Malignant Gliomas and Neural Stem Cells Gone Bad: The Biological and Therapeutic Implications of a Changing Paradigm

National Cancer Advisory Board Meeting; 11/30/06

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National Institute of Neurological Disorders and Stroke
National Institutes of Health

The NIH Neuro-Oncology Branch

Mission: *A joint effort by the National Cancer Institute and the National Institute for Neurological Disorders and Stroke in collaboration with the extramural community, private sector and patient advocates, to develop novel diagnostic and therapeutic modalities for children and adults with brain and spinal cord tumors.*

Strategy: To leverage the unique central position and large resources of the intramural program to synergize with, and add value to new and on-going activities in the extramural community and private sector.

Primary Brain Tumors

- The leading cause of cancer-related deaths in children
- A leading cause of cancer-related deaths in people under the age of 54
- Significant increase in incidence in people over the age of 60 years old (? screening artifact)
- Malignant gliomas the most common primary brain tumor
- Glioblastoma the most common glioma

Therapeutic Advances in Glioblastoma

- **Essentially no highly significant improvement in survival in the last 30 years.**
- **The very few long term survivors (especially children) face significant life-long neuro-cognitive deficits.**

Novel Therapeutic Approaches are Desperately Needed

Neuro-Oncology Branch (NOB) Initiatives: NCAB Presentations and a Quick Update

- **Clinical Trials Program** ***[NCAB, 2003]***
  - **NOB Brain Tumor Clinic**
  - **CTEP-sponsored brain tumor consortia and cooperative groups**
- **New drug development program** ***[NCAB, 2005]***
  - **Pharmaceutical/Biotechnology collaborations**
  - **CTEP**
  - **NOB preclinical screening program**
    - ✓ ***In vitro*** **screens: glioma cells lines, primary glioma cells, primary tumor stem cells**
    - ✓ ***In vivo*** **screen: Animal Brain Tumor Therapeutics Core**
    - ✓ **Bioassays/biomarkers development**
- **GMDI/REMBRANDT** ***[NCAB, 2004]***
  - **Novel target identification**
    - ✓ **"Wet-lab" projects**
- **Translational/Biological Approaches** ***[NCAB, Today]***
  - **Cancer Stem Cells**

{Clinical Trials Working Group: J. Doroshow; H. Fine [NCAB, 2004, 2005]

Clinical Trials/Drug Development Program: Establishment of Extramural Collaborations

- **Is a full member of all three NCI/CTEP-sponsored brain tumor consortia**
  - **NABTC**
  - **NABTT**
  - **PBTC**
- **Preclinical/Translational/Clinical projects with:**
  - **> 20 academic medical/cancer centers nationally**
- **Close collaborative clinical programs with:**
  - **Johns Hopkins Medical Center,**
  - **George Washington Medical Center,**
  - **Georgetown Medical Center**
  - **Fairfax INOVA and**
  - **Washington Hospital Center and a**
  - **Wide array of private neurosurgical, radiation, and oncology practice groups locally and nationally**
  - **Children's National Medical Center**
- **Provides neuro-oncology services for:**
  - **Walter Reed Medical Center**
  - **Portsmouth Naval Hospital**
  - **Naval Medical Center in Bethesda**

Clinical Trials/Drug Development Program: Pharmaceutical/Biotechnology Partners

- Human Genome Sciences
- Infinity
- Ivax
- Merck
- NeoPharm
- NeroSystec
- SibTech, Inc.
- Sugen
- Technion
- The Translational Genomics Research Institute
- Virxsys
- TransMolecular
- Angstrom
- AstraZeneca
- Attenuon
- BBB Technologies, Inc.
- BrainsGate, LTD
- Bristol-Myers Squibb
- Celgene
- Cell Genesys
- Celmed
- Eli Lilly
- Genetec
- 20/20 Gene System, Inc.
- Millennium

• Established 8 Cooperative Research and Development Agreements (CRADAs) between NCI and industry; currently negotiating number of additional CRADAs . Signed > 25 active confidential disclosure agreements with biotechnology and large pharmaceutical companies for co-developing new agents; many will likely lead to additional CRADAs.

The NOB Clinical Research Program: Portfolio Agents in Preclinical and Clinical Development

- CC5013 (A)
- SU5416 (A) *
- LY317615 (A)
- CC8490 (A)
- HTR-Ab1,2(A) [preclinical]
- STI-571 (A)*
- OSI-774 (A)*
- R115777 (A)*
- Talampanel (A+P)
- ZD 6474 (A)
- Depsipeptide (A)*
- Valproic Acid (A)
- GSI 11, 13 (A) [preclinical]
- Bevacizumab (A)
- Bortezomib (A)
- Cyclopropamine (A, P) [preclinical]
- IF 609 (A+P) [preclinical]
- ATN-161 (A)
- TM-601 (A)
- A7 (A) [Preclinical
- ATN-224 (A)
- lapatinib (A) *
- Iressa (A) [Preclinical]
- SAHA (A) *
- GW86034 (A)
- Pegylated IFN (A+P)
- IT Gemcitabine (A+P)
- Cereport (P)
- IT Topotecan (P)
- O6BG/Temodar (P)
- Liposomal doxorubicin (P)
- Phenylbutyrate (P)
- IT Mafosfamide (P) *
- XR-9576 (P)
- BMS-247550 (P)
- ABT-751 (P)
- MLN-518 (A)

A: adults, P: pediatric * Consortia trials

*NOB Clinical and Therapeutics Development Program**

- **43 IRB approved and activated NOB clinical trials for primary brain tumors over the last 5 years. These trials include:**
  - **31 adult studies; 12 pediatric studies**
  - **10 national brain tumor consortia trials chaired by NOB investigators (7 adult; 3 pediatric)**
    - ✓ **NOB Investigator P.I. of the only on-going international phase III registration trial of a systemic agent for recurrent glioblastoma (that was developed by the NOB: Enzastaurin)**
- **Drug development/clinical research program supported by a very active clinical program. Over the last 12 months:**
  - **Accrue 200-300 primary brain tumor patients to NOB IRB-approved clinical trials/yr**
  - **>500 new glioma patients seen/yr**
  - **2000-3000 glioma patients seen in follow-up/yr**
  - **Review 150-200 new patient film consults/yr**

*** NOB has only one lab and two tenure track investigators**

Glioma Molecular Diagnostic Initiative "GMDI"

The goal of the GMDI is to create a publicly accessible web-based glioma data base, and informatics platform consisting of in depth pathologic, molecular and genetic data with detailed clinical corollary data for hundreds of individual brain tumors.

Data base should be invaluable for basic scientists for aiding in:

- ***tumor lineage determination***
- ***gene discovery***
- ***new target identification and validation***

Data base may be invaluable for clinical investigators for:

- ***a prognostically more meaningful classification system.***
- ***toward individual patient-based therapy selection***

.

The Glioma Molecular Diagnostic Initiative

- **A national study through 2 NCI-funded brain tumor consortia**
- **More than 1000 patients with gliomas to be accrued**
- **Extensive prospective clinical data to be correlated with molecular data**

Objectives:

- Produce a **biologically significant pathological classification of gliomas**, with strong correlation to outcome of disease, that would allow for better prognostication and more informed therapeutic decision making.

- Through the informatic mining of extensive molecular/genetic data to be able to **find new molecular targets** for therapy that would be relevant both to the disease type and patient status.

- Produce a **publicly accessible database containing all the aforementioned data, that will contain the analysis tools necessary for all ends of the research spectrum** (clinicians, chemists, geneticist, bioinformaticians, etc) to profit from the wealth of information stored, produce new models of disease, and envision new targeted therapies.

GMDI: Biospecimens Collected

Blood

Tumor

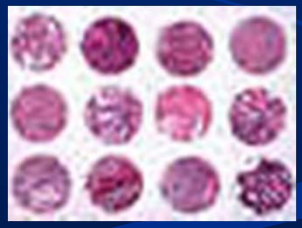
Plasma

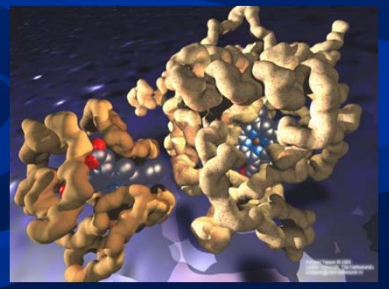
DNA

RNA

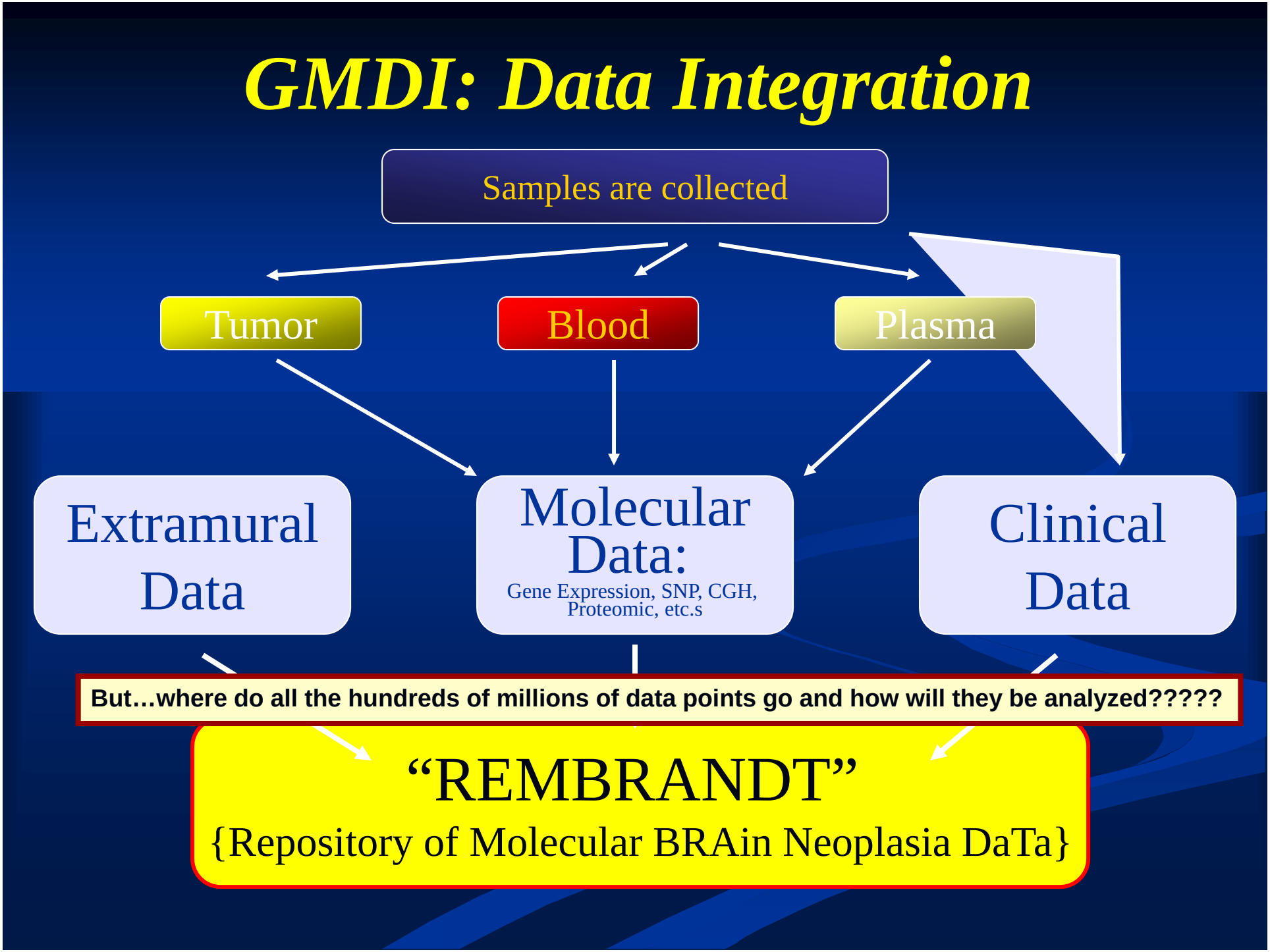
Tumor Core Punch

Proteins





GMDI: Data Integration



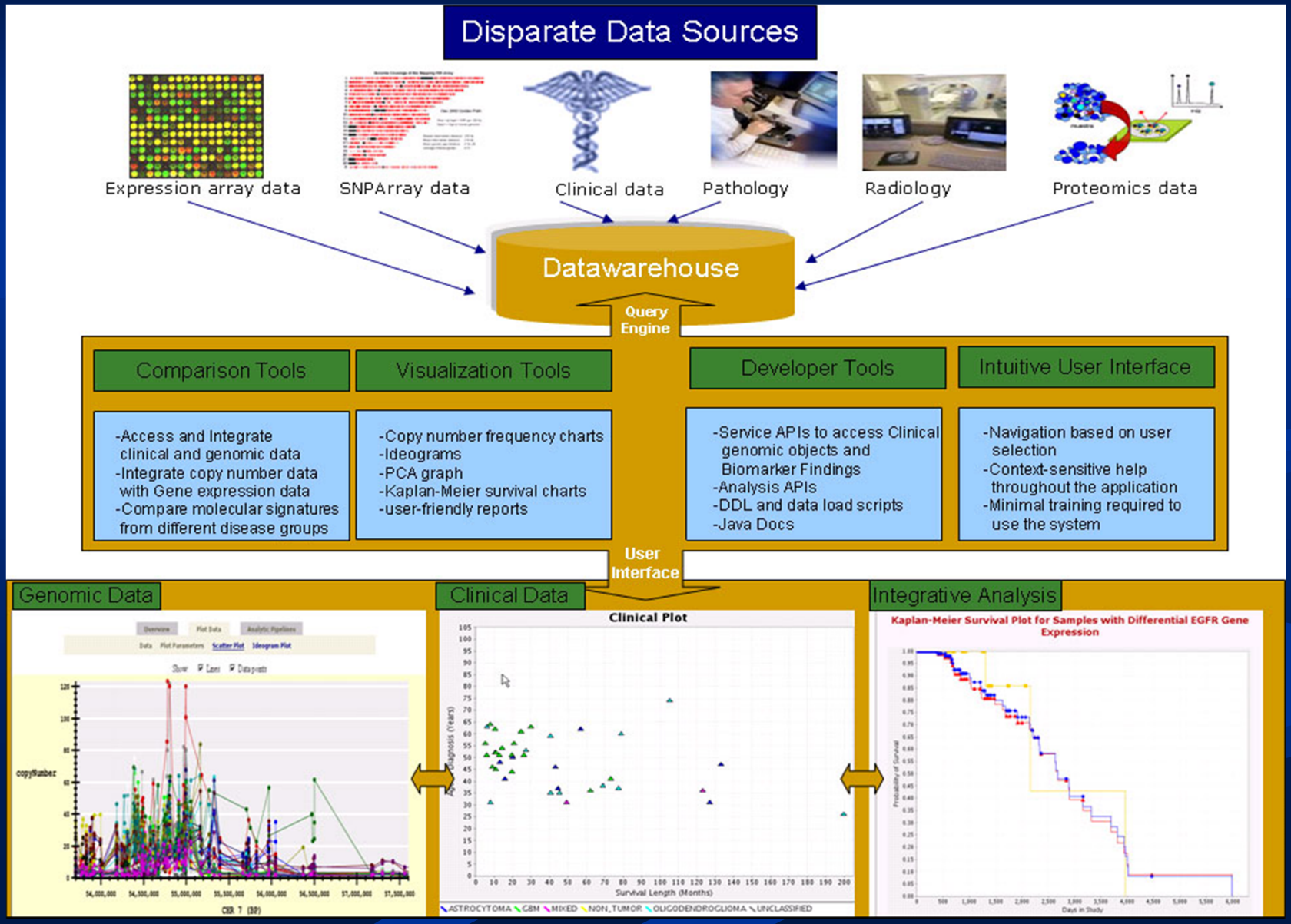
Current Status of the GMDI

- **Retrospective Study**
  - **~300 tumors analyzed for gene expression, 100K SNP and CGH.**
- **Prospective study**
  - **Funding/coordination through the Neuro-Oncology Branch.**
  - **Active Cores**
    - ✓ **Long oligo "Glioma RNA Expression Chip" built and mass produced**
    - ✓ **High-throughput Affymetrix-based gene expression and 100K SNP core.**
    - ✓ **Golden pathway CGH contracted to extramural lab (UCSF).**
    - ✓ **Extramural Neuropathology Core (MDACC/MSKCC/MGH).**
  - **National protocol, NCI intramural and CTEP-approved, and now open at ~18 extramural institutions through both the NABTC and NABTT consortia as well as the brain SPORE institutions and the DC/Bethesda consortia.**
  - **350 glioma Tissue arrays produced**
  - **~ 400 patients have been accrued nationally; molecular data generated, clinical data being gathered (unparalleled by any other tumor type).**
  - **Molecular/genetic data from ~ 500 patients made public…..REMBRANDT**
  - **NINDS collaborating in REMBRANDT funding initiative.**

REMBRANDT Web Site: Gone Live!

http://rembrandt-db.nci.nih.gov/rembrandt/

Data Integration via Rembrandt



REMBRANDT: Current Status

- **Live and running**
- **Number of unique patient samples in the database - ~500**
- **Gene expression data points - 20 million**
- **Copy number data points - 35 million**
- **Hundreds of registered users**
- **Average time spent per session - 45 minutes**
- **Longest visit in June, 2006 – 283 minutes**
- **REMBRANDT and The REMBRANDT team was awarded the Congressional *"Service to America Award"* for science and technology in 2006 (having beat out NASA for the Mars Rover!!!!)**

Lessons Learned over 6 year history of the NOB

The Intramural Program can be used to leverage its unique position and resources to provide clinical expertise for patients with tumors not well understood or cared for in the community and to produce value added to the overall cancer research enterprise.

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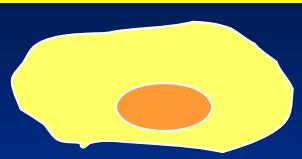
Glioblastomas Originate from Mature Astrocytes: Classical Model



Astrocyte

-EGFR amplification/mutation (7p12)

-INK 4A loss (9q26)

-LOH 10
-PTEN mutation (10q24)

-RB alterations (13q13)

Astrocytoma

Anaplastic Astrocytoma

-PDGF, FGF2 over-expression
-P53 mutation

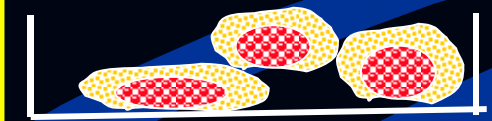
-CDK4 amplification(12q13)
-RB loss
-LOH 19q

-LOH 10q
-PTEN mutation

Primary GBM

Secondary GBM

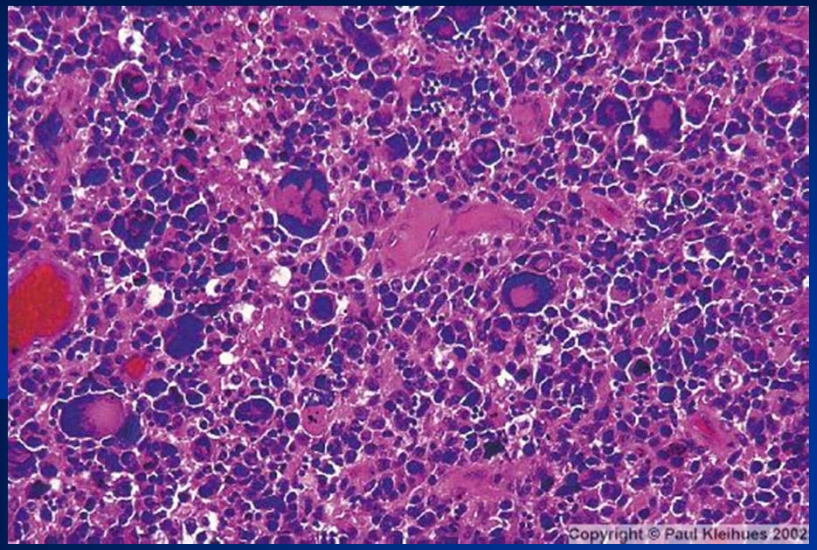
Our current preclinical Cell lines/model

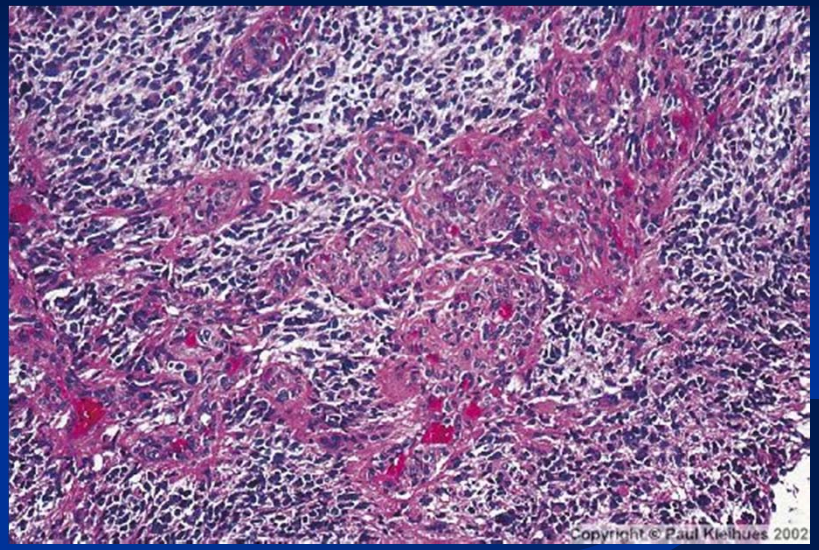


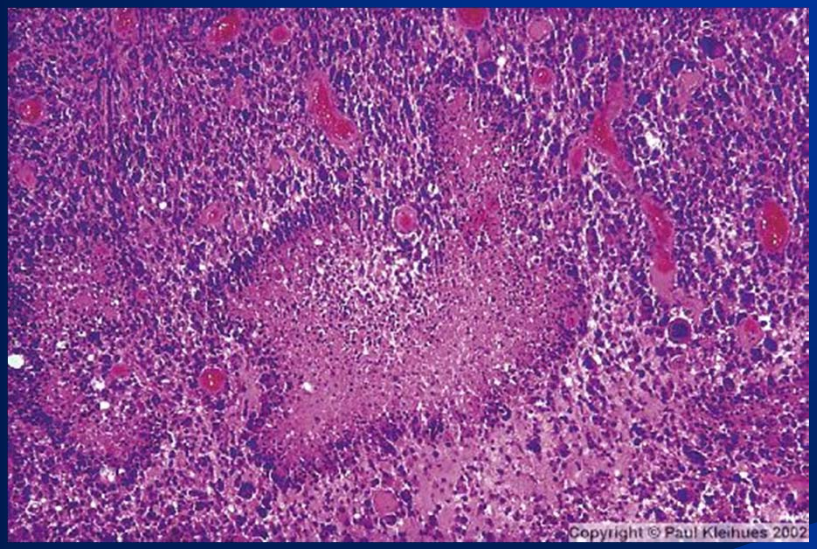
Isolate and study serially passaged tumor cells in serum just like normal astrocytes

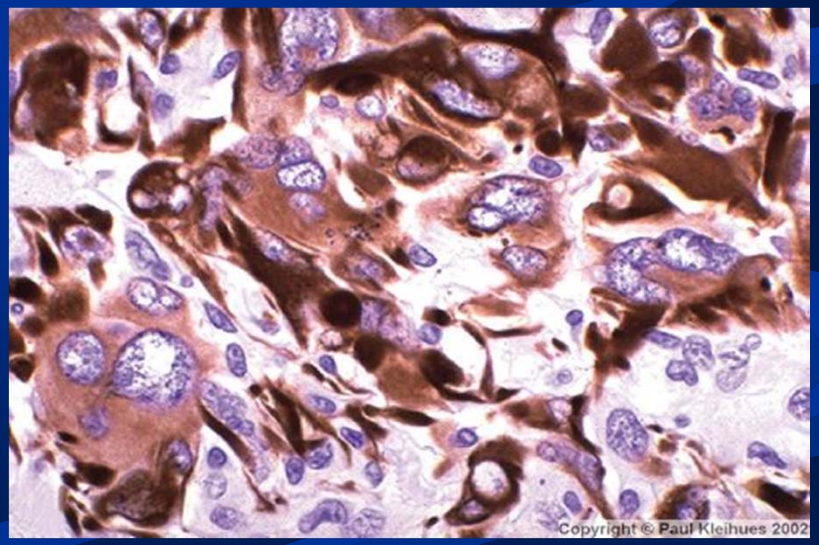
But…….our current in vitro and preclinical xenograft models of human GBMs look nothing like human GBMs

Glioblastoma



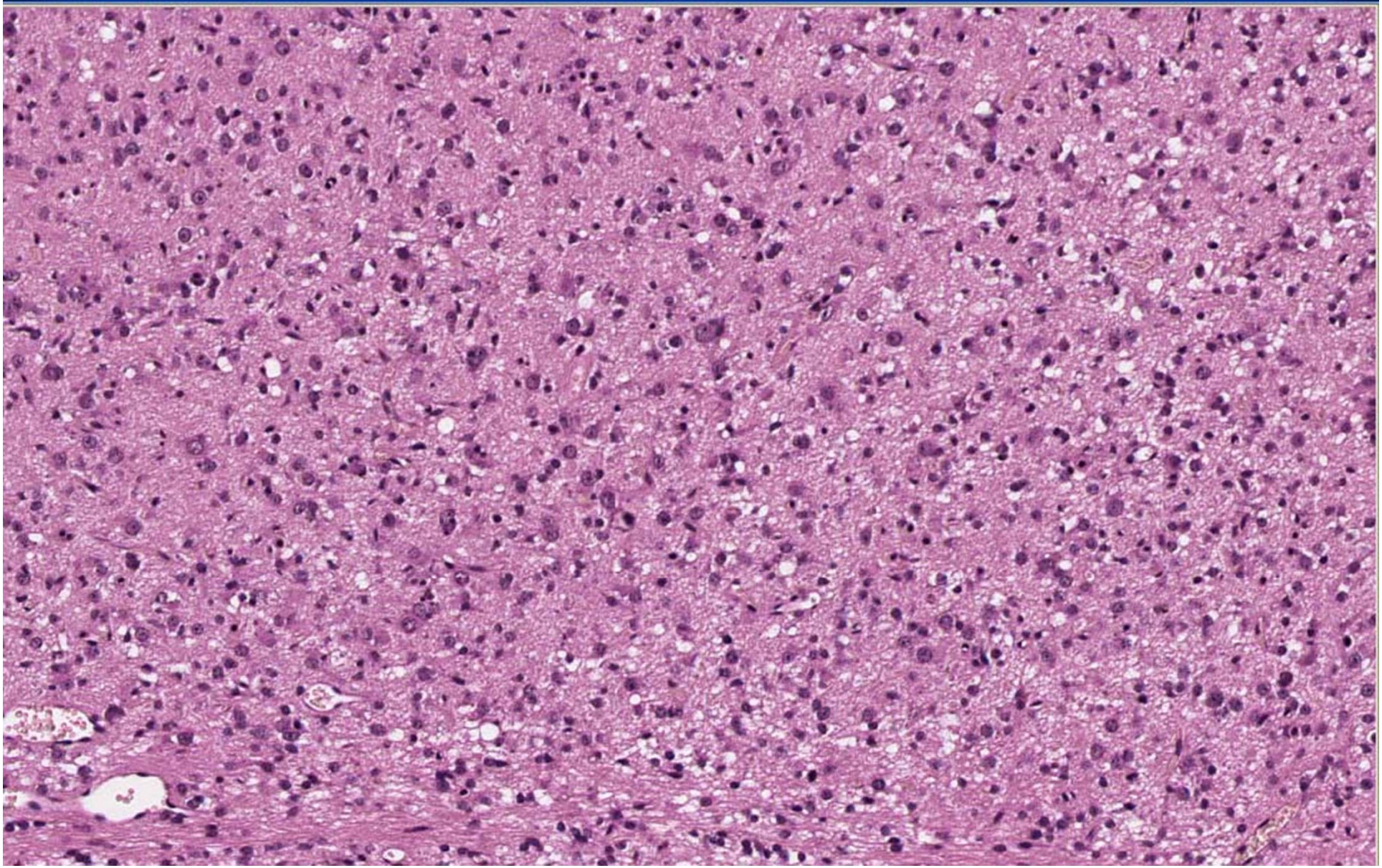




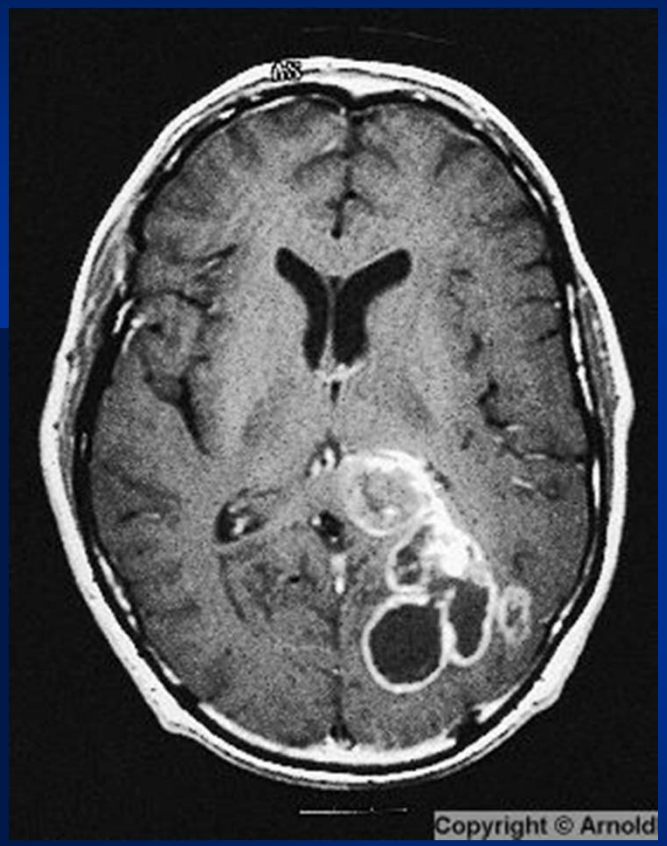


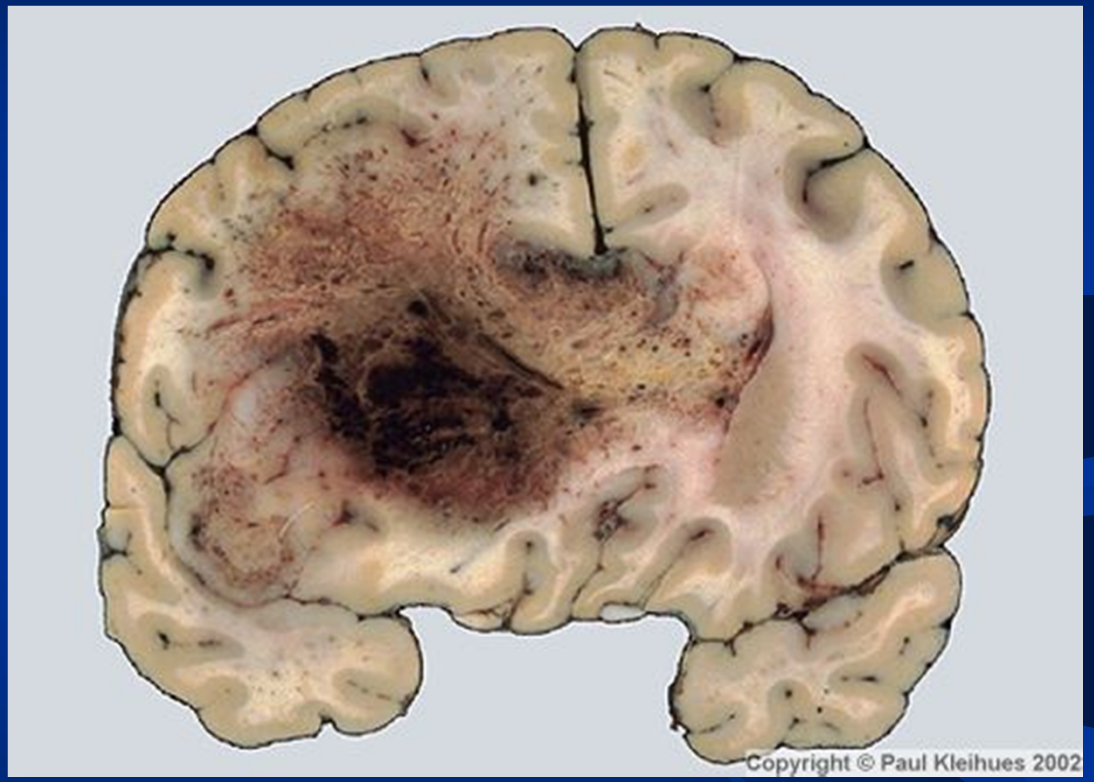
Lantos PL, Louis DN, Rosenblum M, Kleihues P.
Tumours of the nervous system. In: Greenfield's Neuropathology, 2002

Glioma Xenograft



Glioblastoma





Lantos PL, Louis DN, Rosenblum M, Kleihues P.
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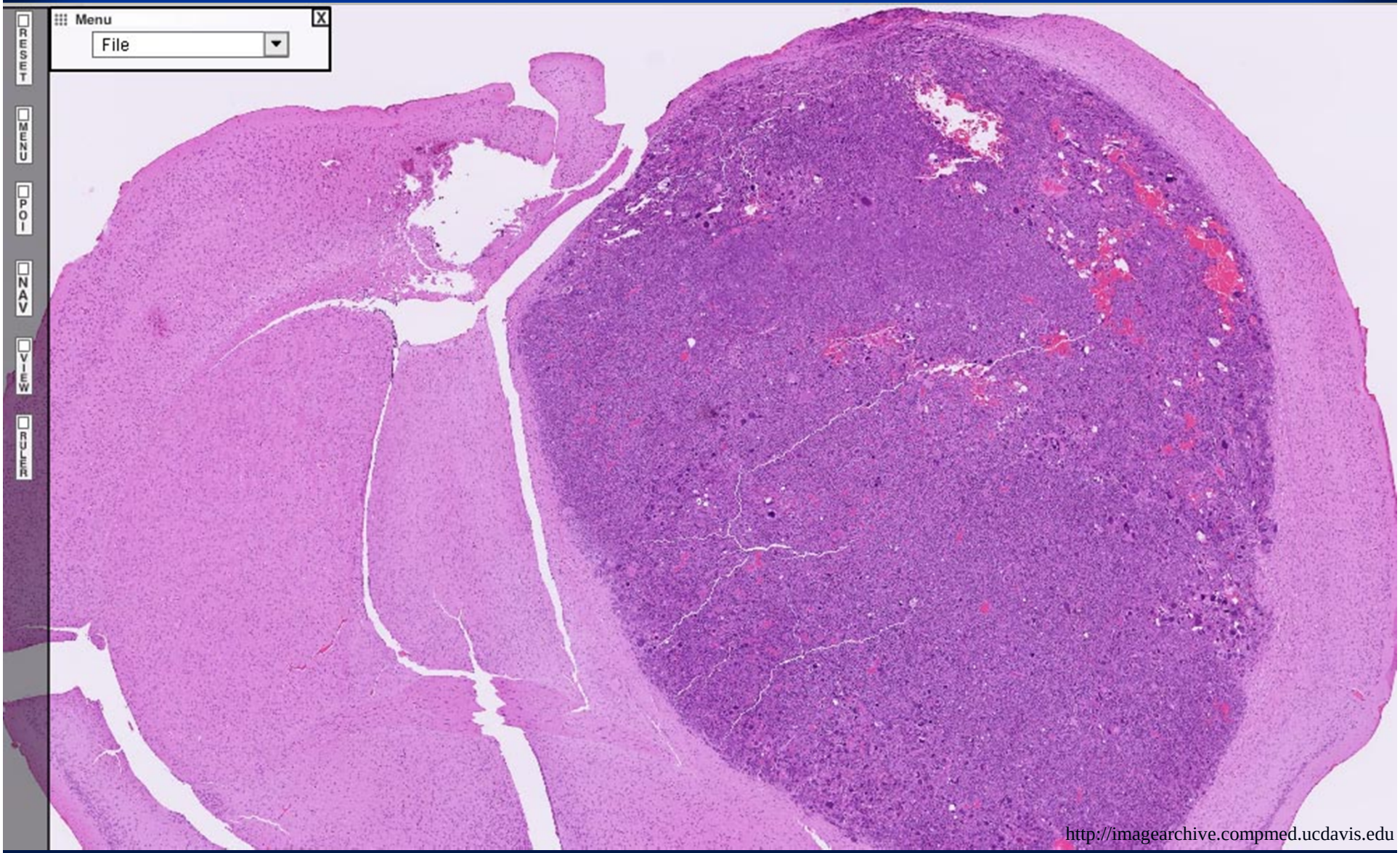
Human Glioma Xenograft



***Premise*: The efficient development of rationale cancer therapy depends on a knowledge of tumor pathogenesis, pathophysiology and predictive preclinical models**

- Most glioma molecular and preclinical studies/models have utilized glioma cell lines that have been created and propagated consistent with their presumed origin from normal astrocytes. **BUT**
- Human gliomas have very few characteristics of normal astrocytes.
  - Morphology (GFAP expression)
- Human gliomas do harbor a number of characteristics of normal neural stem cells (NSC):
  - Self renewal capabilities
  - Immortalization
  - Proliferation
  - Capable of generating progeny that are morphologically and functionally heterogeneous

***Hypothesis:* Gliomas are a stem cell disease and therefore glioma/tumor stem cells may represent a more accurate preclinical model of human tumors than do traditional cancer cell lines?**

Testing the Hypothesis

Grow primary tumor explants under conditions optimal for the proliferation and non-differentiation of normal neural stem cells (NSC

Primary Glioblastoma
from patients

dissociation

Serum-free media
With bFGF + EGF

"NBE" condition
neural stem cells (NSCs)

DMEM with
10 % serum

"Serum" condition
glioma cell lines

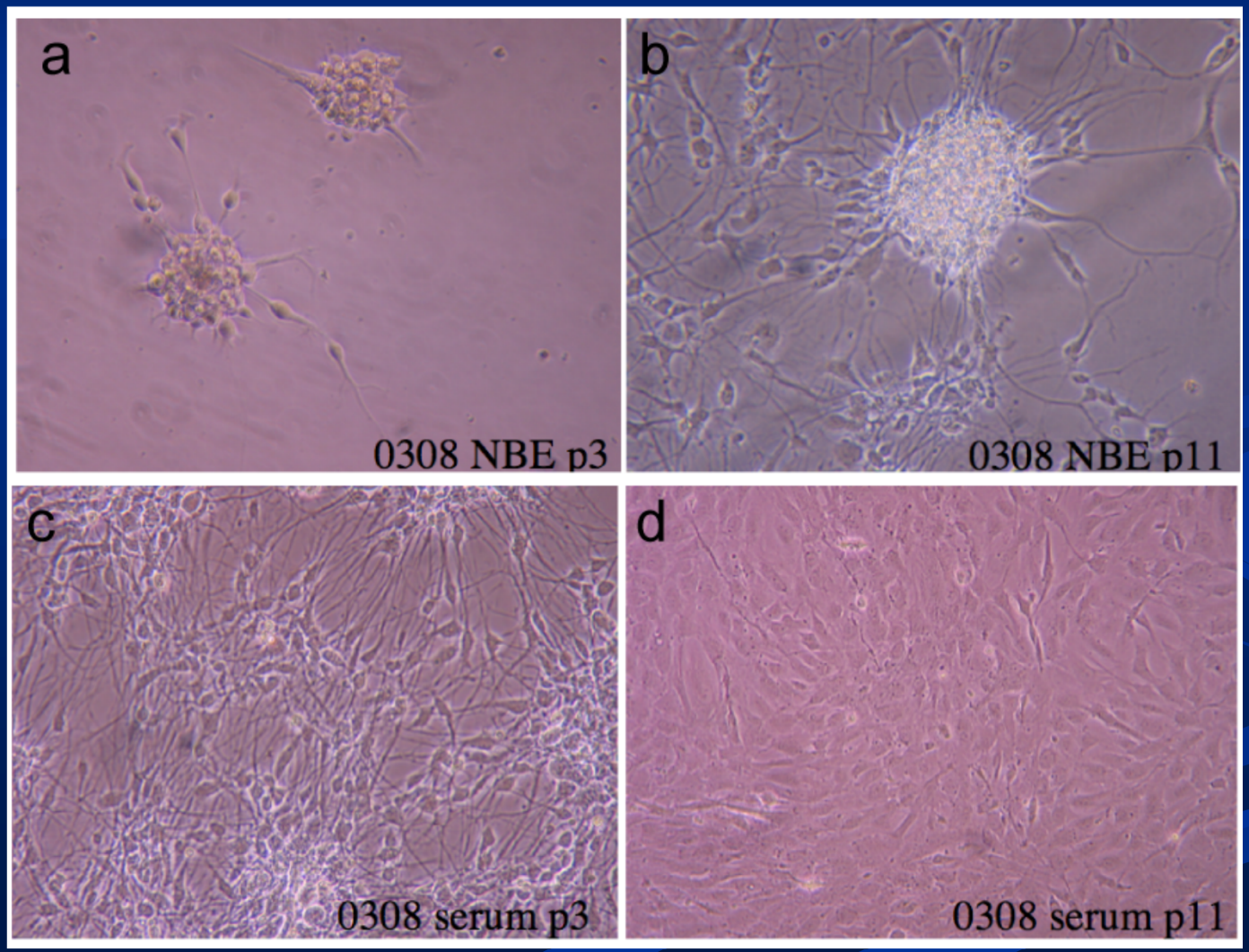
From various primary GBM tissues, we established cells cultured under NBE and serum conditions at the various passages in vitro.
Then, we evaluated:

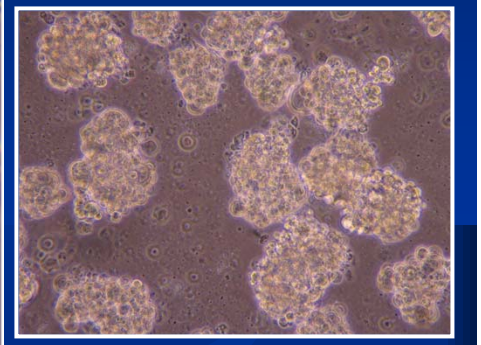
- **Tumorigenic/clonogenic potential**
- **Differentiation potential**
- **Molecular phenotype and genotype**
- **Gene expression profiling**

Lee at al. Cancer Cell 9:391-403; 2006

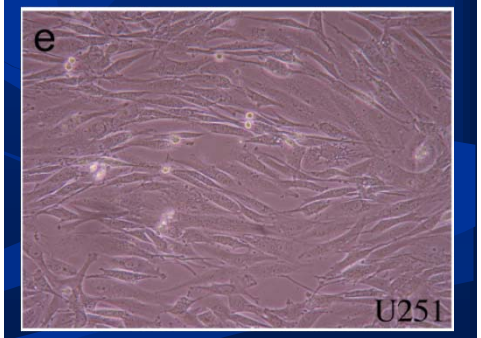
There is a population of glioma cells (? tumor stem cells) within each primary human GBM that when grown under NBE conditions (“NBE cells”) proliferate and function like normal human neural stem cells (NSC) in vitro. Not true for matched glioma cells grown under standard in vitro growth conditions (“serum cells”).

GBM-derived tumor cells cultured in NBE conditions, but not serum conditions in vitro form NSC-like "neurospheres"



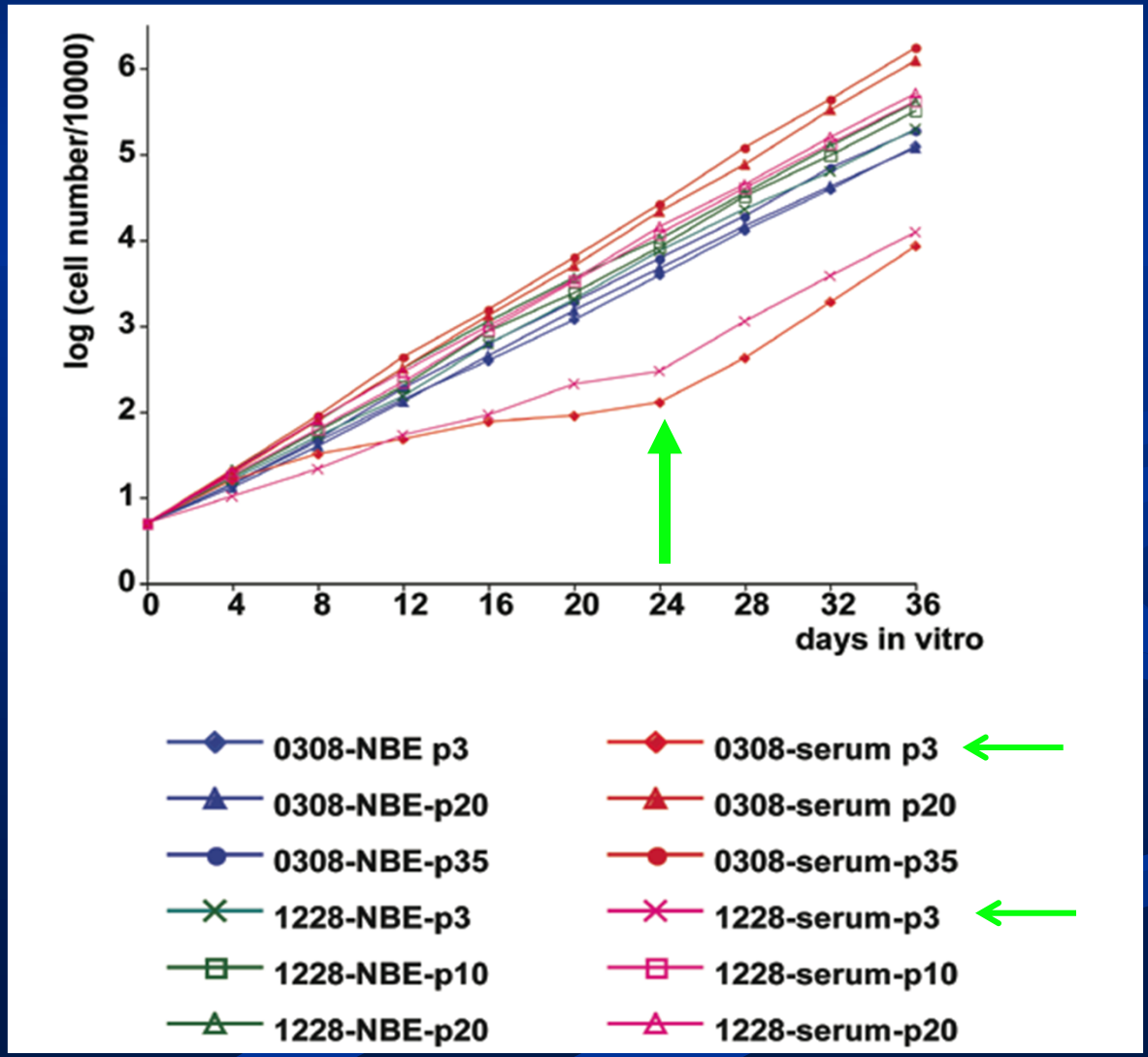


Human fetal Neural stem cells

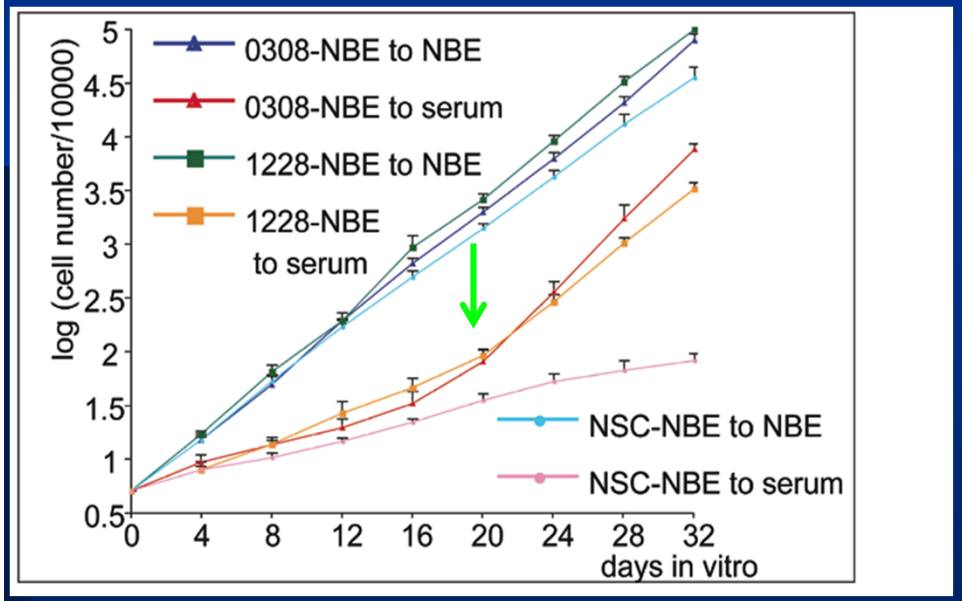


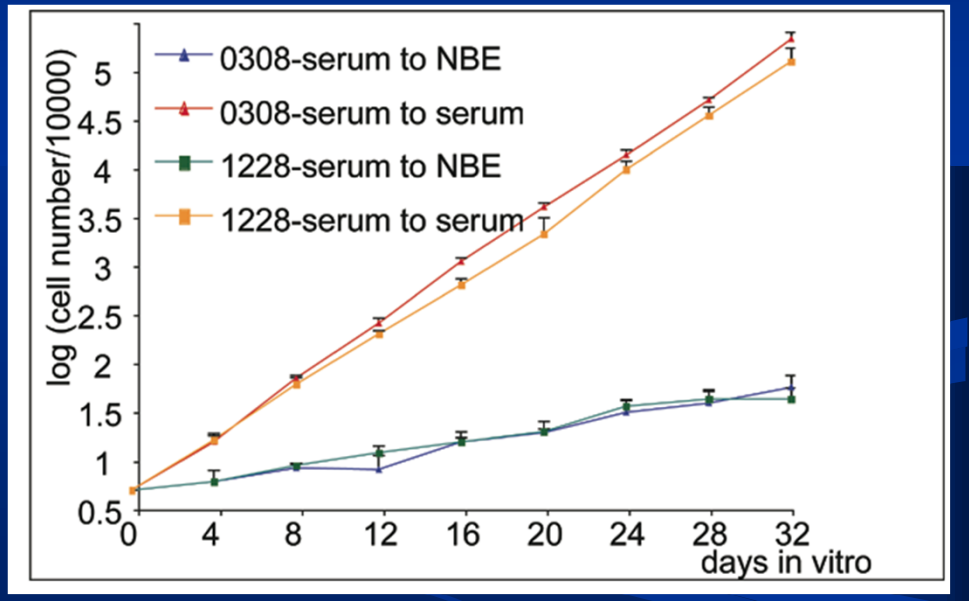
U251: common glioma cell line

NBE cells display linear and constant proliferation kinetics like NSC whereas serum cells start slow and then grow exponentially



Once placed in serum, NBE cells grow permanently like serum cells: just like all other traditional glioma cell lines

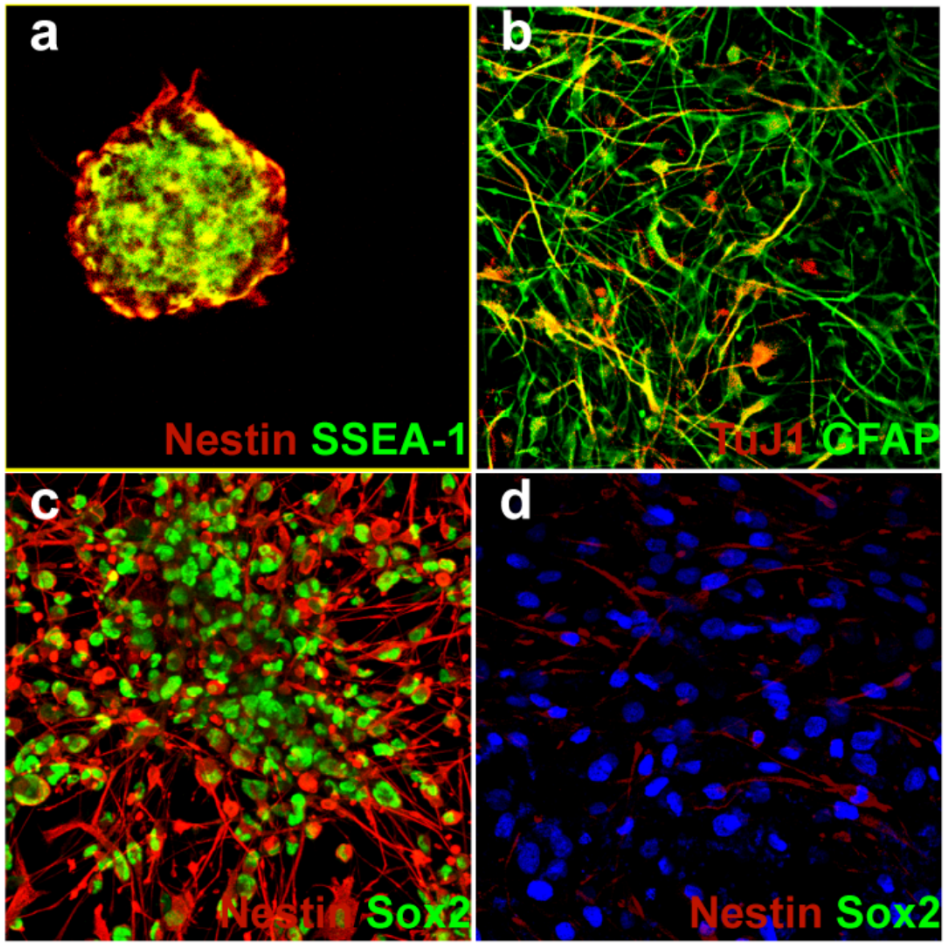


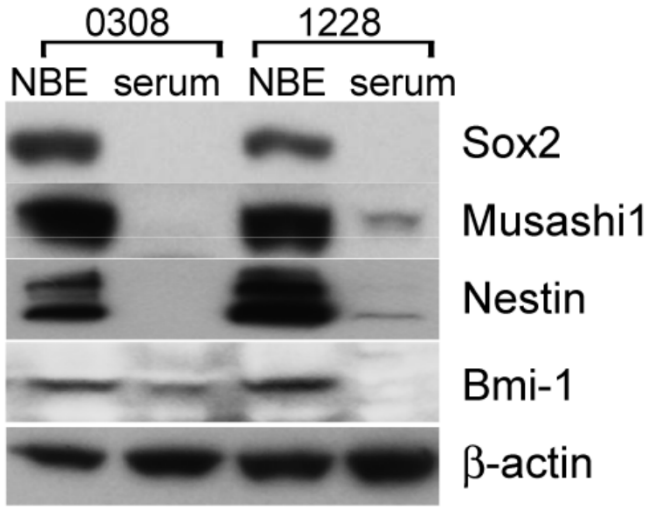


- **Once grown in serum, NBE Cells (a.k.a. TSC) grow exponentially in contrast to NSC.**

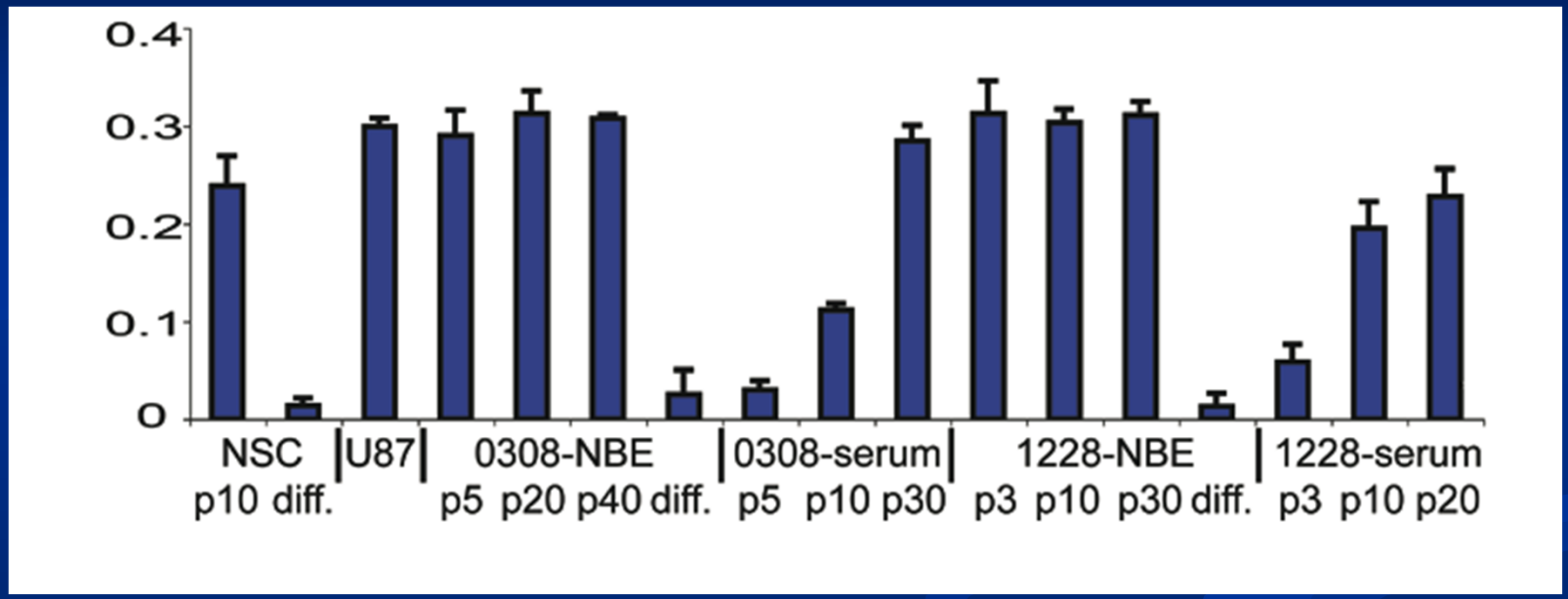
- **Once serum dependent, always serum-dependent**

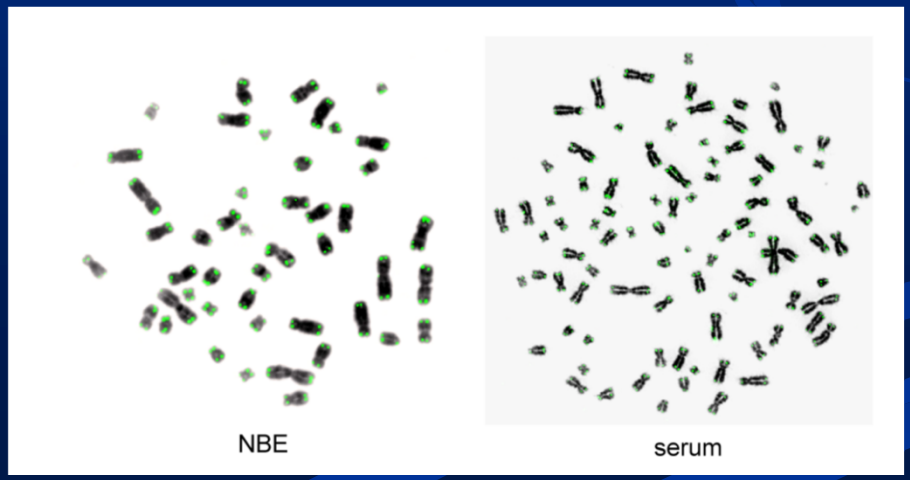
NBE cells, but not in serum cells, express embryonic and neural stem sell-associated genes and can differentiate down both glial and neuronal lineages (just like NSC)





elomerase activation in NBE cells regardless of passage, just li NSC, but only in late passage serum cells ("the change").



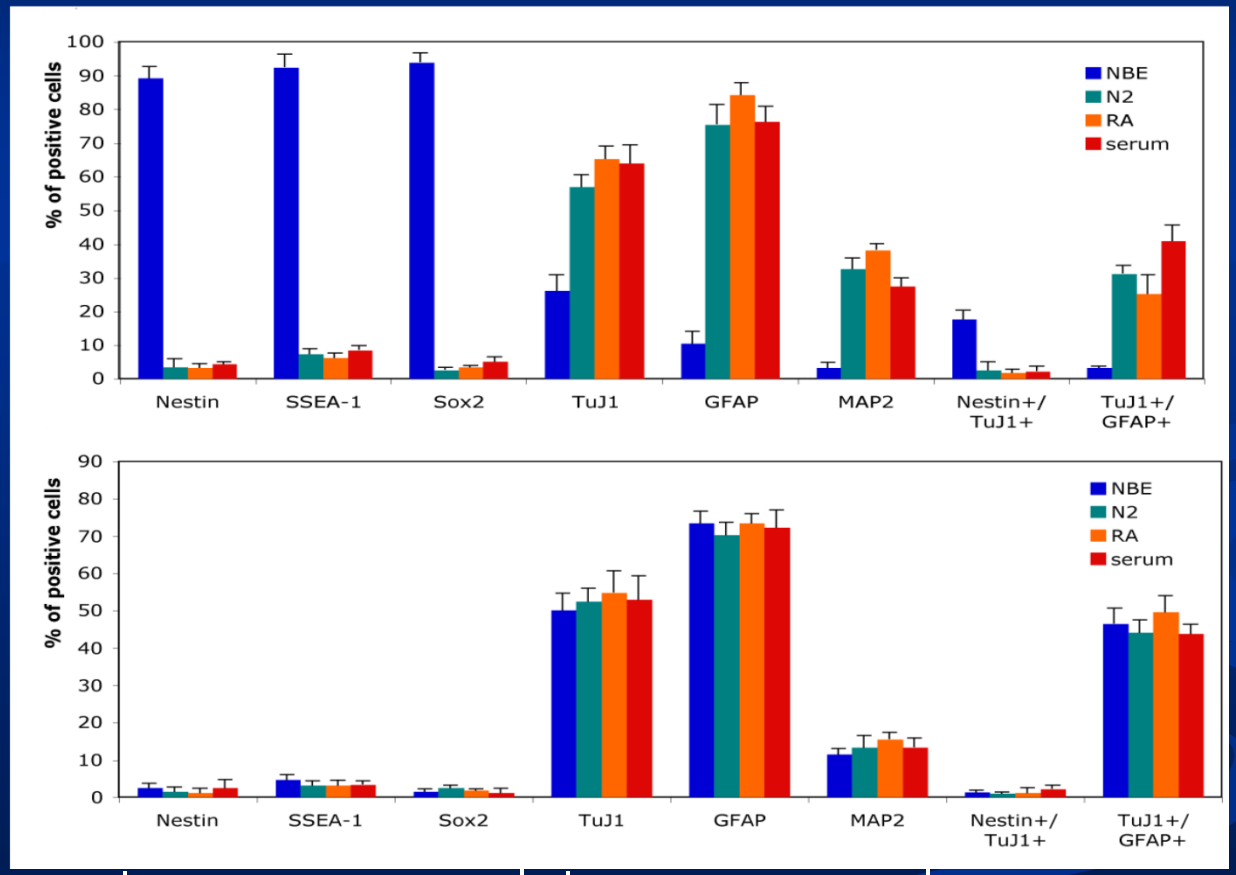


Telomere FISH

NBE cells respond to normal differentiation cues; serum cells do not.

NBE cells

Serum cells



Markers for Stem cell status

Markers for Differentiated cells

Differentiation

- Growth factor withdrawal
- Retinoic acid
- Serum

NBE cells are tumorigenic both in vitro and in vivo. Serum cells are only tumorigenic after they have changed following months of in vitro culture.

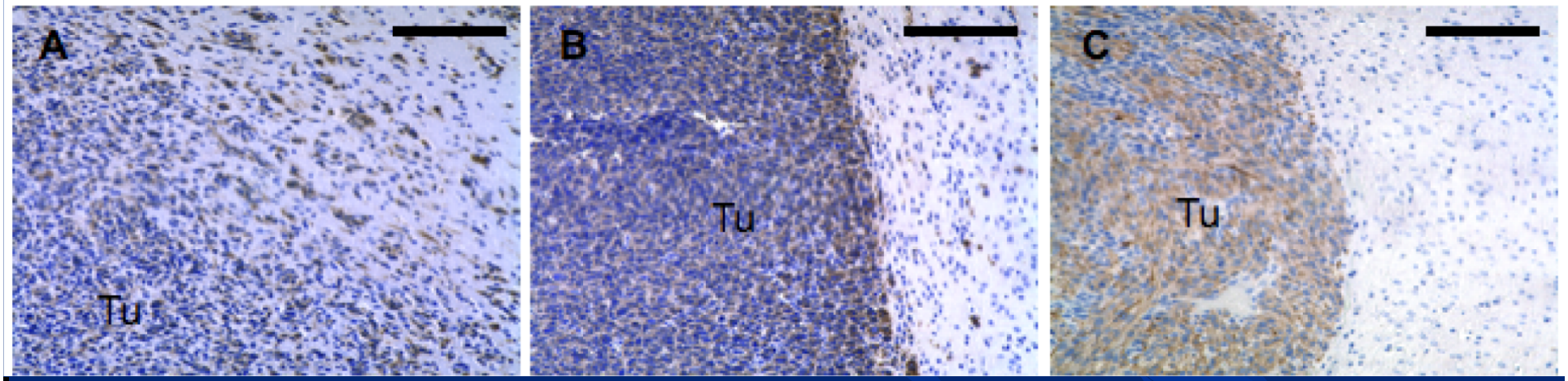
NBE cells are clonogenic; serum cells are not clonogenic until very late passage (the "change).

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

NBE cells are tumorigenic; serum cells are not tumorigenic until very late passage ("the change")

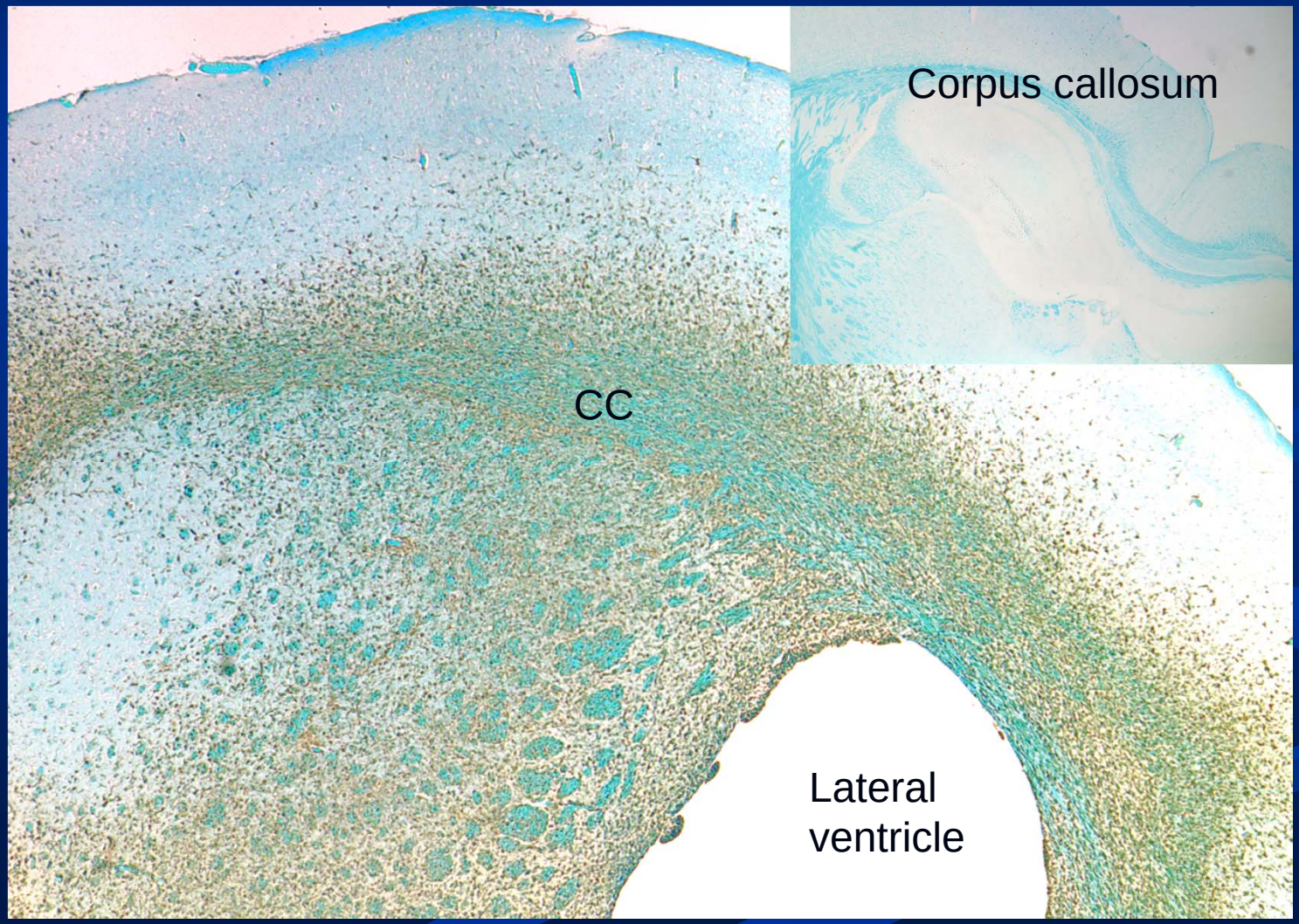
Cells	Injection site	Cell number injected	Death by tumor/ mice	Median survival (weeks)
NSC	brain	20000	0/15	-
0308-NBE p3	brain	20000	9/9	9.6
0308-NBE p3	brain	1000	7/8	15
0308-NBE p10	brain	20000	7/7	11.4
0308-NBE p20	brain	20000	5/5	12.8
0308-NBE p35	brain	20000	10/11	11
0308-NBE p50	brain	20000	8/8	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	-

NBE cell-derived gliomas in mice look pathologically identical to human glioblastomas; serum ("changed") cells and glioma cell lines do not

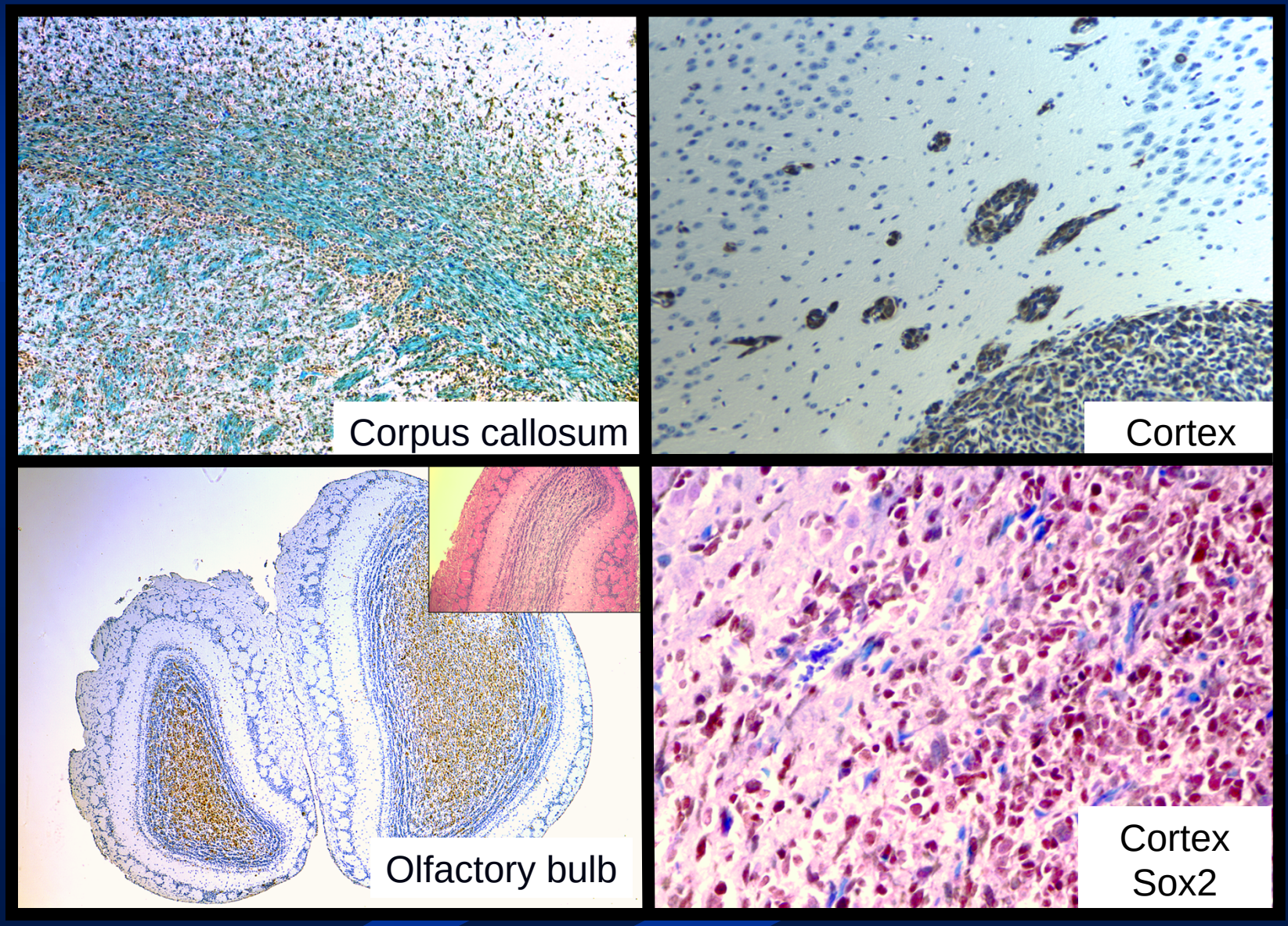


NBE **Serum** **U87**

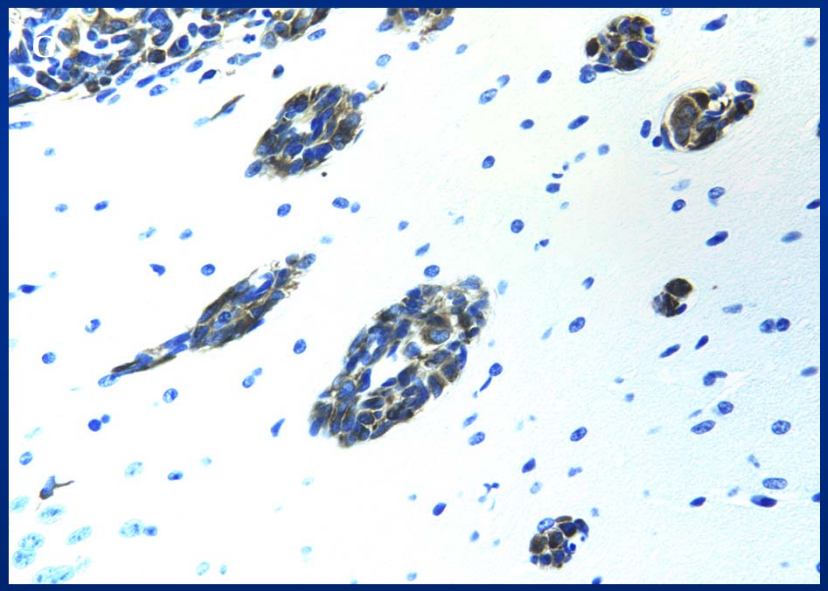
NBE cell-derived tumors are invasive and migrate along white matter tracts, identical to human GBMs



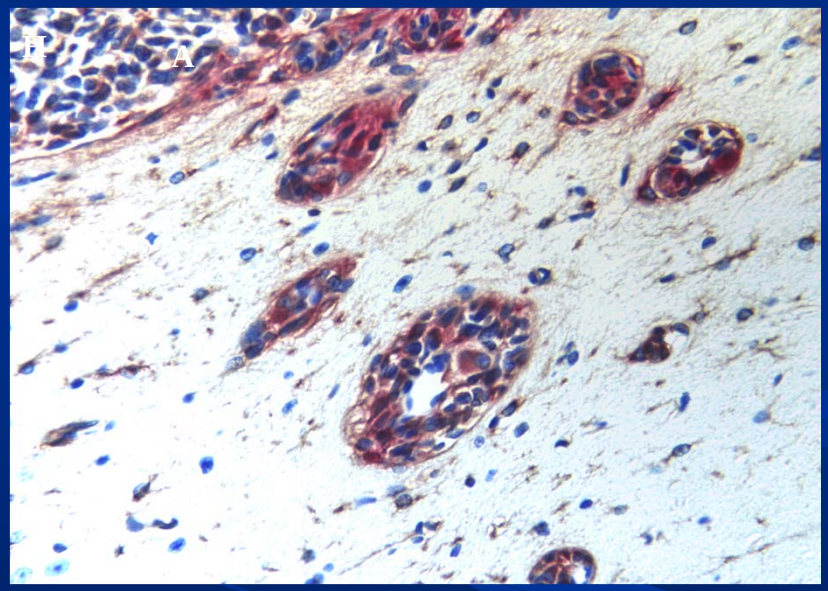
Infiltration and migration of NBE cell-derived tumors along white-fiber tracts and into the cerebral cortex just like human GBMs



NBE cells infiltrate along microvasculature and Express GFAP and/or Nestin just like human GBMs; never seen with standard glioma cell lines



Nestin

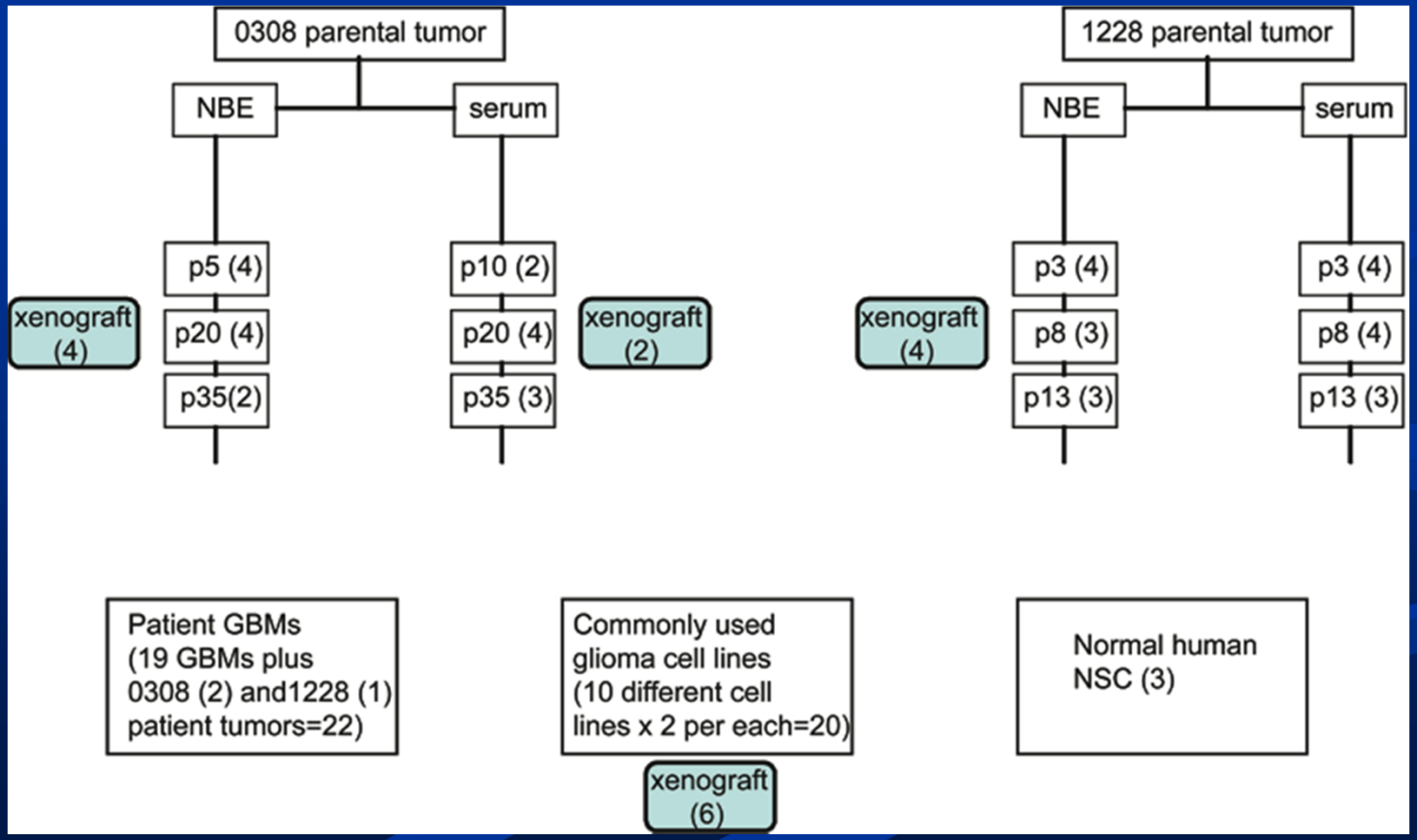


GFAP

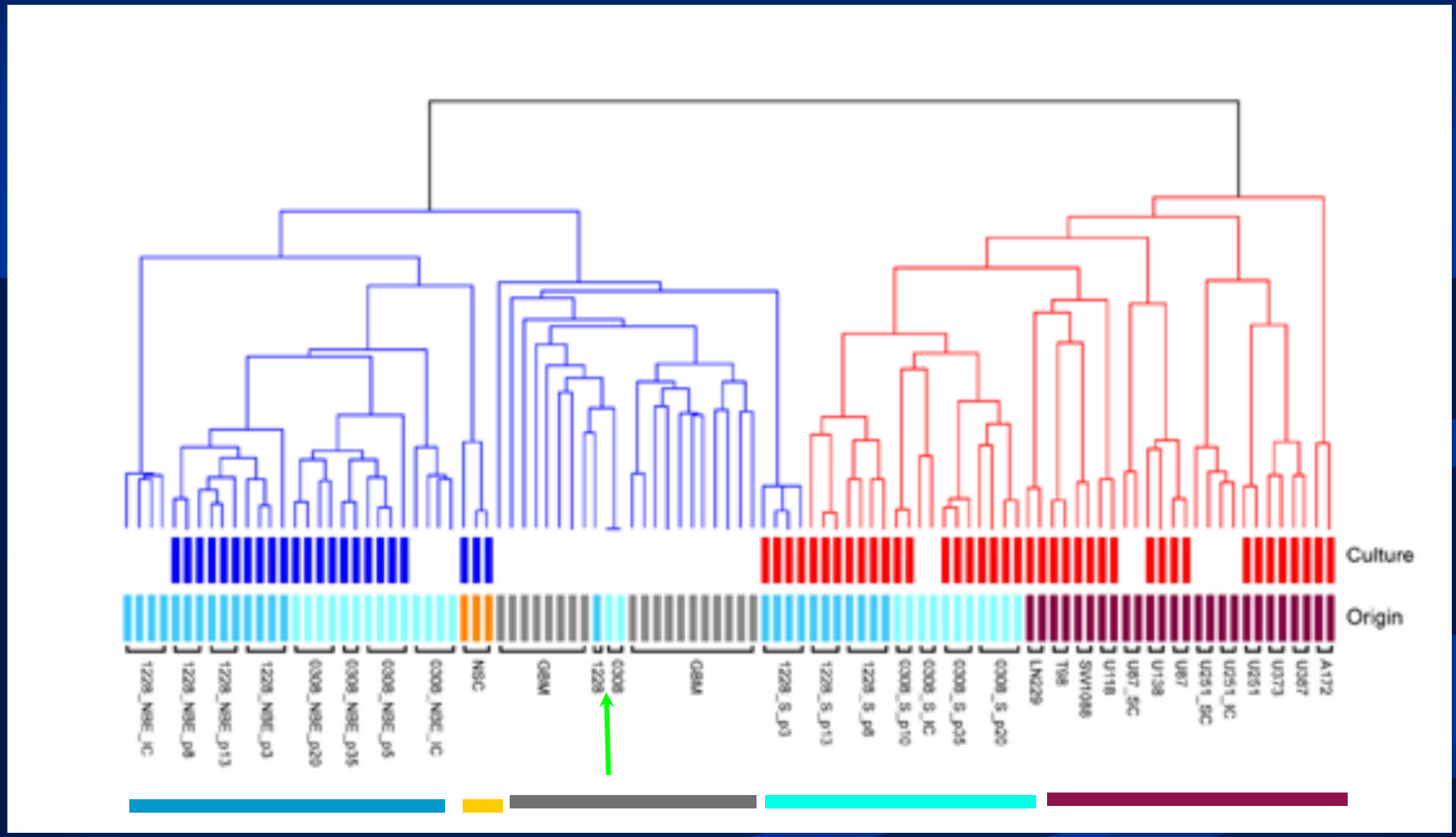
NBE cells have gene expression profiles very similar to the human NSCs and primary human GBMs. Serum cells have gene expression profiles like all other commonly used glioma cell lines, which have little similarity to NSCs or primary human GBMs

Global Gene Expression Profiles of NBE Cells: Experimental Design

1.) Do NBE cells have gene expression profiles similar to NSC?
2.) Do NBE cells have gene expression profiles similar to primary human GBMs?
3.) Do NBE cells generate intracerebral gliomas in immunodeficient mice that have gene expression profiles more similar to primary human GBMs than do the standard glioma cell lines used for the last 20 years?



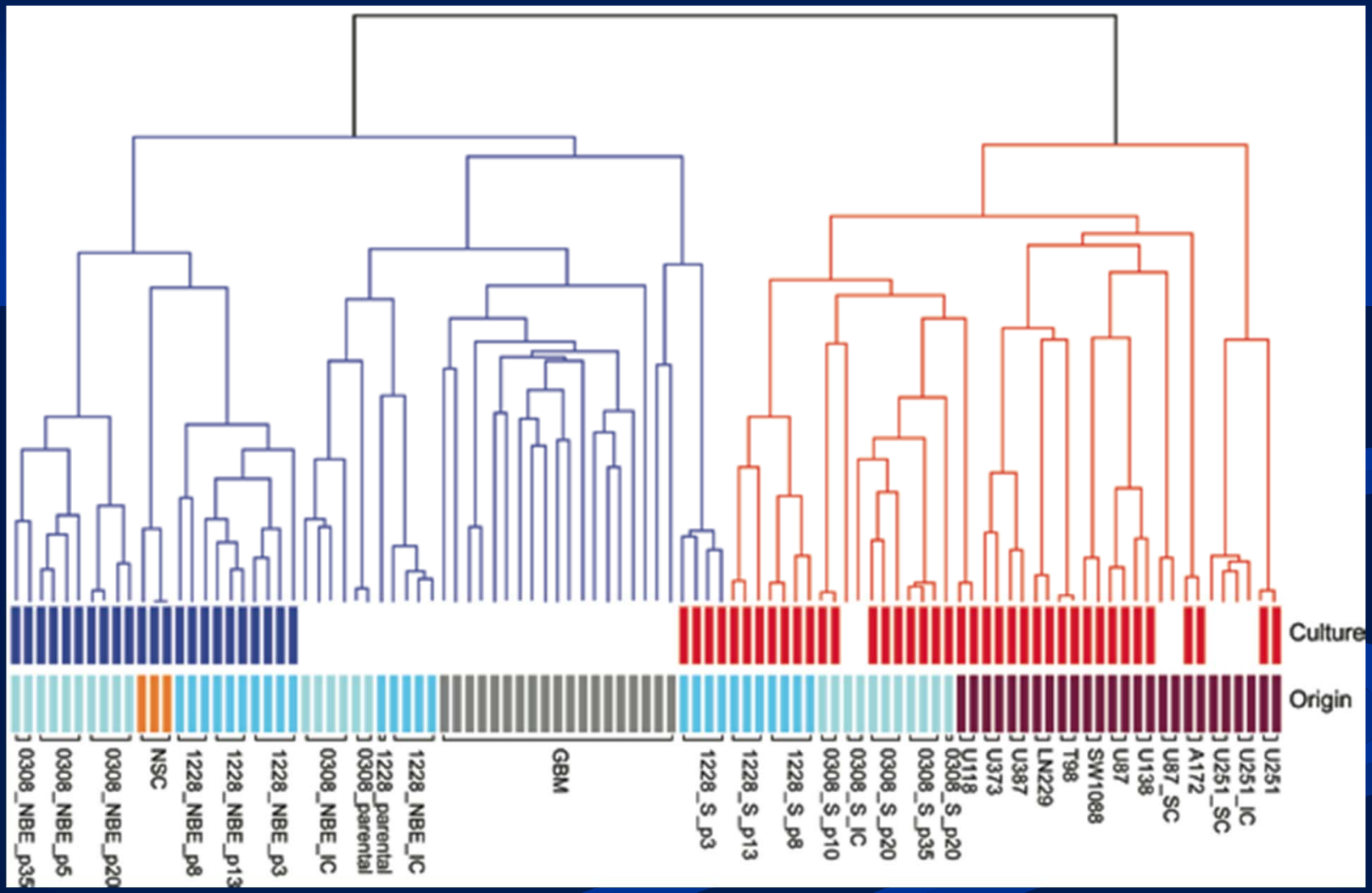
Unsupervised Clustering Analysis: NBE Cells cluster with NSC and primary human GBMs; serum cells cluster with traditional glioma cell lines



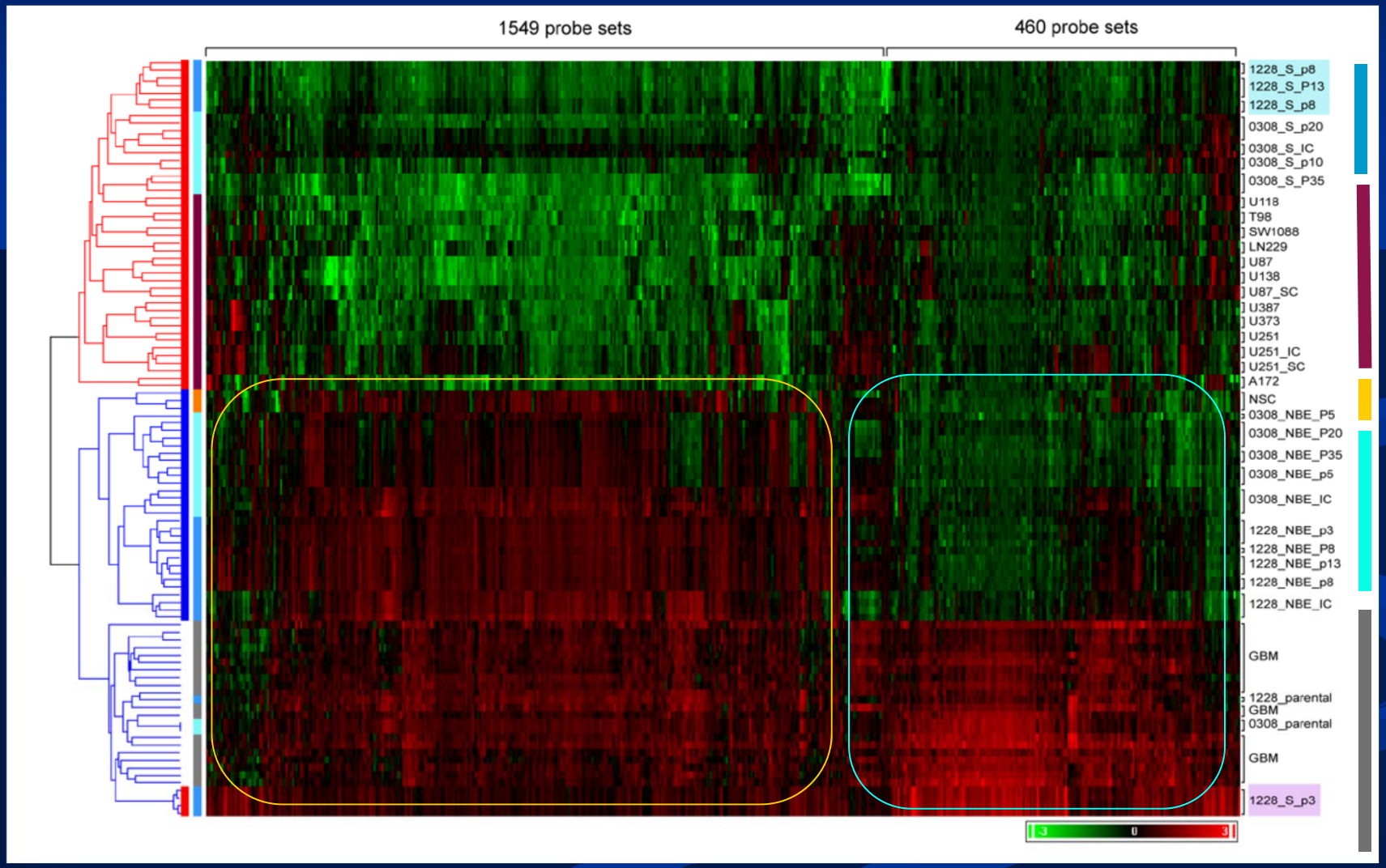
NBE Cells **NSC** **Primary GBMs** **Serum Cells** **Glioma Cell Lines**

{Very early passage 1228 serum cells}

Supervised Hierarchical Cluster Analysis Using "NSC-Enriched Gene Sets "(ortholog list)" As Classifiers



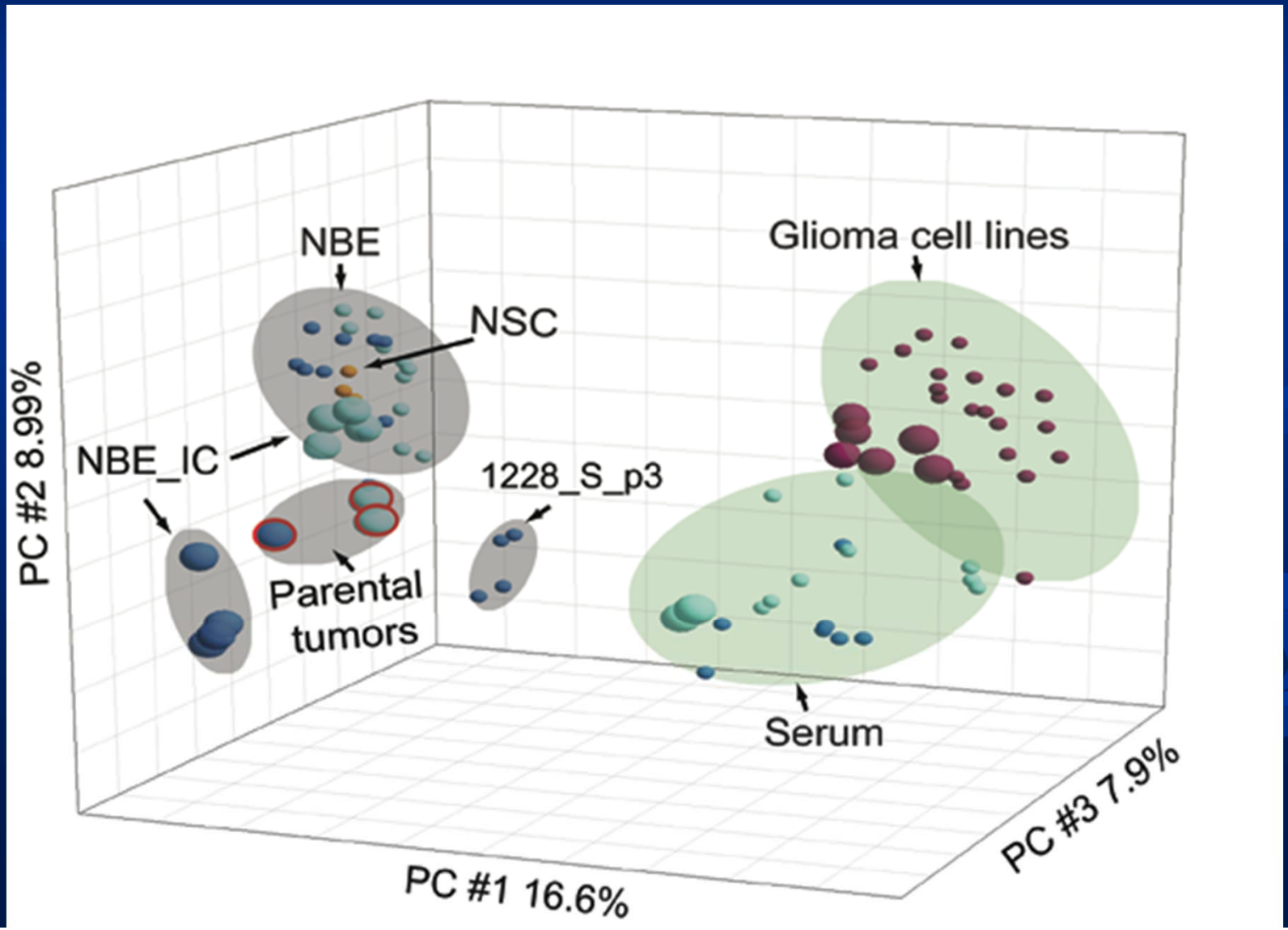
The only major group of genes expressed differently between NBE cells and primary GBMs were immune system genes secondary to contamination of primary tumors with inflammatory cells.



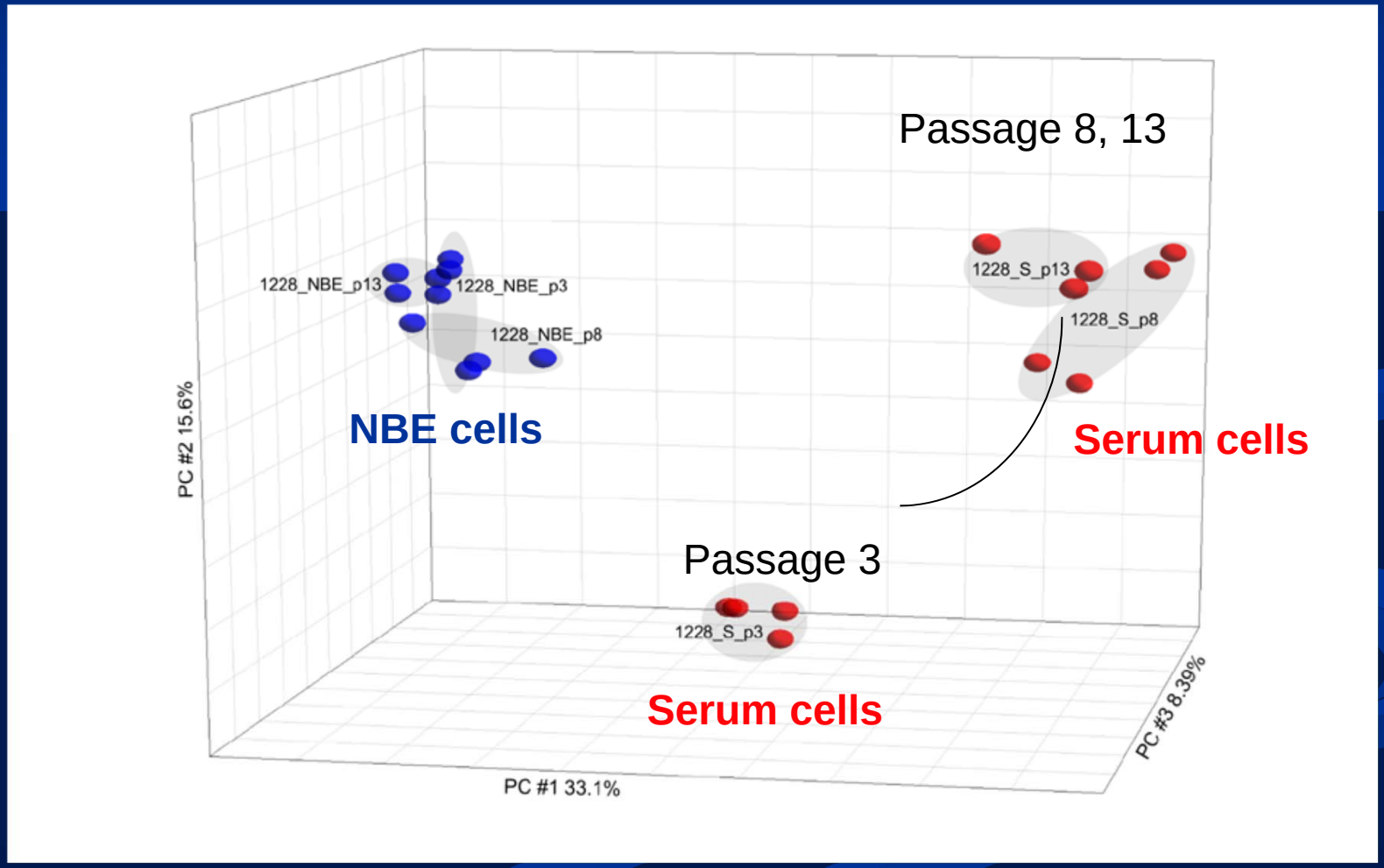
Highly enriched for CNS development genes

Highly enriched for Immune system genes

Principle Component Analysis: NBE cells have similar gene expression to NSC and their original human GBMs; serum cells and glioma cell lines look nothing like primary GBMS

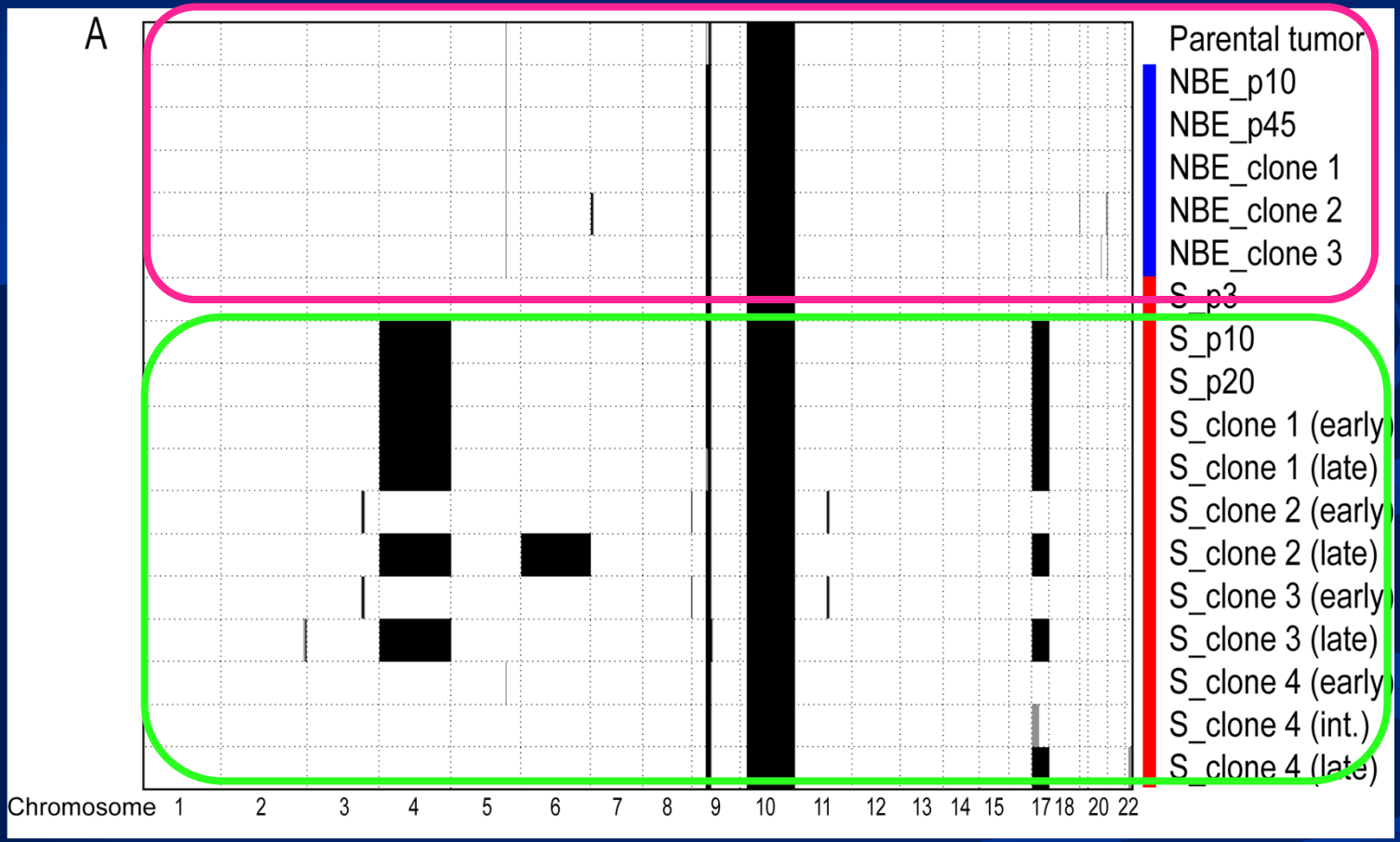


NBE cells maintain their transcriptomes regardless of passages, whereas serum cells change (> 10 fold the number of genes change over passages 3-13 with serum cells compared to NBE cells).



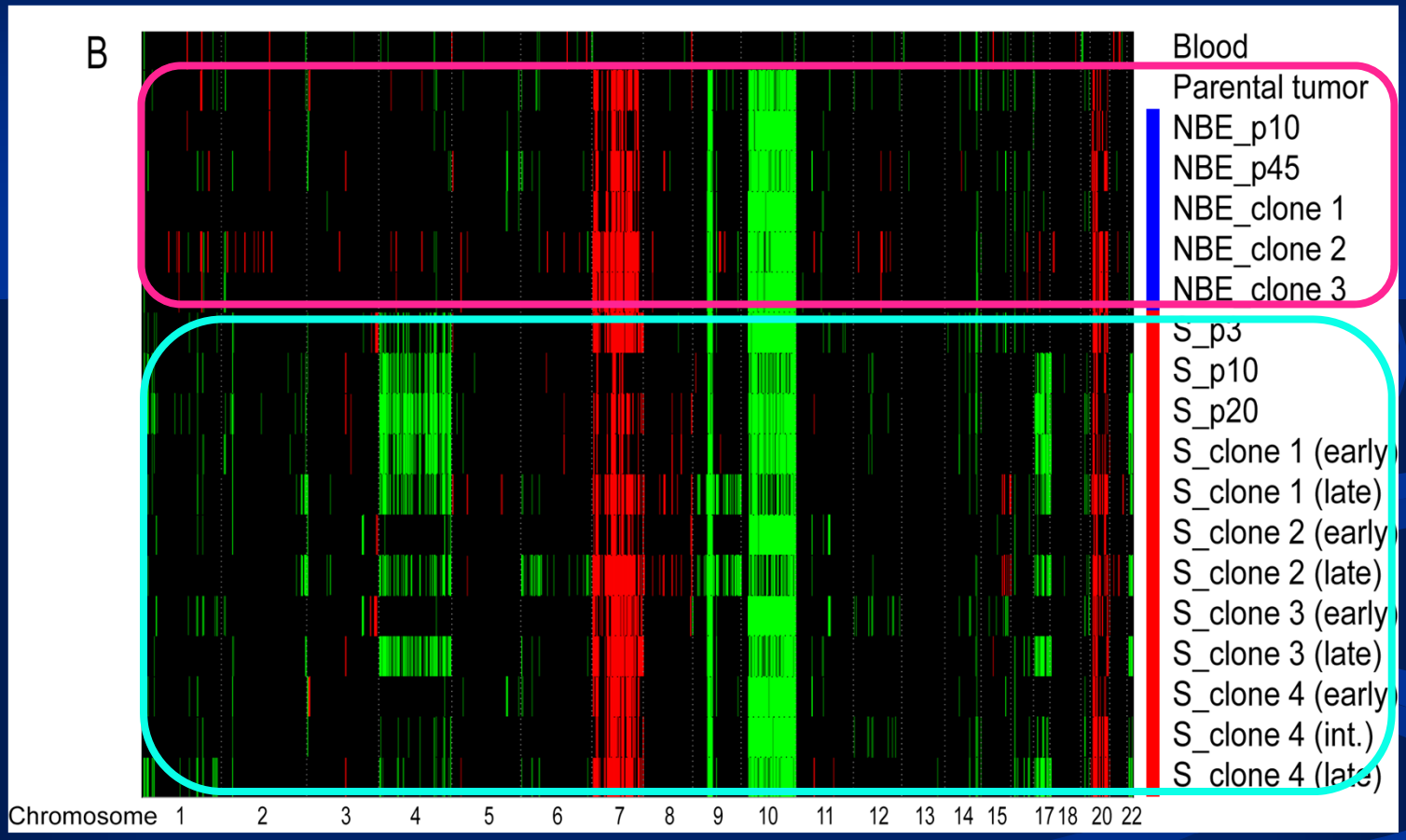
NBE cells maintain the same genotype of the primary human GBM they come from; whereas serum cells develop genomic instability with subsequent mutations not found in the primary human tumor.

NBE cells, but not late passage serum cells maintain genotype of primary parental GBM



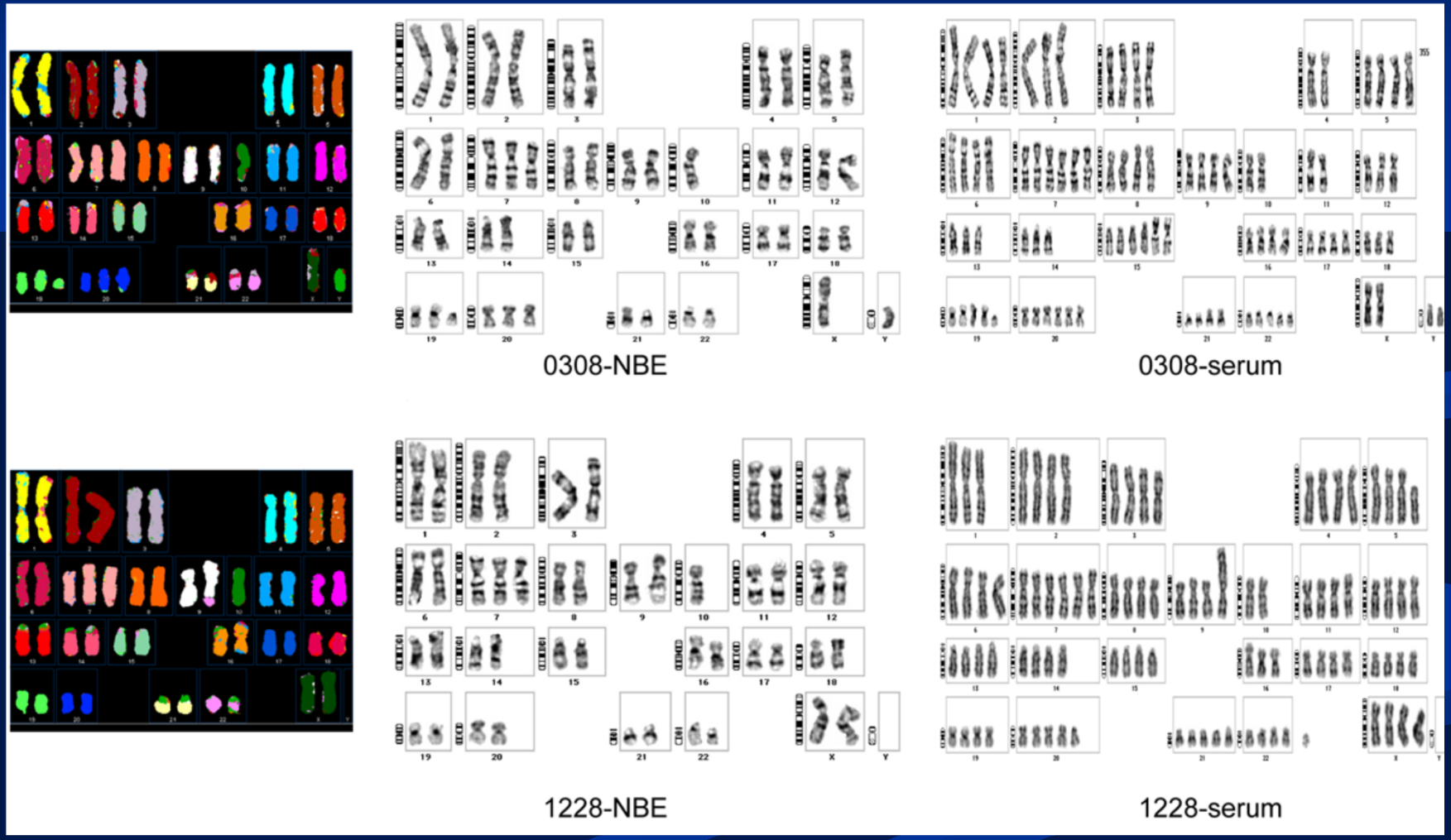
LOH analysis

NBE Cells But Not Serum Cells Maintain Genotype of Parental Tumor



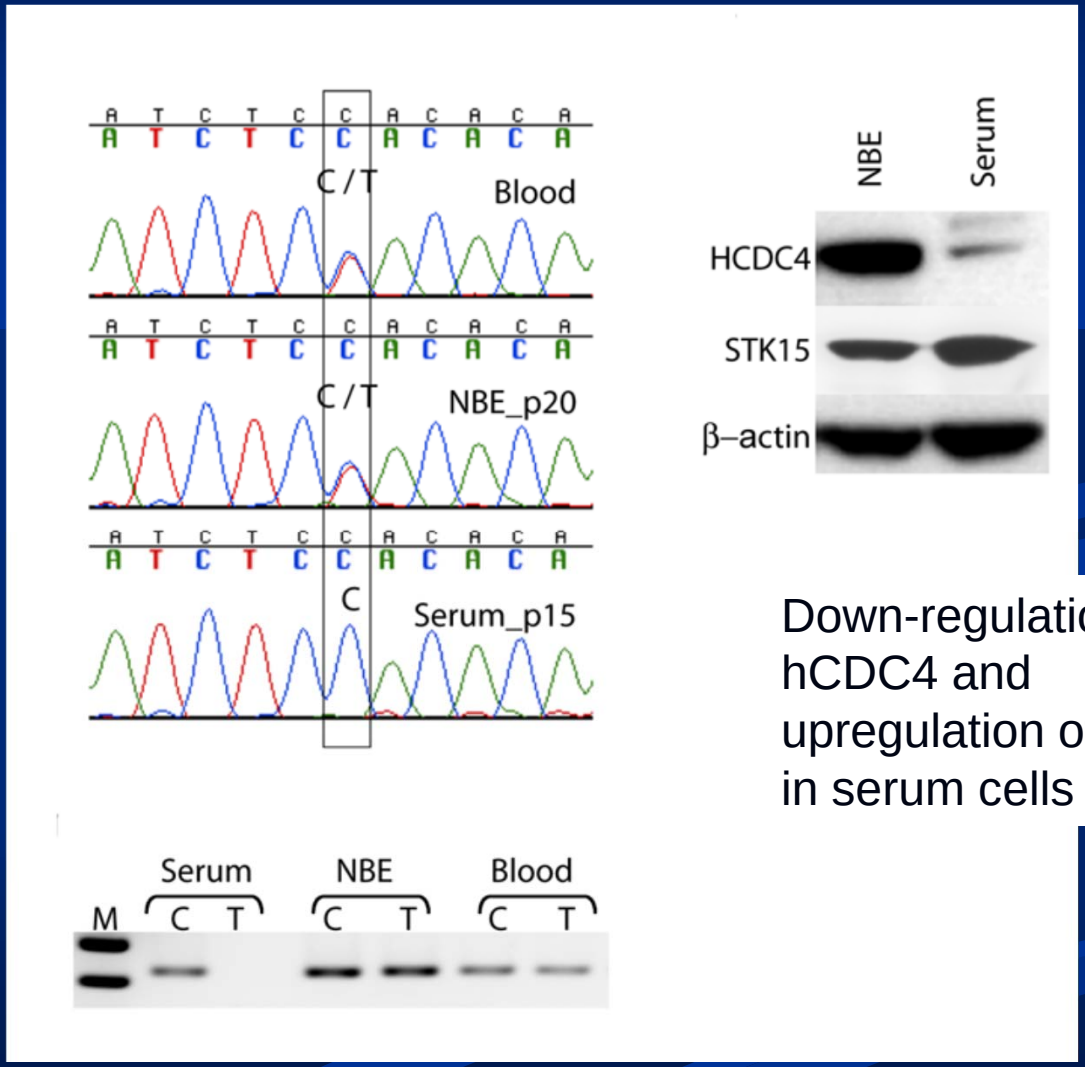
Amplification/deletion

Serum but not NBE cells develop significant genomic instability (aneuploidy) in at late passages



Polyploid genotype In later serum cells coincides with haploinsufficiency of hCDC4 In chromosome 4 with resultant genomic instability in serum-cultured cells

Allele-specific PCR demonstrating LOH of ch. 4 in late serum cells

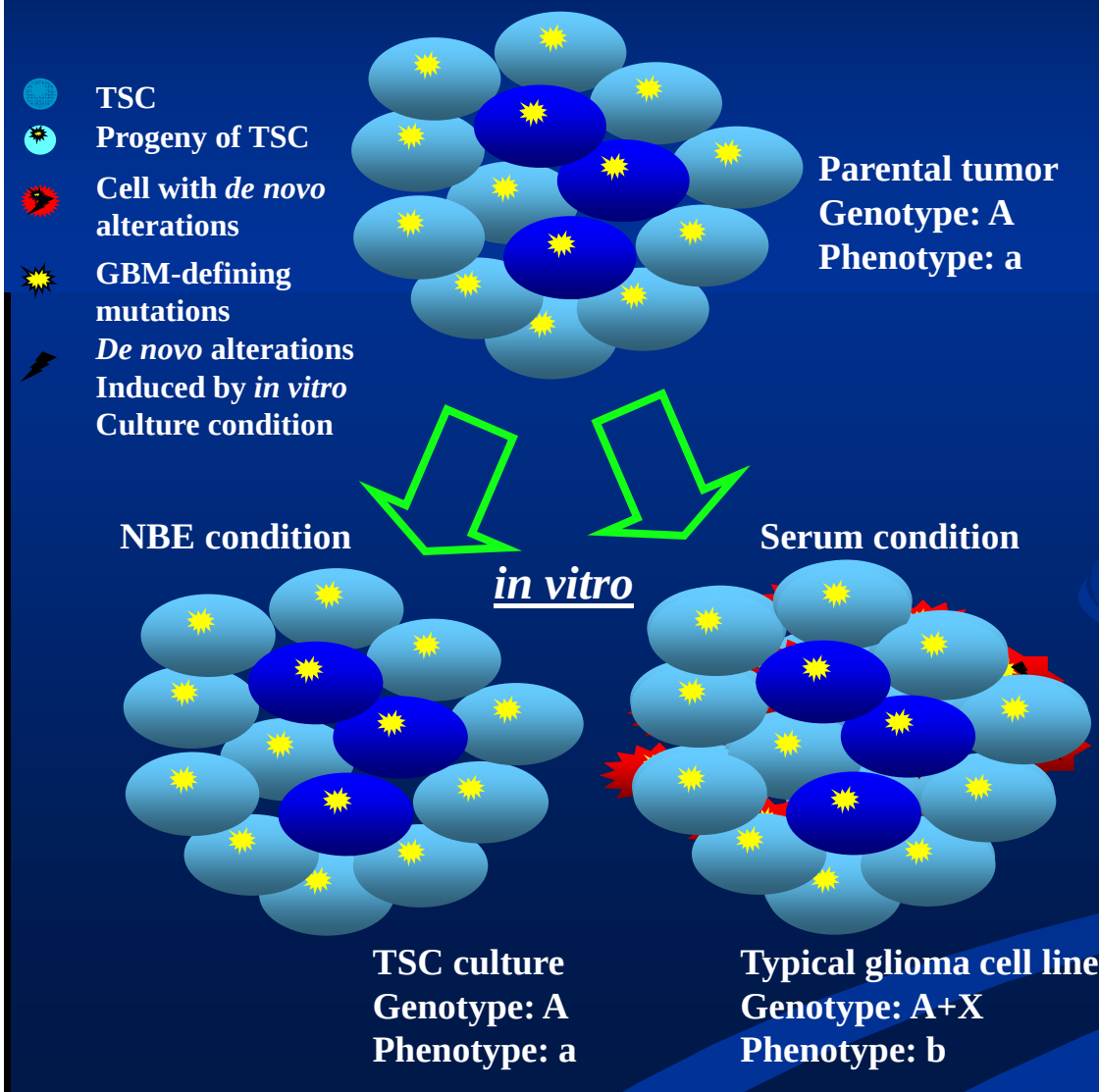


Down-regulation of hCDC4 and upregulation of STK15 in serum cells

Conclusions

- NBE cells represent a subpopulation of cells within primary human GBMs that have the following properties similar to NSC:
  - Self-renewal
  - Telomerase activity
  - Multi-lineage differentiation
  - NSC gene expression profile
- NBE cells represent a subpopulation of cells within primary human GBMs that have the following properties similar to tumor repopulating cells:
  - Similar gene expression profile to primary GBM
  - Clonogenic *in vitro*
  - Tumorigenic *in vivo*
  - NBE tumors *in vivo* are phenotypically similar to primary GBMs.
- **NBE cells are Tumor Stem Cells (TSCs).**

TSC vs. Glioma Cell Lines: A Model

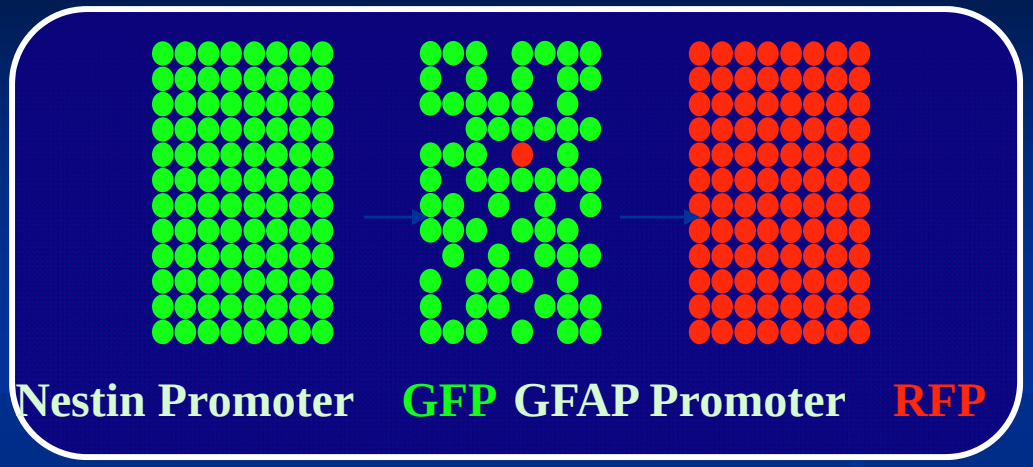


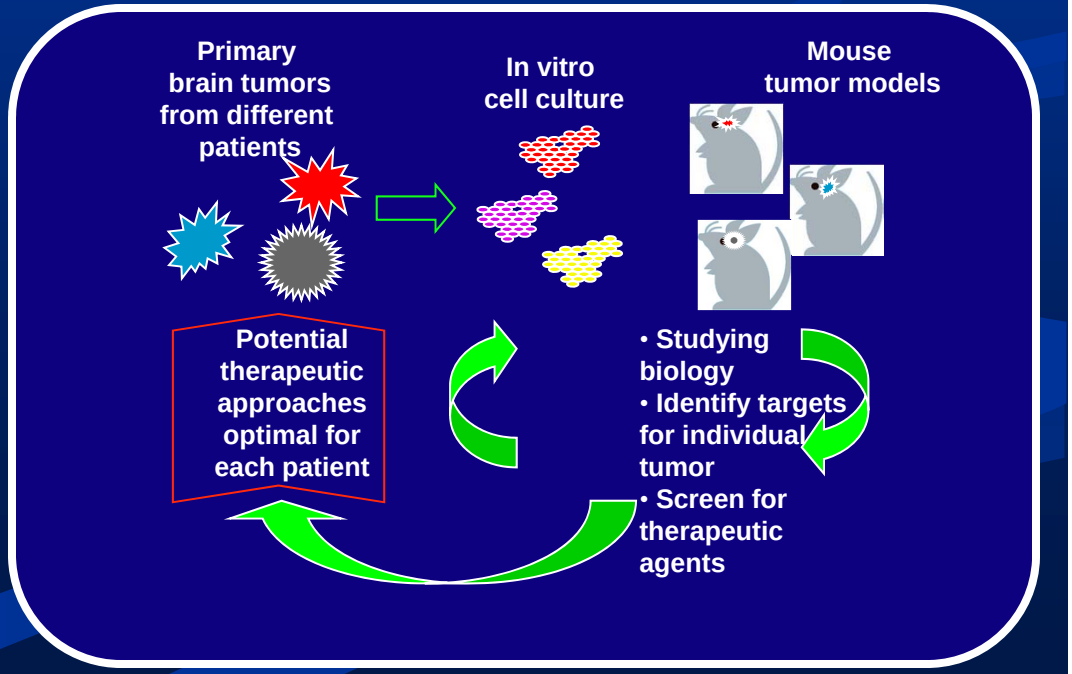
Pivotal Questions

- Are we targeting truly relevant molecular pathways when we study glioma cell lines?
- Are we missing important pathways of tumorigenicity by not studying TSC?
- Will TSC prove to be a more reliable pre-clinical model for studying GBMs than glioma cell lines?

GBM Tumor Stem Cells: Clinical Translation

- High-throughput screen for genes and differentiation-inducing agents using siRNA and large combinatorial chemical libraries.
  - Collaboration with the Broad Institute (MIT/Harvard) and Elli Lilly Pharmaceuticals.
- Establishment of TSC bank for every patient operated on at the NIH.
  - Patient-specific in *vitro*/*in vivo* screening of therapeutic agents
  - Generate data base of the breadth of differentiation/proliferation defects from TSC from different patients.
  - TSC tissue bank for intramural/extramural investigators.
- Molecular/Genetic and functional Atlas of GBM TSC/NSC in the Human Brain
  - Autopsy Study
  - Detailed Molecular study of the entire brain
    - ✓ Infiltrating glioma cells
    - ✓ TSC
    - ✓ NSC





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