg ± SD
p1															
2*n* ±	57	45	44	44	44	44	43	42	—	—	—	—	—	—	45 ± 4
4*n* ±	90	89	88	84	83	78	78	68	67	—	—	—	—	—	81 ± 7
5*n* ±	107	—	—	—	—	—	—	—	—	—	—	—	—	—	
6*n* ±	124	—	—	—	—	—	—	—	—	—	—	—	—	—	
7*n* ±	159	—	—	—	—	—	—	—	—	—	—	—	—	—	
p2															
2*n* ±	55	50	48	43	42	42	42	42	42	41	41	41	37	35	43 ± 5
4*n* ±	88	84	77	75	73	61	73	—	—	—	—	—	—	—	76 ± 9

Abbreviations: Avg, average; M, metaphase; p, passage; SD, standard deviation; 2*n* ±, near diploid.

3.3.2. Clonal copy numbers of intact and clonal marker chromosomes of Focus 10

The copy numbers of most intact chromosomes and of two highly clonal marker chromosomes of Focus 10 were the same in 70–100% of the cells, with the exception of a few outliers (Table 9). The specific copy numbers of the remaining intact chromosomes (e.g., chromosomes 7, 12, 13, and 19) oscillated between two adjacent clonal copy numbers at high combined percentages. The clonal marker der(2;5) was present in 100% of the cells (Table 9). Thus, the copy numbers of the intact and two clonal marker chromosomes of Focus 10 were 70–100% clonal.

In the remaining cells, the copy numbers of the intact and clonal maker chromosomes typically varied ±1 around clonal values, but preferentially again below the clonal numbers (data not shown), as was documented in Table 3 for Ma6 (section 3.1.2).

To test the stability of the chromosomal copy numbers of Focus 10, we compared the chromosome-specific copy numbers of two different passages differing by 10 generations. The copy numbers of the intact and two highly clonal marker chromosomes of two passages of Focus 10 were within 10–30% of each other, with the exception of two outliers (Table 9). Thus, the clonal, chromosome-specific copy numbers were relatively stable for at least 10 cell generations and probably for 34 generations (see section 3.1.1), but not as stable as those of the Ma6 and Mu6 clones.

The stability test also revealed 13 subclonal markers, present in only a minority of the 20 cells of Focus 10 tested per passage. Because these markers were passage-independent they were also quasi-stable, as already suggested (see section 3.1.2). There were more of these subclonal markers in Focus 10 than in Ma6 clones, suggesting higher instability.

3.3.3. Nonclonal marker chromosomes

Any unstable nonclonal and subclonal passage-dependent markers of Focus 10 were again identified by comparisons of two passages differing by 10 cell generations. These comparisons revealed that Focus 10 cells contained on average 3 and a maximum of eight such markers per near diploid cell (Table 10). Thus, the cellular quota of unstable markers per Focus 10 cell was high, compared with those of Ma6 and Mu6 clones (Tables 4 and 7). The simplest explanation for the large numbers of nonclonal and passage-dependent subclonal markers is again a dynamic, karyotype-specific equilibrium of gains and losses without stability selection (see section 3.1.3). Because these markers are not stable and appear in only a minority of transformed cells, we again conclude that such markers are not relevant for oncogenic function.

In sum, the karyotype of Focus 10 was longitudinally stable within relatively narrow limits over 34 cell generations, although the per-cell numerical and structural chromosome heterogeneity at any passage generation would have predicted complete disruption of the karyotype over 34 generations. This result again supports the idea that selection for oncogenic function stabilizes aneuploid neoplastic karyotypes, despite the instability of their chromosomes. We tested this view once more with an established line of SV40 virus–transformed human mesothelial cells.

3.4. Karyotype of a line of human mesothelial cells transformed with SV40 virus

One method of cloning from a mixture of nonidentical cells is to grow it in the same conditions for many generations until a dominant clone or group of clones (alias a cell line), evolves in classical Darwinian fashion. We analyzed the karyotype of a transformed cell line, identified here as M-SV-62, that evolved after 62 passages from a culture of mesothelial cells that were polyclonally infected with SV40 virus [65]. Despite the vitality of this line, a visually detectable share of its cells continued to die while the majority thrived, much like the other clones with the virus-activated genes.

3.4.1. Average chromosome numbers of M-SV-62 stable within ± 1% over many cell generations

A representative karyotype of M-SV-62 is shown in Figure 1D. M-SV-62 had a hyperdiploid stemline with chromosome numbers ranging from 39 to 67; the clonal average was 58 (Fig. 1D; Table 11). Thus, the per-cell

Table 9
Copy number and percentage of intact and marker chromosomes in 14 metaphases of Focus 10 (SV40-infected human dermal fibroblasts) at passages p1 and p2, by ploidy

	Copy number in 14 cells, no. (%)						
	p1					p2	
Chromosome	2*n* ±	4*n* ±	5*n* ± [a]	6*n* ± [a]	7*n* ± [a]	2*n* ±	4*n* ±
1	1/2 (27/63)	3/4 (33/44)	5 (7)	4 (7)	4 (7)	1/2 (28/43)	3/4 (67/17)
2	1 (75)	2 (78)	2 (7)	3 (7)	4 (7)	1 (71)	2 (50)
3	2 (88)	4 (89)	4 (7)	7 (7)	4 (7)	2 (79)	4 (50)
4	2 (88)	4 (44)	6 (7)	6 (7)	8 (7)	2 (86)	4 (83)
5	1 (88)	2 (78)	4 (7)	3 (7)	—	1 (86)	2 (100)
6	2 (100)	4 (56)	6 (7)	6 (7)	6 (7)	2 (79)	4 (50)
7	1/2 (25/75)	3/4 (33/44)	6 (7)	4 (7)	8 (7)	1/2 (64/7)	3/4 (50/33)
8	2 (75)	3/4 (33/44)	3 (7)	5 (7)	8 (7)	2 (79)	3/4 (50/17)
9	1 (75)	2 (89)	2 (7)	3 (7)	—	1 (71)	1/2 (50/33)
10	2 (88)	4 (67)	5 (7)	6 (7)	4 (7)	2 (57)	3/4 (83/17)
11	2 (75)	2/4 (33/44)	6 (7)	6 (7)	5 (7)	2 (57)	2/4 (67/0)
12	1/2 (25/75)	2/4 (56/33)	4 (7)	2 (7)	8 (7)	1/2 (43/43)	2/4 (17/50)
13	1/2 (50/50)	2/3 (22/44)	1 (7)	5 (7)	7 (7)	1/2 (71/7)	2/3 (33/17)
14	1 (75)	2 (33)	1 (7)	3 (7)	4 (7)	1 (71)	2 (67)
15	2 (75)	3/4 (11/56)	4 (7)	4 (7)	4 (7)	2 (43)	3/4 (67/17)
16	2 (88)	4 (67)	4 (7)	6 (7)	7 (7)	2 (64)	4 (67)
17	2 (88)	4 (89)	4 (7)	6 (7)	6 (7)	2 (57)	4 (50)
18	2 (88)	4 (56)	7 (7)	7 (7)	8 (7)	2 (64)	4 (50)
19	1/2 (13/75)	3/4 (22/78)	4 (7)	5 (7)	3 (7)	1/2 (50/22)	3/4 (33/33)
20	2 (88)	4 (78)	6 (7)	6 (7)	8 (7)	2 (86)	4 (50)
21	1 (88)	1/2 (44/11)	3 (7)	—	4 (7)	1 (71)	1/2 (17/50)
22	1 (63)	2 (44)	3 (7)	4 (7)	8 (7)	1 (79)	2 (67)
X	1 (88)	2 (100)	3 (7)	3 (7)	4 (7)	1 (86)	2 (67)
Y	0 (63)	0 (67)	3 (7)	—	1 (7)	—	—
der(2;5)	2 (100)	4 (100)	5 (7)	5 (7)	8 (7)	2 (100)	4 (100)
der(Y;3)	1 (63)	2 (78)	—	3 (7)	3 (7)	1 (86)	2 (100)
der(13;21)	1 (13)	2 (11)	1 (7)	—	1 (7)	—	1 (17)
der(9;21)	1 (13)	1 (11)	—	—	—	1 (14)	—
der(8;21)	—	1 (11)	—	—	—	1 (7)	1 (17)
der(9;12)	—	2 (22)	—	—	—	1 (14)	1 (17)
der(9;14)	—	2 (33)	—	—	—	1 (14)	2 (17)
der(1;13)	—	1 (11)	—	—	—	1 (14)	1 (17)
der(12;14)	—	1 (22)	—	—	—	1 (14)	1 (17)
der(19;22)	—	1 (11)	—	—	—	1 (36)	1 (17)
der(9;22)	—	2 (11)	—	—	—	1 (21)	—
der(8;13)	—	—	1 (7)	—	—	1 (29)	1 (33)
der(14;19)	—	—	—	—	1 (7)	1 (14)	1/2 (33)
der(9)	—	1 (11)	—	1 (7)	—	—	2 (17)
der(2)	—	1 (11)	—	—	—	2 (14)	1/2 (33)

If the specific copy numbers oscillated between adjacent clonal values, two values are reported, separated by a slash mark. For example, chromosome 1 at passage 1, near diploid, had either 1 or 2 copies (1/2), at a clonal percentage of 27% or 63%, respectively (55/35).

Abbreviations: 2*n* ±, near diploid.

[a] Only 1 cell of the 20 exhibited this ploidy at this passage.

chromosome numbers were heterogeneous, varying from −20% to +16% around the clonal average.

To estimate the stability of the average chromosome number of the M-SV-62 clone, we compared two passages of the clone that differed by ~10 cell generations. The average chromosome numbers were 58 at passage 1 and 57 at passage 2, and thus were stable within ± 1% over 10 generations (Table 11).

3.4.2. Copy numbers of intact and clonal marker chromosomes

At any one time, the copy numbers of most intact chromosomes and of 12 marker chromosomes were the same in 70–95% of M-SV-62 cells (Table 12). The copy numbers of the remaining six intact chromosomes were the same in 50–60% of the cells. Thus, the individual percentages of cells with the same copy numbers were more heterogeneous in M-SV-62 than in the clones described in previous sections, suggesting some clonal heterogeneity. The specific copy numbers of four intact chromosomes varied between two adjacent clonal copy numbers at high percentages. Chromosomes 7 and 13 were missing in M-SV-62 (i.e., were nullisomic) in 80–100% of cells; instead, two clonal markers each contained elements of chromosomes 7 and 13. In the remaining cells, the copy numbers of the

Table 10
Nonclonal and passage-dependent subclonar marker chromosomes per metaphase of focus 10 cells, with average for passage and ploidy

p1										**p2**			
2*n* ±		4*n* ±		5*n* ±		6*n* ±		**7*n* ±**		**2*n* ±**		**4*n* ±**	
	NCM		NCM		NCM		NCM	**M**	**NCM**	**M**	**NCM**	**M**	**NCM**
M1	dic(9;19;1)	M1	—	M1	der(14;22)	M1	der(1;9)			M1	der(1;15)	M1	der(1;3)
	der(21;22)[a]	M2	der(9;X)[2]		der(3)		der(1;12)	M1	der(5;19)[4]				der(11;21?)
M2	der(21;22)[a]		der(21;22)[2][a]		der(4;11)		der(10;12;19)		der(9;10)[4]		der(11;16)[a]		der(11;18)
	der(Y;15)		dic(1;5)		der(7;11)		der(15;21)		der(3;9)[3]		der(16;22)		der(5;9)
	der(7;22)		der(3;21)[2]	M2	—		der(9;15)		der(11;17)[2]		der(1;7;13)		der(7;22)
	der(4;9)		der(2;15)	M3	—		dic(2;9;14)		dic(1;21)[3]		der(7;16)		dic(13;19)
M3	der(19;12)		dic(13;13)[a]	M4	—		der(22;21)[2][a]		der(3;14)		der(3)		dic(3;13;22)
	der(19;21)	M3	dic(11;22;12)	M5	—		der(4;22)[a]		dic(14;21)[2]		der(7;19)	M2	der(7;12)[a]
	der(14;22)[a]		der(14;22)[a]	M6	—				der(6;16)	M2	der(7;12)[a]		der(10)
M4	der(14;22)[a]		der(9;11)	M7	—	M2	—		der(9;21)		der(16;19)[a]		der(11;22)
M5	der(8;19)[2][a]		der(8;19)[a]	M8	—	M3	—		der(1;?)	M3	der(?;17)		der(1;19)
	der(13)		der(5;21)	M9	—	M4	—	M2	—		der(7;14)[a]		der(14;20)
	der(14;19)		der(4)	M10	—	M5	—	M3	—	M4	der(7;14)[a]		der(15;19;21)
	der(21;22)[a]		der(3;4)	M11	—	M6	—	M4	—	M5	der(4)		der(5)
	dic(13;13)[a]		der(3)	M12	—	M7	—	M5	—		der(12;21)[a]		der(16)[2]
M6	dic(13;13)[a]		der(7;21)	M13	—	M8	—	M6	—		der(5;14)[a]		der(7;12)[a]
	der(12;19)		der(2;18)	M14	—	M9	—	M7	—		der(7)		der(12;21)[a]
	der(1;7)	M4	der(4;22)[a]			M10	—	M8	—		dic(7;11)	M3	der(4;19)
	der(8)		der(9;22)			M11	—	M9	—	M6	der(7;21)		der(5;12)[2]
	der(9;19)	M5	der(4;10)[2]			M12	—	M10	—		der(12;22)[a]		der(12;22)[a]
	der(Y;13)		der(13;22)[2]			M13	—	M11	—		der(1;16)		der(12)
M7	der(5;13)		der(13;20)[2]			M14	—	M12	—		der(10;17)		der(1;7)
	der(8;13)		der(14;21)[2][a]					M13	—		der(18)		der(7)
	der(9;13)		der(4;13)					M14	—		der(7;12)[a]	M4	der(17;19)
M8	der(14;21)[a]	M6	dic(12;14;22)							M7	der(10;16)		der(20;21)
	der(2;11)		dic(13;21;21)								der(2;11)[2]		der(9;11)[2]
M9	—		der(X)[a]								der(5;21)		dic(6;11)
M10	—		der(15;21)							M8	der(1;12)		dic(7;8)
M11	—		der(11;12)							M9	der(16;19)[a]		der(7;14)[a]
M12	—		der(21;22)[3][a]								der(7;12)[a]	M5	der(13;19)
M13	—		der(11;13)[a]							M10	der(9;19)		der(2;15)
M14	—	M7	der(11;13)[a]								der(7;12;14)		der(7;9)
			der(13;20;14)							M11	der(13;19)	M6	der(5;14)[a]
			der(15;21)								der(14;22)		der(6;18)
			der(X)[a]								der(10;21)		der(18;21)
			dic(1;13;19;20)								der(7;9)		
			der(21;22)[3][a]								der(11)		
		M8	der(17;22)							M12	—		
			der(2;14)							M13	der(1)		
		M9	der(1)								der(3;13)		
		M10	—								der(3;7)		
		M11	—							M14	der(6;11)		

(*Continued*)

Table 10
Continued

p1								p2			
2n ±		4n ±		5n ±		6n ±		7n ±		2n ±		4n ±	
NCM		NCM		NCM		NCM		M	NCM	M	NCM
		M12	—					M14	der(13;15)		
		M13	—					M14	der(15;17)		
		M14	—					M14	der(11;16)[a]		
								M14	der(16)		
								M14	der(11;19)[a]		
								M14	der(19;21)		
Avg ± SD		Avg ± SD		Avg ± SD		Avg ± SD		Avg ± SD		Avg ± SD	
3 ± 2		8 ± 4		4		10		23		3 ± 3		7 ± 3	

Abbreviations: Avg, average; M, metaphase; NCM, nonclonal marker chromosome; p, passage; SD, standard deviation; 2n ±, near diploid.
[a] Shared by ⩾2 cells in the same passage.

intact chromosomes oscillated above and below the clonal values and the clonal markers were missing, as described for other clones.

A preliminary biological cloning experiment confirmed the suspected heterogeneity of M-SV-62; it yielded a subclone, termed F2, with a distinct, spindle-shaped cell morphology and an average chromosome number of only 53 ±3. The chromosomes of F2 had the same copy numbers as M-SV-62, and F2 shared 10 of its 12 highly clonal marker chromosomes with M-SV-62, but the relative clonal percentages of the F2 chromosomes were more homogeneous than those of M-SV-62.

In a test measuring the stability of the chromosome-specific copy numbers of M-SV-62 over 10 cell generations, the percentages of cells with the same chromosome copy numbers were within 20% of each other (Table 12). Thus, the individual copy numbers of most intact chromosomes and highly clonal marker chromosomes of the M-SV-62 line were longitudinally stable for at least 10 cell generations and probably many more, because the line had been passaged 62 times by the time it was analyzed here.

The stability test also revealed 29 subclonal markers, present in small subgroups of M-SV-62 cells, which were also passage-independent and thus stable. Their number is much higher than those observed in the Ma6 and Mu6 clones and higher even than in Focus 10—indicating again greater clonal heterogeneity. This is consistent with the high clonal age and high instability of M-SV-62 compared with the other clones. As we have suggested, the stochastic acquisitions of new subclonal markers by subgroups of cells of an original cancer clone generate stable sidelines, as in natural cancers [3,80].

3.4.3. Nonclonal marker chromosomes

Any unstable nonclonal and subclonal passage-dependent markers of M-SV-62 were identified by comparison of two passages differing by 10 cell generations. The M-SV-62 cells contained on average 11, and a maximum of 16 such markers per cell (Table 13). None of these markers were found in two different passages. The simplest explanation for the large numbers of nonclonal and passage-dependent subclonal markers (annotated in Table 13 as shared by ⩾2 cells) is again a dynamic, karyotype-specific equilibrium of gains and losses without stability selection (see section 3.1.3).

We conclude, as before, that unstable passage-dependent markers of M-SV-62 are not relevant to transformation, because they appear in only a small minority of transformed cells and are not stable. Further, we conclude that the longitudinal stability of the average chromosome numbers and of the specific copy numbers of intact and clonal marker chromosomes support the idea that the M-SV-62 karyotypes are held close to their clonal values, including even their clonal heterogeneity, by stabilizing selections for oncogenic function, despite inherent chromosomal instability.

The structural instability of the chromosomes and the relatively high per-cell mortality of M-SV-62 cells were

Table 11
Chromosome count for 20 metaphases of SV40-transformed human mesothelial cells (M-SV-62)

	Per-cell chromosome count																				
Passage	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	Avg ± SD
p1	67	64	64	63	63	62	62	61	60	59	58	58	58	57	57	54	52	47	46	39[a]	58 ± 7
p2	63	62	62	61	61	61	60	59	59	58	58	57	56	56	54	54	54	52	51	47	57 ± 4

Abbreviations: Avg, average; M, metaphase; p, passage; SD, standard deviation.
[a] Excluded from average.

similar to those of Focus 10, but were higher than those of the Ma6 and Mu6 clones. This result is consistent with the view that the virus-activated genes of SV40 are more destabilizing for chromosomes than the 6 retrovirus–activated genes of the Ma6 and Mu6 clones (see Introduction).

4. Discussion

4.1. Individual quasi-stable karyotypes encode the phenotypes of cancer cells

Each of the seven clones of newly transformed human mammary and muscle cells, as well as an established mesothelial cell line, contained individual clonal karyotypes that were quasi-stable for at least 34 cell generations, within the following limits. Depending on the karyotype, average chromosome numbers of clones were stable within ± 3% and chromosome-specific copy numbers were stable in 70–100% cells. At any one passage, however, per-cell chromosome numbers varied on average within ±18% of clonal means, chromosome-specific copy numbers varied ±1 around clonal averages in 0–30% of cells, and unstable nonclonal markers were found within karyotype-specific quotas of <1% to 20% of the total chromosome numbers. Karyotypic ploidies of two clones also varied. A summary of the rates of karyotypic variations at any one time and the stabilities over time of the seven clones and the cell line is given in Table 14. With these rates of variation, the karyotypes of transformed clones would randomize in a few generations in the absence of selection.

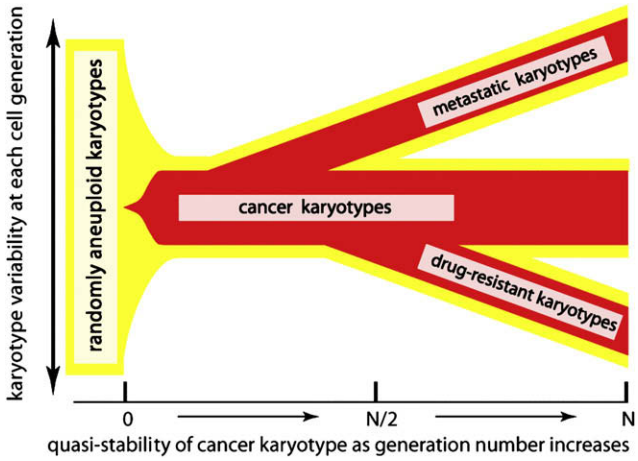

Fig. 2. Cross-sectional variability and longitudinal stability of cancer-causing karyotypes. At each instant the cell population of a transformed clone or cancer shows aneuploidy-dependent diversity in karyotypes, but within certain limits (red zones) the average oncogenic karyotype remains nearly stable over time as the population proliferates. The red tip at generation zero signals the origin of the cancer-causing karyotype from a pool of cells with randomly aneuploid nonneoplastic karyotypes (yellow). The range of oncogenic karyotypic variability of cancer-causing karyotypes (red zones) is flanked at any time by nonneoplastic aneuploid variants from without the quota of cancer-specific variations (flanking yellow zones). Occasionally, there are also stochastic karyotype evolutions, which generate additional, new cancer-specific phenotypes such as drug resistance and metastasis in a process termed tumor progression.

We conclude, therefore, that cancers are initiated and maintained by individual aneuploid karyotypes, much like new species. Such cancer-causing karyotypes are in flexible equilibrium, destabilized by aneuploidy and stabilized (within narrow limits of variation) by selection for oncogenic function. Together, the two competing forces form quasi-stable cancer-causing karyotypes (depicted as red zones in Figure 2). At the same time, destabilizing aneuploidy generates nonneoplastic and nonviable variants (yellow zones flanking the red zones in Figure 2). Occasionally, karyotypic variants evolve that encode new transforming functions such as drug-resistance or metastasis—a process that is typically described as *tumor progression* [19]. Examples such as acquired drug-resistance or metastasis are depicted as branching red zones in Figure 2. We deduce, therefore, that it is the karyotype as a whole that is selected to encode oncogenic function.

Our system, which transforms cells with genes that destabilize the karyotype persistently, lends particular support to these conclusions. This is because the karyotypes of the transformed clones studied here were stable within the stated limits over many cell generations, not only despite the inherent instability of aneuploidy but also despite the instability imposed by the virus-activated genes used to induce transformation (discussed further in the next section).

In the absence of virus-activated aneuploidogenic genes, the karyotypes of cell lines derived even from highly aneuploid cancers are considerably more stable than those of the transformed clones studied here [10,46]. For example, we have found previously that between one third and two thirds of the cells of the established human colon and breast cancer lines HT29, SW480, and MDA 231 had identical karyotypes over many generations, despite coexistence of some unstable marker chromosomes [10,11].

The karyotypic cancer theory resolves the apparent paradoxes of the stability-within-instability of cancers [17], as presented in the Introduction (section 1.2). It explains, for

Table 12
Copy number and percentage of intact and clonal marker chromosomes in 20 metaphases of M-SV-62 at passages p1 and p2

Chromosome	Copies, no.	% at p1	% at p2
1	1	80	90
2	1	75	85
3	2/3	20/70	50/20
4	1/2	25/65	55/40
5	2	80	90
6	4	80	70
7	0	95	100
8	2	45	50
9	2	60	60
10	2	55	65
11	1	90	100
12	1	85	75
13	0	80	90
14	2	50	55
15	3/4	45/20	15/45
16	1	80	60
17	2	40	50
18	1	95	75
19	2	85	70
20	2	80	60
21	2	55	70
22	1	60	40
X	2/3	40/40	20/65
der(1;2)	1	85	85
der(13;14)	1	65	70
der(8;13;18)	1	90	80
der(14;19;20)	1	80	75
der(2;21)	1	95	90
der(4;11)	1	90	75
der(4;7)	1	85	95
der(5;12)	1	90	85
dic(2;7;16)	1	90	90
t(1;10)	1	85	85
t(1;10)	1	85	65
der(14;14)	1	70	75
der(20;22)	1	35	10
der(8;19)	1	35	25
der(X;18)	1	5	5
der(2;15;20)	1	25	5
der(5;12;16)	1	5	5
der(5;12;16;17)	1	10	5
der(X;11)	1	20	5
der(3;14)	1	5	5
der(11;17)	1	10	10
der(16;22)	1	30	30
der(1;11)	1	25	20
der(8;17)	1/2	15/5	20/0
der(19)	1/2	15/5	10/0
der(5;9;12;20)	1	15	5
der(3;20)	1	10	25
der(17;21)	1	5	15
der(1)	1	5	10
der(15;20)	1	5	15
der(8;15)	1/2	0/5	5/0
der(17;19)	1	5	5
der(8;20)	1	5	5
der(17;18)	1	15	10
der(17)	2	5	10
der(15;17)	1	10	10
der(5;9;20)	1	5	20
der(2;20)	1	5	5
der(4;17)	1	5	20
der(19;21)	1	5	5

If the specific copy numbers oscillated between adjacent clonal values, two values are reported, separated by a slash mark. For example, chromosome 3 had either 1 or 2 copies (1/2), at a clonal percentage of 20% or 70%, respectively (20/70), at passage 1 and 50% or 20%, respectively (50/20), at passage 2.

example, why the modal chromosome numbers of cancers remain stable even though the chromosomes making up that count may vary (see section 1.2). Particularly, it explains the observation of Albertson et al. [4] that "The remarkable karyotypic stability of established tumor cell lines in culture over many generations in many different laboratories supports this idea. These cells do show substantial cell-to-cell variability but the average genotype is stable." It also explains many classical idiosyncrasies of cancer and carcinogenesis, as illustrated by the following six examples.

1. *Similar cancers have similar karyotypes and transcriptomes*. It has been demonstrated, especially by comparative genomic hybridizations, that similar cancers from the same tissues of origin have very similar karyotypes [28,29,32–35,81] and transcriptomes (see #6, below). It follows that cancer karyotypes determine cancer phenotypes. Likewise, similar species of the same taxonomic groups have similar karyotypes (e.g. rodents) [55,82].
2. *The proportionality between the degree of aneuploidy and malignancy*. For examples, see reports and reviews by Wilkens et al. [14], Duesberg et al. [18], Foulds [19], Wolman [20], Balaban et al. [21], Mitelman et al. [83], and Doak [84].
3. *Correspondence between high rates of phenotypic and karyotypic alterations*. Take, for example, the high rates at which cancers cells acquire drug and multidrug resistance correlating with high rates of karyotypic alterations [85]. These rates of phenotypic alterations exclude mutations, being orders of magnitude higher than conventional mutations [8,10,15,85].
4. *Immortality of cancer cell populations*. The high rates of karyotype variation coupled with constant selection for proliferative and neoplastic advantages explain how cancer cell populations remain immortal: that is, by replacing fatal karyotypes and mutations by alternate viable karyotypes [18]. Likewise, species achieve immortality by replacing defective individuals.
5. *The loss of the transformed phenotype by alteration of the karyotype*. Consider the obliteration of the oncogenic karyotype by fusion with normal cells or by experimental chromosome transfer, as noted in the Introduction (section 1.3).

Table 13
Nonclonal and passage-dependent subclonal marker chromosomes of M-SV-62 cells per metaphase, by passage

p1				p2	
NCM	NCM	NCM	NCM	NCM	**NCM**
M1 der(6;16)[a]	M9 der(6;16)[a]	M15 1der(6;16)	M1 der(3;4)[a]	M7 der(3;4)a	M13 der(X;21)
der(1;12)[a]	der(15;22)	der(14;19)	der(4;8)	der(15;16)[a]	der(3)[a]
der(1;8;19;18)	der(X;2;5;20;20)	der(15;X)	der(12;20)	der(X;8)	der(8;18;22)
der(9;17;18)	der(8)[a]	der(5;1;20)	der(9;18)	der(5;20;22)	der(3;17)
der(2;5;8;14)	der(1;12)[a]	der(5;8;20)	der(2;10;17)	der(2;11;5)	der(X;9)[a]
der(X;2;5;20;20)	der(X;10)	dic(X;14)	der(10;17)	der(12;5;20;21)	der(2;14;20)
der(X;17;1)	der(12;17;18)	der(18;20)[a]	dic(9;20;22;20;11)	der(16;20;16)	der(9;16;22)
M2 der(5;21)	der(5;20;8)	M16 der(8;14)	der(5;9;14;20;22)	der(X;2;3)	der(9;20;14;5;12)
der(2;22;20)[a]	M10 der(1;9;5)	der(8;16)	der(8;21)[a]	der(8;16;11)	der(8;18)
M3 der(2;22;20)[a]	der(X;5;12;16)	der(1;2;10)	M2 dic(2;8;13;18;21)[a]	dic(3;6)	M14 der(X;20)
der(X;5;18)[a]	der(15;?)	der(1;5;17;4)	der(X;5;17;20;)	dic(13;20;16)	der(14;21)
M4 der(X;5;18)[a]	der(X;17;20)	der(9;22)[a]	der(9;20;22)	dic(2;8;13;18;21)[a]	der(22;20;19;14)
der(1;10;15)	der(X;18;21)	M17 der(9;20;22;18)	der(1;16)	der(X;14;17;19;20)	der(12;5;16)
der(5;13;14)	der(X;X;4;16;16;16;17)	der(1;5;20;17)	der(1)[a]*a*	der(2;12)[a]	der(16;21)[a]
der(20;2;22;16;22;20)	der(5;20;22;2;22;20)	der(1;2;17)	der(5;9;12;20)	der(14;15)[2][a]	der(1;2;15)
dic(10;12)	der(8;19;22)	der(5;22;20;9)	der(1;18;4)	der(16;21)[a]	der(3;8)
M5 der(X;5;5;17;20;22)	der(5;9)[a]	der(X;19;21)	der(3)[a]	M8 der(16;21)[a]	der(2;17)[a]
der(4;9;9;20)	M11 der(5;9)[a]	der(9;17)	der(8;21)[a]	der(15;16)[a]	M15 der(2;17)[a]
der(18;20)[a]	der(11?)	der(8;22)[a]	M3 der(5;20;9)[a]	der(13;22)	der(9;20)[a]
der(8;20;22)[a]	der(13;15)	M18 der(14;20)[a]	der(12)	der(3;16;22;2;20)	der(15)
M6 der(8;20;22)[a]	der(X;5;12;15)	der(8;17;22;2;11;20;9)	der(15;18)	der(1;4;5;10;16;20)	der(1;5;22)
der(17;20)	der(8;15;17)	der(4;16;7)	der(8;9)	M9 der(2;22)	der(X;17)
der(4;21)	der(5;18)	der(21;19;20)	der(3;6)[2]	der(4;22)	der(3;22)[a]
der(5;9;20;20)	M12 der(15;21)	der(9;12)	der(X;14;22)	der(18;21)	der(10;20;22)[a]
der(9;20;9)	der(5;16;20)	der(5;22)	der(X;3)	der(2;18?)	M16 der(8)[a]
M7 der(2;5;7;12;16;16)	der(X;5;12)	M19 der(12)[2]	der(9;16;22;2;20)	der(X;8;13)	der(1;10;22)[a]
der(14;19;20)	der(2;5;8;20)	der(5;20;22)	der(X)	der(9;16;22;13)	der(1;8;10)[a]
der(14;20;17;15)	der(5;14)	der(1;14;19;20)	der(8;21)[a]	der(19;22;16;9)	der(6;7;16)
der(19;22)	der(14;20)[a]	der(X;5;12;?)	der(2;12)[a]	der(9)[a]	der(10;20;22)[a]
der(2;5;9;20;20)	der(1;19)	dic(8;15;20)	M4 der(4;18)	der(11)	der(8;11)[a]
der(5;17;11)	der(8;20;14)	der(12;22;20)	der(3;14)[a]	der(7)	der(3;22)[a]
der(9;22)[a]	der(9;13)	M20 der(6;10)	der(2;9;20;20;22)	der(9;20)[a]	M17 der(X;8;15)
der(X;17)	M13 der(X;1;5;12)	der(18;19)[a]	der(6;7;12;15;20)	der(17;22)	der(2;20;20;22)
der(X;5;18)	der(X;20;22)	der(8;22)[a]	der(1;10;22)[a]	M10 der(14;17)	M18 der(3;13;20)
der(X;5;18)[a]	der(3;19;22)	der(5;18;22)	der(1;8;10)[a]	der(14;4;22)	der(3;6;17)
M8 der(11;17;?)	der(X;9;10)	dic(X;2;5;20;22;22)	der(8;21)[a]	der(16;20;5)	der(5;12;16;17)
der(X;15;19)	M14 der(1;17)	der(5;8;20;22)	der(X;9)[a]	der(8;4;11)	dic(11;20;22;17)
der(2;22;10)	der(10;13;19)	der(17;18;22)	M5 der(2;3;22)	M11 der(X;1;5;12)	der(2;9;9;22)
der(22;?)	der(8;14;14;20)		der(1;5;12;12)	der(19;2;22)	der(6;20)[a]
der(4;12;1)	der(1;2;16)		der(10;20;22)[a]	der(6;20)[a]	der(5;9;14;15;20)
der(5;16;17)	der(X;X;3;8;10;21;22)		der(14;15)[a]	der(6;21)	der(11;15)
	der(5;15)		der(4;8;17)	der(10;20;22)[a]	der(X;15;18)
	der(X;3)		M6 der(17;11;21;11;20)	der(18)[a]	der(?)
	dic(X;8)		der(1;5)	der(8;11)[a]	der(14;15)[2][a]
	der(18;20)[a]		der(5;16;16;18;20)	M12 der(6;15;15)	M19 der(11;22)[a]
			der(2;3)	der(1;8;15)	der(10;20;22)[a]
			der(X;12)	der(8;20;8)	der(1;5;12)[a]
			der(2;5;7;19)	der(3;16;22)	M20 der(1;5;12)[a]
			der(3;4;11;11;20;22)	der(14;20;5;12)	dic(2;8;13;18;21)
			der(4;20)[2]	der(11;22)[a]	der(11;20)
			der(14;15)[a]	der(9)[a]	der(20;21)
			der(3;22)[a]	der(3;6;18)	der(10;20;22)[a]
				der(2;10)	der(3;22)[a]
				der(2;20;22)	der(18)[a]
				der(14;22)	
				der(13;13)	

The average number of nonclonal markers per metaphase (± 1 standard deviation) was 11 ± 2 for passage p1 and 12 ± 4 for passage p2.
[a] Shared by ⩾2 cells in the same passage.

Table 14
Chromosomal variations at any one time, and karyotypic stability over time, of all clones examined, based on their predominant stemlines

	Variability at any one time			Stability over 34 generations
Clone-specific total chromosome numbers	Vary within ±18% of average			Averages stable within ±3%
Clone-specific copy numbers of intact and clonal marker chromosomes	For each specific chromosome: variation of ±1 copy in 0−30% cells.			Copy numbers remain stable in 70−100% cells
Clone-specific numbers of nonclonal marker chromosomes	**Clone**	**Variation**	**Average**	Clonal averages and per-cell variations of nonclonal markers are stable, but individual markers are unstable.
	Ma6	0−5	1	
	Mu6	0−4	0.4	
	Focus 10	0−8	3	
	M-SV-62	2−16	11	

Abbreviations: Focus 10, a focus of SV40-infected human dermal fibroblasts; Ma6, 6 virus−transformed human mammary cell; M-SV-62, SV40-transformed human mesothelial cells; Mu6, 6 virus−transformed human muscle cell; SV40, simian virus 40.

6. *The concordance between the complex phenotypes of cancer cells with the over- or underexpression of thousands of genes*. Examples of complex phenotypes correlating with transcriptomes of thousands of genes include growth autonomy, abnormal nuclear and cellular morphology, highly abnormal metabolism, invasiveness and metastasis, and acquired or inherent multidrug resistance [18,86−89].

4.2. Origin of cancer karyotypes

We have observed here that newly formed clones of transformed cells originated with, and thus were clonal for, the same basic karyotypes that we identified in all subsequent generations (Fig. 2). With this evidence for the karyotypic origin of oncogenic transformation of human cells, and with previous evidence from the literature for preneoplastic aneuploidy both by others and by ourselves [9,18,90,91], we have before us the following two-step mechanism of carcinogenesis (Fig. 3).

Step 1: Induction of random aneuploidy. Two observations suggest that the first step in carcinogenesis is the induction of random aneuploidy. (1) Carcinogens such as the highly aneuploidogenic virus-activated genes studied here, or less aneuploidogenic chemical or physical carcinogens and defective, heritable genes described elsewhere, all induce aneuploidy [9,18,90,92−96]. (2) When tested, random aneuploidy is found to precede neoplastic transformation [9,69,90,91,97−101].

Step 2: Evolution of quasi-stable neoplastic karyotypes from unstable randomly aneuploid karyotypes. Because of the inherent instability of aneuploidy (see section 1.1) and the effects of selection, the karyotypes of aneuploid cells evolve autocatalytically toward two stable endpoints: (1) the quasi-stable karyotypes of immortal cancer cell populations and (2) the lethal karyotypes of cells dying from nonviable combinations of chromosomes and genes (Fig. 3).

Because the odds of generating the new complex functions that define cancer [18] by random alterations of a karyotype are very low, the evolution of new neoplastic karyotypes from randomly aneuploid cells will be rare—comparable again to the evolution of new species. This feature can explain the typically long neoplastic latent periods of years to decades between the induction of aneuploidy by carcinogens and human carcinogenesis [18,101−103].

The relatively high odds and short latent periods of transformation of 1 per 100,000 cells per month observed here (the origin of the clonal stem cell preceded colony formation by 1−2 months) reflects the high and persistent levels of karyotypic fluidity achieved by the virus-activated genes, compared with the relatively low fluidity of cells rendered aneuploid by conventional carcinogens. This is because the virus-activated genes are self-replicating and thus permanent, rather than transient, as are conventional nonbiological carcinogens [75]. The high karyotypic fluidity of cells carrying aneuploidogenic genes also explains their high mortality from fatal karyotypes, as observed here and previously in clones transformed with virus-activated genes [55,104−108] (Fig. 3).

Because the proposed two-step mechanism of transformation generates transforming karyotypes de novo, just like new species, it also explains the proverbial diversity of the cancers induced by the same aneuploidogens, such as SV40 and other polyomaviruses [70,109]. Because of the neoplastic diversity of SV40 tumors in animals, transformation by this virus has recently been called a ‘‘cellular uncertainty principle’’ [70]. The same is true to a lesser degree for the diversity of tumors induced by 6 retrovirus−activated genes [15,66].

The two-step mechanism of transformation also explains why SV40 and other aneuploidogens are necessary only to initiate but not to maintain transformation; that is, they can be switched off experimentally or lost without switching off transformation [67,69,76]. As soon as aneuploidy is induced, its autocatalytic aspect persistently leads to ever-changing candidate karyotypes.

Recent experiments by Heng et al. [51] lend further support to the two-step mechanism of carcinogenesis. They found that randomly aneuploid fibroblasts from patients with the heritable Li−Fraumeni aneuploidy syndrome generated clones of transformed cells in vitro with clonal marker chromosomes that were stable over 180 cell

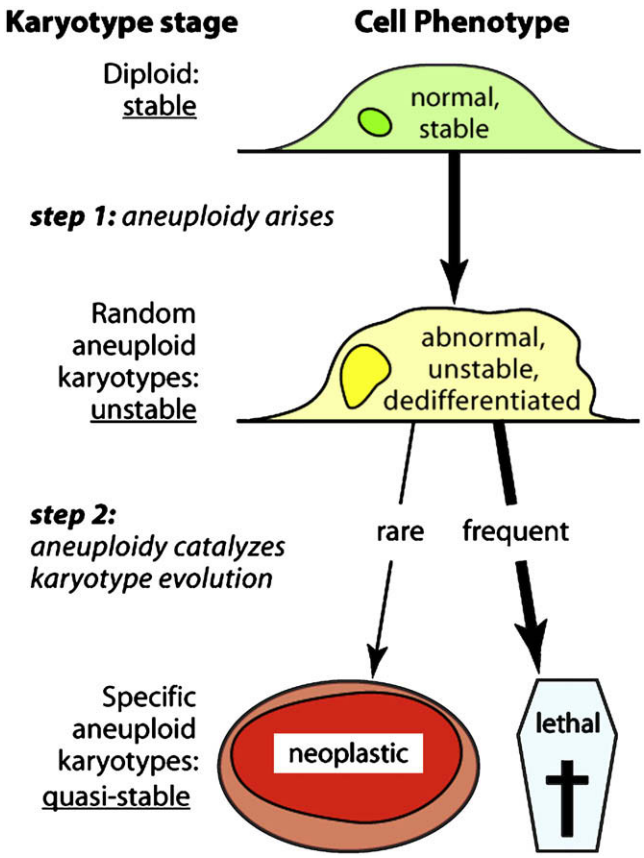

Fig. 3. *The two-step mechanism of carcinogenesis according to the karyotypic cancer theory.* Step 1: The generation of random aneuploidy by carcinogens or spontaneous events. Owing to the inherent instability of aneuploidy, randomly aneuploid karyotypes evolve spontaneously at rates that are proportional to the degrees of aneuploidy. Step 2: Eventually karyotypic evolutions of randomly aneuploid karyotypes reach one of two stable or quasi-stable endpoints: (a) quasi-stable cancer-specific karyotypes, which are rare, or (b) lethal karyotypes, which are common.

generations, despite the simultaneous ‘‘emergence and disappearance’’ of highly unstable marker chromosomes. Furthermore, independent clones from the same parental cells had distinct karyotypes, despite the same heritable mutation or mutations.

The proposed two-step mechanism is thus a coherent and consistent explanation of carcinogenesis.

4.3. Alternative genetic cancer theories

It has long been held that mutations cause cancer. (Various summaries and views of the debate are available [68,69,105,110–114].) The mutation theory holds that three to six specific mutations initiate and maintain cancer, and that karyotypic alterations are unpredictable consequences of transformation [4,105,110,115–119]. Nonetheless, despite enormous efforts, it has not been possible to show that mutated genes from cancers are sufficient to transform normal cells to cancer cells. Even cellular genes that were artificially activated with various viral promoters (such as those studied here) were found to be insufficient to transform normal human and animal cells to cancer cells [15,69,75,120,121]. Instead, only a small fraction of cells carrying such genes were transformed, after delays of several months—and all then had aneuploid karyotypes. As a result of similar efforts, Mahale et al. [122] concluded in 2008 that ‘‘the minimum number of events required for malignant transformation of human fibroblasts is greater than has been enumerated by such oncogene addition strategies.’’

Thus, experiments conducted to test the gene mutation theory of cancer have, instead, confirmed the karyotypic cancer theory advanced here. Indeed, the current study was initiated to test once again whether virus-activated genes are sufficient for oncogenic function or are instead a means of predisposing a karyotype to some other, critical event.

Some investigators have proposed that gains or losses of specific chromosomes or clonal marker chromosomes are selected by cancers, because they increase the expression of hypothetical cancer genes or decrease that of tumor suppressor genes [4,30,89,96,105,118,123]. However, these proposals cannot explain why the entire karyotype of a cancer, rather than just a specific aneusomy, is quasi-stable. It is possible, therefore, that the so-called nonrandom aneusomies observed in many cancers [80] are mere indicators of nonrandom cancer-specific karyotypes such as those described here.

4.4. Conclusions

The present data and those of the cancer literature reviewed here support the theory that specific, aneuploid karyotypes initiate and maintain cancers—behaving much like new species. Thus, individual karyotypes encode the individualities of cancers [15,19], according to this theory, cancer-causing karyotypes represent chromosomal equilibria between destabilizing aneuploidy and stabilizing selections for oncogenic function. This theory explains (a) the individuality of cancers, (b) the fluidity of the cancer karyotypes and phenotypes, and (c) the genetic complexity of cancer-specific phenotypes such as multidrug resistance and metastasis (see sections 1.3 and 4.1). By contrast, cancers caused by a few specific mutations (a) would be uniform rather than individual, (b) would be stable rather than variable, and (c) would have simple rather than complex and multigenic phenotypes.

We conclude that specific karyotypes as a whole, rather than specific mutations, cause cancers. This karyotypic theory explains why specific mutations and even recurrent aneusomies are not sufficient to encode cancers [69,124–126].

Addendum

In an effort to give a visual impression of the karyotypic stability and instability of the transformed human clones described in our paper, we have prepared 3D-karyographs of two clones, Ma6 Clone 10 and M-SV-62, at different clonal generations. 3D-karyographs show parallel lines connecting

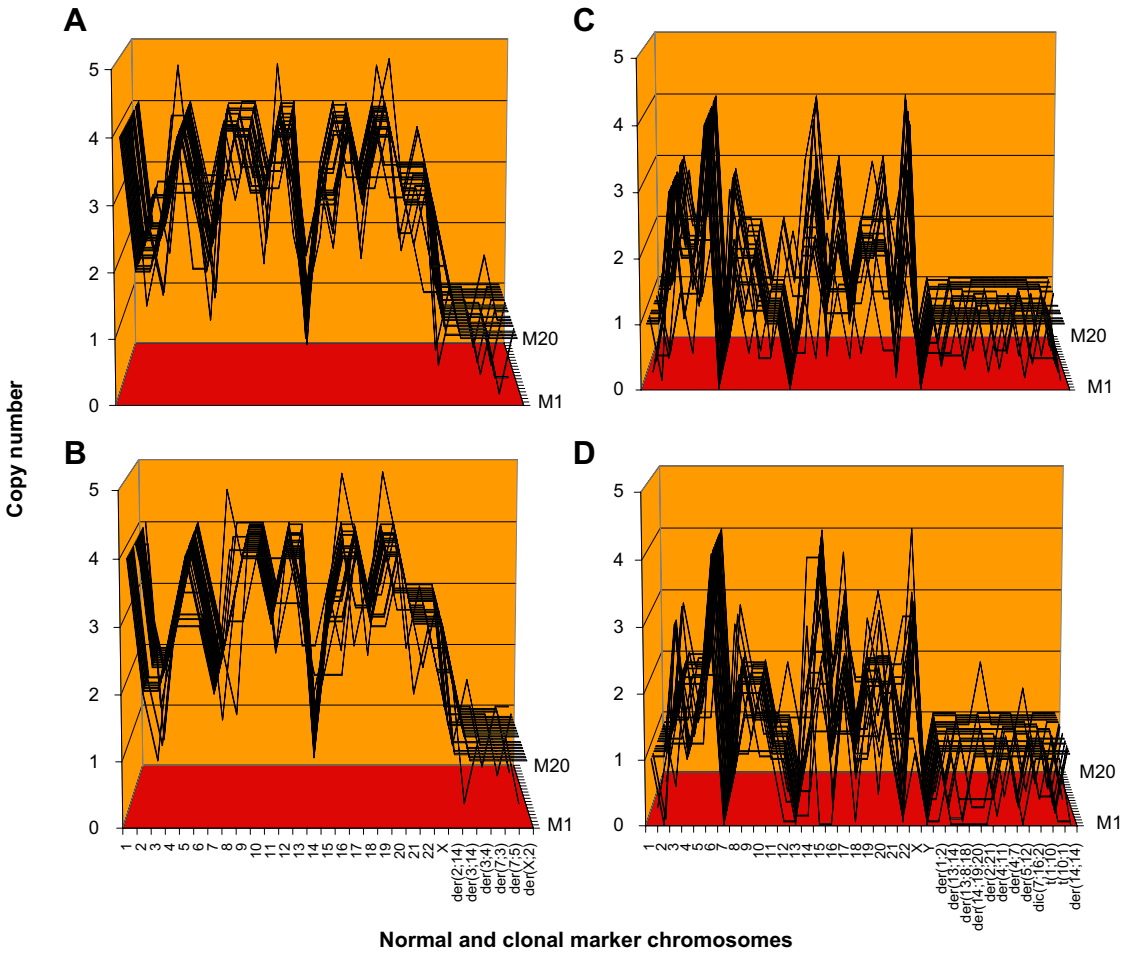

Fig. 4. Karyographs showing chromosomal stability and instability of transformed clones of human mammary cells, Ma6 Clone 10 (A,B), and human mesothelial cells, M-SV-62 (C,D), over 10 cell generations based on the data of Tables 2 and 12.

the copy numbers of intact and clonal marker chromosomes of 20 metaphases. Dimension 1 shows chromosome numbers; dimension 2 shows chromosome copy numbers, and dimension 3 shows metaphase numbers. Comparisons of two passages of 20 metaphases of Ma6 Clone 10 (Figure 4 A, B) and of M-SV-62 (Figure 4 C, D) differing by 10 cell generations show that the average clonality and non-clonality of the metaphases of both clones are stable over time. The data are from Tables 2 and 12.

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