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Experimental control of neoplastic progression in cell populations: Foulds' rules revisited

(spontaneous transformation/apoptosis/NIH 3T3 cells/heterogeneity)

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ABSTRACT **Foulds introduced six rules of tumor progression based on his observations of spontaneous mammary cancer in mice and generalized them to all forms of neoplasia [Foulds, L. (1954) *Cancer Res.* 14, 327–339 and Foulds, L. (1969) *Neoplastic Development* (Academic, New York), Vol. 1, preface and pp. 72–74.] Rules III, IV, and V are considered controversial, and research in animals seems inadequate to resolve the controversies. A subline of NIH 3T3 cells undergoes progressive transformation to produce foci of increasing population density when repeatedly constrained by sequential rounds of growth to and maintenance at confluence. Analysis of the results provides a cellular basis for rules III, IV, and V. Rule III states that progression is independent of the growth of the tumor and occurs in tumors that are arrested. Cell culture shows that progression is actually favored by constraint of growth, a result inconsistent with a major role for point mutations in progression. Indeed, there is a suggestion that the transformation may arise from chromatin changes preceding apoptosis. Rule IV states that progression can be gradual or abrupt but the latter conclusion has been frequently criticized. Cell culture exhibits both forms of progression but, in particular, eliminates the doubt about the abrupt form. Rule V, which is in a sense an extension of rule IV, states that progression follows one of alternative paths of development. The results in culture indicate that every independent transforming event gives rise to foci of unique morphology. Thus, even for the single characteristic of transformed focus morphology, many alternative paths to neoplasia are available to cells. In addition to clarifying the rules of progression, a method is described for pinpointing the time of the occurrence of events that are only expressed as dense foci after a variable lag time. The results in culture reinforce Foulds' conclusion that neoplastic development is primarily an epigenetically driven process and identify some of the cellular interactions that underlie that process.**

The term progression was first introduced in 1935 in a paper by Rous and Beard (1) to describe the development of carcinomas from virus-induced papillomas in rabbits. The concept of tumor progression was later developed and generalized by Foulds (2, 3). He formulated six rules of tumor progression based primarily on the study of mammary neoplasia in mice as follows: *rule I.* Progression occurs independently in different tumors in the same animal. *rule II.* Progression occurs independently in different characters in the same tumor. *rule III.* Progression is *independent of growth.* It occurs in latent tumor cells and in tumors whose growth is arrested. *rule IV.* Progression is *continuous or discontinuous,* by gradual change or by abrupt steps. *rule V.* Progression follows one *of alternative paths of development. rule VI.* Progression does not always reach an end point within the lifetime of the host. The first two rules are widely accepted and considered noncontroversial. Rules III and IV are somewhat controversial. In much contemporary thought neoplastic development is driven by conventional point mutation, which is strongly dependent on the replication of DNA accompanying cell multiplication (4). This dependence on cell multiplication is contrary to rule III, although Strauss (5) has called attention to the possibility of point mutations occurring in the absence of cell multiplication. It is also widely accepted that tumors develop through multiple stages beginning with benign-appearing lesions such as adenomatous polyps in colon cancer (6) or hyperplastic nodules in mammary cancer (7). In keeping with rule IV such development could be the consequence of either a large number of small abrupt steps or of long-lasting, continuous change, a distinction that is difficult to make in the animal. The alternative paths of rule V originally referred to the direct and indirect paths of mammary tumor development in mice (3). The direct path led to unresponsive tumors without traversing the intermediate nodular stage. It has been suggested that neoplastic development is always *indirect* but that the early stages are traversed so early or so quickly that they cannot be detected (8). It has, however, been pointed out repeatedly on the basis of clinical observation that perhaps the majority of human tumors do not develop by progression through intermediate stages (3).

It seems unlikely that the questions that have arisen about rules III, IV, and V can be settled by further work in animals or man. By contrast, cell culture has the potential of providing an understanding at the cellular level of the changes involved in progression. The predetermined rapid transformations induced by retroviruses and oncogenes (9, 10) are not well suited for studying progression, which is often multistep and extended in time. Spontaneous transformation in culture, however, has features that are similar to those of the spontaneous development of tumors in mice that formed the original basis for Foulds' rules of progression. The early studies of spontaneous transformation relied on tumor formation upon injection of cells into animals as the major criterion of transformation combined with morphological changes in cells but did not report on intermediate stages (11). Subsequently, others followed the course of transformation by using various criteria with no clear relation to one another or to Foulds' rules of progression (12, 13). Descriptions of transformation by chemical and physical carcinogens have concentrated on the production of large, dense, transformed foci and ignored the problem of intermediate stages (14, 15). Work from this laboratory has examined various aspects of progression of focus formation but neither the methods nor the materials were well suited to discriminate between the gradual and abrupt progression of Foulds' rule IV (16, 17).

Recently the 28 L subline of NIH 3T3 cells was developed by weekly passages at cloning densities and had properties

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Abbreviations: CS, calf serum; 1°–5° assays, successive assays for focus formation and saturation density.

that were suitable for studying the nature of progression (18). It lost the capacity to make dense, transformed foci in a direct primary (1°) assay but retained for a few months the capacity to make them in a sequential secondary (2°) assay. After 7 months of passage, however, even the capacity for focus formation in the more sensitive 2° assay was usually not expressed. Further study revealed that five serial assays were frequently required for the appearance of large, dense foci. I noticed that small, light foci usually appeared in the earlier assays. These were of a type I tended to ignore because they were difficult to quantitate and were not tumorigenic in nude mice (19). More intensive study revealed information that shed light on the controversial aspects of Foulds' rules of progression. New information was gained about the origin of diversity in the morphology of foci, and a general method was developed for pinpointing the time in a particular sequence of serial assays at which an event occurred that led to the development of dense, transformed foci in a later assay.

MATERIALS AND METHODS

The 28 L subline of NIH 3T3 cells was used throughout. These cells originated by weekly passages of cells seeded at 400 per 100-mm dish in 10% calf serum (CS; vol/vol) in molecular, cell, and developmental biology medium 402 (MCDB 402 medium) (18). About half of the cells formed grossly visible colonies within a week, yielding $>10^4$ cells per colony at the time of passage. The cells had been on this regime for 7 months at the beginning and for 11 months at the end of this series of experiments. One aim of the experiments was to assay all cells from a particular culture for focus formation. Therefore the initial seedings for assay were higher than customarily used—i.e., $1.8–4 \times 10^5$ instead of 1×10^5 per 60-mm dish (21-cm^2 area)—to limit the number of cultures used. During the course of the experiments it was discovered that cells seeded at lower densities usually gave more of the light foci than those seeded at the higher densities. The lowest density that reliably gave the confluent cultures necessary for assaying focus formation in a 2-week assay period was 3×10^4 per dish, and this was used in the later experiments for focus formation in parallel with the higher-density seedings and thereafter to establish separate parallel lineages.

The basic design was to assay the focus-forming capacity of cells sampled from the standard cell passage in five serial assays designated as assays 1°–5°. An important result of the serial assays was to bring out intermediate stages of progression that usually preceded the fully transformed state of tumorigenic dense foci (19). Concentrations of CS were varied in the 1° assay, but all subsequent assays were made in 2% CS. Cells were seeded in all assays at high cell densities of $1.8–4 \times 10^5$ and in many assays at the low density of 3×10^4 per 60-mm dish as well. The high and low seeding densities for the assays are indicated on the lineage diagrams in the figure legends by thick and thin lines, respectively. When the number of focus-forming cells was likely to be too high to give a discrete, countable number of foci, they were diluted and mixed with an excess of 28 L cells from the standard weekly passage that would make a background to display the foci. The standard passage 28 L cells were chosen for background because they were minimally suppressive to focus formation (20). The assays were incubated for 14 days in MCDB 402 medium with the appropriate CS concentration. One dish of each was fixed in Bouin's fluid and stained with Giemsa stain (18). The other was trypsinized, counted, and reseeded for the next sequential assay up to the fifth, when only counting of the second dish without further passage was done. The cell counts, which accompany the photographs, provided an estimate of the saturation density of the culture. The variation in cell counts from dish to dish of the same group of cells was <10%. Controls consisting of 1° assays of cells from the routine weekly passage accompanied every experimental assay. The number and morphology of the transformed foci were recorded when the foci were discrete and well-defined. The culture dishes were then mounted on art boards for display and comparison of complete lineages. Simple counts of cells or foci were totally inadequate in describing the great variety of changes such as mottling and varied focal densities and morphologies. The basic observations are therefore presented as photographs that exhibit the full diversity of foci and background. Saturation densities are shown to the bottom right of each photograph. When the photograph is of a mixture containing some transformed cells with nontransformed cells added for background, the saturation density is of a culture containing only the transformed cells. The branches of the lineage diagrams have been pruned, leaving only those that lead up to the cultures in the photographs. The dishes below the horizontal lines on each are the dishes in the photograph. The thick and thin connecting lines of the lineage diagrams represent seeding densities as described above.

RESULTS

Gradual Progression from Light to Dense Foci in Sequential Assays. Table 1 summarizes the proportion of cell lineages from the three experiments that registered positive for transformation-related changes in each of five serial assays. Although none of the 1° assays in 2% CS had identifiable transformation, some were done in 5% and 10% CS, where light foci would not register, so the 1° assays are not included in Table 1. The proportion of lineages showing *any* transformation-related appearance from small, light foci to large, dense ones went from 22% to 89% between the 2° and 5° assays, while the proportion showing dense foci, with or without light ones, went from 9% to 56%. As discussed later, only 3 of the original 29 lineages showed single-step transformation to dense focus formation with no sign of intermediate stages.

Typical progression through light, moderate, and dense focus formation is illustrated in Fig. 1. The cultures in Fig. 1*A* are controls seeded directly from the exponentially growing routine passages at the same time that the serial assays of the other two series were done. The controls for the 2° and 3°

Table 1. Proportion of cultures with evidence of transformation

	Successive assays							
	2°		3°		4°		5°	
Characteristic	No.*	%	No.*	%	No.*	%	No.*	%
Any morphological sign of transformation	5/23	22	17/33	52	48/63	76	79/89	89
Dense foci	2/23	9	4/33	12	15/63	24	50/89	56

*The number of positives divided by the number of lineages in a particular assay. The number of lineages increased in successive assays as they were split into branches by seeding of cells from the previous assay at low and high density for the next assay.

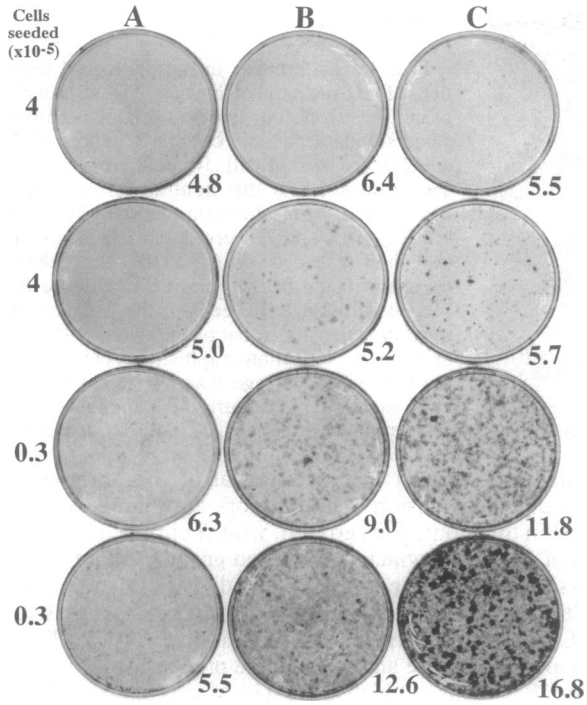

FIG. 1. Gradual progression from light to dense foci in sequential assays. (*A*) Controls, 1° assays in 2% CS from routine weekly passage of 28 L cells at the same time as the sequential assays of *B* and *C*. (*B*) Sequential assays 2°–5° in 2% CS from a 1° assay (not shown) 14 days in 5% CS. (*C*) Sequential assays 2°–5° in 2% CS from a 1° assay (not shown) 10 days in 5% CS, 2 days in 10% CS. The number on the left of each group of three cultures is the seeding density ($\times 10^{-5}$) of that assay. The number to the bottom right of each culture is the saturation density ($\times 10^{-5}$) of its sister culture.

assays were seeded at high density and show no foci. The controls for the 4° and 5° assays were seeded at low density and show the mottling typical of low-density control assays.

The lineages in Fig. 1 *B* and *C* exhibit a few light foci in the early assays, which become darker with each sequential assay, finally producing dense foci in the 5° assay. Comparison of the two lineages supports the idea that the dense foci arise by progression through various stages of light foci. Light foci are seen first in the lineage in Fig. 1*C* in the 2° assay, and these become darker and more numerous in sequential assays. The lineage in Fig. 1*B* lags slightly behind, each assay having fewer and lighter foci than the one on the right. By the 5° assay, Fig. 1*C* lineage has 95 dense foci of medium size on a fairly dense background of moderately stained foci, while the Fig. 1*B* lineage has only 9 small, dense foci on a lighter background. The measurements of saturation density are in keeping with the visual impressions of population density. The entire effect is what would be expected from a gradual, stagewise progression of transformation.

Abrupt Appearance of Dense Foci Without Intermediate Stages. Gradual progression basically like that shown in Fig. 1 was the rule for the 28 L subline during the 4-month period of these experiments. Fig. 2*A*, however, illustrates one of two cases in the same experiment in which large, dense foci suddenly appeared in a 2° assay with no evidence of intermediate stages. Fig. 2*B* is a 3° assay culture with relatively dense foci from a series from the same experiment in which no foci were seen in the 2° assay. The foci in Fig. 2*B* were smaller and lighter than those in Fig. 2*A* but were at a more advanced state than those of Fig. 1 up to but not including the 5° assay of the latter. The saturation density of the culture in Fig. 1*B* was about twice that of control cultures. Assuming that the ≈200 foci were the source of the excess of about 5×10^5 cells over the number in the accompanying focus-free

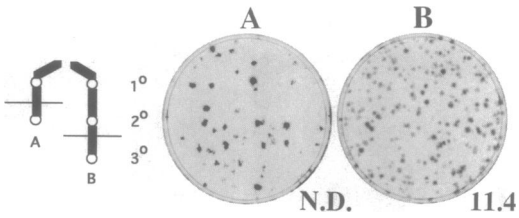

FIG. 2. Abrupt appearance of dense foci without intermediate stages. (*A*) 2° assay in 2% CS from a 1° assay, 14 days in 2% CS. (*B*) 3° assay in 2% CS from a 1° assay, 12 days in 2% CS, 2 days in 10% CS. There were no light foci before or during the assays shown. Saturation density was not determined (N.D.) for sister dish of *A*. Photograph shows cultures below the horizontal line on the lineage diagram.

control assays, there was an average of about 2500 cells per focus. The average focus-initiating cell therefore underwent about 11 divisions in the 14-day assay period as contrasted with only 2 or 3 divisions for the average nontransformed cell in the background. Although cell counts were not done on the sister dish to culture A, previous estimates of comparable foci indicate they consist of $>10^4$ cells for an average of about one division a day. Abrupt transformation appeared in three of the four lineages of this particular group, indicating the cells used to initiate this group were in a state favoring one-step transformation.

Differences in Morphological Detail of Dense Foci from Parallel Lineages. Differences in focal morphologies between lineages can be seen by inspection of the cultures of Figs. 1 and 2. Since there are differences in size and density of these foci, they might be interpreted as stages within a single progressive series. A more convincing comparison can be made of dense foci among parallel lineages started from the same standard passage culture as represented by the 5° assay of the four lineages in Fig. 3. The foci shown in the enlarged photograph are typical of those in the rest of the culture. Three of the assays (Fig. 3 *A*, *C*, and *D*) represent a dilution of the serially assayed experimental cells with an excess of nontransformed control cells to provide a common background and to ensure that most of the dense foci will be discrete. The culture in Fig. 3*A* arose from a different 1° assay than the other three. The lineage of the culture in Fig. 3*B* was split at the 2° assay from those of Fig. 3 *C* and *D*, which were split from each other at the 3° assay. The dense foci of culture *A* were large and stained more darkly in the center than the periphery. Those in culture *B* were fragmented, the result of the retraction of groups of cells into multiple clumps within each focus. The foci in culture *C* were uniformly dense with a relatively well-defined border, while those in the culture *D* had a very irregular shape with diffuse borders. Light foci had appeared before the dense ones in earlier assays of each lineage and were still evident in the 5° assay. It is apparent that each lineage has dense foci of distinctive morphology, suggesting the uniqueness of each event responsible for this advanced form of transformation and its occurrence after the branching of the lineages.

Asynchronous Expression of a Single Transforming Event in Branches of the Same Lineage. A corollary of the uniqueness of foci from each transforming event in different lineages is: if dense foci that arise in two branches of the same lineage *cannot* be distinguished from each other, it is likely they originated from the same transforming event in an earlier assay before the split. Fig. 4 shows the 4° and 5° assays of a lineage split into two branches by high- and low-density passages at the 4° assay. There were no foci in the 2° assay and about 60 very light ones in the 3° assay (not shown). In the 4° assay on the left, seeded at high density, there was an increase in the number of light foci over the number in the 3° assay. In the 4° assay on the right, seeded at low density,

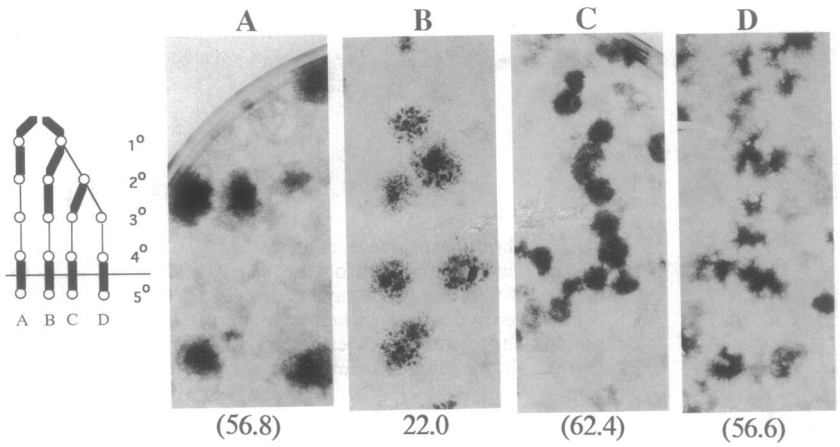

FIG. 3. Differences in morphological detail of dense foci from parallel lineages. 5° assays in 2% CS of cultures from four lineages originating from the same standard passage culture. Cultures of *A*, *C*, and *D* were seeded with 10^4 cells from the lineage shown plus 1.8×10^5 nontransformed cells for background. The saturation densities in parentheses for *A*, *C*, and *D* were determined on cultures (not shown) seeded with 1.8×10^5 cells of the same lineage with no background cells. Culture *B* was seeded with 1.8×10^5 cells of the lineage and the saturation density is that of the sister culture to the one shown. The photograph shows cultures below the line on the lineage diagram at a 4-fold larger scale than the other figures. The numbers of dense foci per dish were 45 (*A*), 20 (*B*), 75 (*C*), and 86 (*D*).

there were 3 dense foci, a half-dozen slightly less dense ones, and a very large number of still lighter ones (though denser than those in the culture on the left). The 5° assay was seeded with 10^4 cells from each branch with an excess of nontransformed control cells to form a background for foci. The lineage on the left, which had no dense foci in the 4° assay, produced 46 of them in the 5° assay, while the one on the right produced twice as many. The morphology of the dense foci was of the same irregular stellate shape in both lineages. The marked similarity of these unusual looking foci in the two branches was strong evidence that they had originated in the same transforming event, which must have occurred before the split at the 4° assay. The fact that no dense foci can be detected in the culture on the left of the 4° assay is a sign that expression of the transformation was delayed by the high-density seeding until the cells could be once again trypsinized and reseeded for the 5° assay.

DISCUSSION

The results reported here serve as a backdrop for those three of Foulds' six rules of progression that are considered somewhat controversial. It can be noted at the outset that the results I have obtained in culture support Foulds' view in each case. Rule III states that progression is independent of growth. Foulds points out "there is little experimental evidence or none that sustained proliferation *per se* enhances progression and some evidence to the contrary" (3). In more recent work with various hormone-induced tumors in rats,

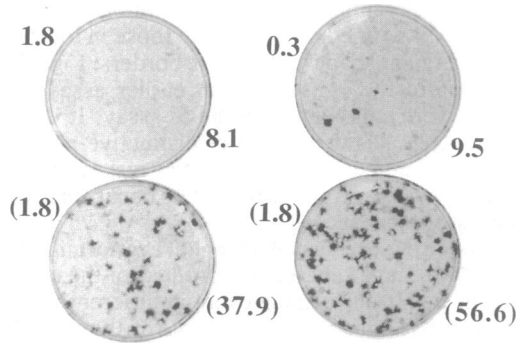

FIG. 4. Asynchronous expression of a single transforming event in two branches of the same lineage: Assays 4° and 5° in 2% CS of a lineage split into two branches at assay 4°. The seeding densities ($\times 10^{-5}$) are at the top left of each dish and the saturation densities ($\times 10^{-5}$) are on the bottom right. The seeding densities in parentheses of the 5° assay refer to the number of nontransformed cells mixed for background with 10^4 cells from the lineage; the saturation densities of the 5° assay were determined on cultures (not shown) seeded with 1.8×10^5 cells of the lineage with no background cells.

Noble (21) reported that withdrawal of the hormone with consequent regression of the tumors accelerated their progression to autonomous growth, while continued administration of hormone, which accelerated growth, minimized progression. We have previously noted that constraint of cell multiplication encourages transformation and progression of BALB 3T3 and NIH 3T3 cells in culture (17, 22, 23). The previous results are amplified in the present work by the use of the 28 L subline, which in most instances requires repeated rounds of constraint by postconfluent incubation and low serum concentration during serial assays to encourage progression. Indeed, I have recently noted that transformation begins only when dead cells begin to appear in the medium. This suggests that chromatin changes preceding apoptosis may contribute to transformation (24, 35). Controls kept at a high rate of multiplication by weekly passage at cloning densities in high serum concentration gave no evidence of progression through the 4-month period of the experiments. The results therefore reinforce Foulds' view that progression can occur with minimal or even negative growth.

Foulds' rule IV states that progression is continuous or discontinuous, by gradual change or by abrupt steps. It has been argued that gradual change might arise from a number of small, abrupt steps or that a seemingly abrupt change may result from traversing intermediate stages so early or quickly as to be imperceptible (3, 8). The controversy is illustrated in the human colon cancer field, where, beginning in 1928, it was generally believed that the malignant tumor arises from adenomatous polyps (25). It was later pointed out that many, if not most colonic cancers, arise in non-polyp-bearing colons (26), so the dual origin of these tumors is likely. In the rat, most experimental cancers of the large intestine arise *de novo* in the flat mucosa without prior adenomatous change (27). The adenoma–carcinoma sequence is more common in mice, but some strains exhibit a predominance of cancer without adenomas (28). Some have argued that overgrowth by the carcinoma obscures the preexisting adenomatous lesion in humans but this view has to be balanced by the failure to find such lesions in a series of very small carcinomas (26). The abrupt origin of dense foci in culture cannot be explained away as overgrowth of a preexisting intermediate stage since the latter would have easily been seen either by its earlier occurrence or by its presence alongside the more advanced foci. No such intermediate stages are seen in the few cases of abrupt origin described here, and since only the dense foci produce sarcomas efficiently in nude mice (19), there is a parallel with the discontinuous or abrupt progression of tumors in animals.

The requirement for multiple serial assays to produce dense foci in most of the 28 L lineages made possible the observation of gradual progression. These serial assays clearly reveal a sequence starting with mottling and small, light focus formation in the 1° and 2° assays, followed by

increases in size and density of the foci in the 3°, 4°, and 5° assays. A particularly suggestive case of the origin of dense focus formers from light ones is seen in Fig. 1, where a tandem relationship is maintained in two lineages, the one with an earlier appearance of light foci maintaining its lead in progression through the later assays and ending with larger, dense foci in the final assay. This does not prove that the dense focus formers arose from the light ones, since an abrupt origin from non-focus-forming cells remains a possibility even in this case, but it provides the strongest evidence yet available for gradual progression.

Rule V is in a sense an extension of rule IV since it states that progression follows one of alternative pathways of development. The terms indirect and direct paths of development are similar to those of gradual and abrupt changes, with the added proviso that there are alternative forms within the gradual and abrupt categories. Indeed, the number of alternative forms is so large that "no two tumors are alike in every respect even when they originate from the same tissue, have the same general properties and have been induced experimentally in the same way" (3, 29). Supportive evidence for a similar conclusion about neoplastic development in culture is provided here by the morphological differences between dense foci from separate lineages (Fig. 3). Such diversity is not restricted to NIH 3T3 cells since it was previously noted that cells derived from each agar colony of BALB 3T3 cells were morphologically unique to that colony (22). Nor is it restricted to established cell lines since it was first described in primary cultures of baby mouse skin fibroblasts, where every one of many spontaneous transformants was described as morphologically unique (30). While recognition of the uniqueness of every tumor requires the perceptive eye of an experienced pathologist, the morphological difference between foci of independent origin is apparent even to a novice. The implication of both observations is that the causal events are unique for every tumor and every morphological type of focus.

Foulds viewed "neoplasia as a developmental process akin to normal development in some respects but differing from it in important particulars" and emphasized the epigenetic basis of neoplastic pathology (3). The changes described here can also be viewed as epigenetic in the original sense of the term, which refers to the origin of new structures in development as a result of interactions among cells and tissues. It should be apparent that this definition does not rule out genetic changes as part of the response to those interactions since chromosomal changes accompany the epigenesis of lymphocytes and germ cells in normal embryological development. The "important particulars" in which neoplastic development differs from normal development are the lack of regularity and predictability of the cellular interactions that result in tumors and the great diversity among tumors of the same class that result from those interactions (31). The diversity of behavior and appearance of solid tumors could have its origin in the karyotypic diversity of solid tumors, as exemplified in human gliomas (32). Even where an oncogene is used transgenically to initiate multiple tumors in a single organ of a mouse, each tumor has a unique, discrete karyotype (33). Neoplastic development should then be characterized as an epigenetic process that results in diverse genetic changes at the chromosomal level (34).

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