What keeps cells in tissues behaving normally in the face of myriad mutations?

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No paradox, no progress (Niels Bohr)

Summary

The use of a reporter gene in transgenic mice indicates that there are many local mutations and large genomic rearrangements per somatic cell that accumulate with age at different rates per organ and without visible effects. Dissociation of the cells for monolayer culture brings out great heterogeneity of size and loss of function among cells that presumably reflect genetic and epigenetic differences among the cells, but are masked in organized tissue. The regulatory power of a mass of contiguous normal cells is expressed in its capacity to normalize the appearance and growth behavior of solitary homophilic neoplastic cells, and to redirect differentiation of solitary heterophilic stem-like cells. Intimate contact between the interacting cells is required to induce these changes. The normalization of the neoplastic phenotype does not require gap junctional communication between cells, though transdifferentiation might. These varied relationships are manifestations of the unifying biological principle of ''order in the large over heterogeneity in the small''.
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Introduction

There have been widely varying estimates of the number of mutations that exist in normal somatic cells of humans. These estimates have largely been based on the number of divisions that cells undergo in a lifetime and on assumptions about the mutation rates per cell division. Some of the estimates have been so high, e.g. 125,000 per colon stem cell in aging humans,[1] that it is difficult to understand how the cells survive, much less how they carry out their normal functions. Such estimates, however, do not take into account the degree of selection among the cells. Nor do they consider the idea of Cairns that stem cells have a mechanism for minimizing the accumulation of mutations in stem cells by keeping only the old template strand of DNA, thereby eliminating copying mistakes.[2] Recently, however, the use of transgenic mice carrying copies of a bacterial reporter gene has allowed measurement of the actual number and type of mutations at various ages in a variety of organs, including those that undergo few if any divisions in most of their cells after birth.[3,4] These observations leave unanswered the question of how the cells maintain their normal morphology and function to the extent that they cannot be distinguished from one another, even in old age. That question can be approached from the behavior of the cells after they have been separated from one another for monolayer cell culture. The results raise related issues such as how isolated neoplastic cells become normalized when surrounded by normal homophilic tissue, and the mechanism of transdifferentiation of isolated normal cells when they are surrounded by normal heterophilic tissue. The findings will be discussed here with regard to the fundamental concept of ''ordered heterogeneity'', namely order in the large over heterogeneity in the small, as proposed independently decades ago by three eminent biological thinkers. How this concept can be applied in the contexts of carcinogenesis and stem cell research will also be discussed.

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Abbreviations: HAN, hyperplastic alveolar nodules; RSV, Rous sarcoma virus.

How many mutations per normal cell in vivo

There has been considerable disagreement whether the large number of mutations that exist in solid epithelial cancers of humans requires an especially high rate of mutation in tumors or can be accounted for by the accumulation of mutations at the normal rate throughout life in the cell that gave rise to the tumor. Those who support the need for a mutator phenotype in tumors argue that the rate of mutations in normal cells is too low to account for the multistage progression of tumors and the thousands of mutations in human tumors. Their estimates of mutation rates are based on measurement of mutations in three genes during culture of cells, assumptions about the number of cell divisions in vivo and the inactivating effect of the

mutations on gene expression.[5,6] These calculations led to the conclusion that the average human cell amasses one to two mutations in a lifetime. Those who oppose the need for a mutator phenotype to account for the number of mutations in human tumors claim that thousands, or tens of thousands of mutations accumulate in stem cells of the colon crypt based on assumptions of a much higher rate of mutation per cell division than those made by proponents of a mutator phenotype.[1,7] In confining their calculations to cells of the colon crypt, they assumed about 5,000 cell divisions in a lifetime in contrast to the value of 100 used by proponents of a mutator phenotype for the average cell of the body.[6,8] Neither of the disparate views was based on direct measurements of mutations in cells in various tissues of the body, and both assumed that mutations only occurred during replication of DNA.

A more direct method became available to measure the actual accumulation of mutations in the DNA of somatic cells during aging in vivo by constructing a transgenic mouse model.[4] The model harbors plasmid vectors containing the β-galactosidase *lac*Z reporter gene integrated head to tail at various chromosomal locations in every cell. The plasmids can be efficiently recovered by transfection into *E. coli* host bacteria. A positive selection system permitting only *E. coli* bacteria with a *lac*Z mutated plasmid to grow allows for accurate determination of mutation frequencies as the ratio of mutant colonies to the total number of plasmid copies recovered. These mutational values can be extrapolated to the entire genome by the ratio of total DNA per cell to the *lac*Z DNA per cell. Cells of the small intestine in old (32 months) mice had about 390 mutations per cell of which some 330 were point mutations and 60 were size changes in DNA, indicating large genomic rearrangements[3] (Table 1).

For comparison with the estimates of mutation frequencies in colon crypt stem cells of older humans,[1,7] the relative size of the mouse and the number of divisions in a lifetime have to be considered. The mouse is less than one thousandth the size of a human, and its small intestine stem cells undergo about 1,000 divisions in its lifetime[9] versus about 5,000 divisions of the human colon crypt cells. The estimate of total mutations for the mouse small intestine stem cells ranges from about 5 to 75 times lower than those of the human colon crypt stem cells. Therefore, the frequency of point mutation in the mouse cells on the basis of the total number of divisions would be in close agreement with the lowest estimates of the human colon crypt cells. Protein-coding DNA and *cis*-regulatory DNA in the mouse are estimated as 5% of total DNA,[10] so there would be about 20 mutations per cell with a reasonable likelihood of affecting its phenotype. However, non-coding DNA, once considered junk or selfish DNA, has now been shown to serve some functions in cells such as loci for centromeres, sources of regulatory RNAs and sites for nuclear matrix attachment[11] The many more mutations in the non-coding than the coding regions might therefore have measurable effects on cell function. That possibility has to be balanced against the evidence that most protein-coding genes in metazoans apparently have no phenotypic expression.[12]

A more serious effect than the point mutations are the 60 large genomic rearrangements in each small intestinal cell, some of which involve millions of base pairs on DNA, which could seriously affect normal regulation through position effects and gene dosage.[3] The mutation loads per cell for four organs raise questions about the relation between the number of cell generations and mutations in general. The three predominantly postmitotic organs, heart, liver and brain, have about 70% as many mutations in 3- to 4-month-old mice as the rapidly multiplying small intestine (Table 1). The accumulated mutations in heart and liver increase 2.5- to 2.8-fold in old (~30 months) mice over those in young mice, while mutations in the intestine increase 3.2-fold, and those in the brain show no increase. The ratio of point mutations to large genomic rearrangements in heart and liver remains the same in young and old mice. In contrast, that ratio increases disproportionately with age in the small intestine and the brain, although the total number of mutations in the brain remains

Table 1. Mutation frequency per cell in organs of young and old mice

		Mutation load per cell		
	Age (months)	All mutations	Point mutations and 1 bp deletions	Large genomic rearrangements
Small intestine[3]	3.3	123	89	34
	32.0	390	332	58
Heart[3]	3.3	81	36	45
	32.0	202	103	99
Liver[4]	4.3	79	42	37
	29.9	224	113	111
Brain[4]	4.3	98	55	43
	29.9	96	74	22

constant. It is evident that many mutations accumulate in cells of all organs during the lifespan of the mouse, even in cells that divide infrequently if at all after birth. Accumulation of mutations at a high rate in non-dividing cells has been reported in bacteria.[13,14] A considerable fraction of the mutations in mouse cells are large chromosome rearrangements that would be expected to induce damaging phenotypic effects in tissues, but none have been reported.

The results from mice leave the unanswered question of the mutation frequency in human cells in vivo where transgenic techniques cannot be used. However, cellular clones were isolated from histologically normal human breasts that allowed identification of nine highly informative microsatellite markers, some of which were at chromosomal regions involved in breast cancer.[15] At least one genetic abnormality was found in half the women examined, with a weighting toward mutations commonly seen in breast cancer. The fact that the regions examined represented only a very small fraction of the entire human genome and yet exhibited cancer-related mutations in many women offers further evidence that cells maintain their normal phenotype despite the presence of many mutated genes of functional significance. Indeed, there is no obligate correlation between loss of heterozygosity or microsatellite instability of human breast and subsequent malignancy.[16]

Young female rats of the Fischer strain have patches of mammary cells with mutations in the *Ha-ras*-1 oncogene, which remain without effect through life unless treated with N-nitroso-N-methylurea.[17,18] Rats treated with this carcinogen develop breast cancers, which are reproducibly associated with the mutated gene. Other examples have been cited of cells in normal tissues that maintain their normal phenotype despite the presence of mutations known to produce pathology under certain selective conditions.[19,20] Presumably the same is true for changes in epigenetically regulated genes, which occur at a much higher rate than somatic mutations.[21] These findings suggest that the architecture of intact tissue averts the potentially abnormal effects of mutated genes and altered epigenetic regulation of genes.

The behavior of animal cells on their dissociation from one another or reduction to very small fragments

If the phenotypic expression of mutated genes in tissues is held in check by the organized structure of the tissue, it might be expected that dissociating the cells from one another and their surroundings would have pathological effects. That this occurs is suggested by the sharp dependence of cell multiplication on the population density of primary cultures of dissociated mouse fibroblasts.[22] Virtually no proliferation occurs where there is little or no contact among the cells, and maximum proliferation occurs where most of the cells are in contact with each other, although at subconfluent density.

A related situation is seen with primary cultures of chicken embryo fibroblasts. Low densities of such cultures multiply very poorly, but if they are seeded on subconfluent feeder layers of x-ray-inactivated fibroblasts, they multiply very well.[23] If contact is prevented between the living and the inactivated cells by placing them on opposed surfaces no matter how close, the full supportive growth of contact cannot be obtained. Hence, the full replicative function of the primary cells is largely dependent on limited contact interactions with other cells.

An early effect of dissociation of newly explanted fibroblasts in growth medium is a 3- to 4-fold increase in size, and in protein and RNA content of the average cell.[24] There is also a shift from a uniformity of cell size in vivo to a marked heterogeneity of their size in vitro. Hepatocytes in the intact liver of rats exhibit a uniform content of albumin from cell to cell,[25] but there is wide diversity of albumin production in a hepatoma cell line.[26] Cartilage cells quickly lose their capacity for making their specific products when they are dispersed for culture, but regain them when they are packed together again.[27]

A minimal size of tissue is required in the early embryo for development to proceed. Small squares of tissue (0.25×0.25 mm) with a relatively homogeneous cell population removed from sites of the flat chick blastoderm developed into central nervous system.[28] The squares also developed into central nervous system when cut into 8 pieces, but not when cut into 16 pieces even though they contained many cells. When the 1/16 fragments were recombined however, they tended to regain their developmental capacity. The critical mass below which the determined character of development is lost is of the same order of magnitude from sponge to chick to mouse—0.1 to 0.2 mm in diameter—or several thousand cells. The data suggest that the developmental capacity of the blastoderm lies more in the pattern of cell interactions than in the cells themselves. A similar conclusion could be drawn from the so-called community effect in development displayed by amphibian blastula cell and tissue recombinations in solid gel culture.[29] Cells from the animal region of the blastula are placed in sandwiches between vegetal regions. The cells must be in a three-dimensional mass, not in a monolayer, to differentiate into muscle. The differentiation occurs in all the cells of the mass at the same time. The result was interpreted as a community effect in which the ability of a cell to respond to induction by differentiating as muscle is greatly increased if it is surrounded by other animal region cells responding in the same way at the same time.

Although these examples illustrate the capacity of organized state of tissue to order the phenotype of its constituent cells, they do not shed light on the effect of genetic or epigenetic changes on the long-term growth behavior of dissociated cells. An opportunity to do so became available in the NIH 3T3 line of mouse cell fibroblasts that proliferates

indefinitely in cell culture. Although the cells are aneuploid, they rarely produce tumors upon subcutaneous injection into athymic mice. The cell line is highly sensitive to contact inhibition and has low saturation densities that are especially evident in low serum concentrations.[30] They produce a uniform, flat monolayer when grown to confluence in 2% calf serum. However, there is a linear increase in saturation densities with serially repeated rounds of prolonged confluence, indicating the continuous appearance of heritable variants with progressively increased capacity for proliferation at high density. The gradual increase in overgrowth capacity of the entire population suggests that the growth behavior of the cells is responsive to many small increments of genetic and/or epigenetic changes. The epigenetic changes could be selected for at high density by suppression of genes involved in growth control through cellular interactions. It is evident that such selection does not ordinarily occur in vivo despite continuous contact among cells in tissues for many years.

Lest it appear that the gradual increases in saturation density under selective conditions only occur in established cell lines, a similar response is seen with fibroblasts freshly explanted from the mouse embryo.[22] Nor is it restricted to fibroblasts since a line of normal diploid liver stem cells increases in saturation density within 2 cycles of selection at high density, and increases many-fold in succeeding cycles.[31,32] Colony formation in agar and capacity to produce tumors with metastases also arise early under selection and increase steadily with each round of growth under selection. It is apparent then that dissociated cells progressively express any of a large number of mutations in contrast to the highly regulated, constant behavior of cells in tissues, despite their many mutations. It is noteworthy that selective conditions for expression of transformation are present in vivo since the intercellular fluid bathing the cells in the form of lymph has much less growth-promoting activity than does the serum used in cell culture.[33]

The effect of cell dissociation can also be observed in vivo by their injection into a site from which they had been completely removed. This was done in the mammary fat pads of a strain of mice bred for a high incidence of mammary cancer. An early stage in the development of mammary cancer is the appearance of hyperplastic alveolar nodules (HAN), which consist of preneoplastic mammary epithelial cells that later develop into mammary tumor cells. The HAN first appear in some of the control mice at 8–9 months and increase in frequency up to 15 months. Removal of the mammary gland epithelial rudiments from the fat pads of 2-month-old mice, followed by enzymatic dissociation of the epithelial cells and transplantation into fat pads of 3-week-old syngeneic mice that have been cleared of mammary epithelium, led to HAN development within 10 weeks.[34] The proportion of positive sites in the host increased with the age of the donor mouse. In contrast, transplantation of intact 1 mm^3 pieces of mammary epithelium from 4-mo-old mice gave no evidence of HAN development. The results showed that the organized state of the developing mammary gland restrains the neoplastic potential of the resident preneoplastic alveolar cells until early adulthood. Similar conclusions were drawn from the higher incidence of mammary tumors when dissociated nodule cells are injected into the gland-free fat pad of mice than when intact fragments of the nodules are injected.[35] Indeed, it had long been known that intact nodules grow much faster when transplanted into gland-free fat pads than into gland-containing fat pads, indicating that normal undisturbed mammary epithelium inhibits nodule growth[36] (Table 2). In contrast, mammary adenocarcinomas grow as well in gland-containing as in gland-free fat pads.[37]

Table 2. Growth of hyperplastic alveolar nodules (HAN) in gland-free versus intact mammary pads of mice

Host age	Fat pad	No. takes	Percentage filling of fat pads
7 mos	Gland-free	18	19 ± 5.5[a]
	Intact	17	1
3 wks	Gland-free	18	25 ± 5.8[a]
	Intact	17	7 ± 3.7[a]

Abstracted from Table 1 of ref. 36.
[a]Standard error of the mean.

The normalizing capacity of specific tissues for neoplastically transformed cells

Despite the deficiency of dispersed cell monolayer cultures for maintaining the differentiated state of most cell types, fibroblasts manage to maintain their major characteristics.[38] The monolayer culture of fibroblasts permitted the development of a quantitative assay for RNA-containing tumor viruses such as Rous sarcoma virus (RSV) by the production of morphologically altered, randomly arranged cells in discrete transformed foci.[39] In a medium containing fetal bovine serum at moderate concentrations ($\geq$ 6%) , or calf serum at high concentrations ($\geq$ 10%), I found that the development of the discrete, multilayered transformed foci was inhibited.[40] However, exposure of the fibroblasts to high-enough concentrations of RSV to infect most of the cells, transformed them regardless of the concentration or type of serum. The results suggested that some proteins in bovine serum inhibited cell surface proteases that were involved in transforming the cells, but prevented transformation only when the infected cells were surrounded by normal fibroblasts.

A systematic study of the effect of normal cells on the transformation of cells infected with a tumor virus was later undertaken by Michael Stoker with the DNA-containing polyoma virus.[41] Polyoma induces tumors in various organs of mice, and transforms a small fraction of cells in a clonally derived population of hamster fibroblasts.[42] Cells that were isolated from a transformed colony failed to multiply when added to a contact-inhibited, confluent sheet of uninfected hamster or mouse cells but did multiply on a sparse layer or a bare surface.[41] Growth inhibition of the polyoma cells requires contact with the confluent sheet of non-transformed cells.[43] Unlike polyoma-transformed cells, RSV-transformed cells can multiply on confluent sheets of normal cells as long as the serum concentration is not too high.[44] The type of normal cell used as confluent background also made a difference since avian cells did not inhibit the growth of polyoma cells. Later studies with spontaneously transformed mouse fibroblasts showed that the lower the saturation density of the confluent background cells, the more effective they are at suppressing the growth and normalizing the appearance of transformed cells.[30,45]

For a time, it was thought that gap junctional communication between transformed and non-transformed fibroblasts, with exchange of molecules less than 1,200 daltons in molecular weight, is required to suppress the transformed state.[46] However, more discriminating co-culture experiments of *Src*-transformed and normal fibroblasts from connexin 43-null transgenic mouse embryos showed that neoplastic suppression occurs despite the absence of gap junction communication.[47] The implication is that adhesion between the cell phenotypes, with stabilization of the transformed cell plasma membrane, is sufficient to normalize cell morphology. This conclusion is strengthened by the capacity of spontaneously transformed fibroblasts to normalize themselves if pushed to the limit of their saturation density.[48] They induced contact inhibition among themselves at about 10 times higher saturation density than that of normal cells. They then assumed a morphology and growth behavior resembling that of normal fibroblasts, which was retained for several days after their subculture at low density. The ultra high density forced extensive surface contact among the transformed cells, presumably restraining their plasma membrane activity, and normalizing their phenotype without the intervention of normal cells.

The problem of the specificity of cell–cell interactions in neoplastic development was addressed in the study of initiation and promotion in epidermal carcinogenesis.[49] Some of the basal epithelial cells or keratinocytes of the mouse epidermis are initiated by a single application of a carcinogenic polycylic aromatic hydrocarbon to mouse skin.[50] The initiated keratinocytes remain without neoplastic expression for the lifetime of the mouse, but can be promoted to form papillomas by repeated painting of the skin with a promoting agent. Initiation is widely considered to be a mutational event and promotion to be an epigenetic process. A major question was what type of cell represses tumor development by the initiated but non-promoted cell. Mixtures were made in cell culture between initiated keratinocytes and a large excess of either normal epidermal keratinocytes or dermal fibroblasts to determine which of these repressed the formation of neoplastic colonies of initiated cells that outgrew the surrounding normal cells.[51] The answer was that the normal keratinocytes suppressed the formation of neoplastic colonies, whereas fibroblasts had no effect on their formation. As was the case with fibroblasts,[47] suppression required intimate contact between the initiated and normal keratinocytes but there was no gap junctional communication between them.[52] In contrast, the normal keratinocytes could not suppress the growth of cells that had progressed to the carcinomatous stage.[51] Application of these techniques in vivo to papilloma formation by grafting the same cell mixtures in the skin of mice revealed similar results in that only normal keratinocytes could suppress growth of the papilloma cells.[53] This was the first clear demonstration that homophilic cells are required to maintain the normal phenotype of initiated cells. It complemented the evidence from genetic skin-grafting experiments that chemical carcinogens act directly on the keratinocytes that give rise to the epidermal tumors, rather than acting indirectly through the underlying dermis,[54] as some had assumed.[55]

Keratinocytes also regulate the growth and morphology of normal melanocytes interspersed as single cells among them in the basal layers of the epidermis.[56] The two closely apposed cell types are in gap-junction communication with each other. Melanoma cells lose their junctional communication with keratinocytes, but gain communication among themselves, and with fibroblasts. The loss of junctional communication between melanoma cells and keratinocytes is associated with disappearance of E-cadherin from the melanoma cells. Forced expression of E-cadherin in the melanoma cells restores gap junctional communication with keratinocytes, and normalizes the growth and morphology of the melanoma cells. While it has been assumed that the restored junctional communication of the melanoma cells with keratinocytes is responsible for normalization of the melanoma phenotype,[56] the suppression of growth of papilloma cells by normal keratinocytes in the absence of junctional communication[52] suggests that the increased adhesiveness of melanoma cells to keratinocytes through the increase in E-cadherins accounts for both the renewal of junctional communication and normalization. This conclusion is reinforced by the aforementioned self-normalization of transformed fibroblasts at extremely high saturation density.[47] The fact that gap junctional communication of the melanoma cell with fibroblasts has no effect on its neoplastic phenotype[56] is additional evidence of the specificity of the normalizing interaction.

Features of the in vivo normalization of hepatocarcinoma cells by normal liver

A comprehensive study of the regulatory effect of intact organs on the morphology and behavior of neoplastic cells was described in a series of papers on the fate of two lines of aneuploid rat hepatocarcinoma cells and their normal diploid, stem-like progenitors after transplantation into the liver of syngeneic rats of different ages.[57–60] All the transplanted cells could be identified by a chromosomally integrated *lac*Z reporter gene. Individual cells of the less aggressive tumor line migrated into the hepatic plates (Fig. 1B) where they resembled normal stem-like cells that had differentiated into hepatocytes (Fig. 1A).[57] No tumors developed from this line in young rats, either at the site of injection or in the hepatic plates, but tumors did appear with increasing frequency in rats injected at later stages of life (Table 3). Cells of the liver tumors that developed from the more aggressive line of hepatocarcinoma cells were plump, and had an epithelial morphology (Fig. 1D). Subcutaneous or intraperitoneal transplantation of both tumor lines developed tumors regardless of age. The cells of the tumors at the subcutaneous site had a spindle-shaped morphology that was similar to that of mesenchymal cells (Fig. 1C).

The significant results of these experiments can be summarized as follows. The capacity of the liver to normalize the morphology and growth capacity of the malignant cells decreased as the rats aged. The extent of normalization was greatest in solitary cells that had migrated into the hepatic plates and were in intimate contact with the normal hepatocytes (as in Fig. 1B). The greater the number of malignant cells at a site, the more likely they were to undergo some degree of proliferation.[58,60] The greatest success in normalization

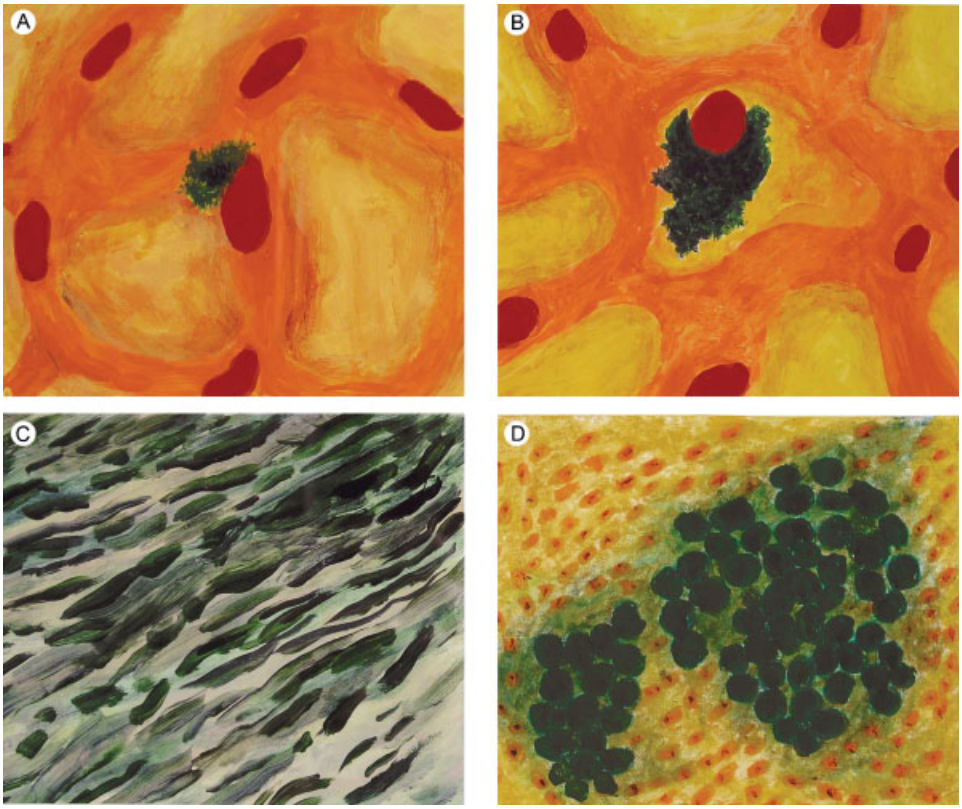

Figure 1. Artistic representations of frozen sections of normal diploid or transformed aneuploid rat liver cells transplanted into the liver parenchyma or subcutaneous region of young rats. The transplanted cells carried copies of a chromosomally integrated *lac*Z reporter gene and are green while the nuclei of the liver parenchyma are red and cytoplasms are orange. **A:** A normal diploid, stem-like liver cell 30 days after transplantation of many such cells into the liver. The cell had differentiated into a normal liver parenchymal cell. **B:** One of many moderately aggressive transformed liver cells transplanted into the liver integrated into hepatic plates and morphologically differentiated with no tumor formation 87 days after transplantation. **C:** Spindle cell tumor arising with short latency (17–21 days) after subcutaneous transplantation of malignant liver cells. Both moderately and highly aggressive transformed cells form tumors quickly when transplanted subcutaneously. **D:** Tumor formed in liver after intrahepatic injection of highly aggressive transformed cells. The tumors exhibited a higher degree of differentiation than those observed at subcutaneous sites. **A** and **B** are at higher magnification than **C** and **D**. The paintings were adapted by Dorothy M. Rubin, with permission, from photomicrographs in Figures 2 and 4 of Ref. 37.

Table 3. Frequency of hepatic tumor formation after transplantation of hepatocarcinoma cells into liver of young and old rats

	Animals with tumors/Animals transplanted	
Days after transplantation	**Young rats (3–9 months)**	**Old rats (18–24 months)**
7	9/9	5/5
14	10/10	5/6
85	0/18	17/19

Abstracted from McCullough et al.[60]

occurred when cells were transplanted into the spleen and filtered as solitary cells into the liver.[58] Malignant cells that leaked from the liver into the lungs formed tumors there even in young rats. Not surprisingly, the more aggressive line of cells was more likely to escape the normalizing effect of the liver microenvironment. The cells of the less aggressive line retained their normalized appearance and quiescent state in the young rats, but as the rats aged, the cells began to multiply, just as they did when initially transplanted into the old rats. The normalized cells could be recovered by explantation to cell culture where they reverted to an undifferentiated, aggressively tumorigenic phenotype.[58] The overall results illustrate the capacity of the young liver to regulate the behavior of malignant stem-like cells, and the loss of this capacity with age, suggesting a causal relationship to the well-known increased incidence with age of solid epithelial cancer. In contrast to the decreasing capacity of liver for regulation with age in male rats, aging female rats retained that capacity.[61] This sexual difference may be related to the much greater resistance of females to chemical induction of liver cancer in animal models, and to the dramatically lower incidence of primary liver cancers in women than in men.

Transdifferentiation of stem cells in ectopic transplantation

The essential role of the intact liver in maintaining the normal tissue-specific behavior of its cells is also illustrated by the epigenetic effect of transplanting the normal diploid stem-like liver cell line described earlier into the heart muscle of adult female rats.[62] The transplanted cells took on the phenotype of cardiomyocytes, with well-organized sarcomeres and myofibrils. They also formed intercalated disks and gap junctions with host-derived myocytes, suggesting that they participate in the function of the cardiac syncytium. The same effect was obtained in cell culture by growing a high density of dissociated cardiomyocytes from neonatal rats or mice on a coverslip, then adding a small number of the liver cells for 4–14 days.[63] The liver cells in contact with the cardiomyocytes expressed cardiac-specific proteins, myofibrils, sarcomeres and nascent sarcoplasmic reticulum. They also started beating rhythmically after coupling with adjacent neonatal cardiomyocytes. These results support the conclusion that adult-derived liver stem cells acquire a cardiac phenotype and function when they are in contact with an overwhelming majority of the myocytes. Direct contact is necessary since non-contacting groups of the liver cells in the same culture retain their identity, and myocyte-conditioned medium is without effect on the liver cells. The results suggested that metabolic exchange between cardiomyocytes and the liver stem cells may be required for transdifferentiation of the latter. Fusion of the different cell types was not necessary for transdifferentiation. The conclusion was that the contacting cellular microenvironment of either rat or mouse cardiomyocytes can alter the germ-layer specificity of adult liver stem-like cells of the rat. There are many other examples of the plasticity of stem cells derived from other tissues when placed in contact with a variety of different tissues.[64] It is not surprising therefore that somatic cells can maintain their differentiated state in situ despite myriad mutations.

Although the effect of the microenvironment on the appearance and behavior of cells has been known since Spemann's demonstration of the inducing effect of surgically displacing the organizer region in early embryogenesis,[65] little was known until recently about the molecular biology of such responses. That situation has begun to change with the advent of microarray analysis to define gene expression profiles. A paradigmatic example was provided in the use of two human glioma cell lines that were cultured as monolayers or were injected subcutaneously and intracerebrally into immunologically deficient mice to obtain gene expression profiles in differing microenvironments.[66] The expression profiles were significantly different depending on the location in which the glioma cells were grown. In addition, the two lines differed from each other when grown in cell culture and subcutaneously, but had similar expression profiles when grown intracerebrally. The results suggest that gene expression of cells and thus their phenotype is affected not only by their growth in vitro versus in vivo, but also by their orthotopic versus ectopic location in vivo. In this case, the glioma cells did produce tumors in both in vivo locations. No attempt was made to determine whether gene expression of the intracerebrally injected cells resembled that of normal glial cells, but the experiment established, unequivocally, the sensitivity of gene expression to the microenvironment in which the cells grow.

Ordered heterogeneity as the fundamental and unique feature of the living state

The accumulated results from diverse aspects of cell and organismic biology leave no doubt that the hierarchy of cells, tissues and organisms maintains increasing stability at each

level over the disorder at the corresponding lower levels. Since these relationships are apparent in all organisms in both the normal and the neoplastic state, it suggests a broad biological principle that is characteristic and distinctive of the living state yet, perhaps because of its self-evident nature, such a principle is not widely acknowledged as such in biological science. It was in fact first formulated in a closed meeting of a dozen distinguished American biologists in 1955.[67] The meeting was called by the Biology Council of the U.S. National Academy of Sciences and the National Research Council to consider the dearth of concepts in biology, despite the superabundance of empirical results. The principle was articulated by Rollin Hotchkiss, a pioneer in the establishment of DNA as the genetic material responsible for the transformation of genetic characteristics of bacteria, in his definition of life as "the repetitive production of ordered heterogeneity".

The concept of ordered heterogeneity was seconded and elaborated by the prominent embryologist Paul Weiss, who had been thinking along similar lines in his application of systems thinking to developmental biology. In such thinking, the variance of a whole system is much less than the sum of variances of its components, which is equivalent to saying that the basic characteristic of a living system is its essential constancy with respect to the fluctuations of its parts.[68] There seemed to be general agreement among the participants in the validity of the concept of ordered heterogeneity, as reflected in later remarks by the chairman.[69] Although it proved to be the only concrete concept to emerge from the meeting, it was largely ignored in the wider community of biological scientists.

During the same period, Walter Elsasser, who had made epochal contributions to three of the most fundamental problems of physical science,[70] had also formulated a biological principle virtually identical to ordered heterogeneity, while totally unaware of the concepts meeting.[71] His conclusion was the fruit of a decades-long effort to develop a theory of organisms based on the complexity and radical heterogeneity of molecules making up every cell. Most of these molecules are constantly undergoing enzymatic changes that proceed isoenergetically at great speed with energy changes only slightly larger than thermal noise. Such a relation would lead to chaos in the inorganic world, but are associated with great morphological stability in organisms, both phylogenetically and ontogenetically. This consideration, plus the great variation from person to person in details of anatomy and chemical concentrations while stably maintaining characteristic species morphology, led him, independently, to the organismic principle that there can be regularity in the large where there is heterogeneity in the small. He later adopted Hotchkiss' phrase of ordered heterogeneity as the keystone in his autonomous theory of organisms,[72] which does not violate physical law, and, most emphatically, provides no support for vitalism.

Ordered heterogeneity therefore serves as an axiom or a logical primitive, defined as a self-evident proposition that is not derived from, or reducible to something else. The ideas of complexity and heterogeneity seem so obvious and intrinsic to the living state that the necessity for their ordering seems trivial to many observers. In that sense, ordered heterogeneity can be compared to the "axiom of choice" in mathematical set theory. All the axiomatic systems in set theory require the proof of the axiom of choice, but it cannot be derived from the others.[73] If it is removed from mathematics, a large number of mathematical proofs would be incorrect. So it is, that without ordered heterogeneity, organisms could not exist. Despite its concurrent independent derivation by three outstanding contributors to biological thought, and its acceptance by the small group of other leading biologists, the concept never gained traction in the field, possibly obscured by the stampede to molecular biology that followed resolution of the structure of DNA. However, the same era witnessed the widespread use of monolayer culture of dispersed cells in cell biology, which revealed their loss of differentiated traits, while opening up quantitative studies of viral–cell interactions. As an unrecognized sideline, these experiments provided support for ordered heterogeneity without acknowledging its existence. Had it been more widely perceived, it would have served as a unifying concept for observations made in diverse biological fields, and suggested directions for further exploration. It is therefore reassuring that propositions similar to ordered heterogeneity are appearing with increasing frequency in cancer biology and embryogenesis.[45,74–77]

Conclusions

1. Intact animal tissues carry many mutations in every cell without recognizably affecting the morphology or function of the tissue. Dispersal of the cells from tissues for monolayer cell culture reveals much heterogeneity and abnormal function among them, which may result from the endogenous mutations or epigenetic effects. Drastic reduction of size in early developing tissue, or change from a three-dimensional to a monolayered distribution prevents differentiation, which indicates that the developmental capacity of early embryogenesis depends more on the pattern of cell interactions than on the cells themselves.
2. Neoplastic development of cells in vitro and in vivo can be suppressed by contact of the individual cells with an excess of normal homophilic cells. Although such normalization of neoplastic expression was at first attributed to the establishment of gap junctional communication among the cells, more recent experiments show that intimate contact, which is of course required for such communication, is itself sufficient for such normalization. The effectiveness of normalizing interactions varies inversely with the aggressiveness of the neoplastic cells, and directly with their

adhesiveness to the surrounding normal cells. Given the capacity of the normal tissues to normalize frankly neoplastic cells, it is not surprising that they can suppress transformation of initiated cells that carry oncogenic mutations.

3. The liver is an ideal in vivo tissue for normalizing homophilic carcinoma cells, probably because of the uniformity and predominance of parenchymal structure, and the technique of filtering carcinoma cells through the spleen so they are widely distributed in the liver as solitary cells. The greater the local density of the transplanted neoplastic cells, the lower is the efficiency of their normalization. The capacity for normalization by the liver decreases as the animal ages, suggesting a causal relationship to the increase of cancer with age.
4. Although transdifferentiation of cells requires contact with a different tissue, it is not known whether gap junctional communication is required.
5. The principle of ordered heterogeneity, proposed independently by Hotchkiss, Weiss and Elsasser, provides an overarching theoretical framework for unifying the varied homeostatic relations among cells in tissues.
6. Cytocidal therapy of cancer resembles a zero sum game because it damages both the cancer cell and the surrounding cells that modulate its neoplastic expression. Testing chemotherapeutic drugs should include evaluation of their effects on the capacity of normal cells and tissues to suppress in vitro and in vivo growth of added neoplastic cells.
7. Since the phenotype of normal stem cells varies with their microenvironment, and microenvironments change with age and disease, systematic studies of these variables should be undertaken before regenerative stem-cell therapy is more widely applied in humans.

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