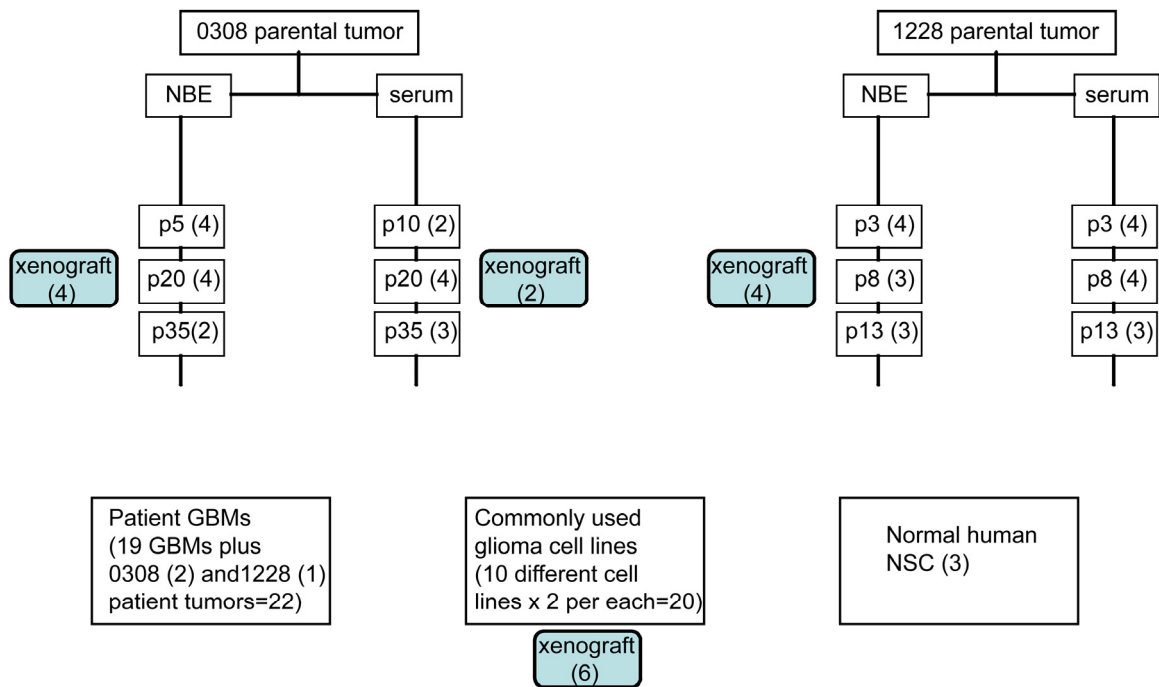


## Supplemental data

### 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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Figure S1. Experimental schema for microarray



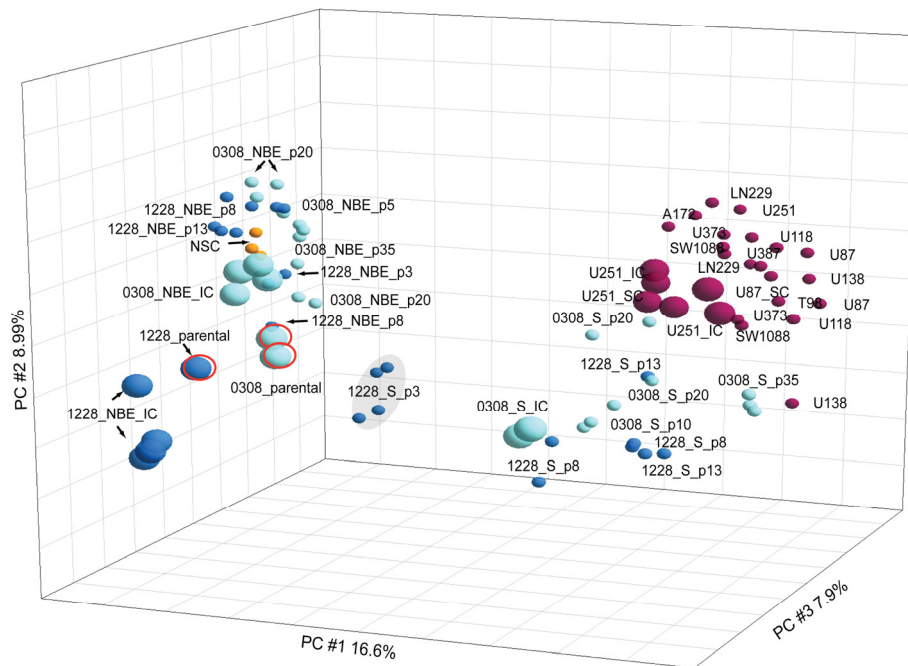


**Figure S3.** Principal component analysis (PCA) of NBE- and serum-cultured GBM cells

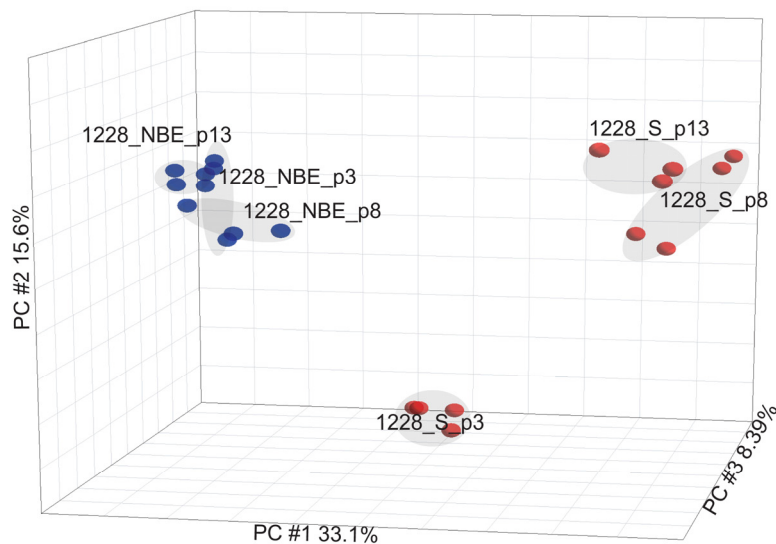
**A:** Figure 4B with detailed sample information.

**B:** PCA of *in vitro* 1228-NBE and serum cells at various passages. Blue and red balls indicate NBE-cells and serum-cells, respectively. Gray circles encompass samples at the given passage (n= 3 or 4 per each group). Note that early passage 1228-serum cells (1228\_S\_p3) were intermediate between the NBE group and the serum group.

**A**

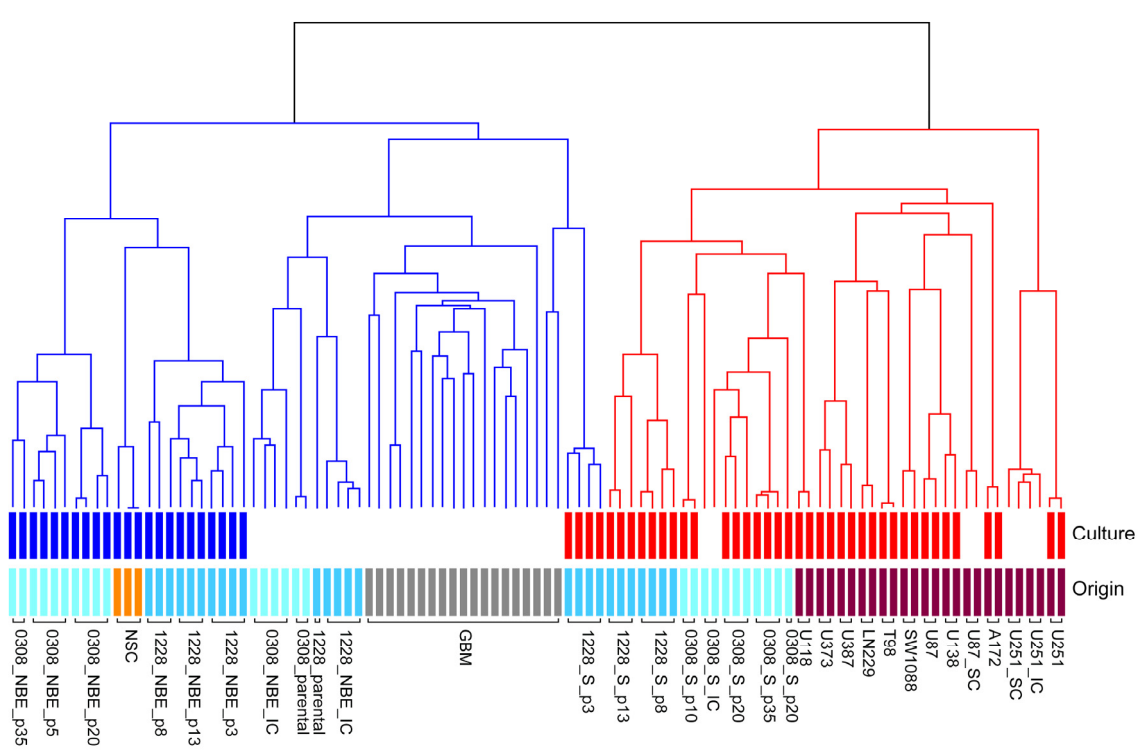


**B**



**Figure S4.** Dendrogram of cluster determined by supervised hierarchical cluster analysis using “NSC-enriched gene sets” as classifiers

See text for the detail.



**Table S1.** Clonogenicity of GBM cells cultured in NBE and serum conditions

Description of cells		Numbers of colonies ( $\pm$ s.d)	
Origin	Passage	NBE-agar	serum-agar
0308-NBE	p3	254.8 ( $\pm$ 23.4)	0
0308-NBE	p10	218.5 ( $\pm$ 48.2)	0
0308-NBE	p20	207.3 ( $\pm$ 34.2)	1.3 ( $\pm$ 1.3)
0308-NBE	p50	294.0 ( $\pm$ 25.7)	2.0 ( $\pm$ 0.8)
0308-serum	p3	1.3 ( $\pm$ 1.0)	0
0308-serum	p10	0	74.0 ( $\pm$ 32.5)
0308-serum	p20	23.8 ( $\pm$ 6.2)	348.5 ( $\pm$ 35.7)
0308-serum	p50	10.9 ( $\pm$ 4.5)	405.9 ( $\pm$ 15.1)
1228-NBE	p3	224.8 ( $\pm$ 13.4)	0
1228-NBE	p10	263.9 ( $\pm$ 14.0)	1.5 ( $\pm$ 0.7)
1228-NBE	p30	285.8 ( $\pm$ 22.6)	0
1228-serum	p3	0	11.5 ( $\pm$ 4.5)
1228-serum	p10	11.2 ( $\pm$ 5.3)	311.2 ( $\pm$ 15.3)
1228-serum	p30	4.5 ( $\pm$ 2.7)	318.5 ( $\pm$ 25.1)
U87MG (glioma cell line)		9.5 ( $\pm$ 4.5)	372.3 ( $\pm$ 52.5)
U251 (glioma cell line)		89.3 ( $\pm$ 39.9)	455.3 ( $\pm$ 51.7)

Three weeks after seeding the cells into NBE-agar and serum-agar, we counted the number of colonies (> 100  $\mu$ m diameter) in 3 random fields per sample in triplicates. Data from a representative experiment are shown.

**Table S2.** Tumorigenicity of NBE- and serum-cultured GBM cells at various passages

	Cells	Injection site	Cell number injected	Death by tumor/ mice	Median survival (weeks)
	NSC	brain	20000	0/15	-
	0308-NBE p3	brain	20000	9/9	9.6
	0308-NBE p3	brain	1000	7/8	15
	0308-NBE p10	brain	20000	7/7	11.4
	0308-NBE p20	brain	20000	5/5	12.8
	0308-NBE p35	brain	20000	10/11	11
	0308-NBE p50	brain	20000	8/8	13.8
	0308-serum p3	brain	20000	0/6	-
	0308-serum p3	brain	100000	0/7	-
	0308-serum p5	brain	100000	0/7	-
	0308-serum p10	brain	100000	1/13	*
	0308-serum p15	brain	100000	2/6	**
	0308-serum p20	brain	100000	8/14	16
	0308-serum p25	brain	100000	11/12	14.4
	0308-serum p35	brain	100000	7/7	10.8
	0308-serum p10	s.c	2 million	0/5	
	0308-serum p32	s.c	2 million	4/4	
	1228-NBE p3	brain	20000	7/7	11
	1228-NBE p3	brain	1000	8/8	12.8
	1228-NBE p20	brain	20000	5/5	10.6
	1228-serum p3	brain	100000	0/8	-
	1228-serum p10	brain	100000	0/7	-
	1228-serum p20	brain	100000	0/7	-

\* Tumor detected 18 weeks after injection (\*\* 15, 17 weeks).

- Tumor not detected more than 6 months after injection.

**Table S3.** Serial transplantation of 0308-NBE cell-derived tumors

Serial transplantation of 0308-NBE cell-derived tumors				
	Cells	Injection site	Cell number	Incidence of tumor/ mice
Secondary transfer	Orthotopic xenograft tumor derived from 0308-NBE-p3 cells; without culture	brain	N.D.	7/7
	additionally cultured in NBE condition for 3 passages	brain	20000	8/8
	additionally cultured in serum condition for 3 passages	brain	20000	0/11*
	additionally cultured in serum condition for 10 passages	brain	100000	2/7
Tertiary transfer	Dissociated from secondary xenograft tumor tissues,			
	additionally cultured in NBE condition for 3 passages	brain	20000	7/7
	additionally cultured in serum condition for 3 passages	brain	20000	0/12*

\* Tumors not detected 6 months after injection.

N.D. Not determined

**Table S4.** Two-way ANOVA analysis of 1228-NBE and serum cells at different passages

	Number of probe sets differentially expressed between samples					
	NBE p3	NBE p8	NBE p13	Serum p3	Serum p8	Serum p13
NBE p3	-					
NBE p8	602	-				
NBE p13	476	28	-			
Serum p3				-		
Serum p8				4410	-	
Serum p13				5637	1410	-

In order to determine the potential effects of the culture conditions (NBE versus serum conditions) and *in vitro* passage on gene expression of 1228-GBM cells, we performed two-way ANOVA analysis. The numbers of probe sets differentially expressed (more than 2-fold up or down-regulation) between the samples are presented. Note that over 4000 probe sets are differentially regulated in early (p3) versus later (p8 and p13) passage serum cells. We could not perform similar analysis on 0308 cells because of limited amount of early passage serum cells.

**Table S5.** Functional annotation of 2029 probe sets highly expressed in early compared to late passage 1228-serum cells

	<b>Function</b>	<b>No. of genes</b>
1549 probe sets	Nervous system development and function	168
	Organ development	120
	Cell to cell signaling and interaction	116
	Cell morphology	89
	Cellular assembly and organization	86
	Cellular development	80
	Cellular movement	77
	Tissue development	71
	Neurological diseases	49
	Cancer	49
480 probe sets	Immune response	104
	Hematological system development and function	86
	Immune and lymphatic system development	82
	Cell to cell signaling and interaction	77
	Cell death	70
	Cellular growth and proliferation	65
	Cellular development	57
	Cellular movement	55
	Cell signaling	55
	Cancer	53

Based on Ingenuity pathway analysis (<http://www.ingenuity.com>).

Biological functions were assigned to each gene network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base. The biological functions assigned to each network are ranked according to the significance of that biological function to the network. A Fischer's exact test is used to calculate a p-value determining the probability that the biological function assigned to that network is explained by chance alone. All listed pathways were predicted with  $p < 0.001$ .

**Table S6.** Functional annotation of probe sets differentially regulated in NBE- versus serum-cultured GBM cells

	<b>Function</b>	<b>No. of genes</b>
1864 probe sets (> 2 fold expression in both 0308-and 1228-NBE cells compared to serum-cells)	Nervous system development and function	174
	Organ development	140
	Tissue development	114
	Cell to cell signaling and interaction	106
	Cellular development	86
	Cellular assembly and organization	85
	Cell morphology	81
	Neurological diseases	59
1444 probe sets (> 2 fold expression in both 0308-and 1228-serum cells compared to NBE-cells)	Cellular growth and proliferation	229
	Cancer	199
	Cell death	186
	Cardiovascular development and function	157
	Cellular development	144
	Cellular movement	141
	Cell to cell signaling and interaction	138
	Tissue morphology	131

Based on Ingenuity pathway analysis.

All listed pathways were predicted with  $p < 0.001$ .

**Table S7.** Representative gene lists selected from “NBE-specific probe sets”

Gene name	Official Symbol	In vitro			Xenograft			Patient GBM	tissues 0308+1228
		NBE	NSC	Cell lines	NBE_IC	S_IC	cell_line_tumor		
ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	2.6	1.9	1	2	1.9	1.1	2.9	2.7
ATP-binding cassette, sub-family G (WHITE), member 1	ABCG1	3.9	2.1	1.5	4.1	2.2	1.3	8.7	12.0
Abi interactor 2	ABI2	3.5	3.4	1.4	4.1	1.8	1.6	3.0	3.8
ankyrin 2, neuronal	ANK2	4.1	8.4	0.8	3.0	1.9	1.4	5.0	4.2
aquaporin 4	AQP4	79.9	51.6	0.9	711.9	16.6	1.3	1305.3	947.1
bone morphogenetic protein 7 (osteogenic protein 1)	BMP7	19.2	44.8	2.8	48.1	4.6	1.6	17.1	25.3
calbindin 1, 28kDa	CALB1	20.4	3.2	1.4	7.3	8.2	1.7	4	8.1
cyclin D2	CCND2	63.3	84.0	0.3	34.3	13.0	0.5	39.9	45.1
cortixin 1	CTXN1	3.7	4.4	0.7	4.3	1.4	0.6	2.5	4.8
chemokine (C-X-C motif) receptor 4	CXCR4	15.6	11.1	0.2	3.9	3.2	1.9	14.6	11.4
delta-like 1 (Drosophila)	DLL1	33.5	119.7	1	79.2	14.4	16.2	52	68.9
delta-like 3 (Drosophila)	DLL3	3.4	11.1	0.9	12.1	1.3	1	4.2	13.2
E2F transcription factor 5, p130-binding	E2F5	3.3	4	1.1	2.6	1.2	1	1.5	1.9
ephrin-A1	EFNA1	7	1	3.9	3.2	3.2	2.9	10.5	8.1
ephrin-A3	EFNA3	79.3	61.8	16.9	189.1	129.8	101.6	27.6	168.5
FYN oncogene related to SRC, FGR, YES	FYN	2.7	2.3	0.9	3.4	2.2	1.3	2.9	3.4
histone deacetylase 4	HDAC4	2.4	2.9	1.7	2.7	1.9	1.9	3	4
Homer homolog 1 (Drosophila)	HOMER1	5.5	6.8	1.3	7.3	3.6	1.8	5.5	4.3
inhibitor of DNA binding 4, dominant negative Helix-loop-helix protein	ID4	7.3	8.2	0.9	11.4	2.8	0.3	13.2	9.8
LIM domain only 2 (rhombotin-like 1)	LMO2	9.7	17	0.8	7.9	4.6	1.6	18.4	18.2
myeloid ecotropic viral integration site 1 homolog (mouse)	MEIS1	6.6	1.9	1.3	5	2.2	1.9	1.8	2.7
musashi homolog 1 (Drosophila)	MSI1	15.9	19.3	0.9	15.5	1	1.4	8.9	14.1
Musashi homolog 2 (Drosophila)	MSI2	4.2	3.5	1.4	4.5	2.1	2.6	3.9	3.5
nestin	NES	3.1	2.1	0.4	2.6	1.1	2	2.2	2.1
Notch homolog 1, translocation-associated (Drosophila)	NOTCH1	3.8	3.1	1.1	5.5	1.1	1.1	2.8	4.1
Notch homolog 4 (Drosophila)	NOTCH4	2.3	1.5	0.7	2.7	1	1.1	2.5	2.2
oligodendrocyte transcription factor 1	OLIG1	49.7	23.3	0.9	83.2	14.6	1.1	54.9	72.1
oligodendrocyte lineage transcription factor 2	OLIG2	48.3	7.3	1.1	68	7.3	0.3	36.1	68.6
orthodenticle homolog 2 (Drosophila)	OTX2	7.7	12.8	1.1	20	11.3	11.5	14.9	9.1
P300/CBP-associated factor	PCAF	2.5	2.5	1.1	4.0	1.5	1.7	4.8	3.5
POU domain, class 3, transcription factor 2	POU3F2	5.2	3.6	0.8	6	1.1	1.8	4.1	4.4
protein kinase C, zeta	PRKCCZ	5.1	4.4	0.9	7.8	1.4	1.7	3.3	11
prominin 1	PROM1	7.9	8.2	1.7	18.3	6.4	1.8	20	76
Prospero-related homeobox 1	PROX1	8.3	3.3	1.1	4.1	1.3	1.8	5.7	4.5
Patched homolog (Drosophila)	PTCH	4.7	9.8	2.1	5.1	2	1.6	4.7	3.7
patched domain containing 1	PTCHD1	6.9	0.9	1	10.2	5.6	1.9	7.3	9
RAS p21 protein activator (GTPase activating protein) 1	RASA1	3	2	2.9	4.6	1.8	3.8	13.4	6.6
roundabout, axon guidance receptor, homolog 2 (Drosophila)	ROBO2	5.1	14.4	0.4	16.9	6.5	0.4	8.3	8.6
SMAD, mothers against DPP homolog 1 (Drosophila)	SMAD1	3.6	2.2	0.8	2.8	1.9	1.8	3.2	2.6
SRY (sex determining region Y)-box 11	SOX11	3.8	6.8	0.1	7.2	1.3	0.1	2.0	7.3
SRY (sex determining region Y)-box 13	SOX13	3.2	0.3	1.5	3.2	1.1	2.4	3.2	2.9
SRY (sex determining region Y)-box 2	SOX2	10.9	14.5	0.7	14.5	4.9	3.1	8.3	12.1
SRY (sex determining region Y)-box 6	SOX6	1.9	2.4	1.2	2.4	1.3	1.7	2.5	2.9
SRY (sex determining region Y)-box 9 (campomelic dysplasia)	SOX9	2.7	3.4	0.7	3.4	1.3	2.4	3.3	3.5
tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase	TNKS	2.4	2.2	1.1	2.3	1.4	1.3	1.7	2.3

Expression levels of 45 unique NBE-specific genes in various samples were evaluated compared to late passage serum cells.

All 101 samples were divided into subgroups; NBE represents the average signal intensity of 0308- and 1228-NBE cells at all passages *in vitro*.

Cell lines indicate the average of 10 glioma cell lines. Serum represents the average signal intensity of all serum-cultured cells except 1228-serum p3 cell.

Fold changes of all samples were calculated relative to the value of serum cells (set to 1).

**Table S8.** Representative gene lists selected from “NSC-enriched probe sets”

Gene name	Official Symbol	In vitro			Xenograft			Patient tissues	
		NBE	NSC	Cell lines	NBE_IC	S_IC	cell_line_tumor	GBM	0308+1228
calcium/calmodulin-dependent serine protein kinase (MAGUK family)	CASK	1.9	2.2	1.2	3.3	1.2	0.7	1.4	2.3
chondroitin sulfate proteoglycan 3 (neurocan)	CSPG3	22.3	67.9	0.3	55.3	5.2	0.4	34.0	51.6
CUG triplet repeat, RNA binding protein 2	CUGBP2	2.1	2.4	0.8	2.3	1.7	1.2	2.4	2.7
discoidin domain receptor family, member 1	DDR1	3.3	3.0	0.9	3.5	2.2	1.7	3.5	3.4
delta-like 1 (Drosophila)	DLL1	23.5	56.1	2.4	44.0	6.4	7.5	34.4	33.7
dual specificity phosphatase 16	DUSP16	1.9	1.4	1.3	1.5	1.2	1.3	2.3	2.0
FYN oncogene related to SRC, FGR, YES	FYN	2.9	2.5	1.0	4.0	2.0	1.3	3.7	3.5
GRB2-associated binding protein 1	GAB1	4.2	4.5	1.0	3.9	1.6	1.4	4.1	4.3
G protein-coupled receptor 56	GPR56	3.5	4.9	1.2	5.2	1.6	2.3	4.4	4.9
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	HMGCS1	3.0	3.4	0.6	2.2	0.8	0.5	0.9	1.4
inhibin, beta B (activin AB beta polypeptide)	INHBB	13.4	1.9	1.1	18.5	1.8	8.1	25.2	23.0
LIM domain only 1 (rhombotin 1)	LMO1	3.7	8.9	0.7	7.2	1.5	1.4	2.1	5.7
myosin X	MYO10	1.6	2.4	1.0	1.6	0.9	0.8	1.1	1.1
nestin	NES	2.7	1.8	0.5	2.3	1.0	1.8	2.0	1.8
Notch homolog 1, translocation-associated (Drosophila)	NOTCH1	3.5	2.8	1.1	5.4	1.0	1.1	2.8	3.9
Pellino homolog 1 (Drosophila)	PELL1	3.0	3.4	1.5	4.6	1.7	2.9	4.8	5.2
pleiotrophin (heparin binding growth factor 8)	PTN	5.4	5.0	0.7	5.2	2.8	2.1	4.1	4.3
protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	11.8	11.0	0.4	14.8	5.4	3.3	12.0	13.9
sal-like 3 (Drosophila)	SALL3	3.7	9.1	1.0	11.2	1.5	2.6	6.8	4.2
signal-induced proliferation-associated 1 like 2	SIPA1L2	2.6	3.2	1.7	2.5	2.3	1.3	2.8	2.6
solute carrier family 15 (H+/peptide transporter), member 2	SLC15A2	15.0	28.7	0.9	19.6	3.2	2.4	14.1	14.9
SRY (sex determining region Y)-box 2	SOX2	7.6	9.9	1.8	10.2	3.4	4.4	6.4	8.4
Spermatogenesis associated 6	SPATA6	13.9	12.8	2.1	13.8	2.8	7.6	8.1	9.7
transcription factor 8 (represses interleukin 2 expression)	TCF8	2.2	3.6	0.9	3.0	1.2	1.1	2.4	3.0

Expression levels of 24 unique "NSC-enriched probe sets" in various samples were evaluated compared to late passage serum cells.

All 101 samples were divided into subgroups; NBE represents the average signal intensity of 0308- and 1228-NBE cells at all passages *in vitro*.

Cell lines indicate the average of 10 glioma cell lines. Serum represents the average signal intensity of all serum-cultured cells except 1228-serum p3

Fold changes of all samples were calculated relative to the value of serum cells (set to 1).

**Table S9.** Characterization of 0308- and 1228-GBM cells

Patient information		Description
0308	37 y.o. male	Homozygous deletion of INK4a/ARF locus (chromosome 9) Loss of chromosome 10q Trisomy of chromosomes 7 and 20 Partial trisomy of chromosome 19 Translocation t(10;21) Local amplification of EGFR (about 6 copies of EGFR/cell) PTEN mutation (nonsense mutation at amino acid 76) P53 mutation (M237V, point mutation in DNA binding domain)
1228	64 y.o. female	Homozygous deletion of INK4a/ARF locus (chromosome 9) Loss of the entire chromosome 10 Trisomy of chromosome 7 Translocation t(9;22) Local amplification of EGFR (about 6 copies of EGFR/cell) PTEN mutation (Q171L, point mutation in phosphatase domain) Wild type p53