Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines

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Summary

The concept of tumor stem cells (TSCs) provides a new paradigm for understanding tumor biology, although it remains unclear whether TSCs will prove to be a more robust model than traditional cancer cell lines. We demonstrate marked phenotypic and genotypic differences between primary human tumor-derived TSCs and their matched glioma cell lines. Unlike the matched, traditionally grown tumor cell lines, TSCs derived directly from primary glioblastomas harbor extensive similarities to normal neural stem cells and recapitulate the genotype, gene expression patterns, and in vivo biology of human glioblastomas. These findings suggest that TSCs may be a more reliable model than many commonly utilized cancer cell lines for understanding the biology of primary human tumors.

Introduction

Cancer cell lines have been the historical standard both for exploring the biology of human tumors and as preclinical models for screening potential therapeutic agents. It has become increasingly clear, however, that phenotypic characteristics and the multitude of genetic aberrations found within repeatedly in vitro passaged cancer cell lines often bear little resemblance to those found within the corresponding primary human tumor (Deschavanne and Fertil, 1996; Hecht et al., 1995; Pardal et al., 2003; Romer and Curran, 2005). This not only in part explains why both in vitro and in vivo cancer cell line-based preclinical therapeutic screening models have been poorly predictive for identifying clinically useful therapeutic agents, but may also have led to some important misinterpretations regarding the relevance of aberrant signaling pathways within cell lines compared to primary tumors. This realization led us on a search for a more biologically relevant model system for exploring glioma biology and for the screening of new therapeutic agents.

Neural stem cells (NSCs) and in situ glioma cells share the characteristics of continuous self-renewal, extensive brain parenchymal migration/infiltration, and potential for full or partial differentiation—properties not found in glioma cell lines (Morshead and van der Kooy, 2004; Sanai et al., 2005). These observations suggest that at least some in situ glioma cells may harbor features consistent with tumor stem cells (TSCs) (Pardal et al., 2003; Phillips et al., 2006; Reya et al., 2001; Sanai et al., 2005). Brain tumor stem-like cells have been recently reported; however, besides the morphological similarities and differentiation capacity of these cells, little else is understood regarding the actual similarities between glioma TSCs, normal human NSCs, and the primary tumors (Galli et al., 2004; Hemmati et al., 2003; Singh et al., 2003, 2004; Taylor et al., 2005; Yuan et al., 2004; Zhu et al., 2005). We therefore undertook a series of studies to readily identify and better characterize glioma TSCs in an attempt to understand the relationship between these TSCs, the primary tumor, and traditional glioma cell lines.

One obvious difference in the in vitro propagation of NSCs compared to the commonly used glioma cell lines is the requirement for serum. All established glioma cell lines (like most other cancer cell lines) are grown in media containing serum, whereas NSCs are grown in serum-free media, since serum causes irreversible differentiation of NSCs (Gage et al., 1995; McKay, 1997; Reynolds et al., 1992). To explore how growth under these two

SIGNIFICANCE

We developed and characterized a model system in which primary human glioblastoma cells are propagated in vitro under conditions optimal for either normal NSCs or typical glioma cell lines. We demonstrated that TSCs closely mimic the genotype, gene expression profile, and biology of their parental primary tumors. By contrast, tumor cells grown under standard serum-containing culture conditions result in the loss of TSCs and ultimately lead to the outgrowth of cells that are vastly different both genetically and biologically from their parental tumors. These observations bring into question the relevance of standard cancer cell lines for studying the biology of human cancer and for screening new therapeutic agents. TSCs may prove to be a more reliable model system.

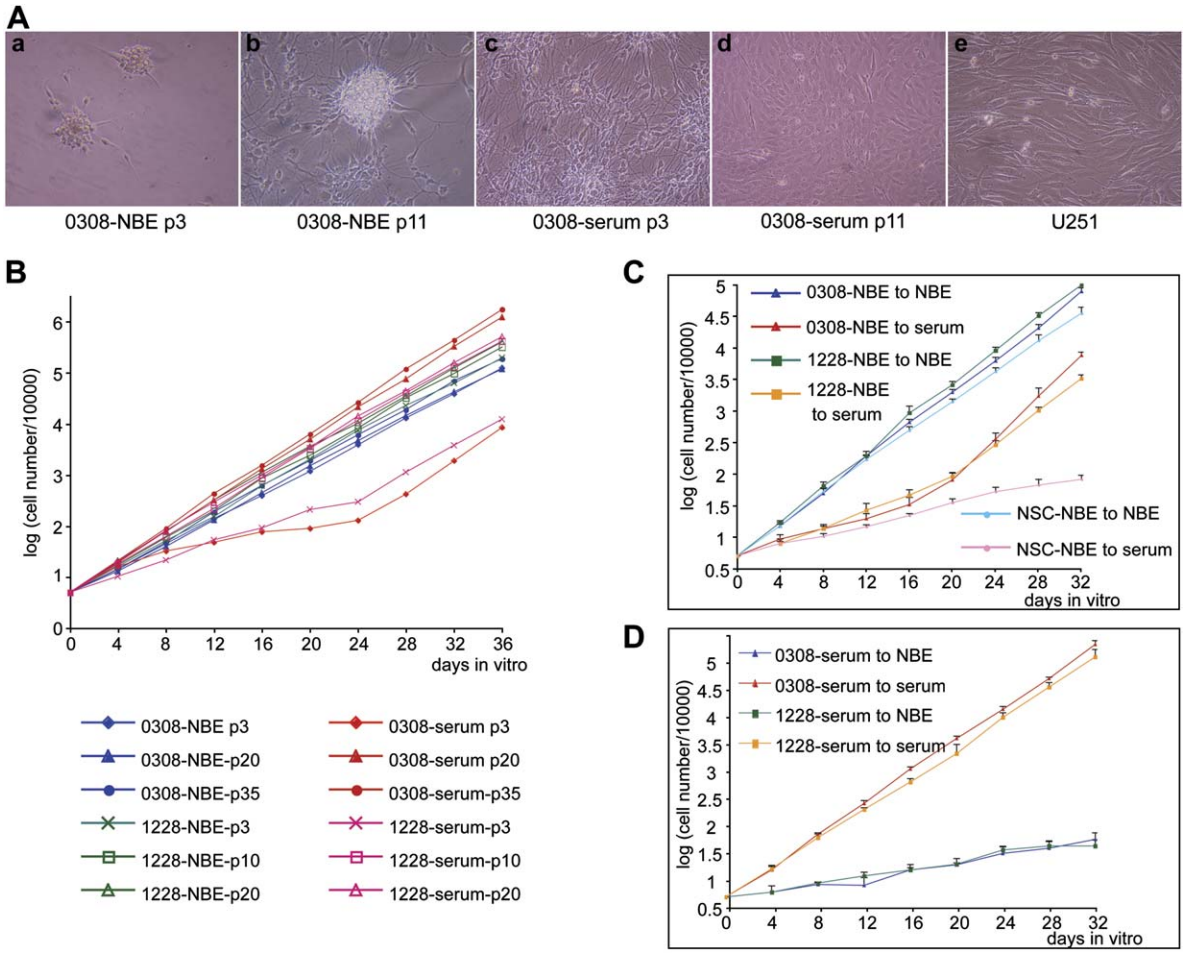

Figure 1. In vitro characterization of tumor cells cultured in NBE and serum conditions

A: Microphotographs of NBE-cultured glioblastoma (GBM) cells at early passage (**Aa**) and late passage (**Ab**) and serum-cultured GBM cells at early passage (**Ac**) and late passage (**Ad**). Note that the late passage serum cells are morphologically similar to U251 cells, a common glioma cell line (**Ae**).

B: Proliferation kinetics of NBE-cultured and serum-cultured GBM cells. 0308-NBE cells (blue lines) and 1228-NBE cells (green lines) at all passages, but only late passage 0308-serum cells (red lines with filled triangle and filled circles) and late passage 1228-serum cells (pink lines with open rectangles and open triangles) proliferated at constant rate. By contrast, early passage 0308-serum cells (red line with diamond markers) and early passage 1228-serum cells (pink line with X markers) proliferated very slowly initially followed by a plateau phase, before their exponential growth phase.

C and D: Proliferation kinetics of clonally derived cells grown under NBE condition (**C**) and serum condition (**D**). Monoclonal populations of 0308 (triangle, n = 3), 1228 (rectangle, n = 3), and polyclonal normal human NSCs (circle, n = 3) initially expanded in NBE conditions (**C**) or serum conditions (**D**) were further cultured in NBE and serum conditions for a month, and cell numbers were counted. Error bars represent standard deviation.

different in vitro conditions affects primary tumor cells, we prepared single cell suspensions of freshly resected and dissociated glioblastoma (GBM) tissues from patients. These cells were cultured under conditions optimal for propagation and nondifferentiation of normal NSCs ("NBE" conditions: serum-free *N*eurobasal media supplemented with *b*asic FGF and *E*GF), or conditions optimal for growth of glioma and most other cancer cell lines ("serum" conditions: DMEM media containing 10% fetal bovine *serum*). We have established a number of GBM-derived NBE- and serum-cultured cells from this protocol, two of which (lines 0308 and 1228) are described in detail here.

Results

In vitro characterization of GBM cells cultured in NBE and serum conditions

Glioma cells cultured in NBE and serum conditions displayed profound biological differences in vitro (Figure 1). First, cells cultured in NBE conditions (0308-NBE and 1228-NBE) readily proliferated both as nonadherent, multicellular spheres in uncoated plates ("neurospheres") and as an adherent monolayer in poly-L-lysine/laminin-coated plates, as is seen with normal NSCs. In contrast to the NBE cells, cells cultured in serum conditions (0308-serum and 1228-serum) formed a monolayer that initially had a highly heterogeneous morphology but within a month became homogeneous with a morphology reminiscent of fibroblasts or epithelial cells (Figure 1A). Although this dramatic morphological change of serum-cultured primary glioma cells has been routinely observed, it has never been understood. Second, cells cultured in NBE proliferated at a constant rate regardless of passage number, whereas cells cultured in serum showed initial growth followed by a plateau phase, only to eventually proliferate at a much greater rate in later passages (Figure 1B). To determine whether the differences in the growth kinetics between 0308- and 1228-serum cells were secondary to the different growth conditions or whether the differences were secondary

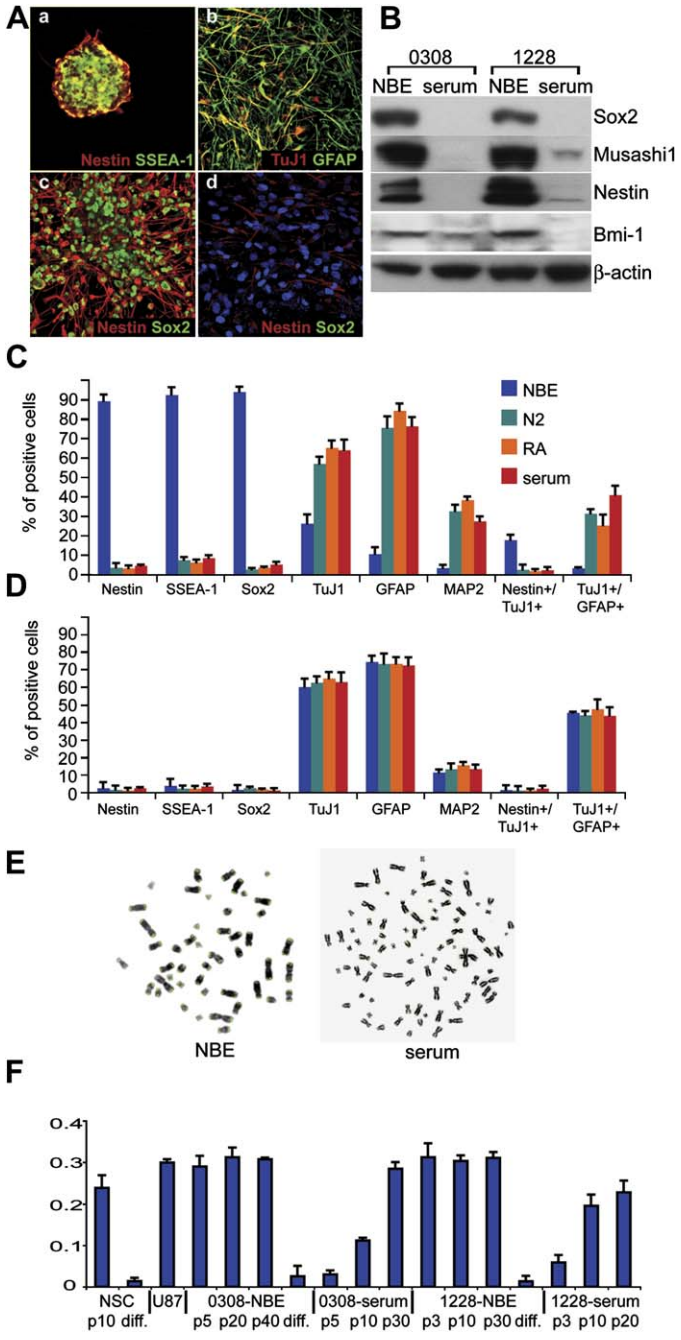

Figure 2. Evaluation of stem cell-like characteristics of GBM cells cultured in NBE and serum conditions

A: Immunohistochemical analysis of NBE and serum GBM cells (passage 10). The majority of NBE cells cultured under NBE conditions are immunopositive for Nestin, SSEA-1, and Sox2 (**Aa** and **Ac**). When these cells were cultured in serum condition for 10 days, they lost Nestin expression and became positive for GFAP and TuJ1 (**Ab**). About 40% of these cells were positive for both TuJ1 and GFAP (shown as yellow in **Ab**). Serum cells do not express either Nestin or Sox2 (**Ad**). DAPI staining (shown as blue) was performed in **Ad** to identify cells.

B: Western blot analysis of various NSC markers in NBE and serum GBM cells. All cell lysates were obtained from cells at passage 15. β-actin blot was used as a loading control.

C and D: Differentiation potential of NBE- and serum-cultured GBM cells in various conditions. Cells (**C**, 0308-NBE cells p15; **D**, 0308-serum cells p15) were cultured in the indicated conditions for 10 days and processed for immunohistochemistry, and then immunopositive cells were counted. N2 condition indicates the withdrawal of bFGF and EGF from NBE media. RA condition indicates the addition of all-*trans*-retinoic acid (2 μM) in N2 media. Double-positive cells for two antigens were counted positive for each antigen and also represented separately (e.g., TuJ1+/GFAP+; double-positive cells for both antigens). Error bars represent standard deviation.

E: Preserved telomeres in 0308-NBE and 0308-serum cells (passage 15) by telomere-PNA-FISH analysis; telomeres were labeled as green dots.

F: Quantitative ELISA for measuring telomerase activities in various cells as determined by the telomeric repeat amplification protocol assay (Roche). y axis represents absorbance at 450 nm, which is proportional to the telomerase activity. When NSCs and NBE cells were cultured in serum conditions for 10 days (marked as diff.), their telomerase activities decreased to very low levels. The experiments were performed in triplicates, and error bars represent standard deviation.

to the inadvertent in vitro selection of a clonal population of cells, we evaluated the proliferative kinetics of cells derived from multiple different single clones. The cells were initially derived from either NBE or serum conditions and then transferred to both NBE and serum conditions (Figures 1C and 1D). Upon the addition of serum, NBE cells showed a decreased rate of proliferation initially, followed by late exponential growth, recapitulating the growth pattern seen in the polyclonal serum cell population (Figure 1C). By contrast, cellular proliferation of serum cells nearly ceased following transfer to NBE conditions (Figure 1D). Thus, it appears that the initial culture of primary tumor cells under serum conditions leads to changes that cannot be subsequently reversed following transition to NBE conditions.

A third way in which NBE- versus serum-cultured cells differed was that NBE-cultured cells but not serum-cultured cells had the potential for multilineage differentiation and clonogenicity, central features of NSCs (Figure 2). Approximately 90% of NBE cells were positive by immunohistochemical analysis for the NSC markers Nestin, Sox2, and SSEA-1 (Brazel et al., 2005; Capela and Temple, 2002; Lendahl et al., 1990; Park et al., 2003). Western blot analysis confirmed abundant expression of these NSC markers in NBE cells (Figure 2B) (Brazel et al., 2005; Park et al., 2003; Sakakibara et al., 1996). After removal of growth factors (bFGF and EGF), or the addition of retinoic acid (RA) and serum, NBE cells began to lose their NSC markers and developed morphologies and immunohistochemical staining patterns consistent with cells of glial and neuronal lineages. Unlike normal NSCs, however, about 40% of cells costained for both glial (GFAP) and neuronal (TuJ1) markers, suggesting that differentiation pathways in these NBE cells are not entirely normal (Galli et al., 2004; Hemmati et al., 2003). By contrast, 0308- and 1228-serum cells expressed few or no NSC markers (Figures 2A, 2B, and 2D). Cells were only weakly positive for GFAP and TuJ1 and did not respond to differentiation cues such as RA and CNTF (Figure 2D and data not shown). Next, we performed single cell neurosphere formation assays in order to determine the clonogenicity of NBE- and serum-cultured cells. 0308-NBE and 1228-NBE cells had clonogenic frequencies of 23.4% (±2.4%) and 17.4% (±3.8%), respectively, reminiscent of NSC-derived neurospheres, whereas 0308- and 1228-serum cells failed to form neurosphere-like cells when plated in NBE conditions. Finally, a fourth way that NBE- and serum-cultured glioma cells differed was in their telomerase activities, although both cells retained telomeres as determined by fluorescence in situ hybridization (FISH) using telomere-specific probes (Figure 2E). 0308-NBE and 1228-NBE cells had consistent telomerase activity regardless of passage number, whereas telomerase

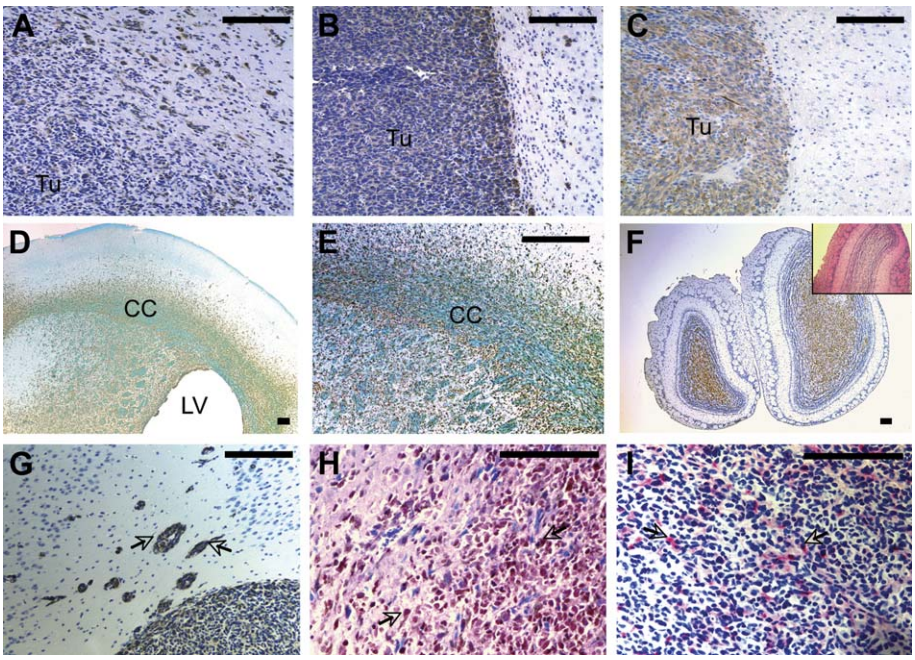

Figure 3. Phenotypic characterization of tumors derived from NBE- and late passage serum-cultured GBM cells

A–C: Representative microphotographs of NBE-cultured cell-derived intracranial xenograft tumor (0308-NBE p20) (**A**), serum-cultured cell-derived tumor (0308-serum p35) (**B**), and tumor generated from U87MG glioma cell lines (**C**). Note the extensive infiltration into the surrounding cerebral cortex in **A** only. Tu indicates central tumor mass.

D–F: Infiltrative/migratory nature of xenograft tumors derived from NBE-cultured GBM cells. Numerous NBE GBM cells (stained by brown color) diffusely infiltrated adjacent cortex (**D**) and migrated along the corpus callosum (CC, blue-stained by luxol fast blue dye) (**E**) and olfactory bulb (**F**). Inset in **F** is a hematoxylin/eosin (H/E)-stained section depicting normal olfactory bulbs. LV in **D** indicates lateral ventricle.

G–I: Immunohistochemical staining of NBE cell-derived tumors by using antibodies for Nestin (dark brown in **G**), Sox2 (red/purple in **H**), and GFAP (red/purple in **I**). Scale bar, 100 μm.

activity was lost when these cells were cultured in serum-containing media, consistent with what occurs in normal NSCs (Haik et al., 2000; Ostenfeld et al., 2000). Likewise, early passage 0308-serum and 1228-serum cells did not have appreciable telomerase activity. Interestingly, however, serum-cultured cells regained telomerase activity in later passages, coincident with their late passage exponential growth phase (Yang et al., 2004) (Figure 2F). Taken together, these data demonstrate that both 0308- and 1228-NBE cells contain many of the self-renewal and differentiation characteristics of NSCs, whereas serum-cultured cells do not.

Tumorigenic potential of GBM cells cultured in NBE or serum conditions

In order to evaluate the tumorigenic potential of the NBE cells compared to the serum cells, we first determined their ability to grow in soft agar (Table S1 in the Supplemental Data available with this article online). Cells were seeded into two different soft agar layers, one prepared with NBE media and the other with serum media, and then colonies were counted 3 weeks later. Both early and late passage NBE cells consistently formed colonies in soft agar without serum, but not in the agar with serum, suggesting that the clonal expansion of these cells requires lack of differentiation. Conversely, early passage serum cells failed to form colonies under either soft agar condition. Late passage, exponentially growing serum cells, however, readily formed large colonies in serum-containing agar but not in NBE-agar.

To test in vivo tumorigenic potential of NBE and serum cells at different passages, we stereotactically injected the cells into the brains of neonatal immunodeficient SCID mice. Two to three months later, nearly all mice (16/17) injected with 0308-NBE cells developed tumors, whereas none of the 13 mice injected with 0308-serum cells developed tumors (Table S2). 0308-NBE and 1228-NBE cells retain a consistent tumorigenic potential independent of passage number (as low as 1000 cells), whereas serum-cultured cells at early passages are nontumorigenic. When established NBE cell-derived intracranial xenograft tumors were dissociated, recultured under NBE conditions, and then injected back into the brains of new recipient mice, there was no loss of tumorigenic potential (Table S3). By contrast, when the same xenograft-derived cells were grown under serum conditions, all subsequent tumorigenic potential was lost, suggesting that the loss of tumorigenicity of these glioma cells is due to the serum-culture condition. Interestingly, although early passage serum-cultured cells did not form tumors, the late passage, exponentially growing, telomerase-positive 0308-serum cells began to produce intracranial tumors at an increasing rate with progressive passage number. By contrast, no tumors were generated from passage ten 1228-serum cells 6 months after injection.

Phenotypic characterization of tumors derived from NBE- and late passage serum-cultured GBM cells

There were significant histopathological differences between the intracranial tumors derived from NBE-cultured cells and those derived from late passage serum-cultured cells (Figure 3). Intracranial tumors generated by 0308-NBE and 1228-NBE cells demonstrated extensive infiltration into the surrounding cerebral cortex, a pathognomonic feature of human GBMs (Galli et al., 2004; Maher et al., 2001; Sanai et al., 2005; Singh et al., 2004). NBE GBM cells showed a predilection for migrating along white matter tracts such as the corpus callosum, as is characteristically seen with GBM cells in the brains of patients (Figures 3A, 3D, and 3E). Additionally, a significant number of cells were seen migrating toward the olfactory bulb (Figure 3F). By stark contrast, all of the tumors generated from late passage 0308-serum cells were well delineated with little tumor cell infiltration into the surrounding normal brain, a characteristic phenotypically identical to the human tumor xenografts generated from the standard glioma cell lines (e.g., U251, U87MG cells) (Figures 3B and 3C). These data demonstrate that tumors derived from

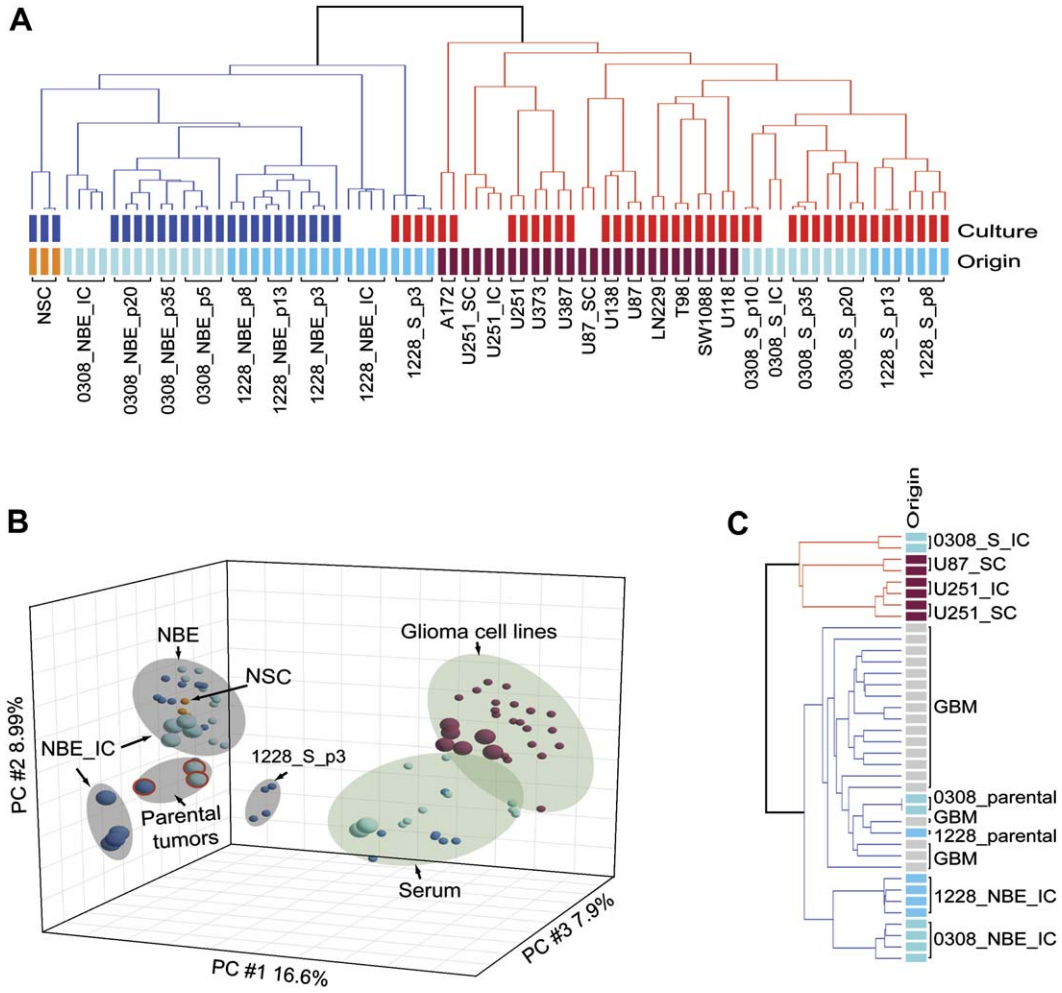

Figure 4. Global expression profiling analysis of NBE- and serum-cultured GBM cells

A: Dendrogram of cluster determined by unsupervised hierarchical cluster analysis of 79 in vitro and in vivo xenograft samples. Pearson correlation-based algorithm was applied on the basis of similarity in the expression pattern over all genes (over 42,000 probe sets). All samples represent independent biological samples. 1228_S_p8 indicates the cells at passage 8 (p8) cultured in serum condition (S), derived from the patient tumor 1228 (1228). IC and SC indicate xenograft tumors generated by intracranial and subcutaneous injection of the cells, respectively. Detailed descriptions about the samples are provided in the Supplemental Data (experimental schema in Figure S1). Two main clusters were marked by different colors (blue and red lines). Blue and red bars on the upper row indicate in vitro samples cultured in NBE and serum conditions, respectively. Color bars on the lower row indicate the origin of the corresponding samples. 0308 and 1228 cells are distinguished by two different tones of light blue. Purple and orange bars represent commonly used glioma cell lines and NSCs, respectively. The above labeling schemes are consistently used in subsequent figures.

B: Principal component analysis (PCA) of the previous data sets including the parental primary GBMs. Small and large balls indicate in vitro and in vivo xenograft samples, respectively. Colors of balls indicate the origin of samples (see above). Parental patient tumors are marked as balls with red circle. x, y, and z axes represent three major principal components (PC). Note two distinct clusters; one cluster consists of NBE cells and their derivative xenograft tumors, NSCs, and parental patient tumors, whereas the other cluster consists of serum cells, ten commonly used glioma cell lines, and their derivative tumors. Detailed descriptions about the samples are provided in Figure S3.

C: Dendrogram of cluster determined by unsupervised hierarchical cluster analysis of in vivo xenograft and primary GBM samples. Gray bars indicate patient GBM samples (except 0308 and 1228 patient tumors). Blue and red lines mark two main clusters, respectively.

NBE cells, but not serum cells, phenocopy the critically important histopathological features of the original human GBM tumor.

Transcriptome analysis of NBE- and serum-cultured GBM cells

To further understand the differences between NBE- and serum-cultured GBM cells, we generated gene expression profiles of NBE cells, serum cells, their derived xenograft tumors, and the original GBMs taken directly from the patients (see experimental schema in Figure S1). NBE- and serum-cultured GBM cells from different passages were also included in the analyses in order to evaluate the potential effects of in vitro passage number on gene expression.

Unsupervised hierarchical cluster analysis of all in vitro and in vivo xenograft samples (n = 79) revealed two distinct clusters; one cluster consisted of NBE cells and NSCs, and the other consisted of serum cells and ten commonly utilized glioma cell lines (with the one exception of 1228-serum passage 3 cells; see below) (Figures 4A and 4B). Gene expression profiles of xenograft tumors derived from both 0308- and 1228-NBE cells were similar to those of NBE cells and NSCs, but distinct from those of serum-cultured cells and their derivative tumors

(0308-serum-p25-derived tumors), suggesting that the distinct expression profiles of NBE-cultured and serum-cultured cells reflect intrinsic biological differences between the cells rather than some transient effect of serum on cellular gene expression (i.e., serum-responsive genes) in the serum-cultured cells.

Since NBE-cultured GBM cells reproduced the histopathology of human GBMs in situ, we next evaluated whether the global expression profiles of NBE cell-derived tumors resembled those of primary human tumors (Figure 4C). To this end, we compared the expression profiles of xenograft tumors derived from NBE- and serum-cultured GBM cells to those of 21 randomly chosen GBMs, including the parental 0308 and 1228 GBMs. Unsupervised hierarchical cluster analysis revealed that both 0308- and 1228-NBE-derived tumors were closely related to their parental tumors and other primary GBMs (Figure 4C). Tumors derived from late passage 0308-serum cells, however, had markedly different profiles from their matched NBE-derived tumor xenografts and from parental 0308 tumors as well as from other primary GBMs, clustering with xenograft tumors derived from the U87MG and U251 traditional glioma cell lines (Figure 4C and Figure S2).

Passage-dependent gene expression profile changes in NBE and serum GBM cells

Our in vitro and in vivo findings demonstrate that the phenotype of NBE cells was well maintained regardless of passage number, while that of serum cells was changed dramatically upon serial passages. Consistent with this, principle component analysis (PCA) indicated that early passage (p3) 1228-serum cells were intermediate between the NBE group and the serum group, whereas later passage 1228-serum cells (p8 and p13) were tightly clustered with all other traditional glioma cell lines (Figures 4A and 4B). Thus, gene expression profiles of serum-cultured cells progressively diverge away from those of primary GBMs and NSCs toward those that resemble established glioma cell lines with increasing passage (Figure 4B). In order to further analyze gene expression changes in NBE and serum cells with increasing passage number, we performed PCA and two-way ANOVA analysis (Figure S3). All 1228-NBE cells, regardless of passage, clustered tightly, and the numbers of genes differentially expressed between different passages of cells were small (Table S4). By contrast, approximately 5000 probe sets were differentially regulated in early (p3) versus late passage 1228-serum cells (p8 and p13), raising the possibility that these significant gene expression changes in serially passaged serum cells may, in part, be responsible for the different phenotype of late passage serum cells and NBE cells as well as primary GBMs (i.e., does one progressively lose expression of GBM-defining genes with serial passage in serum?). To test this hypothesis, we performed a supervised hierarchical cluster analysis of the entire data sets by using 2029 probe sets that were expressed more than 2-fold in 1228-serum p3 cells compared to the later passages (p8 and p13) (Figure 5A). Expression levels of almost all of these genes were very high in primary GBMs, but not in the later passage 0308- and 1228-serum cells or glioma cell lines. Interestingly, about 75% of these 2029 probe sets were also highly expressed in NSCs and NBE cells regardless of passage number. Functional annotation of this gene set by Ingenuity Pathway Knowledge Base predicted that many of these genes are involved in CNS development, CNS function, and cell-to-cell signaling (Table S5). On the other hand, a quarter of the probe sets were represented only in primary GBMs but not in NBE or serum groups. These probe sets were mainly immune system-related genes, consistent with the minor contamination of CD45-positive lymphocytes in early passage glioma cultures. Conversely, we identified 945 probe sets that were upregulated in later passage (p8 and p13) compared to early passage (p3) of 1228-serum cells. Supervised analysis using these probe sets indicated that these genes are also upregulated in 0308-serum cells and glioma cell lines, but not in NBE cells, NSCs, and primary GBMs (data not shown). Taken together, these data support the notion that serum-cultured cells rapidly lose characteristics of the primary GBMs upon passage in vitro, whereas NBE-cultured GBM cells maintain their parental phenotypes.

Analysis of NBE- and serum-specific genes and their expression in primary GBMs

We next evaluated the genes that were differentially expressed between NBE- and serum-cultured cells. As determined by two-way ANOVA (with cell origin and culture condition as the factors), 5795 and 6895 probe sets were differentially expressed in 0308- and 1228-NBE cells compared to 0308- and 1228-serum cells, respectively. Among them, 1864 probe sets were commonly upregulated in both 0308- and 1228-NBE cells, and 1444 probe sets were commonly upregulated in serum cells. Supervised hierarchical cluster analysis using these probe sets revealed that the 1864 NBE-specific probe sets were highly expressed in primary GBM tumors as well as NSCs, but not in glioma cell lines (Figure 5B). By contrast, most of 1444 serum-specific probe sets were highly expressed in glioma cell lines, but not in GBM tumors. Functional annotation of the NBE-specific probe sets using Ingenuity and gene ontology analysis tool GOSTAT predicted that a majority of these genes are involved in CNS function and development, consistent with the previous analysis (Table S6 and data not shown). Representative gene lists of these probe sets are presented in Table S7. Notably, numerous stem cell-associated genes were found in the NBE-specific sets, but not in serum-specific sets.

Based on high expression of a number of stem cell-associated genes in the NBE cells, we performed a stratified analysis of all of the in vitro and in vivo NBE and serum cells, as well as the primary GBMs, using probe sets highly enriched for stem cell-related genes. The gene set was selected by first finding the genes that overlapped between three distinct murine NSC-enriched gene lists reported in three different studies, and then converting these genes (approximately 200) to their human orthologs (Fortunel et al., 2003; Ivanova et al., 2002; Ramalho-Santos et al., 2002). Clustering data showed that these stem cell-associated genes are very similarly regulated in both NBE cells and NSCs as well as in primary GBMs in contrast to serum cells and glioma cell lines (Figure S4 and Table S8).

Taken together, these data demonstrate that NBE cells are remarkably similar to normal NSCs and that NBE cells and their derivative tumors properly maintain many biological characteristics of the parental GBMs and other primary GBMs, whereas traditionally grown, serum-cultured cancer cell lines do not.

Genotypic characterization of NBE- and serum-cultured GBM cells

In order to evaluate genomic changes in NBE- and serum-cultured glioma cells in detail, we performed single nucleotide

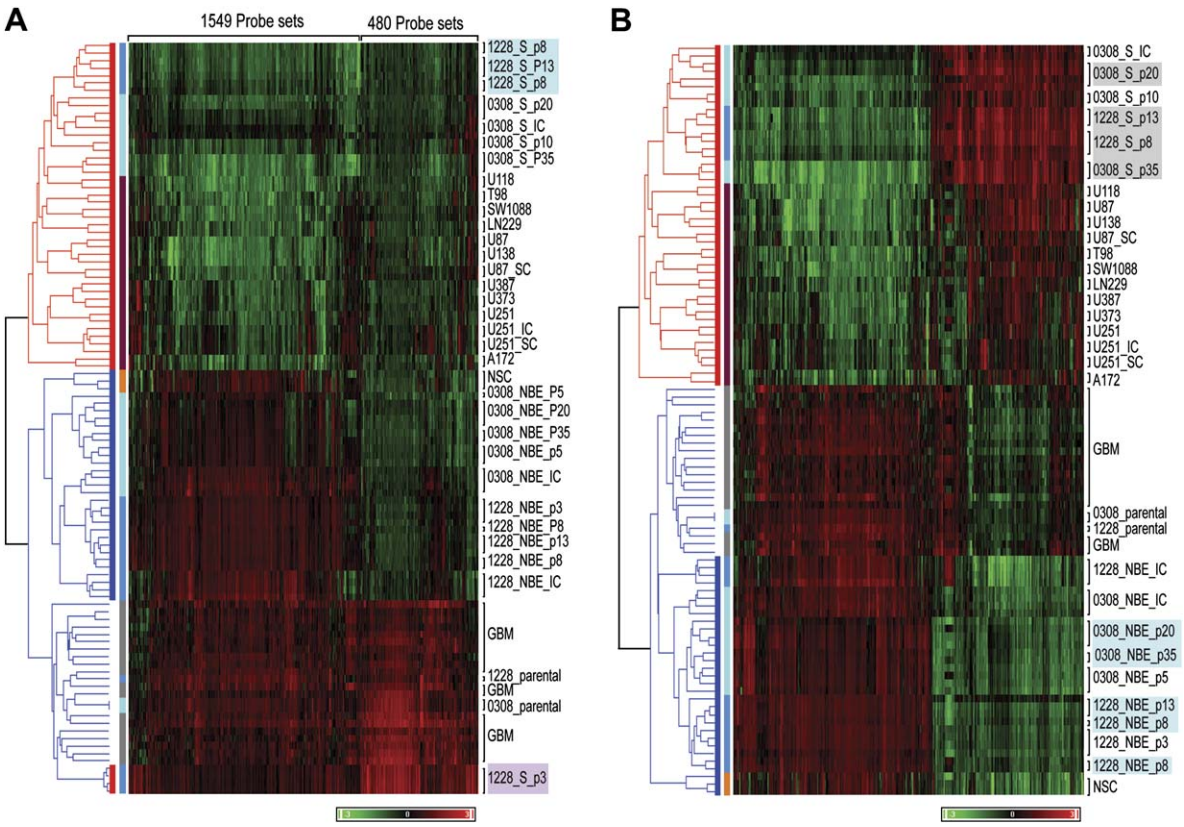

Figure 5. Supervised hierarchical analyses of NBE- and serum-cultured GBM cells

A: Heatmap of supervised hierarchical cluster analysis using ''passage-dependent gene sets'' as classifiers. See text for the detail. Dendrogram of cluster is shown on the left of the heatmap. Expression level of a given gene is indicated by red (high) and green (low) in the heatmap. Samples used for generating ''passage-dependent gene sets'' are marked by colored boxes on the right.

B: Heatmap of supervised hierarchical cluster analysis of ''NBE-specific'' and ''serum-specific'' probe sets. Samples used for generating these probe sets are shown in colored boxes on the right of the heatmap.

polymorphism (SNP) analysis as well as spectral karyotyping (SKY) and Giemsa banding analysis (Figure 6). We identified homozygous deletion of the *INK4a/ARF* locus at chromosome 9, loss of chromosome 10q, trisomy of chromosome 7, and local amplification of *EGFR* locus in both 0308 and 1228 surgical samples. These genetic alterations are common genomic features in a significant percentage of primary GBMs (Vogelstein and Kinzler, 2004; Wiltshire et al., 2000). The detailed genomic characteristics of the 0308 and 1228 cells are summarized in Table S9.

Given the changes in growth rates, morphology, tumorigenicity, and gene expression over time in the serum-cultured cells, we evaluated serial genomic DNA profiles of NBE- and serum-cultured cells at various passage numbers by SNP analysis (Figures 6A and 6B). Even after 1 year of maintenance in NBE culture condition (more than 70 passages so far), both polyclonal and monoclonal populations of 0308-NBE cells largely maintained their parental tumor genotype. By sharp contrast, 0308-serum cells underwent significant genomic rearrangements as early as passage 10 (less than 2 months of culture) represented by pseudotetraploidy, loss of heterozygosity (LOH), and deletion of the entire chromosome 4 and chromosome 17 (Figure 6). Different subclones of 0308-serum cells showed other unique changes, including LOH of the entire chromosome 6 (Figures 6A and 6B). None of these genomic alterations were detected in the 0308 parental tumor, nor in early passage cells, suggesting that these additional genomic changes occurred during the in vitro culture (Figures 6A and 6B). Giemsa banding data revealed that over 95% of late passage 0308-serum cells were pseudotriploid or tetraploid (more than 200 nuclei examined) with a modal chromosome number of 77 ranging from 63 to 89. By contrast, more than 90% of 0308-NBE cells at passage 40 were near diploid. Similarly, about 70% of 1228-serum cells at passage 20 were pseudotetraploid, whereas passage 20 1228-NBE cells were near diploid (Figures 6E and 6F).

Intriguingly, LOH in chromosomes 4 and 17 found in most of the late passage 0308-serum cells corresponds to the chromosomal locations of the *hCDC4* (*Fbxw7*) and *p53* (*TP53*) genes, respectively (Figure 7). Coincident with the onset of increased proliferation, tumorigenicity, and aneuploidy, late passage 0308-serum cells were found to ultimately downregulate the remaining wild-type hCDC4 gene, essentially eliminating expression of hCDC4 protein. HCDC4, an E3 ubiquitin ligase, is reported to be involved in degradation of aurora kinase A (*STK15* [*AURKA*]) (Mao et al., 2004; Marumoto et al., 2005; Yang et al., 2004).

Indeed, late passage 0308-serum cells not only lose hCDC4 expression but also have significant upregulation of STK15 (Figure 7C). This is of potential importance given the recent description of hCDC4 as a haploinsufficient tumor suppressor

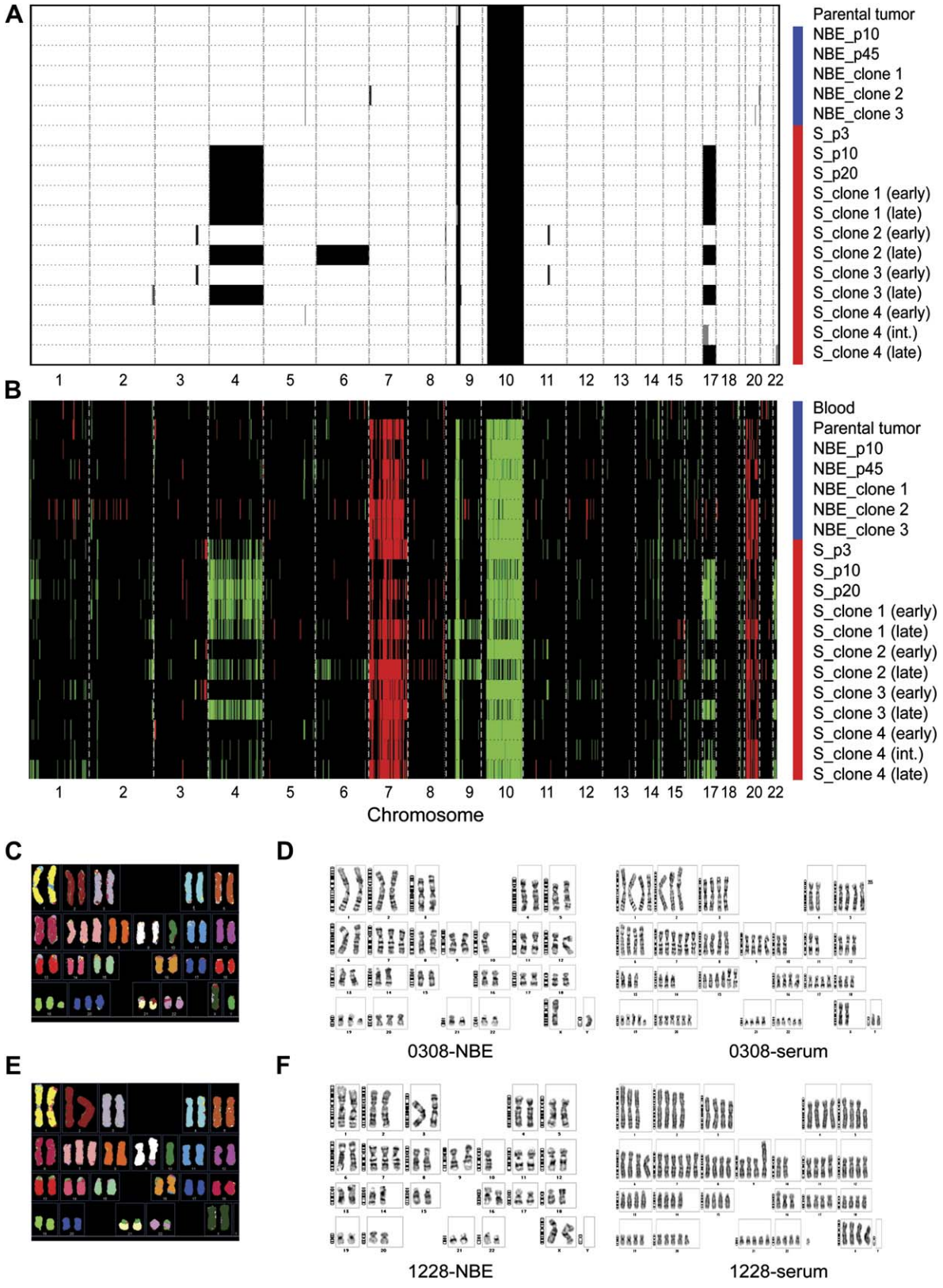

Figure 6. Genomic analysis of NBE- and serum-cultured GBM cells

A: Heatmap representing loss-of-heterozygosity (LOH) areas in 0308-NBE and serum cells at different passages. LOH areas were determined by comparing SNP allelic calls of genomic DNA from tumor cells to those of peripheral blood DNA from the same patient. The calculation and smoothing were performed using dChip software. x axis represents chromosomes in numerical order. Chromosome X was omitted, since it is not informative in LOH analysis. Black areas represent LOH. Blue and red bars on the right of the heatmap represent the cells cultured in NBE and serum conditions, respectively. For clonally derived cells

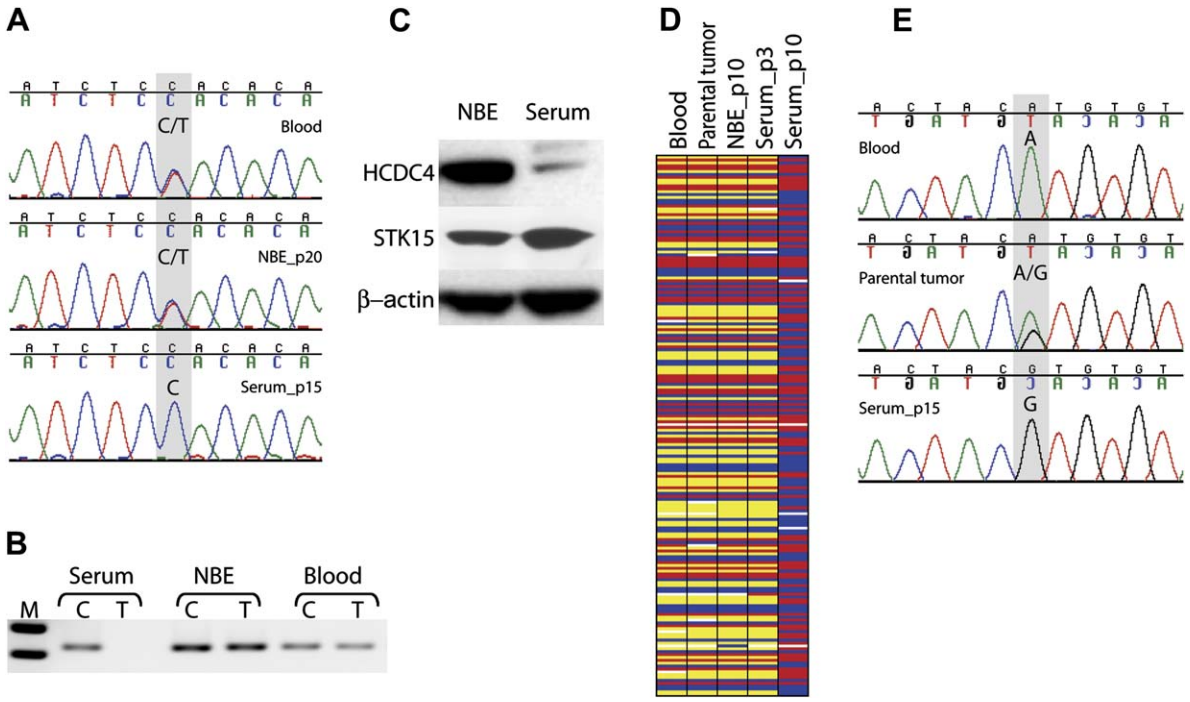

Figure 7. Genomic alterations of GBM cells cultured in serum condition in vitro

A and B: LOH of chromosome 4 in later passage 0308-serum cells was confirmed by genomic PCR sequencing (**A**) and allelic-specific PCR (**B**). Boxed area in **A** indicates genomic sequence of SNP_A-1510430 (hCDC4 locus in chromosome 4). Note the loss of DNA polymorphism in 0308-serum cells (C only instead of C/T sequences). **B:** DNAs from serum cells, NBE cells, and blood from the 0308 patients were PCR amplified by using allelic-specific primers. C and T represent 3′ end sequences of allelic-specific primers (sequences shown in Experimental Procedures). Note the absence of DNA amplification in serum cells by allelic-specific PCR. DNA marker is indicated by M.

C: Western blot analysis of hCDC4 and STK15 in 0308-NBE and serum cells. β-actin Western blot was used as a loading control.

D: LOH of entire chromosome 17 in later passage 0308-serum cells (p10) determined by SNP calls. All SNPs located in chromosome 17 are pseudocolored by heterozygous calls (AB calls; yellow) and homozygous calls (AA and BB calls; red and blue, respectively). Absence of heterozygous calls indicates the loss of either maternal or paternal chromosome. Note the complete loss of heterozygous calls (yellow) in serum cells at passage 10.

E: Genomic sequence of the p53 gene in 0308-serum cells. Boxed area indicates genomic DNA sequence difference. DNA from peripheral blood contained wild-type sequence, whereas parental tumors have both wild-type and mutant sequences. Note the absence of wild-type sequence in serum cells.

gene, inactivation of which results in aneuploidy (Mao et al., 2004; Rajagopalan et al., 2004). In addition, late passage 0308-serum cells lose the entire chromosome 17, which contains the wild-type p53 allele, leaving only the mutant p53 allele found in both the 0308-NBE cells and the parental tumor (Figures 7D and 7E). Loss of p53 function has also been associated with genomic instability (Duensing and Duensing, 2005; Fujiwara et al., 2005). Thus, the loss of both hCDC4 and p53 function in 0308-serum cells, with their potentially important biological consequences, further demonstrates the significant differences between serum cells and their matched parental tumors.

Discussion

The TSC concept has potentially profound implications both for basic biological studies and for the development of new therapeutic strategies (Dean et al., 2005; Huntly and Gilliland, 2005). However, the true relatedness of previously reported TSCs to normal stem cells and their exact relevance to our current in vitro and in vivo models of cancer, as well as to primary human tumors in situ, remain unclear (Dontu et al., 2003; Huntly and Gilliland, 2005; Kim et al., 2005; Pardal et al., 2003; Reya et al., 2001). Using a model system derived from primary GBMs, we have demonstrated that NBE-cultured cells derived from primary GBMs bear remarkable similarity to normal NSCs. These similarities include the ability of NBE cells to form neurospheres in vitro, potential for indefinite self-renewal, ability for terminal differentiation into glial and neuronal lineages, the possession of gene expression profiles similar to NSCs, and genetic stability over serial passage in vitro. Nevertheless, NBE cells also harbor all of the genetic aberrations found within the primary tumor, have gene expression profiles similar to the

(labeled with clone number), passage number was not assigned. The culture time difference between early and late clones is approximately 1 month. Note that LOH of chromosomes 9 and 10 is evident in all of samples including the parental 0308 patient tumor. NBE-cultured cells maintain parental genotype regardless of passage and clones, whereas most of the late passage serum cells revealed LOH of chromosomes 4 and 17.

B: Genomic amplification (red) and deletion (green) areas in 0308-NBE and serum cells at different passages. Copy number values were calculated using Affymetrix chromosome copy number analysis tool and smoothed by running average over 15 consecutive SNPs. Note the amplification of chromosomes 7 and 20 and deletion of chromosomes 9 and 10 in parental tumors. Late passage serum cells revealed significant genomic alterations including deletion of chromosomes 4, 17, and 22.

C–F: Spectral karyotyping (SKY) (**C** and **E**) and Giemsa banding analysis (**D** and **F**) revealed that 0308-NBE (passage 40) and 1228-NBE cells (passage 20) are near diploid, whereas 0308-serum cells (passage 15) and 1228-serum cells (passage 20) are predominantly pseudotriploid or tetraploid.

A

	NBE-cultured GBM cells	Serum-cultured GBM cells
Proliferation	Constant	Limited growth, reaches plateau, followed by exponential growth
Clonogenicity Tumorigenicity	Clonogenic and tumorigenic regardless of passages	Not at early passages
Differentiation potential	Induce to become glial and neuronal lineages	Do not respond to differentiation stimuli
Telomerase activity	Positive	Negative initially, but became positive at late passages
Tumor histology	Extensive migration/infiltration Phenocopy primary human GBMs	Fail to show infiltration similar to common glioma lines
Global gene expression	Similar to primary human GBMs	Different from primary tumors similar to common glioma lines
NSC-related genes	Nestin, Sox2, CD133, Musashi, Bmi	-
Genotype	Same as parental tumors regardless of passages	Additional genomic alterations not found in parental tumors

B

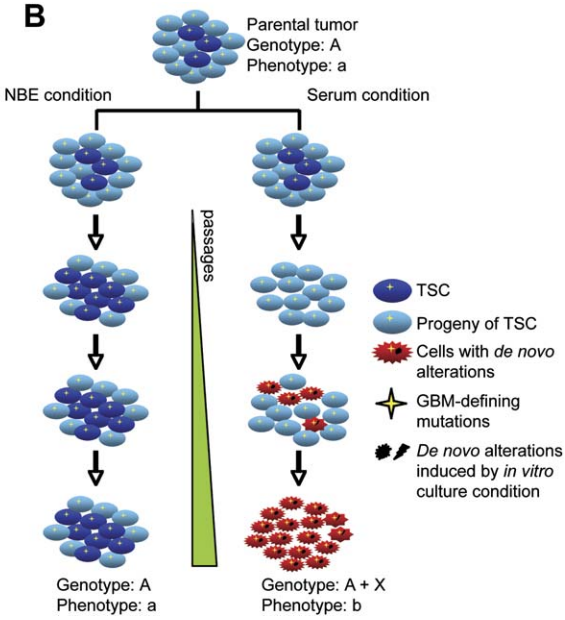

Figure 8. A hypothetical model depicting the relationship between primary patient tumors, TSC cultures, and commonly used glioma cell lines

A: Summary of our findings in 0308 and 1228 GBM cells.

B: NBE culture conditions maintain the properties of primary tumors by preserving the putative TSC populations (shown as dark blue). Rather than diverting TSCs away from NSC-like behavior, it appears that the GBM-defining genetic perturbations (e.g., INK4a/ARF deletion, loss of chromosome 10q, EGFR amplification; marked as yellow stars) merely endow oncogenic properties onto a population of cells with inherent stem cell properties (i.e., NSCs). By contrast, the TSC population is quickly lost through differentiation following growth in serum, similar to that seen in normal NSCs. TSC progenies (shown as light blue) are neither clonogenic nor tumorigenic despite the fact that they harbor the same genetic changes (marked as yellow stars) found in the corresponding TSCs and primary tumor. Continuous cultures of these cells in serum conditions result in the outgrowth of a subpopulation(s) of cells harboring additional genetic and/or epigenetic changes (marked as black spots) not found in the TSCs or primary GBMs. These late passage serum-grown cells (shown as red) are vastly different both genetically and biologically compared to the primary tumors from which they are derived, but similar to commonly used glioma cell lines. Vertical green bar represents the increasing number of passages in vitro.

GBMs they were derived from, and appear to be the principal tumorigenic cell type that can recapitulate the overall in vivo phenotype of the parental GBM. Therefore, cells fulfilling the criteria of TSCs are maintained in NBE conditions (Reya et al., 2001).

By contrast, cells derived from the same GBM specimens as the NBE cells, but grown under standard in vitro conditions using serum-containing media, lose their self-renewing capabilities, have no ability to differentiate, have gene expression profiles that are similar to neither NSCs nor the primary GBMs they were derived from, and are neither clonogenic nor tumorigenic. While 0308 serum-cultured cells did ultimately regain tumorigenic potential in later passages, these late passage cells do not recapitulate the tumorigenic phenotype of the original tumor and undergo significant de novo genomic alterations. This occurrence precisely matches the phenotypic and genotypic pattern found in most of the commonly used glioma cell lines in that only a small subset of these cell lines are tumorigenic in vivo, and of those, none demonstrate the tumor invasiveness seen with the NBE-derived tumors and that characterize human gliomas (Giannini et al., 2005). Additionally, it has been reported that most primary gliomas are near diploid, whereas established glioma cell lines are predominantly polyploid (Bigner and Vogelstein, 1990; Hecht et al., 1995; Kubota et al., 2001; Wiltshire et al., 2000). Therefore, we propose that the inherent "TSC" population within primary GBMs is quickly lost in typical glioma culture conditions, and the cells found following prolonged in vitro passage are the product of an outgrowth of a cell clone(s) that has undergone profound "de novo" genetic and/or epigenetic changes (Figure 8). In this context, it is not surprising that the gene expression profiles and the in vitro and in vivo behavior of serum-cultured cells appear remarkably similar to other established glioma cells lines but highly different from their matched NBE-cultured cells, parental tumors, and other primary human GBMs (Mehrian Shai et al., 2005).

Several groups have reported that TSC-like cells can be isolated from the established tumor cell lines by culturing these cells in serum-free media with selected growth factors such as PDGF, bFGF, and EGF (Kondo et al., 2004; Patrawala et al., 2005). If a subpopulation of TSCs can persist even after years of culture in vitro, it would be interesting to understand how these cells maintain their TSC-like properties under differentiation-inducing conditions. Alternatively, it is possible that cells with stem-like properties reemerge from some established cell line populations, through epigenetic reprogramming and/or selection of a subpopulation of cells with genomic instability as seen in our 0308 serum-cultured cells (Fujiwara et al., 2005; Kondo and Raff, 2004; Shin et al., 2003). In either case, further studies evaluating the relatedness of these established cell line-derived "stem cell-like" cells to their parental tumors and a thorough phenotypic/genotypic characterization of these cells would ultimately be required to truly understand how closely they fulfill the criteria of TSCs.

In summary, TSCs derived from primary GBMs bear remarkable phenotypic similarity to human NSCs and appear to be the tumorigenic component of at least a subset of human GBMs. Since TSCs maintain the ability to terminally differentiate,

agents that induce differentiation may have a therapeutic role in the treatment of the TSC compartment. Thus, NBE cells may be an optimal model system for the preclinical screening of such agents. By contrast, tumor cells grown under standard serum-containing cell culture conditions result in the loss of TSCs and ultimately lead to the outgrowth of a population of cells that are vastly different both genetically and biologically from the primary tumors from which they were derived. These observations bring into question the relevance of standard cancer cell lines for studying the biology of human cancer.

As we increasingly come to appreciate the significant heterogeneity within specific subtypes of solid tumors like GBMs and as we develop more rationally based, molecularly targeted drugs, we will move increasingly toward the era of personalized therapy for individual tumors. Such an individualized therapeutic approach will require a model system for identifying and understanding the basic genotype, gene expression profiles, and/or in vitro and in vivo biological characteristics of unique tumors from individual patients. The overarching implication of our findings is that many traditionally utilized cancer cell lines may be unreliable model systems for understanding the biology of primary human tumors, for screening new therapeutic agents, and ultimately for guiding personalized tumor therapy. TSCs, however, may prove to be a more reliable model system.

Experimental procedures

Tumor specimens and primary tumor cultures

Following informed consent, tumor samples classified as GBM based on World Health Organization (WHO) criteria were obtained from patients undergoing surgical treatment at the National Institutes of Health in accordance with the appropriate Institutional Review Boards (Kleihues et al., 2002). Within 1 to 3 hr after surgical removal, tumors were washed and enzymatically dissociated into single cells. Red blood cells were removed by differential centrifugation. Tumor cells were cultured in either NBE media consisting of Neurobasal media (Invitrogen), N2 and B27 supplements (0.5× each; Invitrogen), human recombinant bFGF and EGF (50 ng/ml each; R&D Systems), or serum media consisting of DMEM media (Invitrogen) with 10% fetal bovine serum (Cellgro). For neurosphere culture of NBE cells, uncoated plastic dishes were used. For adherent culture of NBE cells, the plates were precoated with poly-L-lysine/laminin mixture (Invitrogen).

Immunohistochemistry

Immunohistochemistry of paraffin sections was performed as previously described. The following antibodies were used as primary antibodies: GFAP (DAKO), Sox2, Nestin (R&D Systems), and SSEA-1 (Developmental Studies Hybridoma Bank). The corpus callosum was stained by luxol fast blue (Sigma), according to the manufacturer's recommendation.

Intracranial tumor cell injection into SCID mice

For evaluation of tumorigenicity, cells cultured in either NBE and serum conditions were resuspended in 2 μl of HBSS and injected stereotactically into neonatal SCID mice. For the analysis of tumor histology, both neonatal and 6- to 8-week-old mice were used. Coordinates for stereotactical injections into the adult mice were 3 mm distal to the midline, 2 mm anterior to the coronal suture, and 2.5 mm deep from the dura.

FISH analysis and SKY of tumor cells

For cytogenetic studies, cells were arrested at the metaphase by colcemid (Sigma), incubated in hypotonic buffer, and then fixed in methanol/acetic acid mixture. For Giemsa banding of chromosomes, more than 100 nuclei were analyzed for each cell population. Telomere FISH analysis was performed according to the manufacturer's instructions (Dako). EGFR FISH was performed using centromere 7 and an EGFR probe set (Vysis).

RNA expression array and data analysis

Total RNAs were isolated using TRIZOL (Invitrogen) and further purified using RNeasy Mini Kit (Qiagen). Samples were processed and hybridized to Affymetrix U-133 plus2 GeneChip Arrays according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix). Briefly, 6 μg of total RNA was converted to cDNA with Superscript reverse transcriptase (Invitrogen), using T7-linked Oligo (dT) primer. Complementary DNA was transcribed in vitro using the T7 Bioarray High Yield RNA Transcript Labeling Kit (ENZO Diagnostics) to generate biotinylated cRNA. Purified cRNA (20 μg) was fragmented and hybridized to GeneChip arrays. All arrays were confirmed to be within acceptable minimal quality control parameters including the signal intensity ratio of the 5′ and 3′ ends of the internal control genes of β-actin and GAPDH less than 2. The initial gene expression analysis data files (CEL files) were generated using Affymetrix GeneChip Operating Software (GCOS) version 1.1.

The gene expression CEL files were normalized using dChip invariant method and PM-MM difference model was used to obtain the expression values (Li and Wong, 2001). Probe sets with zero variance and only "absent calls" across all the samples were removed prior to data analysis. The hierarchical cluster analysis, principal component analysis (PCA), and two-way analysis of variance (ANOVA) were performed using Partek software 6.0 (Partek Inc.). The Pearson correlation with average linkage and Euclidean distance with average linkage was used for unsupervised hierarchical clustering and for supervised hierarchical clustering, respectively. For ANOVA, the false discovery rate of 0.05 and 2-fold change were applied as thresholds to select the up- or downregulated gene lists.

SNP array and data analysis

Genomic DNA from patient tumor samples and cultured cells was prepared using the QiaAmp DNA kit (Qiagen). DNA from patients' blood was prepared by QiaAmp DNA blood mini kit (Qiagen). Array experiments were performed according to the manufacturer's recommendations (Affymetrix). Briefly, 250 ng of genomic DNA was digested with XbaI (New England Biolabs), ligated to XbaI adaptors, and subsequently amplified by PCR. Fragmented PCR products were then labeled, denatured, and hybridized to the arrays. After hybridization, the arrays were stained on the fluidics station 450 and scanned using a high-resolution microarray scanner 3000.

GeneChip Human Mapping 10K arrays (Affymetrix) covers 11,555 single-nucleotide polymorphism (SNP) loci in the human genome with average resolution of one SNP every 210 kb. SNP calls were determined by GDAS version 2.0. Chromosome copy numbers were estimated using Affymetrix GeneChip Chromosome Copy Number Tool version 1.1.1(Huang et al., 2004).

PCR sequencing and quantitative real-time PCR

Genomic alterations identified by the SNP analysis were verified by PCR and subsequent sequencing. The presence of LOH of chromosome 4 in later passage 0308-serum cells was confirmed by PCR sequencing of several SNPs including SNP_A-1510430 and SNP_A-1515580 (Affymetrix). The following primers were used: 5′-AGTCCCTTGAGGCATGTTGG-3′ and 5′-GTCAACTTGAGCAAAAAAATCTAGC-3′ (for SNP_A-1510430), 5′-GATTCATTGTTGTTGCTAAAAGTG-3′ and 5′- TGCCTCTCTTCTCTCTTCCC-3′ (for SNP_A-1515580). For allele-specific PCR for the above SNPs, the following primers were used: 5′-CGCAACAACAATGAATCTC<u>C</u>-3′ and 5′-CGCAACAACAATGAATCTC<u>T</u>-3′ (SNP_A-1510430), 5′-GAGCCCTTGGCTTCTGATAT<u>A</u>-3′ and 5′-GAGCCCTTGGCTTCTGATAT<u>G</u>-3′ (SNP_A-1515580).

Real-time PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. All samples including no template controls were assayed in triplicates. The relative amount of targets transcripts was normalized to the number of human GAPDH transcripts found in the same sample. The human reference RNA (Qiagen) was used as the calibrator. The relative quantitation of target gene expression was performed with the standard curve or comparative cycle threshold (C_T) method.

Supplemental data

The Supplemental Data include four supplemental figures and nine supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/5/391/DC1/.

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Accession numbers

The microarray data have been submitted to the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/; the accession number is GSE4536.