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Letter to the Editor

Correspondence re: R. Grundel and H. Rubin, Effect of Interclonal Heterogeneity on the Progressive, Confluence-mediated Acquisition of the Focus-forming Phenotype in NIH-3T3 Populations. Cancer Res., *51:* 1003–1013, 1991.

In their recent paper in *Cancer Research* (1), Grundel and Rubin propose that epigenetic phenomena occurring during confluence play a causal role in progression of malignant transformation of NIH-3T3 cells. This conclusion is partially based on experiments showing that NIH-3T3 cells maintained at confluence show a time-dependent increase in focus formation upon subculture and reassay. The process of progression is poorly understood and may in fact involve a multiplicity of mechanisms including the sort of epigenetic phenomena postulated by the authors. In recent years, increasing emphasis has focused on the molecular nature of progression-related mechanisms, including activation of cellular oncogenes and loss of tumor suppressor genes. The data presented by Rubin and his colleagues in this and previous publications are not, in fact, inconsistent with the hypothesis that initiation and even progression of cells to neoplasia occur by means of rare mutational or other genetic events.

Grundel and Rubin show that one half of the dishes seeded with 1×10^5 cells will contain a transformed cell at confluence (preassay dishes) and the other half will not. If the total probability that at least one cell will be transformed from the time of seeding 1×10^5 cells until confluence is reached 4–5 cell doublings later is about 0.5, then it can be calculated that the probability (P) of a mutation that converts a cell to a focus-forming (transformed) cell is $\sim 2.25 \times 10^{-7}$/cell/generation. This value is very close to estimated mutation rates ($1.8–2.2 \times 10^{-7}$/cell/generation) for mammalian genes (2). At confluence normal cells stop growing, while transformed cells continue to grow; this is what forms a focus. The single transformed cell present in one-half of the dishes will therefore divide at a specific generation time until the dishes are passaged for the postconfluent assay.

The number of foci seen in the postconfluent assay will then be roughly equal to the size of the expanded transformed clone derived from the original transformed cell divided by 30. The latter factor is taken from the fact that at passage only 1×10^5 cells from a total of about 3×10^6 cells in the confluent dish are reseeded in the postconfluent assay dishes. If one assumes a generation time for the transformed cells in the confluent dishes of ~50 h, then the number of transformed cells seeded in each assay dish will vary as a function of time kept in confluence as shown in Fig. 1. The *dashed line* is the calculated curve, shown along with the experimental data presented by Grundel and Rubin (1). One-half of the dishes will produce no foci, a result also presented in the paper; after 17 days in confluent culture, one half of the reassay dishes exhibit large numbers of homogeneously appearing foci, and the other half show none. In contrast to this result for the dark staining type III foci, lighter staining, less malignant foci were seen in high frequency in all the dishes. The authors report an average of about 6 light staining foci/dish in the preassays, implying a transformation probability of $\sim 2 \times 10^{-6}$. When these dishes are replated, the number of resulting light foci in the postconfluent assay dishes will depend on the number of cells/focus but will clearly be high. After 14 days in confluent culture, assuming a generation time of 1.5–2 days, the number of foci seen in postconfluent assays would be between 25 and 125, if the original plate contained 6 transformed clones.

The morphological appearance of the foci led the authors to conclude that they share a monoclonal origin, a hypothesis that is more consistent with the conversion of a single cell to a transformed phenotype than with the idea of simultaneous progression of a large number of cells. It should be noted that the influence of nongenetic mechanisms of progression in this system, as in many other *in vitro* and *in vivo* models, may also be of critical importance. As pointed out by the authors, the dramatic difference observed in focus yield between 14- and 17-day preassays cannot be explained simply by mutational or other activation mechanisms operating in a single cell. Notwithstanding the possible contributory role of epigenetic progression mechanisms, it remains entirely possible that initiating and early steps in the progression of NIH-3T3 cell transformation proceed via the expansion of clones of individual cells that have probably undergone rare genetic alterations.

The authors refer to previous work from their laboratory demonstrating that NIH-3T3 cells passaged in 2% calf serum produced more foci than cells passaged in 10% serum (3). Assays for both groups were done in 2% serum. The authors concluded from this and other experiments that growth in low serum favored a population-wide shift in focus-forming potential of the culture. However, these data can just as easily be explained by assuming a rare (1 in 10^5) transformation of a single cell followed by selectively advantageous growth. If 1×10^5 cells are seeded for assay after various periods of growth in 2% serum and if 1 of these cells is a transformant, the clonal expansion of the transformant can be calculated using a Gompertz model. Fig. 1 of Ref. 3 provides growth rate and saturation density values for normal and transformed cell lines grown in 2% serum. Using these values in the Gompertz formula, the size of the transformed clone as a function of time in 2% serum

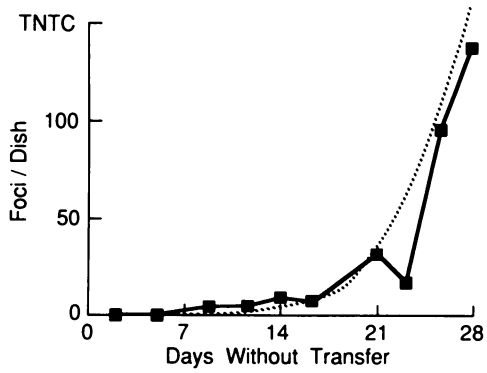

Fig. 1. Experimental and calculated values for focus yield/postconfluent assay dish as a function of time in confluent culture (days without transfer). The *solid line* and data points are taken from Fig. 9 of Ref. 1; the dashed line was calculated as

$$N_t = \frac{N_0 \cdot 2^{t/G}}{30}$$

where the number of foci is assumed to be equal to the number of cells (N_t) in an expanded transformed clone at the time of transfer, t. This cell number is a function of the number of cells (N_0) at $t = 0$ (assumed to be 1), and the generation time G, assumed here to be 52 h. *TNTC*, too numerous to count.

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can be calculated. These calculated values are shown in Table 1 along with the experimental data of Rubin *et al.* (3). As in the present paper, not all the data presented in the earlier work (3) can be so easily accounted for by clonal selection and expansion of rare transformed cells. The point is that in many cases, results interpreted to support an epigenetic mechanism can as easily be applied to selection processes associated with rare genetic events.

Table 1 *Increase of focus-forming cells as a function of time in 2% serum as calculated assuming clonal expansion*

Wk	From Ref. 2	Calculated[a]
0	0.001	0.001
2	0.075	0.076
3	0.27	0.40
5	0.65	0.55
6	0.59	0.55
7	0.35	0.55
8	0.56	0.55

[a] Calculated assuming that normal cells exhibit a growth rate of 0.75/day and a saturation density of 6.5×10^5; transformed cells grow at a rate of 0.85/day and have a saturation density of 1.5×10^6. These values were extracted from Fig. 1 of Ref. 2. Data shown was calculated using a competitive Gompertz model of the form

$$N_{i,t} = N_{i,0} \exp\left(\ln \frac{i}{M_{i,t}}\right) \cdot (1 - e^{-kt})$$

where the number of type i cells at time t, $N_{i,t}$ is a function of the initial number of cells ($N_{i,0}$), the saturation density M_i, and the growth rate k. The total number of cells in the culture is represented by $N_{i,t}$.

Dr. Rubin's contributions have clearly demonstrated the inherent complexity of cellular processes involved in neoplastic transformation. However, current research into molecular mechanisms associated with initiation, as well as progression of the transformed phenotype, demonstrates that mutational and related genetic mechanisms may be at least as complex as putative epigenetic phenomena (4, 5). It is obvious that such phenomena must play some role in carcinogenesis, but genetic changes are difficult to rule out in any system and, based on the wealth of data from many models, are likely to be critical events during multistage malignant transformation.

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Reply

In the preceding Letter to the Editor (1), Garte questions our proposal that nonrandom, adaptive changes underlie the neoplastic transformation and progression of NIH-3T3 cells (2, 3). He prominently states that the appearance of foci in our experimental cultures can be explained by rare mutational events followed by a selective growth advantage of the mutant cell. He only passingly acknowledges that much of our data cannot be explained by such clonal expansion of rare mutant cells.

The main part of our work is, in fact, inconsistent with this mutation-driven mechanism. For instance, we find that the probability of transformation of NIH-3T3 cells is strongly influenced by the nutrient and growth factor environment of the cells (4). Also, the transformed state of NIH-3T3 cells exhibits characteristics of diversity and reversibility which are unlikely to be mutationally based.

The dependence of transformation on the cellular environment is well illustrated in an experiment mentioned by Grundel and Rubin (3). In that experiment, a clonal population of NIH-3T3 cells was continually maintained in log phase growth for 21 weeks and was assayed repeatedly during that period for the ability to form transformed foci. Not a single focus formed in any of 50 assays performed during this period. Near the end of this experiment an aliquot of these non-focus-forming cells was transferred to a medium containing a substantially lower concentration of serum. After only 2 days of growth in this low serum environment these NIH-3T3 cells were assayed for focus formation. Numerous foci formed. The cells maintained in low serum and the cells maintained in higher serum were assayed for focus formation using an identical assay procedure. Therefore, the only reason for differences in focus-forming ability between the two groups of cells was the environment to which the cells had been exposed prior to assay. Cells made foci after a short period of growth in low serum; cells maintained in high serum consistently did not make foci. Obviously the ability to express the focus-forming, or transformed, phenotype was dependent on the environmental history of the cells. This is not the hallmark of a process in which mutation is the necessary and sufficient explanation of causation.

Garte does admit that feasible mutation and cell proliferation rates cannot explain certain changes we have observed, such as an NIH-3T3 population whose ability to form foci increased over 1000-fold in just 3 days (3). Nonetheless, he has produced a graph, his Fig. 1, which purports to show that other results we present can be simply explained by mutation and cell proliferation. In that figure, Garte predicts that the number of foci per culture should reach an uncountable level by 28 days after the appearance of the first focus-making cell in the population. This matches what we observed. However, his selected curve ignores a >50% decline in number of foci that occurs after the 28-day cutoff on his graph (see Ref. 3, Fig. 9). Thus his prediction does not describe what we observed. This decline can hardly be explained on his assumption of selective growth of mutated cells. This decline is consistent, however, with the suppression or reversal of the ability to form foci which we have already documented to occur in the NIH-3T3 system (2, 4, 5).

Garte takes data on rates of focus formation from two of our papers and states that these data could have been generated from populations of cells which mutated at rates of 2×10^{-7}

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