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DEFINED HUMAN CELLULAR SYSTEMS IN THE STUDY OF GLIOMA DEVELOPMENT

Russell O. Pieper

Brain Tumor Research Center, Dept. of Neurological Surgery, and The UCSF Cancer Center, University of California – San Francisco, San Francisco, CA

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1. ABSTRACT

The creation and characterization of permanent cell lines derived from primary human gliomas in the 1960s gave scientists access to unlimited, renewable material in which to study the development of brain tumors. These cells, however, were already tumorigenic and selected for growth in culture, limiting the amount of information that could be gathered about the events that led to the formation of their tumors of origin. In response to these limitations, investigators moved to the study of primary tumors to identify in a correlative fashion the lesions important in tumor formation, and to the use of animal models to gain information about the transformation process. While these approaches have been unarguably successful, they too are limited by their correlative nature (in the case of cytogenetic studies) and their potential lack of direct relevance to human cancer (in the case of mouse models). Recent developments in the isolation and manipulation of human cells have allowed investigators a fresh chance to address questions about brain tumors in a direct fashion. The present review serves as a summary of important recent findings derived from the use of defined human cells in the study of gliomas.

2. INTRODUCTION

Gliomas remain among the most fatal of human tumors (1). Although empirically-derived treatments can be developed in the absence of knowledge about the underlying basis of the disease, the development of optimal, targeted glioma therapies depends on a better understanding of the origin of human gliomas. To date the investigation of the origin of human gliomas has focussed primarily on the identification of genetic gains and/or losses consistently seen in primary human tumors, and in identification of the protein or proteins associated with these alterations. While this approach has been extremely successful in defining the key genetic events associated with glioma formation and progression, an exact definition of how these alterations contribute to glioma formation still relies on introduction of these alterations into normal glial or glial precursor cells, and on monitoring of the subsequent effects on growth and transformation. Ideally, the analysis of consequences of genetic alterations would take place in human cells. Normal human astrocytes and other human glial precursors, however, have been relatively difficult to obtain, and even harder to propagate and genetically modify, particularly in comparison to rodent cells which are readily available and which can be altered in a defined fashion by transgenic and gene knock out techniques. As a consequence, rodent cells have become an important system in which to monitor the effects of genetic change on cellular transformation. Recent improvements in gene introduction techniques and cell availability, however, have begun to attract investigators back to human cell systems, and have resulted in several studies in which the importance of various genetic events in astrocytic transformation have been examined (2-4). The results of such studies, which will be reviewed here, provide key information on the susceptibility of normal human glial cells to immortalization and transformation, as well as pointing to interesting and perhaps important differences in the alterations which are critical in the transformation of human versus rodent cells. These studies suggest that while questions of glioma biology at the whole

Table 1. Summary of the genetic alterations commonly seen in grade II- IV human gliomas

	Grade II	Grade III	Grade IV
hTERT activity	Absent	Present	Present
PDGFR expression	+	++	+++
p53 pathway	Disrupted	Disrupted	Disrupted
pRb pathway	Intact	Disrupted	Disrupted
Ras activation	+	++	+++
Akt activation	--	--	++
EGFR activation	--	--	
VEGF expression	--	--	++

organ level may only be addressable in rodents, the development of human cell systems for the analysis of gene function may provide an important and instructive alternative for the identification of lesions important in the formation and progression of human gliomas.

3. OVERVIEW OF GENETIC LESIONS ASSOCIATED WITH GLIOMA FORMATION AND PROGRESSION

Gliomas have historically been classified by their histological appearance and their resemblance to astrocytes, oligodendrocytes, or ependymal cells. These histological characteristics in turn form the basis of a grading system intended to describe the tumor and help predict its behavior. Presently, the most widely accepted grading system is that established by the WHO which classifies gliomas into 4 categories (grades I-IV)(5). While this classification scheme provides a basis for distinguishing one tumor from another, and also for the planning of therapeutic options, it also provides a framework on which the genetic differences that underlie the different tumor grades can begin to be examined. Over the years a number of investigators have used an array of techniques to uncover genetic alterations both common and unique to primary tumors of each tumor grade. These studies suggest a multi-hit model of gliomagenesis in which genetic alterations can be separated into early events noted in all grades of tumors, intermediate events that accompany the change from LGG to AA and GBM, and late events that appear unique to GBM.

The early, intermediate, and late genetic events associated with the various histologic grades of glioma have been described in several recent reviews are summarized in Table 1. LGG are characterized by alterations in the PDGF pathway, displaying increased expression of both the PDGF-alpha receptor, and to a lesser extent, PDGFR-alpha itself (6-8). PDGFR activates several downstream growth-promoting signaling pathways including Ras and PI3kinase (reviewed in 8). In addition, the Ras pathway itself may also be upregulated in low grade gliomas (9) in a manner unrelated to Ras mutation (10). LGG also display inactivation of the p53 pathway, which includes the p53 downstream targets p21 and bax and the regulators of p53 stability p14ARF and HDM2 (the human xenolog of MDM2)(11). Grade III anaplastic astrocytomas (AA) are characterized by PDGF-A and PDFGR alpha upregulation, Ras pathway activation, and p53 pathway alterations similar to that seen in LGG (11). There are, however, two additional pathways that appear to be uniquely affected in AA. First, the pathway controlled by the retinoblastoma protein (pRb), which is consistently intact in LGG, is inactivated in some 20% of AA, and in nearly 90% of AA with p53 alterations (11). In most cases the pRb pathway inactivation is a consequence of deletion or methylation of the CDKN2A/INK4A locus (11), inactivation of which eliminates the ability of pRb to serve as a regulator of the G1 checkpoint. A second important alteration noted in AA is the activation of the reverse transcriptase component of telomerase (hTERT), noted in approximately 50% of AA but only 10% or LGG (12). hTERT associates with telomeres and appears to play a critical role in protecting chromosome ends from initiating DNA damage-associated signals that can activate cellular senescence pathways.

Grade IV GBM are divided into two groups, one of which (*de novo* or primary GBM) appears to arise spontaneously with all the characteristics of the GBM phenotype, the other (progressive or secondary GBM) develops slowly over a period of years from unresected or incompletely resected lower grade gliomas (5). The prognosis for these tumors appears to be nearly identical, and the genetic alterations associated with each are also similar. Both *de novo* and progressive GBM share alterations common to LGG and AA including PDGF and Ras pathway activation, pRb and p53 pathway inactivation, and hTERT activation, although in each case the percentage of tumors with these characteristics is increased over AA (7, 8, 11-13). Both GBM sub-types, however, also have additional alterations unique to the grade IV category, the most notable being the activation of the PI3K/Akt pathway by inactivation of PTEN. PTEN is lost in approximately 30-40% of GBM, while Akt activity, perhaps in response to PTEN loss or IGF-1 over-expression (14), is upregulated in approximately 80% of GBM (15-17). *de novo* GBM, but not secondary GBM, also exhibit activation of EGFR which is closely linked to gene amplification and rearrangement (18).

In summary, genetic analyses of primary human tumors have suggested that the development of even LGG requires multiple genetic alterations. The accumulation of these alterations is associated with the progression of the tumor to a more aggressive phenotype, or in some cases is sufficient to alone allow the emergence of aggressive de novo GBM. The alterations noted effect growth and growth suppression pathways in a manner that in most cases appears to be non-redundant, i.e. most pathways are only targeted once. In other cases, however, alterations appear to be potentially redundant, e.g. alterations in both the EGFR and PDGFR pathways, suggesting that our level of our understanding of cellular communications and signaling may not yet be complete.

4. ASSESSING THE IMPORTANCE OF GENETIC LESIONS IN GLIOMA FORMATION IN HUMAN AND RODENT SYSTEMS

While the study of primary human gliomas and the linkage of their genetic characteristics to their histology

and classification has proven to be tremendously enlightening, it has, for the most part, neither changed the way gliomas are treated, nor the ultimate survival of patients with these tumors. To better answer questions related to mechanisms of tumorigenesis, and to better identify potential therapeutic targets, a variety of systems have been used to study the function of genes which, by virtue of their alterations in a significant percentage of primary tumors, are presumed to be important in glioma development. Historically, the significance of specific genetic alterations has been studied in tumor cell lines derived from primary tumors. The logic to these studies is that if a tumor cell line derived from a tumor lacks a particular protein of interest, then replacement of the factor or factors should inhibit growth and/or change the characteristics of the tumor toward a more normal state. Alternatively, the introduction of a suspected growth factor into a tumor cell line, even if the factor is already expressed, should further enhance the tumorigenic phenotype. There are, however, limitations to such systems. First, because most tumor lines were established by a process that selects for the ability of cells to grow *in vitro*, the resultant cultured cells may only minimally resemble the primary tumor of origin. Secondly, because most tumor cell lines have accumulated culture-induced alterations as a consequence of their extended period of *in vitro* growth, initial similarities to the primary tumor of origin may be lost over time. Finally, the factors required for growth *in vitro* may simply be different than those required *in vivo*, lessening the likelihood of finding molecules relevant to the *in vivo* disease. While the evaluation of the function of suspected tumor-related genes in tumor cell lines has been reasonably successful and has shed light on the function of a variety of genes, the use of normal human astrocytes (NHA) for this purpose has been less popular. Conceptually, if one could introduce alterations into NHA or perhaps even other more primitive glial precursors that might play a role in gliomagenesis, and if these alterations could result in the creation of cells that could grow as tumors, the contribution of each introduced molecule could be examined in a stable and defined genetic environment. Normal human astrocytes, however, have historically been available only from autopsy cases or from embryonic material, and are thus in limited supply. Additionally, because NHA in culture have a finite lifespan (2, 3), little time exists in which to make genetic alterations and evaluate the consequences. Finally, because there has until recently been no precedent for the transformation of normal human cells, the use of normal human glial cells to assess events important in glial transformation has been limited.

As an alternative, investigators have turned to rodent systems, both using normal mouse astrocytes and whole animal systems (reviewed in 19, 20). These techniques have allowed investigators to generate animals deficient in a specific gene or genes or expressing a target gene or genes in every cell in the animal. More sophisticated methodology has further allowed expression/knockout of genes in specific tissues e.g. GFAP+ astrocytes and nestin + precursors, or even conditional expression and/or recombination based on the presence or absence of inducing agents. Furthermore, successfully manipulated cells can clearly be shown to contain the desired alteration and can be isolated and examined in either in culture for targeted behavior or in the intact animal for effects on tumor behavior *in vivo*. For these reasons, rodent systems have in a relatively short period of time come to dominate the study of events important in gliomagenesis.

5. ADVANCES IN HUMAN CELL TECHNOLOGY

Although the study of gliomagenesis in rodents has yielded significant answers with regard to important events in tumor formation, there is a growing interest in complementing studies in rodent systems with studies in human cells. As noted, however, the ability to use human cells has been limited by issues of availability, difficulties in propagating and manipulating human cells, and uncertainty about whether and how such cells could be transformed. Perhaps the biggest break in this area occurred in 1999 with the publication of a paper suggesting that normal human fibroblasts (NHF) could be converted into cells that were, by multiple criteria, tumorigenic (21). This work was the culmination of at least three decades of studies examining the type and number of lesions required to transform normal human cells. Previous studies consistently showed that while two genetic alterations were sufficient to transform so-called “normal” rodent cells such as NIH3T3 (which in fact lack both p16 and p19ARF)(22), human cells were remarkably resistant to transformation (23, 24). Hahn and Counter succeeded in transforming human cells (in this case human fibroblasts and later epithelial cells)(21, 25) at least in part because of an improved understanding of how the human cellular lifespan was controlled. While inactivation of growth suppressing pathways in combination with activation of growth stimulatory pathways in rodent cells was sufficient for cellular transformation, telomere-based limits imposed on lifespan prohibited the transformation process in human cells. The provision of hTERT, along with the growth-stimulatory properties provided by activated Ras and the elimination of p53 and Rb pathways (by T antigen) proved to be the appropriate combination to extend lifespan, promote growth, and bring about transformation of normal human cells. Coincident with this development was the improved availability of NHA primarily through commercial sources, eliminating the need for direct isolation and characterization of astrocytes. Additionally, because NHA proved to be suitable for retroviral infection and because retroviral constructs were increasingly available, investigators were finally in a position in which there was hope of introducing alterations into NHA and sorting out which events might result in glial transformation. The results of such studies have made for interesting comparisons between the study of gliomagenesis in human versus rodent cells

6. DIFFERENCES BETWEEN RODENT AND HUMAN ASTROCYTES WITH REGARD TO TRANSFORMATION, AND WHAT THEY TELL US ABOUT GLIOMAGENESIS

The publication of the first studies examining genetic events sufficient for transformation of human glial

cells in 2001 (2-4), along with an ever increasing number of similar studies in mice and rodent cultures has allowed for a more clear definition of the differences between rodent and human glial cells with regard to transformation, and by implication, differences between rodent and human gliomagenesis. These differences can most readily be seen by comparing the requirement for telomerase, the role of genetic stability, and the number and type of genetic lesions required in glial transformation.

6.1. TERT requirement

Normal human cells, including NHA, have a finite lifespan in culture. This finite lifespan appears to be associated with telomeres, large nucleoprotein structures comprised of tandem repeats of TTAGGG sequence complexed with reverse transcriptase (hTERT), telomerase RNA (hTER), and a various other proteins (26). The telomeres in NHA are relatively long compared to those in normal human fibroblasts (NHF) (27, 28), but appear to shorten with age and to ultimately shorten, at least in culture, to a length that triggers a senescence-like phenomenon and permanent growth arrest. The mechanisms that control this process remain poorly understood in both human and rodent astrocytes. A key point, however, is that rodent cells, including astrocytes, have extremely long telomeric sequences, and appear not to use telomere length to mark lifespan or to control the cellular aging process (29, 30). Additionally, rodent cells in culture are far more likely to escape from controls that limit lifespan than are comparable human cells, the difference being estimated at 7 orders of magnitude (29). The relevance of this difference to the *in vivo* development of cancer remains an intensely debated issue, and it is unclear if there is an *in vivo* correlate to senescence (31, 32). It is clear, however, that the unlimited growth potential that is characteristic of human tumors requires telomerase reactivation, whereas elimination of any of a number of negative growth regulators including p53 and p16 allows mouse fibroblasts and astrocytes to escape senescence, to proliferate indefinitely, to accumulate genetic alterations, and to take on characteristics of transformed cells, even in the apparent absence of telomerase (33, 34). Telomerase reactivation is clearly important in gliomas *in vivo* as 50% and 90% of grade III and grade IV gliomas, respectively express telomerase, while NHA do not (2-4). It therefore appears that the importance of telomerase in human gliomagenesis can best be assessed in cells of human origin. As an alternative, however, mTERT or TER-deficient mice aged to the point at which their telomeres are eroded have been suggested as an alternative system in which to study telomeric function in gliomagenesis *in vivo* (26, 35). Because mouse cells do not, however, use telomere length to monitor lifespan, and furthermore are highly efficient at using recombination mechanisms to avoid the problems associated with shortened telomeres, even these models may not entirely represent the importance of telomerase in human glioma development.

The current human cell based systems clearly suggest that hTERT reactivation is critical in the transformation of glial cells and perhaps the development of high grade gliomas. The role of telomerase in LGG development, however, remains unanswered (and perhaps unanswerable) using existing human and rodent systems. The LGG are unique among gliomas in that they grow slowly for long periods of time, yet rarely express telomerase (12). It is tempting to speculate that these tumors represent disregulated, telomerase-negative astrocytes destined for a crisis-like event, and that conversion of the LGG to a higher grade glioma requires telomerase reactivation. The creation of a LGG model in which to test this idea, however has been complicated by the fact that intracranially implanted genetically modified human astrocytes grow relatively rapidly and have a high mitotic index (40-60%)(3) inconsistent with that of LGG. For reasons described, mouse models of LGG are also unlikely to be able to address the importance of telomerase in the conversion of LGG to higher grade tumors. A second unresolved issue is whether the telomerase-negative status of LGG gives any clues as to the cell of origin of the tumor. NHA are telomerase negative and therefore could give rise to telomerase-negative LGG lesions. The expression of telomerase in CNS stem cells such as those in the subventricular area (36-38) has not, however, been reported. Furthermore, while telomerase expression has not been reported to be affected by differentiation, it remains possible that telomerase negative LGG could arise from either telomerase-negative cells, or from telomerase positive cells which lose telomerase activity upon block or reversal of differentiation. Given the important developments in the area of neural stem cell development and the possible linkage of stem cells to gliomagenesis, human cell systems employing various precursor cells may be of some use in addressing the role telomerase and telomerase reactivation play in the earliest stages of glioma development.

6.2. Genetic Stability

Studies of susceptibility to genetic rearrangement in rodent and human cells suggest that inherent genetic instability may influence the interpretation of results derived from studies of gliomagenesis in human and rodent systems. The earliest studies in rodents showed that transgenic expression of T antigen, a powerful viral protein capable of disabling both the p53 and pRb pathways, could induce tumor formation at multiple sites including the brain when targeted by a GFAP promoter (39). The genomic instability noted in the subsequent tumors was typically ascribed not to the inherent instability of the mouse genome, but rather to the known ability of T antigen to inactivate p53 and induce genomic instability. An increasing number of studies in rodents, however, suggest that single genetic alterations not involving p53 can also lead to significant genomic instability in rodent cells. As an example, cdk4 over-expression in GFAP+ astrocytes led to the development of cells with a near tetraploid DNA content (34). Additionally, expression of an activated Ras in GFAP+ astrocytes also led to the formation of astrocytes which could undergo spontaneous immortalization, accompanied by the development of aneuploidy (40). In this regard it is worth noting that while the genetic alterations introduced in most transgenic glioma animal models are well defined, the genetic composition of the

resultant tumors is rarely examined. In limited instances in which this analysis has been performed, however, the tumor inevitably contains far more alterations than the single intended lesion (41). These results suggest that in rodent systems, genetic alterations that result in tumor formation may rely on inherent rodent genome instability to drive the creation of the ultimate tumor genotype.

In contrast to rodent cells, human fibroblasts and astrocytes appear to be considerably more genetically stable. Recent studies in p53-deficient fibroblasts created by knock-out techniques demonstrated that complete loss of p53 was insufficient to drive aneuploidy or to transform cells (42). These findings are consistent with studies in telomerase-positive NHA in which loss of p53 in combination with inactivation of pRb was sufficient to immortalize NHA, but was insufficient to alter chromosome number or genomic integrity (at least at the level detectable by CGH), or to drive transformation (3). While genetically modified normal human cells appear to retain genomic integrity, it should be noted that as in the rodent studies, a complete analysis of the ploidy of tumors derived from genetically modified NHA has not been reported. Nonetheless, these results suggest that transformation of NHA is not dependent on genomic instability, and that individual events thought to be important in gliomagenesis might be more readily studied in the human cell setting.

6.3. The number and types of genetic lesions required

The large number of studies concerning the transformation process in rodent and human cells *in vitro* and *in vivo* have pointed out significant differences between rodent and human cells, particularly with regard to the number and types of lesions required for transformation.

Single genetic alterations introduced into mice by transgenic means have been shown to result in immortalization and/or transformation of rodent astrocytes in culture, and occasionally in the formation of gliomas in animals. As an example, deletion of p53 (33), over-expression of cdk4 (43), or over-expression of MDM2 (34) all led to the creation of astrocytes which, when placed in culture, spontaneously underwent immortalization accompanied by accumulation of genetic alterations. More dramatically, expression of PDGF-B in GFAP-positive or nestin-positive cells led to oligodendrogliomas (44), while over-expression of activated Ras similarly led to astrocytic transformation and the formation of gliomas *in vivo* (12). There are, however, conflicting reports as to whether activated Ras can in and of itself lead to glioma formation *in vivo* (45), and it appears that the strain of animal used as well as the means of introducing the genetic alteration may influence the experimental outcome. Furthermore, a variety of single alterations targeted to GFAP+ or nestin+ cells did not in and of themselves lead to transformation *in vitro* or *in vivo* including pRb deletion (46), ARF deletion (47), or Akt activation (45). These results suggest that, by and large, single genetic alterations, while sufficient to transform mouse astrocytes, rarely give rise to gliomas *in vivo.*

In contrast to the results from single genetic alteration studies, gliomas can be generated *in vivo* in animals in which any of a number of lesions were combined. As an example, animals with GFAP-targeted expression of T antigen (which inactivates both the p53 and pRb pathways) develop astrocytomas (39), INK4a deficient mice with nestin-targeted mutant EGFR expression develop astrocytomas (48), and mice with nestin-targeted K-Ras and Akt expression develop glioblastoma-like tumors (45). Again, it is unclear if these were the only alterations noted in the subsequent tumors or if these were simply the initiating lesions. These results have been taken to suggest that multiple genomic alterations, often of the same variety as noted in primary human tumors, are required for tumor formation in mice. These studies (and those in human cells as well), however, rarely take the approach of asking if alterations not commonly seen in gliomas are also sufficient to induce gliomas and if any perturbence targeted to GFAP+ or nestin+ cells drive the system toward transformation. Additionally, because a variety of strains of mice are used in modeling studies, the results are not always consistent. As an example, while deletion of the INK/ARF locus did not result in glioma formation in one study (49), deletion of the ARF locus alone in a slightly different strain of animal was sufficient (47). Furthermore, while one study suggests that inactivation of the both the p53 and pRb pathways is key for glioma development in mice (48), other studies suggest that activation of Ras and Akt is sufficient for glioma formation (45). Nonetheless, these studies on the whole suggest that a limited number of alterations in rodent systems can lead to gliomagenesis.

The number and type of lesions required for transformation of human astrocytes appear to differ from those described in the rodent system. As noted, a fundamental difference in the paths in which rodent and human cells take to transformation is the reliance upon telomerase. In two studies with NHA, it was demonstrated that immortalization was a strict pre-requisite for transformation, and that in the absence of telomerase reactivation, the growth of cells, regardless of other genetic alterations induced, was very limited (2-4). The question of whether telomerase activation alone was sufficient for immortalization remains somewhat open as the growth rate of NHA is quite slow in the presence of hTERT. Two studies, however suggest that hTERT reactivation alone is sufficient to allow for indefinite growth of NHA in culture, but not *in vivo* (2-4). Telomerase reactivation therefore appears to be a key early step in the development of the transformed phenotype in human glial cells. Again however, because LGG do not typically express telomerase, it would appear that the very earliest step of human transformation rely on growth stimulation and/or elimination of tumor suppression prior to resolution of the telomere issue.

Other single genetic alterations in NHA were also incapable of generating the types of transformed phenotypes occasionally seen in rodent models. Specifically, inactivation of the pRb or p53 pathways by the viral oncoproteins HPV16 E6 or HPV16 E7 extended

Table 2. Transforming capabilities of combinations of genetic alterations in NHA

Cell types	Colony numbers [a]	% Tumor incidence (n) [b]
NHA		
E6/E7/hTERT/Ras	1018 ± 205	ND
E6/E7/hTERT/wtEGFR	0	ND
E6/E7/hTERT/EGFRvIII	11 ± 4	ND
E6/E7/hTERT/Akt	0	ND
E6/E7/hTERT/wtEGFR/Akt	95 ± 31	91 (11)
E6/E7/hTERT/EGFRvIII/Akt	105 ± 31	ND
E6/E7/hTERT/Ras/Akt	1318 ± 205	80 (5)
E6/E7/Ras	0	ND
E7/hTERT/Ras	0	ND
E6/ E7/hTERT	0	ND
T antigen	0	0 (ref 2)
T/hTERT	<100	0 (ref 2)
T/Ras	<100	0 (ref 2)
T/hTERT/Ras	~700	95 (18) (ref 2)

[a] Cells (1×10^4, or 5×10^4 for T antigen experiments) were plated in soft agar, and colonies were scored after 3 weeks. Results represent mean ± SD from two separate experiments. [b] Cells were injected s.c. into nude mice. n, number of animals treated. ND, not done.

the lifespan of NHA from approximately 10 to 20 population doublings, although these alterations were not sufficient to immortalize or transform cells (3)(Table 2). Furthermore, while inactivation of both the p53 and pRb pathway extended the lifespan of astroctyes even further and increased their rate of growth, no immortalized clones developed (2, 3). It should be noted that based on studies in fibroblasts, if larger numbers of cells were carried through this procedure, the genetic alterations allowing for other changes that mediated transformation would be expected to occur in approximately 1 in 10^8 cells (29). None of the changes introduced alone, however, were sufficient to immortalize cells at rates that approximated those in rodent astrocytes. Similarly, stimulation of the Ras pathway at the level of Raf also did not lead to transformation but rather led to premature senescence in NHA (50). While it remains unclear if stimulation of other growth factor pathways would similarly lead to growth arrest, the data in the human system suggest that immortalization and transformation in human cells requires more than single genetic alterations.

Unlike results from rodent systems, studies of transformtion in NHA show that pairs of genetic alterations typically do not lead to transformation. The introduction of T antigen, which functionally inactivates both p53 and pRb, leads to transformation of rodent astrocytes in culture, and drives the formation of gliomas *in vivo* in mice (39), did not lead to transformation of NHA (2), nor did similar inactivation of the p53 and pRb pathways by E6/E7 (3). Additionally while combined expression of T antigen or E6/E7 with hTERT immortalized NHA, it did not lead to their transformation (2-4)(Table 2). Rather, transformation was accomplished only by the combined alteration of multiple cellular pathways including those controlled by telomerase, p53, pRb, and activated Ras. The order of introduction of these alterations may be important, although this has only loosely been examined. In studies of NHA transformation, hTERT reactivation is typically performed first to allow more time for subsequent manipulations. Furthermore, because activation of the Ras pathway leads to cellular senescence in the presence of p53 and pRb, and growth arrest in cells lacking pRb (50), Ras alterations are typically made last. The typical order of introduction of genetic alterations in these studies, although driven by convenience, may in fact mimic the order that occurs naturally as the same growth inhibitory checkpoints may need to by passed in a specific order *in vivo* as well as *in vitro*. Regardless of their ordering, however, the number of pathway alterations necessary to bring about transformation of human cells appears to be larger than that in rodent cells.

The types of pathways involved, and their interchangeability, also appears to be different in human cells than in rodent cells. While any of a number of activated growth pathways (Ras, EGFR, Akt) can co-operate with other pathways (pRb, p53, INK4a) to bring about tumorigenesis in mouse cells and in animal models of gliomagenesis, in NHA, the requirement for activated Ras cannot be met by wild type EGFR, constitutively activated EGFR, or constitutively activated Akt (3, 4)(Table 2). Why activated Ras appears to be irreplaceable in the transformation of NHA remains unclear. Ras signals down multiple pathways, the most important considered to be the Raf, PI3K, and RalGDS (51). Activated EGFR, however, signals down the Ras pathway (52), and Akt signaling is the consequence of activation of the PI3K arm of the Ras pathway, making it less clear why neither activated EGFR nor Akt can replace the function of Ras in transformation of NHA. There is some suggestion that of the major signaling pathways that Ras activates, perhaps the Ral GDS pathway is most critical in mediating cellular transformation (53). Studies of Ras pathway activation, however, typically rely on the use of Ras point mutants which, while selective for activation of Raf, PI3K, or RalGDS pathways, are by no means exclusive for these pathways (51). Overall,

Table 3. Summary of the strengths and weaknesses of the study of gliomagenesis in rodent and human systems

	Strengths	Weaknesses
Rodent systems	• Relatively easy to introduce defined genetic alterations by knockout strategies	• Relative genetically instability makes cells more susceptible to transformation than comparable human cells
	• Optimal for studying invasion and angiogenesis	• May differ from human cells in fundamental control of growth
	• Can address role of multiple in vivo cell types in the tumorigensis process	• Identification of in vivo cell types still in infancy
Human systems	• Relatively gentically stable	• Very hard to introduce defined alterations by knockout strategies
	• Allow for direct study of pathways that control human cell growth	• Difficult to study some in vivo processes
	• Can address roles of multiple cell types in the tumorigenesis process	• Isolation of different cell types still in infancy

however, the analysis of the number and type of alterations necessary to transform NHA appear to reinforce the idea that normal human astrocytes have more stringent limitations placed on their transformation than do GFAP+ mouse cells, which in turn may be more difficult to transform than their nestin+ counterparts.

Finally, differences appear to exist between human cell models of glioma and mouse models of glioma with regard to lesions required for the progression to GBM. GBM-like tumors have been created in mice by the mutation of NF1 and p53 (54), but also by the expression of K-Ras and activated Akt in nestin-positive cells (45). These results suggest that while alterations of multiple pathways are required to generate the GBM phenotype in mice, any of a number of alterations may drive the process. In human cells, the situation is a bit different. While expression of hTERT, inactivation of the p53 and pRb pathways (by E6/E7), and expression of an activated Ras in NHA resulted in cells that formed tumors when implanted intracranially, these tumors clearly had AA-like characteristics and lacked the defining features of GBM including necrosis and microvascular proliferation. GBM-like tumors could, however, be created by the additional expression of activated Akt (4), suggesting that Akt activation (by expression of an activated Akt construct or by loss of PTEN in primary GBM) drives the final stages of GBM formation. Notably, however, other genetic alterations frequently found in GBM such as VEGF over-expression could not similarly drive GBM formation in NHA (55). These results suggest that while Akt appears to play a key role in GBM formation, it's role appears more readily replaceable in mouse models than in human systems.

7. PERSPECTIVE – WHAT IT ALL MEANS

The study of glial transformation in mouse and human glial cells has provided considerable insight into the processes that control cell growth and lifespan, and into the alterations that can send cells spinning toward glioma formation. With regard to gaining a better understanding of human gliomas, both human and mouse systems appear to offer different strengths and weaknesses, which are summarized in Table 3. While mouse astrocytes can be isolated and studied, their genetic instability in culture, their propensity to transform, and their lack of dependence on telomere-related cues to control lifespan makes these cells the most distant from the human setting. These cells will undoubtedly still be important in situations in which comparable genetically altered human astrocytes cannot be obtained or created. *In vivo* studies of mouse gliomagenesis also appear to be of considerable value, with mouse glial cells *in vivo* transformed by lesions that in many cases resemble alterations seen in human primary glioma. The biggest strength of the mouse models, however, is that they allow the study of aspects of glioma biology such as invasion and angiogenesis that can only be studied *in vivo.* In this regard, animal models of gliomagenesis are likely to be important for some time to come. Into this mix now enters the use of human glial systems, which offer their own combination of advantages and disadvantages. Human astrocytes have characteristics not found in mouse cells, including a reliance on telomerase, the presence of redundant pathways that help insure close control of genomic stability, and the requirement for a defined series of events perhaps occurring in a defined order, for transformation. While phenomenon such as invasion and necrosis may be less suitable for study in human cell systems, and while there certainly may be differences in the behavior of NHA in culture versus those *in vivo*, NHA appear to be an ideal complement to mice in the study of transformation and gliomagenesis.

A final question is if and how results derived from mouse models can be incorporated or made compatible with those derived from human systems. There clearly are differences in the results derived from the two systems, and it is likely that some of these differences will truly represent divergence in the way in which human and mouse cells control growth and lifespan. Some differences, however, may merely be the consequence of the population of cells examined. While the NHA used in published studies to date are relatively defined with regard to background and origin, the target cells in mouse models are defined only in terms of our understanding of which cells are targeted by GFAP- and nestin-specific promoters. It

may therefore be possible that an as yet undefined subset of mouse astrocytes behave in a manner entirely consistent with NHA. Alternatively, the human glial populations used in studies to date may represent somewhat mixed populations of cells and perhaps even some stem cell-like precursors. As stem cell isolation techniques improve and as means to better define glial and glial progenitor populations are developed, the results from *in vitro* studies of better defined populations of human astrocytes or astrocyte progenitors may more closely resemble those derived from mouse models. Therefore, while it cannot be determined at present if studies in mice or human cells best mimic the human setting *in vivo*, it is clear that both human and rodent based systems will be useful in identifying pathways important in controlling growth and differentiation, in determining how these pathway act, and in identifying potentially new and exciting targets for glioma therapy.

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Send correspondence to: Dr Russell O. Pieper, Brain Tumor Research Center, Dept. of Neurological Surgery, and The UCSF Cancer Center, University of California – San Francisco, San Francisco, CA, UCSF Cancer Center, 2340 Sutter St., Rm N261, San Francisco, CA 94115-0875, Tel: 415-502-7132, Fax: 415-502-6779, E-mail: rpieper@cc.ucsf.edu