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Human Cell Hybrids: Analysis of Transformation and Tumorigenicity

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In what would now be accepted as a farsighted hypothesis, Theodor Boveri (*1*) proposed that neoplastic cells, as a consequence of chromosomal imbalance, arise from normal cells. This theory has been refined to involve somatic mutation (*2*) as the precipitating cause of neoplastic transformation. The somatic mutation theory now vies strongly with viral (*3*) and epigenetic (*4*) theories of cancer.

The concept that the transition of a normal cell to a neoplastic one is not a simple one-step mutational event but rather a series of progressive events has gained credence in recent years (*5, 6*). Genetic analysis of these events has been aided by the utilization of somatic cell hybridization techniques (*7*). Examination of hybrid cells resulting from the fusion of a tumorigenic cell with a normal one should allow the determination of whether tumorigenicity behaves as a dominant or recessive trait. The answer to this seemingly simple question, however, has been the subject of considerable controversy.

Early investigators (*8*) isolated hybrid cells derived from the fusion of mouse cells of low malignant potential with those of high malignant potential. The hybrids were as malignant as the highly malignant parent, thereby leading to the interpretation that tumorigenicity behaves as a dominant trait. However, Harris, Klein, and their colleagues on the basis of an extensive series of experiments came to the opposite conclusion (*9*). Their experiments indicated that, when highly malignant mouse cells were fused with other mouse cells of low malignant potential, the resulting hybrids were transiently suppressed in their ability to form tumors. Tumorigenic segregants, which had regained the malignant potential of the highly malignant parent, arose rapidly from these hybrids. Analyses of the chromosomal complements of the parental and hybrid cell populations indicated that the tumorigenic segregants had lost substantial numbers of chromosomes, including those originating from the normal parent. Similar investigations, using other intraspecific rodent hybrids, have essentially confirmed the findings of Harris *et al.* (*10*), although there are certainly notable exceptions to this generalization (*11*). The major drawback in all of these studies has been the chromosomal instability of intraspecific rodent hybrids, where a significant proportion of the total chromosomal complement is rapidly lost. This rapidity of

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chromosome loss, in addition to making the initial premise of suppression of malignancy hard to evaluate, renders the identification of the specific chromosome or chromosomes that possibly control the expression of the tumorigenic phenotype an extremely arduous task.

Interspecific human-mouse hybrids have been used in an attempt to identify the regulatory control of the tumorigenic phenotype. Here, the situation is at least as controversial as with intraspecific rodent cell hybrids—different investigators working with the same cell combinations have again reached opposite conclusions. Jonasson and Harris (*12*) saw complete suppression of tumorigenic potential in hybrids formed between mouse A9 cells and human fibroblasts, whereas Kucherlapati and Shin found no such suppression in their A9 X human fibroblast hybrids (*13*). It should be pointed out that Kucherlapati and Shin had to inject up to 1000 times more hybrid cells to achieve the 100 percent tumor incidence seen with parental A9 cells. Finally, Croce and colleagues (*14*) showed that, when SV40-transformed human fibroblasts are fused with normal mouse cells, the tumorigenic phenotype continues to be expressed until the integrated SV40 genome is lost by chromosome segregation. Once again, the general rule, from the above studies, appears to be that chromosomes rapidly segregate from interspecific hybrids.

In order for meaningful progress to be made in the somatic cell genetic analysis of transformation and tumorigenicity, it is necessary to develop hybrid cell systems that are chromosomally, and therefore phenotypically, stable. Slow, controlled chromosomal segregation should then permit identification of those chromosomes associated with the expression or suppression of tumorigenicity. Also, hybrid cells with stable phenotype expression will allow one to probe with confidence into those traits expressed in vitro which specifically correlate with tumorigenicity. In this article we discuss the development of an intraspecific human cell hybrid system that meets many of these goals.

Human Cell Hybrids

Most of the studies discussed here were undertaken with human diploid fibroblasts as the normal parental population and HeLa cells as the tumorigenic one. At first glance it may seem unusual to use HeLa cells as a parental population since it has been assumed that this cell line, and other long-term cell lines, are karyotypically unstable in continuous culture. This is, in fact, not the case with certain clonal derivatives of HeLa, including the two clones, D98/AH-2 and $D98^{OR}$ (*15*), which we have used extensively. In our experience the karyotypes of these clonal populations have remained stable over hundreds of population doublings and remain similar to the karyotype of the D98/AH-2 clone (*16*). In these initial studies the choice of HeLa was dictated by the necessity of using mutant cell lines for hybrid selection in the HAT (hypoxanthine, aminopterin, thymidine) selective medium system (*17*). We have recently developed other mutant cell lines (described below) that have allowed us to expand the number of different parental tumorigenic and normal cell lines for hybridization. The HeLa/fibroblast (H/F) hybrids discussed below and their parent populations are outlined in Table 1. Several different human fibroblast cell lines were used as the normal parent. The in vitro properties of the hybrids derived from these different parental crosses are essentially identical.

Summary. Intraspecific human-human cell hybrids provide a stable model system with which to investigate the genetic control of transformed and tumorigenic phenotypes. Using this system it has been shown that these phenotypes are under separate genetic control. Furthermore, the tumorigenic phenotype can be complemented by fusion of different tumorigenic cells, resulting in nontumorigenic hybrids. This system also provides information on the control of differentiated function. Molecular cytogenetic techniques should reveal the nature of the chromosomal control of neoplastic transformation.

Table 1. Description of parental and hybrid human cell lines. Abbreviations: HGPRT $^-$, hypoxanthine guanine phosphoribosyl transferase–deficient; OUA^R, ouabain-resistant.

Cell line designation	Genetic constitution
	Parental lines
IMR-90	Normal human fibroblast
WI-38	Normal human fibroblast
$75\text{-}18^{OR}$	Normal human fibroblast ($HGPRT^-$, OUA^R)
GM0077	Normal human fibroblast (hexosaminidase A–deficient)
D98/AH-2	HeLa variant ($HGPRT^-$)
$D98^{OR}$	HeLa variant ($HGPRT^-$, OUA^R)
	Hybrid lines
ESH2	D98/AH-2 × WI-38
ESH3	D98/AH-2 × WI-38
ESH5	D98/AH-2 × GM0077
ESH15	D98/AH-2 × $75\text{-}18^{OR}$ ($HGPRT^-$, OUA^R)
ESH20	D98/AH-2 × $75\text{-}18^{OR}$ ($HGPRT^-$, OUA^R)
ESH39	$D98^{OR}$ × IMR-90 (OUA^R)

Hybrid Morphology and Growth Characteristics

HeLa/fibroblast clones arising in selective medium have a morphology intermediate between that of the epithelial HeLa and fibroblastic normal parents (Fig. 1). The H/F cells grow rapidly in medium containing 5 percent calf serum and reach high population densities, similar to those attained by parental HeLa cells. In addition, the H/F hybrids grow well in Eagle's minimal essential medium containing 0.1 percent serum, an environment in which normal cells fail to proliferate. These initial observations indicated that the hybrid populations retained in vitro properties characteristic of their HeLa parent.

Tumorigenic Potential of HeLa/Fibroblast Hybrids

The primary objective of our early studies was to determine whether the tumorigenic phenotype of the H/F hybrids was suppressed or continued to be expressed. Because they are intraspecific human cell hybrids, the use of appropriately immunosuppressed animals was necessary for tumorigenicity assays. Immunosuppressed mice were chosen and included mice that were (i) thymectomized at birth and treated with antithymocyte antiserum; (ii) adult mice that were thymectomized, whole body irradiated, and then reconstituted with bone marrow (T^-B^+ mice); and (iii) congenitally athymic nude mice, which lack functional T cells (*18*). In all cases, the same result was obtained; that is, whereas 1×10^5 parental HeLa cells injected subcutaneously produced progressively growing tumors in 100 percent of the animals, no tumors were formed when as many as 1×10^8 H/F hybrid cells were injected (*19*). However, when the nontumorigenic hybrids were grown in culture for extended periods of time, tumorigenic segregants appeared which were as tumorigenic as the HeLa parent (*19*). The appearance of these segregants occurred as a rare event and was associat-

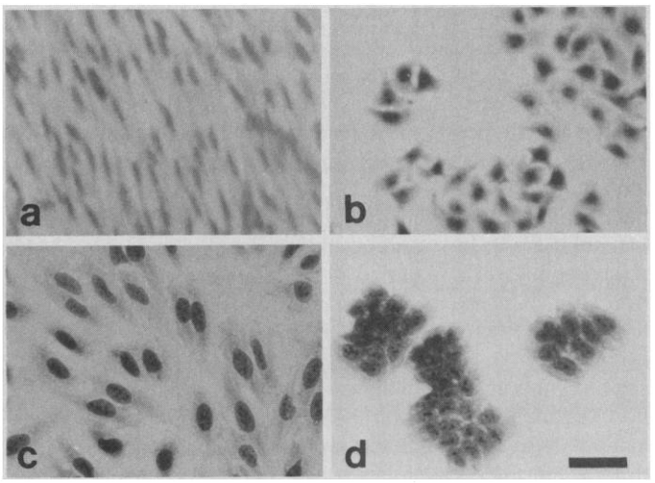

Fig. 1. Morphologies of parental and hybrid cells. (a) Normal human fibroblast; (b) HeLa; (c) nontumorigenic HeLa/fibroblast hybrid; (d) tumorigenic segregant of the hybrid in (c). Bar, 25 μm; Giemsa-stained.

ed with the loss of relatively few chromosomes (Fig. 2).

Most of our tumorigenicity assays were performed in nude mice. The use of nude mice as an adequate model for tumorigenicity testing has recently been questioned (*20*). In particular, nude mice have high levels of natural killer (NK) cells or natural cell-mediated cytotoxic activity. This NK activity has been shown to inhibit the growth of certain tumorigenic cells in nude mice (*21*). The route of injection is also important; certain lymphoid cell lines form tumors when injected intracerebrally but not when injected subcutaneously (*22*). We have examined all of these factors and have found that none of them are responsible for the lack of proliferation of the nontumorigenic H/F hybrids (Table 2). The lack of growth of the nontumorigenic hybrids and the corresponding active proliferation of the tumorigenic segregants, even when injected into the same sites in nude mice, is due to their differential responses to a putative host-mediated growth regulatory signal (*19*). Both cell types initially proliferate actively for a period of 3 to 4 days. Nontumorigenic hybrid cells then apparently respond to host-mediated regulatory factors which rapidly inhibit mitotic activity. Cessation of cell division is not accompanied by cell death—the cells remain as a viable nondividing tissue in the animal, and they can be biopsied and reestablished in cell culture. In contrast, the tumorigenic segregants continue to proliferate and form progressively growing tumors.

The complete suppression of the tumorigenic phenotype in H/F cells (Table 2) is in clear contrast to that observed with interspecific human-mouse hybrids or intraspecific rodent hybrid cells. As discussed below, this difference is probably due to the greater chromosomal stability of intraspecific human cell hybrids.

Karyotypic Analysis of H/F Hybrids

The lack of tumorigenicity in these H/F hybrids would suggest that this property is suppressed by the introduction of regulatory elements encoded by a gene (or genes) present on a specific chromosome (or chromosomes) derived from the normal parental cell. Loss of the specific chromosome would then lead to removal of the regulatory control and subsequent reexpression of tumorigenicity.

The HeLa parents used in our studies, D98/AH-2 and $D98^{OR}$, have a strong modal distribution of 61 chromosomes. Fusions between these cells and normal diploid fibroblasts should result in hybrids containing a theoretical complement of 107 chromosomes, if all parental chromosomes are retained. In fact, when hybrid cell populations were analyzed within a few weeks after fusion, the chromosome complements ranged from 86 to 104 chromosomes. Individual clones, however, had strong modal distributions. After the initial loss of chromosomes soon after fusion, the chromosomes in the hybrid cells were stable for many population doublings. The finding that nontumorigenic clones of H/F hybrids had modal chromosome numbers ranging from the mid-80's to almost the total expected chromosome complement indicates that chromosome loss alone is not sufficient for tumorigenic expression. The extreme chromosomal stability of these hybrids, unlike that of interspecific human-mouse hybrids (*12, 13, 23*) and intraspecific rodent hybrids (*9–11*), is the most likely explanation for the rare appearance of tumorigenic segregants.

Whether specific chromosomal loss is associated with the appearance of tumorigenic segregants is a difficult question to answer when we are dealing with intraspecific hybrids. Preliminary analysis of paired nontumorigenic H/F hybrid clones and tumorigenic segregant clones arising from them, when chromosome banding techniques are used, has indicated that the loss of chromosomes 11 and 14, respectively, is associated with the reexpression of tumorigenicity (*24*). At present we are unable to unequivocally identify the parental origin of these two chromosomes that are missing in the tumorigenic segregants. More definitive analyses will require the use of parental cells with specific chromosomal translocations and characteristic isoenzyme patterns. However, if this relation between loss of multiple chromosomes and control of expression of tumorigenicity is confirmed in further studies it will indicate a complex, genetically determined, regulatory interaction operating in the control of neoplastic expression. Such multiple genetically determined control seems feasible when considered in light of epidemiological studies (*25*) and experimental transformation systems (*26*).

Table 2. Tumorigenicity of representative HeLa/fibroblast hybrids in nude mice.

Cell line	Cells inoculated (No.)	Nude mice used for tumorigenicity assays (No. of tumors/No. inoculated)						
		Newborn	*nu/nu* × *nu/nu* newborn	Subcutaneous	Weanling		Intracerebral*	Irradiated
					Intramuscular	Intraperitoneal		
ESH5	2×10^7	0/4	0/3	0/3	0/3	0/3	0/4	0/6
ESH5T†	1×10^6	3/3	2/2	3/3	3/3	3/3	2/2	4/4
ESH39	1×10^7	0/4	0/3	0/6	0/3	0/3	0/3	0/6
ESH39T†	5×10^6	2/2	2/2	3/4			2/3	4/4

*Approximately 1×10^5 cells in a volume of 0.03 ml injected. †Tumorigenic segregants (adapted from Stanbridge and Ceredig).

In vitro Phenotypic Characteristics of H/F Hybrids

The suppression of tumorigenicity in H/F hybrids and the subsequent appearance of tumorigenic segregants provide a

Table 3. Summary of in vitro properties of parental and hybrid human cells.

In vitro phenotype	Parental cells		Nontumorigenic HeLa/fibroblast hybrids	Tumorigenic HeLa/fibroblast segregants
	HeLa	Fibroblast		
Morphology	Epithelial	Fibroblastic	Intermediate	Epithelial
Density-dependent inhibition of growth	No	Yes	No	No
Requirement for serum growth factors	Reduced	High	Reduced	Reduced
Lectin agglutination	+++	±	+++	+++
Anchorage-independent growth in soft agar and methylcellulose	Yes	No*	Yes	Yes
Fibronectin expression	None	High	Reduced (short branched filaments)	Reduced (unbranched stitch pattern)
Cytoskeleton				
Microtubules	Organized	Organized	Organized	Organized
Microfilaments	Poorly organized	Organized	Organized	Poorly organized
Placental alkaline phosphatase	High	Low	High	High
Ganglioside analysis	Simple	Complex	Relatively complex	Relatively complex
Human chorionic gonadotrophin synthesis	Present	Absent	Absent	Present

*Under certain nutritional conditions normal fibroblasts are capable of anchorage-independent growth in methylcellulose (*46*).

useful system in which to evaluate the specific association of a particular in vitro phenotypic marker with in vivo growth potential. A comparison of the properties of the nontumorigenic hybrids with their tumorigenic derivatives should permit identification of properties that can be correlated with tumorigenicity.

Experimentally it has been shown that the process of neoplastic transformation is accompanied by an array of in vitro phenotypic changes. These changes include altered cellular morphology, loss of density-dependent inhibition of growth, reduced requirement for serum growth factors, enhanced proteolytic activity, altered metabolic rates, expression of new products and surface antigens, anchorage independence, and alterations in cytoskeletal and membrane architecture (*26*).

We have examined a number of these factors in our hybrid system and have found that most of them do not distinguish nontumorigenic hybrids from their tumorigenic segregants (Table 3). They include reduced requirement for serum growth factors, decreased expression of fibronectin, and anchorage independence (*19, 27*), all of which have received favor as specific correlates to tumorigenicity (*28*). Since the nontumorigenic hybrids behaved in many respects like their tumorigenic parental HeLa population we were initially only able to detect tumorigenic segregants by repeated injection of serially cultivated H/F hybrid cell lines into nude mice and observing for tumor formation.

Cells reconstituted in cell culture from the rare tumors that formed showed certain differences from the mass population, which was originally injected into the animal. The morphology of the cells more closely approximated that of the HeLa parent and grew as tightly adherent islands on the plastic surface of the culture flasks (Fig. 1). Once identified, the tumorigenic segregants were now recognizable as discrete clusters even when they were present as a very minor fraction of the mass population of nontumorigenic hybrid cells (*27, 29*). From the time rare segregants were detected (usually at less than 0.1 percent of the total population) several weeks of continuous subculture were necessary before they took over as the major population. This takeover occurs although the tumorigenic segregants exhibit no apparent growth advantage in growth curve analyses except for a slightly elevated population density at confluency, which may be related to their tighter packing in these conditions (Fig. 3).

Another alteration that has occurred in all of the tumorigenic segregants that have been isolated is a distinctive pattern of fibronectin expression on the cell surface when visualized by immunofluorescence (*29*). Fibronectin is a 240-kilodalton glycoprotein present in large amounts on the surface of normal cells. The level of expression of this extracellular matrix protein is greatly reduced on neoplastic transformation of normal cells (*28, 30*). Both nontumorigenic hybrids and tumorigenic segregants express only small amounts of fibronectin relative to the normal fibroblast parent; the former express an incomplete branched fibrillar network extending over the areas of cell-cell contact, whereas the latter express a short stitchlike pattern located exclusively at cell-cell junctions (Fig. 4). An

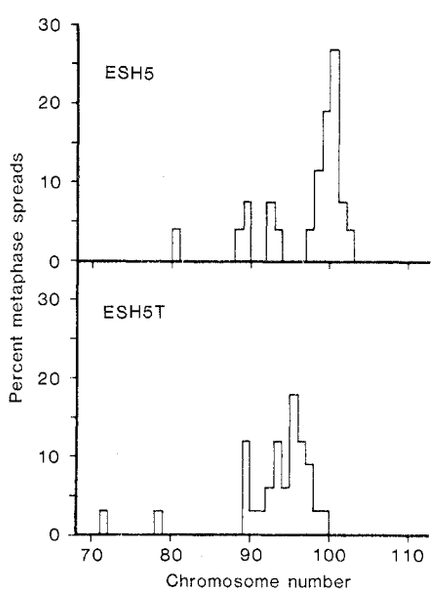

Fig. 2. Histogram of the chromosome distribution of HeLa/fibroblast hybrid ESH5. The upper panel is the nontumorigenic parent and the lower panel is a tumorigenic segregant derived from it. The relatively small loss of chromosomes in the tumorigenic segregant is characteristic of these hybrid paired lines.

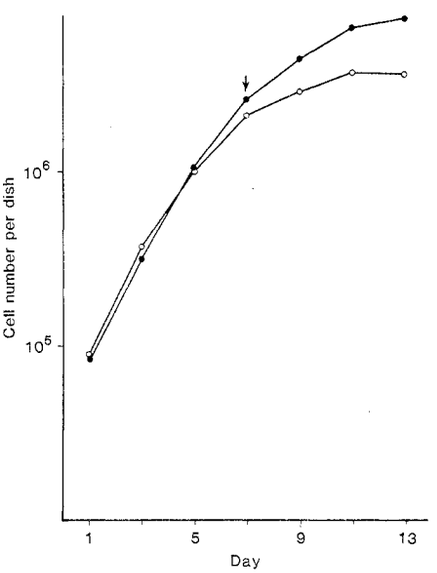

Fig. 3. Growth curves of the nontumorigenic HeLa/fibroblast hybrid, ESH39, and its tumorigenic segregant ESH39T. Cells were grown in Eagle's MEM plus 5 percent calf serum. Each point is the mean of triplicate counts; ○, ESH39; ●, ESH39T. Arrow represents the level of confluency at which the cells would normally be subcultivated.

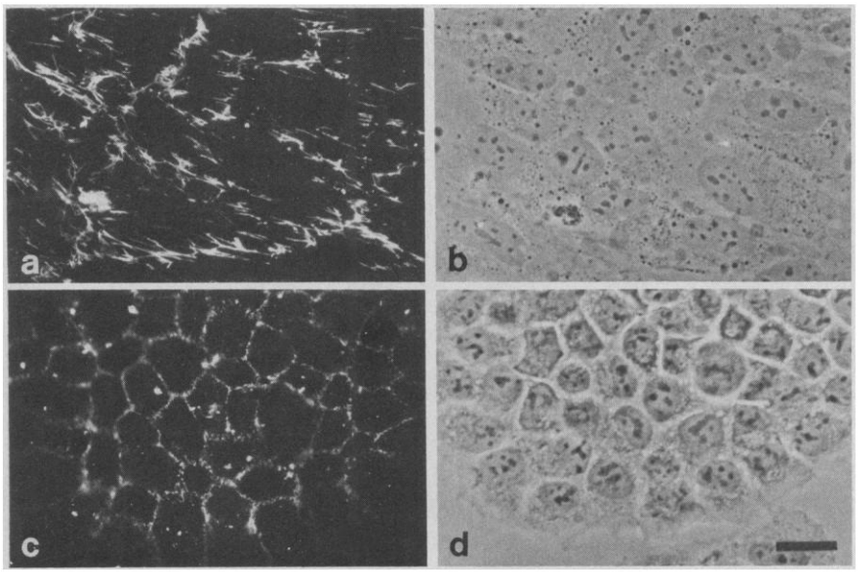

Fig. 4. Distribution of fibronectin on the surface of HeLa/fibroblast hybrids. Fluorescence and corresponding phase-contrast photomicrographs of (a and b) a nontumorigenic hybrid and (c and d) its corresponding tumorigenic segregant. Bar, 10 μm.

analysis of collagen organization on the cell surface also revealed an identical shift to this stitchlike fibronectin pattern seen in tumorigenic segregants. The organization of the extracellular matrix has been suggested (*26*) to play a vital role in neoplastic growth behavior; thus the coordinate alteration in two major extracellular matrix components in H/F tumorigenic segregants may well be critical to in vivo growth potential. The changes in cell morphology, together with the altered distribution of fibronectin and collagen, have been unequivocal correlates of H/F tumorigenic segregant populations.

A final distinguishing feature of the tumorigenic segregants is the reexpression of the alpha subunit of the growth hormone, human chorionic gonadotropin (α-hCG). HeLa cells ectopically synthesize this subunit, whereas normal fibroblasts do not. In all of the nontumorigenic H/F hybrids screened for α-hCG by radioimmunoassay the synthesis of this hormone has been extinguished. However, in every case analyzed, tumorigenic segregant clones reverted to synthesizing this subunit in amounts approximating that of the HeLa parent (*31*). Although the synthesis of this growth hormone may play a role in the neoplastic nature of these cells, an alternative assumption, and perhaps the more likely one, is that there is a regulatory gene controlling the expression of α-hCG on the same fibroblast chromosome as the regulatory gene governing the suppression of the tumorigenic phenotype of the hybrid cells. Thus, when tumorigenicity is suppressed, α-hCG synthesis is extinguished; and upon loss of the relevant chromosome (possibly two in the case of an autosome) coordinate expression of tumorigenicity and α-hCG synthesis occurs.

Table 4. Characteristics of SV40-transformed fibroblasts and hybrids derived from them.

Cell line	Modal chromosome number	Growth in selective media		SV40 T Ag	Tumorigenicity*
		HAT	6-Thioguanine		
Parental					
WI-18/VA-2	67 (48–83)†	−	+	+	10/10
IMR-90	46	+	−	−	0/6
Hybrid					
WI-18/VA-2 × IMR-90					
Clone 1	93, 98 (59–104)	+	−	+	3/8
Clone 2	102 (74–109)	+	−	+	6/14
Clone 3	101 (83–109)	+	−	+	4/4
Clone 4	105 (83–122)	+	−	+	1/7
Clone 5	96 (66–101)	+	−	+	0/5

*Shown as the ratio of the number of animals showing tumors to the number inoculated. †Range in parentheses.

Generality of the Phenomenon of Suppression of Tumorigenicity

Our initial studies were conducted with a single subpopulation of HeLa and two different fibroblast parents (*19*). We have now extended these studies to include several other subpopulations of HeLa and normal fibroblast strains. In all cases, suppression of the tumorigenic phenotype has been observed. This result has been independently confirmed (*32, 33*). Hybrids have also been generated from fusions between HeLa and human epidermal keratinocytes. These hybrids are again initially suppressed for their tumorigenic phenotype, but tumorigenic segregants appear quite rapidly. Certain interesting features of these segregants are described below.

We have also attempted to develop hybrids between other malignant parental cells and normal fibroblasts, with the following intriguing results. In most instances, particularly when the karyotype of the malignant parental population is close to diploid, we have been unable to obtain long-term hybrid cell populations from fusions between these cells and normal human fibroblasts. Clones do arise in the selective medium, but apparently undergo a limited number of population doublings and then appear to enter a senescent phase. Similar observations have been reported (*34*). In one case fusion between A549, a human lung carcinoma line, and normal fibroblasts has resulted in continuously proliferating hybrid cells that are nontumorigenic.

We have successfully obtained hybrids from fusions between sarcoma cell lines and normal fibroblasts. In these cases, the hybrids contain at least a tetraploid, and often a hexaploid, chromosomal complement of the tumorigenic parent. One sarcoma cell line, HT1080, has been studied extensively. When pseudodiploid clones of this cell line were used for fusion with diploid fibroblasts, hybrid clones were rarely isolated. These clones all contained a tetraploid complement of HT1080 chromosomes. Fusions between tetraploid clones of HT1080 and normal fibroblasts resulted in a much higher frequency of hybrid populations. Again, the hybrid clones contained a diploid set of normal chromosomes and at least a tetraploid set of HT1080 chromosomes. From these preliminary data, it seems possible that most diploid clones of HT1080 also cannot give rise to continuously proliferating hybrids when fused with normal fibroblasts, unless an increase in ploidy occurs (*35*).

Several of the HT1080/fibroblast hybrid clones were tumorigenic when as-

sayed in nude mice. Croce and colleagues (*32*) also studied HT1080/fibroblast hybrids extensively and concluded that tumorigenicity behaves as a dominant trait in this combination of cells. An alternative explanation, and one which we subscribe to, is that a gene dosage effect may be operating in this system, similar to the "balance of chromosomes" theory (*36*). Thus, when there is a diploid complement of both HT1080 and normal fibroblast chromosomes, hybrid clones are capable of only a limited proliferative capacity, perhaps dictated by the proliferative capacity of the normal parent. However, when there is a change in the balance of chromosomes, for example $4n$ for HT1080 and $2n$ for normal fibroblast, then the hybrid cell is both transformed and tumorigenic. This possibility must await further experimentation.

Complementation of the Tumorigenic Phenotype

If we accept for the moment the recessive nature of the tumorigenic phenotype, then an interesting question is whether a single genetic locus or multiple genetic loci are involved. An approach to this question is to produce hybrids between different tumor cell populations. If a single, common genetic locus is responsible for the tumorigenic phenotype of different malignant cells, then the hybrid will be tumorigenic no matter which combinations of cells are used. However, if more than one genetic locus is involved, then complementation may occur between different malignant cells resulting in a nontumorigenic hybrid. This hypothesis was tested (*37*) with a number of tumorigenic mouse cell lines that had been shown to be suppressed for their tumorigenic phenotype when fused with normal mouse cells. The surprising result was that, in 21 of 22 combinations of malignant × malignant crosses, the hybrids retained their tumorigenic status. One explanation, on the basis of the premise above, would be that a single genetic locus is responsible for the tumorigenic phenotype of mouse cells. However, these hybrids exhibited the same chromosomal instability seen with intraspecific mouse hybrids of malignant and normal cells, which could obviously influence this interpretation.

Using a similar experimental approach as that of Harris and his colleagues (*37*), we hybridized several combinations of human malignant cell lines with the following results. When different carcinoma cell lines were fused together, the

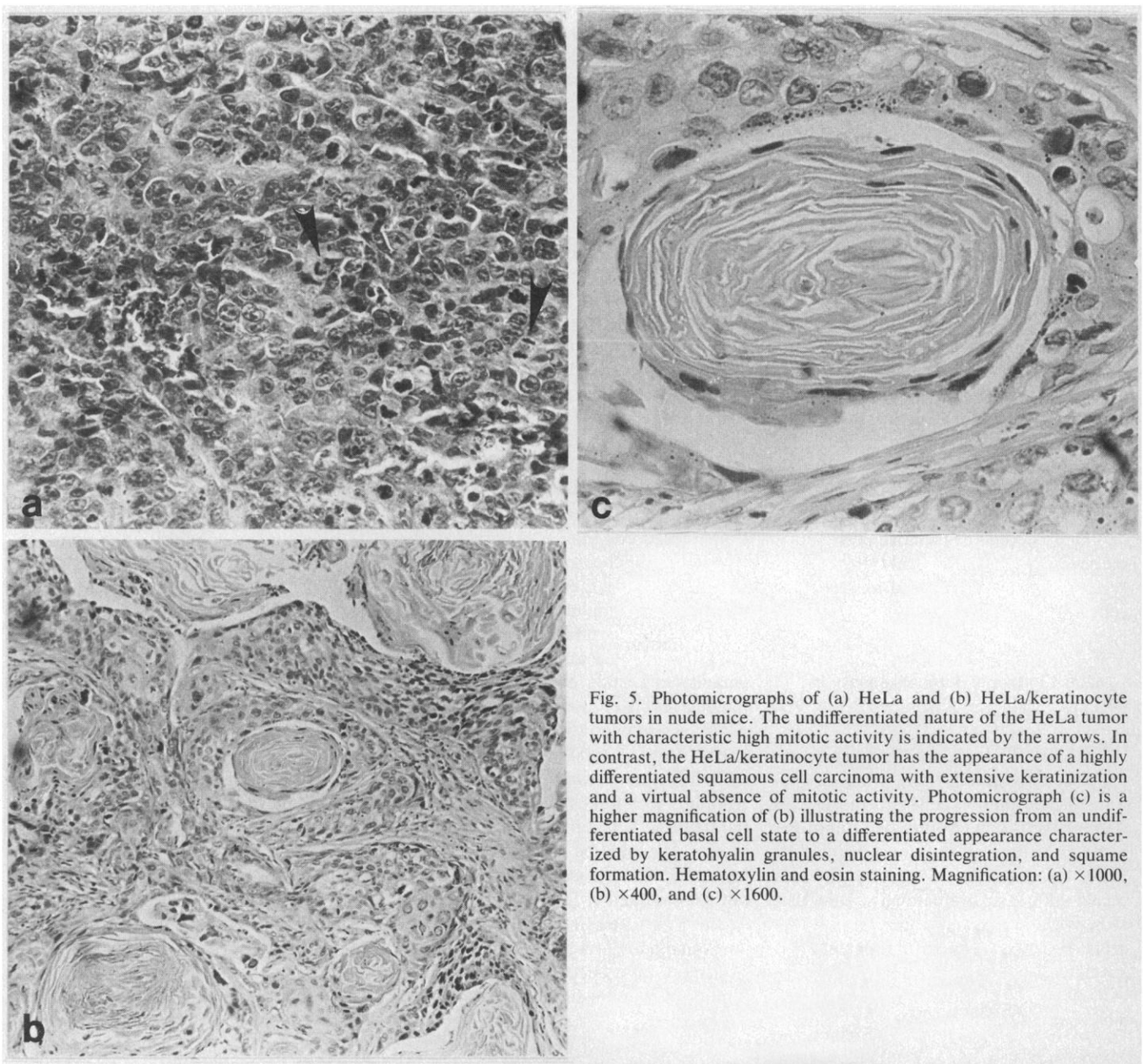

Fig. 5. Photomicrographs of (a) HeLa and (b) HeLa/keratinocyte tumors in nude mice. The undifferentiated nature of the HeLa tumor with characteristic high mitotic activity is indicated by the arrows. In contrast, the HeLa/keratinocyte tumor has the appearance of a highly differentiated squamous cell carcinoma with extensive keratinization and a virtual absence of mitotic activity. Photomicrograph (c) is a higher magnification of (b) illustrating the progression from an undifferentiated basal cell state to a differentiated appearance characterized by keratohyalin granules, nuclear disintegration, and squame formation. Hematoxylin and eosin staining. Magnification: (a) ×1000, (b) ×400, and (c) ×1600.

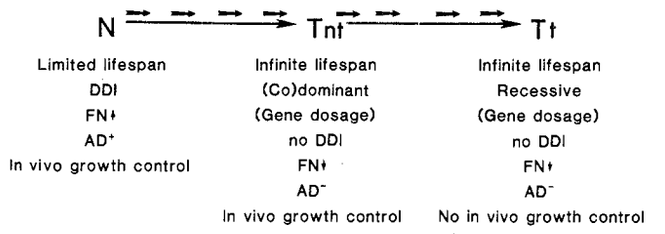

Fig. 6. A model for the progression of the normal cell (*N*) to a neoplastic cell (*Tt*) via a transformed nontumorigenic intermediate (*Tnt*). Characteristic phenotypic traits are illustrated below each cell type. Abbreviations: *DDI*, density-dependent inhibition of growth; *FN*, fibronectin level; AD^+, anchorage-dependent growth; and AD^-, anchorage-independent growth.

resulting hybrids were still highly tumorigenic. Fusions between carcinoma and lymphoblastoid cell lines also resulted in tumorigenic hybrids. However, in this case the lymphoblastoid cells contained Epstein-Barr viral genomes, which may influence the regulatory control of the tumorigenic phenotype (as discussed later). In contradistinction to the hybrids described above, when carcinoma cells were fused with sarcoma cells, the resulting hybrids were nontumorigenic. Moreover, the same was also true with carcinoma × melanoma hybrids.

The simple interpretation of these data is that a common genetic locus governs the expression of tumorigenicity in carcinoma cells; hence complementation does not occur in hybrids derived from malignant cells of the same somatic origin. In contrast, different loci presumably govern the tumorigenic expression of sarcoma and melanoma cells, thereby leading to complementation and suppression of tumorigenicity in hybrids between these neoplastic cells and carcinoma cells.

Thus, in human cells there appears to be a family of genes controlling the expression of tumorigenicity, possibly a distinct one for each somatic cell type. The situation is somewhat analogous to the retroviral *src* and *onc* genes which transform different cell types. This analogy may become even more pertinent if the suspicion that retroviral transforming genes are actually acquired host cellular genes is confirmed.

Genetic Control of Tumorigenicity in Virus-Transformed Cells

Croce *et al.* (*14, 23, 38*) have convincingly demonstrated that, when SV40-transformed human cells are fused with normal mouse macrophages, tumorigenicity is expressed as a dominant trait. There are at least two reasons why this result differs so dramatically from that seen with intraspecific human cell hybrids. First, their studies deal with interspecific hybrids in which regulatory control of gene expression across species may not occur because of a lack of recognition of regulatory signals. Second, SV40 virus transforms both human and mouse cells, and therefore its transforming-gene product (or products) may supersede the putative regulatory control exerted by products of the normal mouse genes. Support for this latter interpretation comes from the finding that the SV40-transformed human cells used by Croce *et al.* are only weakly tumorigenic; injection of as many as 1×10^8 cells into nude mice resulted in occasional small tumors that did not progress (*38*). However, interspecific hybrids derived from the fusion of these human cells with mouse macrophages were highly tumorigenic, with large progressive tumors forming in 100 percent of the animals injected with as few as 1×10^7 cells.

We have examined the influence of chromosomally integrated SV40 genomes on the expression of tumorigenicity in intraspecific human cell hybrids. Several clones of a tumorigenic SV40-transformed fibroblast cell line, WI-18/VA-2, were fused with normal diploid fibroblasts, and the hybrids were tested for tumorigenicity in nude mice. A full spectrum, ranging from complete suppression to full expression of tumorigenicity, including hybrid clones that were less tumorigenic than the parental SV40-transformed cell line, was obtained (Table 4). Thus, the regulatory control of tumorigenicity in these hybrids is considerably more complex than that noted in human-mouse hybrids. Possible reasons for the continued expression of tumorigenicity in certain of the hybrids could include excision and reintegration of SV40 genomes into different chromosomal sites, or viral gene amplification, including tandem duplication. Molecular mapping techniques will be needed to unravel the questions raised by these results. It should be noted that the presence of SV40 viral genetic information is not itself sufficient for tumorigenicity in many human cell lines. In fact, most of the SV40-transformed human fibroblasts examined have been found not to form tumors in immunosuppressed mice (*18, 39*).

HeLa × Human Keratinocyte Hybrids: An Exercise in Differentiation

Having clearly demonstrated that the fusion of normal fibroblasts with HeLa leads to suppression of tumorigenicity, we expanded these studies to include normal epithelial cells. To accomplish this, pure populations of human epidermal keratinocytes were established in culture and then fused with HeLa cells. Preliminary characterization of the HeLa/keratinocyte hybrids indicates that the tumorigenic phenotype is initially suppressed (*40*). Tumorigenic segregants were isolated with greater frequency than was noted with H/F hybrids. The tumors that formed in nude mice were slow growing and highly differentiated. Whereas both HeLa and tumorigenic H/F segregants produced anaplastic carcinomas with high mitotic activity, the HeLa-keratinocyte hybrids formed highly or moderately differentiated squamous cell carcinomas (Fig. 5) with only low mitotic activity. It is reasonable to suppose that the highly differentiated state of these tumor cells is a consequence of developmentally programmed events orchestrated by normal keratinocyte genetic information. Keratinocytes themselves terminally differentiate in culture (*41*). Thus, these hybrids may be a useful model for the study of squamous cell carcinoma, which is very difficult to establish in culture. The system also provides the opportunity for analyzing the interrelation of differentiation and tumorigenicity.

Conclusions and Future Prospects

There have been many investigations into the genetic analysis of tumorigenicity by means of somatic cell fusion techniques. However, most of these studies have been hampered by chromosomal instability of the hybrid cells. These studies have included primarily intraspecific rodent and interspecific human-rodent combinations. The intraspecific human cell hybrid system described in this article offers distinct advantages. In particular, the chromosomal stability, and hence the phenotypic stability, of the hybrids permits examination of large numbers of cultured cells that are homogeneous for the given trait being studied. It has been possible to determine that in H/F hybrids there is stable and complete suppression of tumorigenicity, whereas many transformed phenotypic traits continue to be expressed. The analysis of paired nontumorigenic hybrids and their corresponding tumorigenic segregants

provides an excellent approach to defining those traits specifically correlated with tumorigenicity. In particular, the characterization of the cell surface composition of these genetically similar paired hybrids may facilitate the identification of markers of neoplasia.

One feature of these paired nontumorigenic and tumorigenic cell lines is particularly interesting—although both populations proliferate with essentially identical growth kinetics in vitro, they differ dramatically in their growth potential in nude mice. This finding has led us to consider that, although there is much interest in growth factors that are necessary for the proliferation of normal as compared to transformed cells in vitro (*42*), the crucial level of growth control, the one that determines the neoplastic state of a cell, occurs only in the intact animal and is separate and distinct from those now measured under conditions of in vitro culture (*19*).

Our analysis of paired nontumorigenic and tumorigenic hybrids has indicated that specific chromosome loss is associated with the reexpression of tumorigenicity. However, chromosomal analysis of intraspecific hybrids is arduous and allows only presumptive interpretations of the link between retention or loss of specific chromosomes and suppression or expression of the tumorigenic phenotype. Further refinements will be necessary, including the use of parental cell lines containing specific chromosomal translocations and also cell lines with appropriate isoenzyme polymorphisms.

Our studies permit us to postulate the following model (Fig. 6) for the progression of a normal cell to a neoplastic one. There is at least a two-step progression, each being under separate genetic control (*19*). The possibility certainly exists that a multistep progression occurs, but our studies have as yet not revealed further events. In vitro phenotypic traits such as a lack of density-dependent inhibition of growth, decreased fibronectin expression, and anchorage-independent growth are apparently necessary, but not sufficient, determinants for tumorigenicity. The key difference between the transformed nontumorigenic cells and their tumorigenic segregants is their response to growth regulatory signals in the intact animal (*19*).

Investigations into the genetic and biochemical basis of the regulatory control of transformation and tumorigenicity are rapidly increasing. Relatively few scientific groups using human cell experimental systems have tackled this problem. This may, in fact, be due to a mistaken impression that human cell systems are difficult to work with because of the paucity of genetic markers necessary for genetic analysis. There are now many gene loci which have been assigned to specific human chromosomes (*43*), and individual chromosomes can be identified by banding techniques. Mutant human cell lines are also available for hybrid selection. Aside from the obvious biomedical relevance of working with human cells, the chromosomal and phenotype stability of intraspecific human cell hybrids render them an attractive model system for the genetic analysis of transformation and tumorigenicity. Recent technological advances in gene transfer with metaphase chromosomes (*44*), microcells, and DNA (*45*) should complement somatic cell hybridization techniques and help to accelerate the quest for the identification of the genetic basis of human cancer in this experimental model system.

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