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Rethinking "Cancer as a Dynamic Developmental Disorder" a Quarter Century Later

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Abstract

In a Perspectives article of 1985 in *Cancer Research*, I discussed evidence for a nonmutational origin of teratocarcinoma, and cited related claims in liver carcinogenesis, which I later broadened to include spontaneous transformation in cell culture. Further research on spontaneous transformation cast doubt on that interpretation, and motivated re-examination of the cases for teratocarcinomas and liver cancer. This re-examination led to the conclusion that mutation and selection underlie the development of neoplasia in all three cases. At the same time, it affirmed the primary role of hierarchical tissue structure and intercellular relations in maintaining homeostasis, which was the major theme of the 1985 article. Tumor promotion disrupts intercellular relations to allow progressively selective proliferation of initiated cells and accompanying clonal accumulation of genetic change. [Cancer Res 2009;69(6):2171–5]

Introduction

In 1984, Peter Magee, the Editor of *Cancer Research*, asked me to write a Perspectives article on the cell biology of cancer. I wrote about hierarchical relations between cells and how disruption of those relations might result in neoplastic development. The resulting article (1) considered as a prime example of such relations the experimental induction of teratocarcinoma by the transplantation of early mouse embryos to the testis of adult mice (2), and the totipotent normalizing effect of cells from a euploid teratocarcinoma into the blastocyst of the developing embryo (3). The latter result, in particular, led to the conclusion of a nonmutational origin of the teratocarcinoma (3). A similar interpretation was cited for chemical carcinogenesis of the liver in an addendum to my article. More detailed support for this interpretation of liver carcinogenesis appeared in a 1991 Perspectives article, to which I contributed a section on spontaneous transformation in cell culture again suggesting a nonmutational origin of transformation (4). However, as more information became available and the data were more critically analyzed, it became apparent that the neoplastic transformation and its reversion in the three cases cited were more correctly described as special cases of genetic change and selection. At the same time, the results provided insights on hierarchical relations in tissues, which help to resolve the clonal and field theories of carcinogenesis.

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Evidence Linked to a Nonmutational Origin of Cancer

Teratocarcinoma. The strongest case for a nonmutational origin of cancer in the 1985 article came from the transplantation of a small number of embryonal cancer cells from a euploid line of teratocarcinoma cells into the blastocyst of a genetically distinct strain of mice, and after development of the embryos to term (3). A few of the mice that developed from the injected blastocysts were genetic mosaics with substantial contributions of tumor-derived cells in many developmentally unrelated tissues, including the germline that formed reproductively functional spermatozoa. The results were interpreted as "an unequivocal example of a nonmutational basis for transformation to malignancy and reversal to normalcy." The origin of the teratocarcinoma from disorganized embryonic cells that had been transplanted to adult testis suggested that "malignancies of some other more specialized stem cells might arise comparably through tissue disorganization leading to developmental aberrations rather than changes in gene structure" (3).

Aneuploid sublines of the same teratocarcinoma lost their multipotentiality (5, 6). Another line of embryonal cancer cells that maintained its euploid karyotype maintained its multipotentiality (7). However, euploid embryonal cancer cells of different origin failed to develop beyond colonization of the yolk sac (8). Neither a high rate of colonization nor a normal karyotype ensures full reversion of embryonal cancer cells to normal behavior (9). It was conjectured that the normal karyotype of the cases that failed to develop fully could have masked genetic changes below the resolution of karyotyping (7). An opportunity to distinguish between reversible epigenetic-physiologic changes and mutation selection in the origin of teratocarcinomas was afforded by the development of the nuclear transfer technique to enucleated oocytes, which can reset the developmental state of the injected nucleus to that of the zygote.

The technique was applied to embryonal cancer nuclei from three teratocarcinomas that differed in their carcinogenicity and differentiative potential (10). Fresh teratocarcinomas were obtained by the injection of nuclear transfer embryonic stem cells from the three lines s.c. into mice. Embryonal cancer cells of each line and their respective nuclear transfer embryonic stem cells displayed exactly the same potential when tested for tumor formation and chimera development. Comparative genome hybridization identified the same genetic lesions in the embryonal cancer and nuclear transfer embryonic stem cells of each line, with the number of such lesions inversely proportional to the differentiative potential of each of the three lines. Although it could not be concluded that those lesions were directly responsible for the differing behavior of the cell lines, they were consistent with the idea that genetic changes that could not be reprogrammed determine the developmental potential of each line. Other results show that tumors of known genetic origin besides

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teratocarcinomas can produce a variety of normal differentiated cells that retain the genetic changes associated with tumorigenesis (11). It is noteworthy, however, that the nuclei of leukemia, lymphoma, and breast cancer cells failed to produce embryonic stem cells.

Liver cancer. Evidence for a nonmutational origin of experimental liver cancer was briefly cited in the addendum to my 1985 article (1). Liver cancer arises from hepatocyte nodules that are induced by chemical carcinogens (12). However, 95% of the nodules are remodeled into normal-appearing liver by a process thought to be redifferentiation (13). Farber thought of "the hepatocyte nodule as a physiological response to environmental perturbations. . . and not as aberration or a mutation" (1, 14). The nodules remodeled to the point that they could no longer be grossly distinguished from normal liver. The 5% of nodules that persisted were uniformly stained, kept the 2-cell-thick plates, and were precursors of hepatocarcinomas (15).

The remodeled nodules were considered redifferentiated, therefore essentially normal. Remodeling of the hepatocyte nodules was the linchpin of "the most telling evidence that points overwhelmingly to the physiological adaptive nature of the hepatocyte nodule" (4). However, the nodules arise from resistant hepatocytes that first form irreversibly altered microscopic foci that are promoted to nodules (16). The remodeled nodules exhibit the irregular, patchy staining for nodule-associated enzymes that is characteristic of unpromoted microscopic foci for at least 36 weeks (16). At 28 weeks, the remodeled nodules proliferate at one-fourth the rate of persistent nodules, but that is 4 times higher than normal hepatocytes (15). They are therefore clearly abnormal in several respects, which is consistent with their mutational origin; so is the one-hit nature of the dose response of hepatocyte focus formation to carcinogen application (17). Beginning at 6 months, nodules of characteristically malignant cells appear in a few of the persistent nodules (18), indicative of further mutations that drive progression to malignancy.

Farber himself frequently calls attention to similarities in the pathogenesis of liver and skin carcinogenesis, emphasizing the disappearance of nodules in the former and papillomas in the latter as examples of reversibility (14, 19). However, the incompleteness of reversion of papilloma is well-established, as is their mutational origin. Labeled papillomas that have fully regressed after cessation of repeated carcinogen treatment of rabbit skin recur when the carcinogen treatment is repeated, or promoting treatment is instituted (20). Papillomas of mouse skin that regress after cessation of promotional treatment of initiated skin appear as papillomas much faster than they do in initiated but previously nonpromoted skin when promoter is applied (21). The evidence for persistence of remodeled nodules and regressed papillomas is therefore consistent with their mutational origin.

Spontaneous Transformation of Cultured Cells

I wrote the second half of the 1991 Perspectives article in which I supported a physiologically adaptive origin for the neoplastic transformation of cells in culture (4). I was influenced by the arguments for a nonmutagenic initiation of teratocarcinomas and liver cancer described above. At that time, my laboratory had begun working on spontaneous transformation of NIH 3T3 cells (22), an aneuploid line of mouse fibroblasts that had come into prominence as the most responsive target for transformation by transfection of DNA from a line of cells derived from a human bladder cancer (23). Even without transfection, however, the NIH 3T3 cells spontaneously produced a few transformed foci when incubated to confluence (24, 25). Because spontaneous transformation was driven by reducing the growth rate of cells, either by confluence or low-density passage in low calf serum (CS), the possibility of classic point mutation as the causal agency seemed remote, and the idea of nonmutational physiologic adaptation became even more attractive than before.

The nature of spontaneous transformation was further explored by isolating cells from transformed foci and subculturing them in low-density passages with high CS to determine whether the transformation was reversible (26). The retrieval of the transformed cells was carried out by trypsinizing the foci that were set off from surrounding cells by a cloning cylinder. Repeated low-density passage of the isolated cells in high CS resulted in the apparent reversal of their transformed state. Further support for the

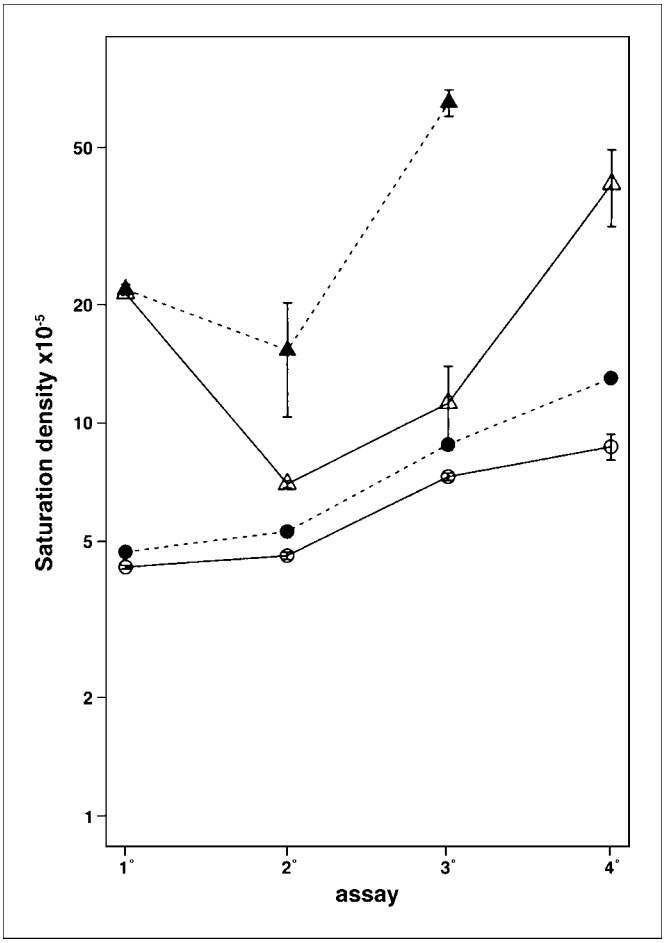

Figure 1. Saturation densities proportional to CS concentration in a 1° assay are reflected in serial assays in a single low CS concentration. Saturation densities were obtained for NIH 3T3 cells seeded in multilineage 1° assays in 2% CS for 2 wk (○—) and 3 wk (●--), or 10% CS for 2 wk (△—) and 3 wk (▲- -). All the lineages were then subcultured in 2% CS for 2 wk in 2°, 3°, and 4° serial assays to determine the cumulative effects of the total number of cell divisions in the 1° assays. The higher the total number of cell divisions and the longer the time in the 1° assay, the greater were the cumulative saturation densities in the 2°, 3°, and 4° assays. Note the small SDs in all the saturation densities derived from the 1° assays in 2% CS, and in the 2° assay derived from the 1° assay in 10% CS for 2 wk. That uniformity from culture to culture indicates that there were many growth variants in the starting material. The SDs become high when large, dense foci appeared differentially in the lineages within each category, indicating the progressive generation of new mutations (30). Reproduced with permission from PNAS.

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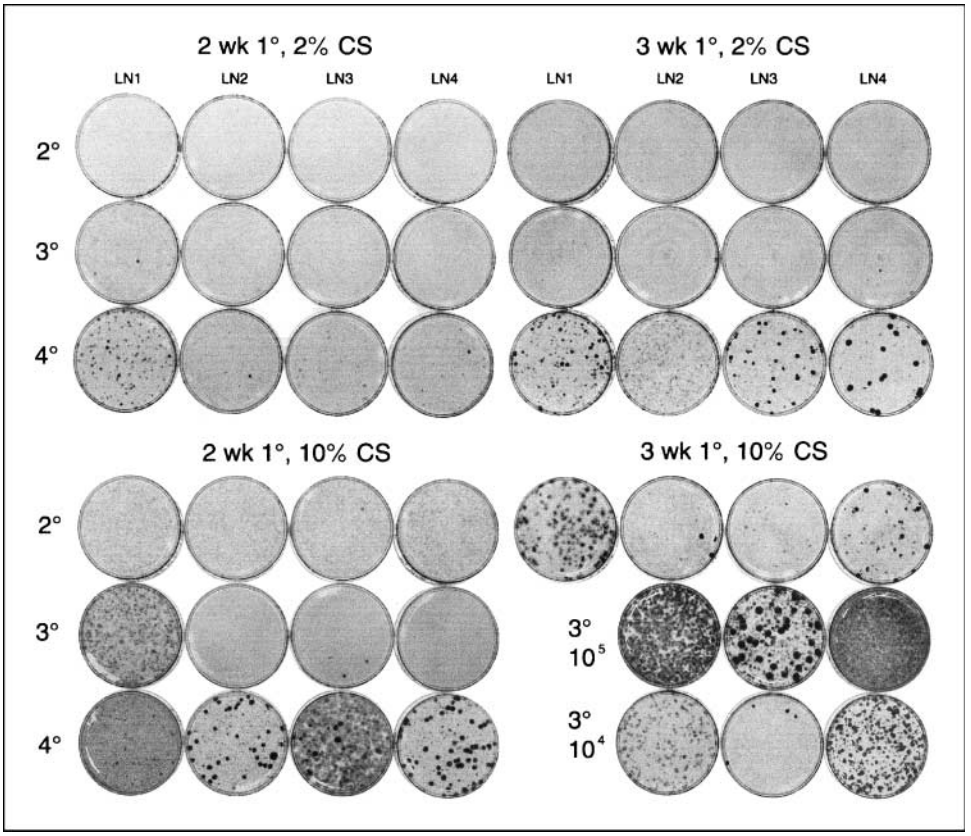

Figure 2. Appearance of transformed foci in the later assays depends on conditions of the 1° assay. The 2°, 3°, and 4° serial assays were all in 2% CS for 2 wk. Multiple lineages (*LN 1, 2, 3, 4*) in each of the 4 categories were derived from different CS (2% and 10%) and incubation periods (2 and 3 wk) of the 1° assay. Note that the transformed foci appear earlier and are larger the higher the CS concentration and the longer the time in the 1° assay. Also, note the hundreds of light, pinpoint foci in the 2° assays derived from 2 wk 1° assays in 10% CS. The progeny cells from those foci of the 2° assay initiate so many foci in the 3° assay that they coalesce to make a thicker, but uniform, background in LN 2, 3, and 4 of the 3° assay, in which LN1 has started to produce well-defined foci. LN1 transformed foci become confluent in the 4° assay, whereas large, dense foci appear in the other 3 lineages. Large, dense tumorigenic foci appear in the 3° assay derived from the maximum conditions (10% CS for 3 wk) in the 1° assay. When the cells used for the undiluted (10^5) cells of this 3° assay were diluted 10-fold (10^4 cells) and seeded with 10^5 cells partly resistant to spontaneous transformation, the dense foci seen in the undiluted assay decreased in size, and the lighter foci disappeared. This showed that contact with confluent, partly resistant cells suppressed growth of the transformed cells (30). Reproduced with permission from PNAS.

physiologic adaptation model was sought in an experiment in which multiple cell lineages were prepared for a single round of confluence in either low or high CS (27). Each lineage was then separately cultured through further rounds of confluence in the low CS to determine the variance within each category for saturation density and transformed focus formation. There was no variation in the early increases in saturation density of lineages within each category without foci (Fig. 1), which conformed to the physiologic adaptation model. However, the appearance of dense transformed foci in later rounds of confluence, originating from the first round in high CS for an extended period, showed a wide range of variation (Fig. 2) consistent with a mutational origin. The overall results seemed to be consistent with Farber's view that the multiple early foci and nodules in liver carcinogenesis are physiologically adaptive in origin, but the later rare carcinomas are mutational (4).

The physiologic adaptation model began to unravel with the finding that the spontaneously transformed cells derived from foci in confluent cultures proliferated at a lower rate than non-transformed cells in low-density passages with high CS (28). This difference in growth rate raised the possibility that any normal cells included during the retrieval of cells from transformed foci would be selected in the subsequent low-density passages, and give the false impression of the reversal of the transformed state. Indeed, when nontransformed cells were excluded by isolation of single transformed cells on glass chips, the transformed state of their progeny proved to be irreversible and, therefore, characteristic of a genetic origin (29).

The final tipping point in the realization that even the early increases in saturation density were of genetic origin came from a more careful re-examination of the cultures in Fig. 2. What had been taken as smooth monolayers in the secondary assays in the lower left quadrant of the photograph actually consisted of many uniformly distributed, barely visible small foci. It was then realized that a selection had been made of cells in the first round of confluence with a capacity for proliferation at high density. If there were too many such selected cells, as in the third round of confluence, they formed a thicker but smooth sheet and, in the fourth round, led to further variant selection to produce the fully transformed dense foci seen in the lower right quadrant of Fig. 2 (30). The implication is that both the increases in saturation density without foci and the later appearance of foci arise by selection of genetic variants.

Given that the uniform, within-category increases in saturation density resulted from selection, there must have been a relatively high frequency in the starting material of genetic variants for capacity of cells to grow at high density. A high frequency source of such variants is provided by heterogeneity in capacity for development of transformed foci seen in Fig. 3 (31). A contribution to such frequent genetic variation is likely to be the chromosomal rearrangements and aneuploidy that were distinctive and unique for each of the NIH 3T3 cells that were examined karyotypically (32).

Cell-Cell Contact Interactions Conditionally Determine Suppression or Selection of the Neoplastic Phenotype

Re-examination of the experimental teratocarcinoma literature unearthed the early insight of Damjanóv and Solter (33) that the disorganization of mouse embryos transplanted to extrauterine sites in adults allows uncontrolled proliferation of embryonic stem cells, which then undergo a mutation-selection process resulting in

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teratocarcinomas. The authors suggested that "the clonal theory of cancer. . .should at least be supplemented with the field theory that emphasizes the intercellular relations as the crucial ones." Although this insight was largely forgotten in the excitement generated by the totipotency of a single subline of euploid teratocarcinoma cells shown a year later (3), that very demonstration should have highlighted the importance of intercellular relations in maintaining (and restoring) tissue homeostasis.

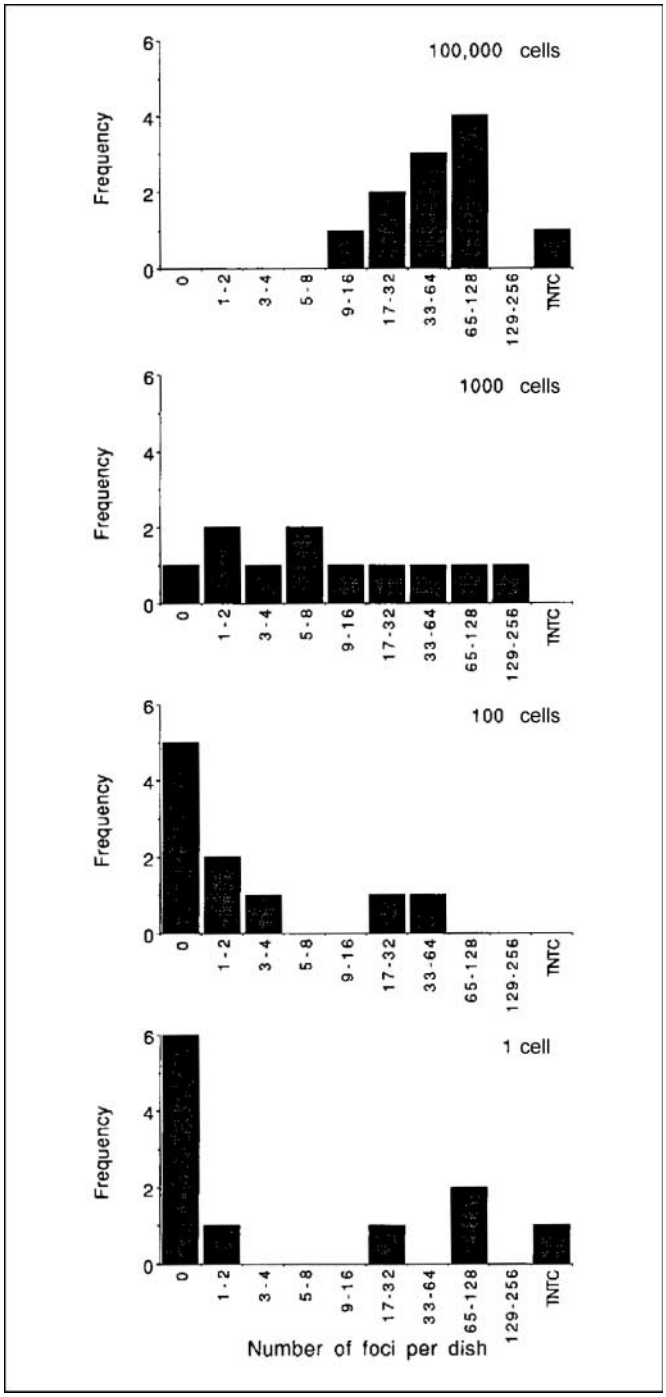

Figure 3. Heterogeneity of the numbers of dense foci in 2° assays of cultures originally derived from a wide range of starting cell numbers. Numerous cultures were started from 10^5, 10^3, 10^2, and 1 cells. When their cell numbers became sufficient, each of the cultures was used in a 1° assay of 10^5 cells in 2% CS for 21 d, and some of them were stained for transformed focus formation. Cells were harvested separately from each of the remaining cultures and seeded in 2 cultures at 10^5 cells for a 2° assay under the same conditions, all of which were stained at 21 d, and dense foci were counted. "Frequency" on the Y-axis refers to the number of starting cultures displaying the number of foci per culture in the 2° assay shown on the X-axis (31).

A largely forgotten illustration of the normalizing influence of hierarchical structure came from studies of mouse prostate in organ culture (34). Treatment of organotypic fragments of prostate with carcinogen produced histologic effects compatible with malignancy, including massive hyperplasia, anaplasia, abnormal mitoses, and invasion through the basement membrane. However, implantations of 872 of the transformed pieces into as many mice under a variety of conditions for extended periods of time failed to produce a single tumor. Indeed, all the implanted pieces recovered from the mice were highly differentiated, and resembled normal prostate. In contrast, disaggregation of cells from the carcinogen-treated prostate and their repeated passage in monolayer culture resulted in their multistep development into permanent cell lines that produced progressively growing transplantable tumors in mice (35).

A recent example of the transformation-suppressing role of tissue organization was seen in organotypic culture of mammary acinar structures (36). Activation of an oncogene in structurally unorganized acinar structures leads to their hyperproliferation and transformation. However, oncogene activation in mature, quiescent acini with established epithelial architecture does not reinitiate the cell cycle or transform these structures. The capacity of oncogene activation to reinitiate cell proliferation is restored by the loss of a cell polarity regulator protein. Such results may explain the estimate that the probability per cell cycle of carcinogen treatment *in vivo* giving rise to a tumor is many orders of magnitude lower than that observed for transformation of cells in monolayer culture (37).

Other examples of the normalizing effect of cell-cell interactions were given in my 1985 Perspectives article, but oddly enough, the most widely studied of such interactions were unintentionally omitted. "Oddly" because I had reported the first of these in the normalization of chicken fibroblasts transformed by infection with Rous sarcoma virus when seeded on a contact-inhibited monolayer of normal fibroblasts (38). There were other studies of such normalizing effects among fibroblasts (39), but the full significance of these observations was not appreciated until it was shown that normal epidermal cells suppress the growth of adjacent papilloma cells in culture (40) and in skin grafts on mice (41). Those findings of cell-cell contact suppression of potentially neoplastic cells provided a physical basis for the failure of carcinogen-initiated epidermal cells to produce tumors for the lifetime of the mouse unless treated repeatedly with a promoting agent (42, 43).

A particularly significant aspect of cell-cell interactions was discovered with the introduction of rat hepatocarcinoma cells into the normal liver of rats. Moderately malignant hepatocarcinoma cells injected into the liver of young rats differentiated into mature hepatocytes and failed to proliferate until the rat aged (44). Proliferation also occurred when the hepatocarcinoma cells were initially injected into aged rats. The normalization of the hepatocarcinoma cells was most effective when they were introduced into the liver as single cells, which allowed maximum surface contact with the surrounding hepatocytes (45). S.c. injection of the hepatocarcinoma cells allowed their maximal proliferation into malignant tumors (44). The increase in their proliferation in the liver with age of the recipient rats suggests that a decline in the normalizing capacity of tissue parenchyma contributes to the marked increase of solid epithelial cancers with age (46).

Conclusions

1. Reconsideration of the case for a nonmutational, physiologically adaptive origin of teratocarcinoma, hepatocarcinoma, and spontaneous transformation of cells in culture (1, 4) supports a process of mutation and selection as their underlying mechanism.
2. The postulated role of hierarchical structure of tissue in suppressing tumor development is reinforced. The primary mode of the suppression is adhesive contact between incipient neoplastic cells and their surrounding normal, homotypic cells. The teratocarcinogenic effect of disorganizing embryos (33) counsels caution in the use of disaggregated embryonic stem cells in regenerating normal function in defective adult tissue (47). The role of tissue organization in maintaining homeostasis is formalized in the biological principle of ordered heterogeneity, in which there can be regularity in the large where there is heterogeneity in the small (48).
3. The effectiveness of the NIH 3T3 cell line as a target for demonstrating the transforming capacity of oncogenes (23) depends on its partially transformed state, which needs only a nudge from an added oncogene to progress to more advanced transformation (24, 25).
4. Promotion allows selective clonal expansion of mutated cells with increased capacity for proliferation under physiologically inhibitory conditions. That expansion permits further mutations to accumulate. This enrichment step, rather than the initial mutation, is the crucial, numerically limiting factor in tumor development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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