Definition of the immunological properties of cancer stem cells isolated from human glioblastoma

Cristina Maccalli

Unit of Immuno-biotherapy of Melanoma and Solid Tumors

Division of Molecular Oncology

San Raffaele Scientific Institute, Milan, Italy

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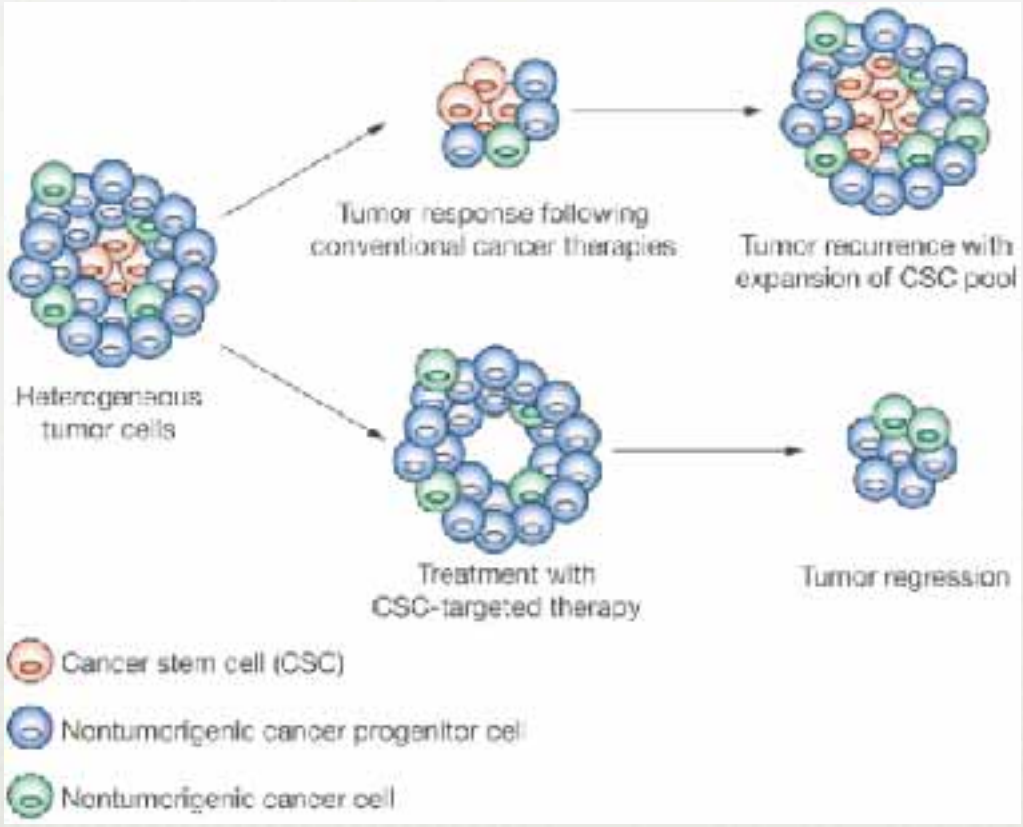
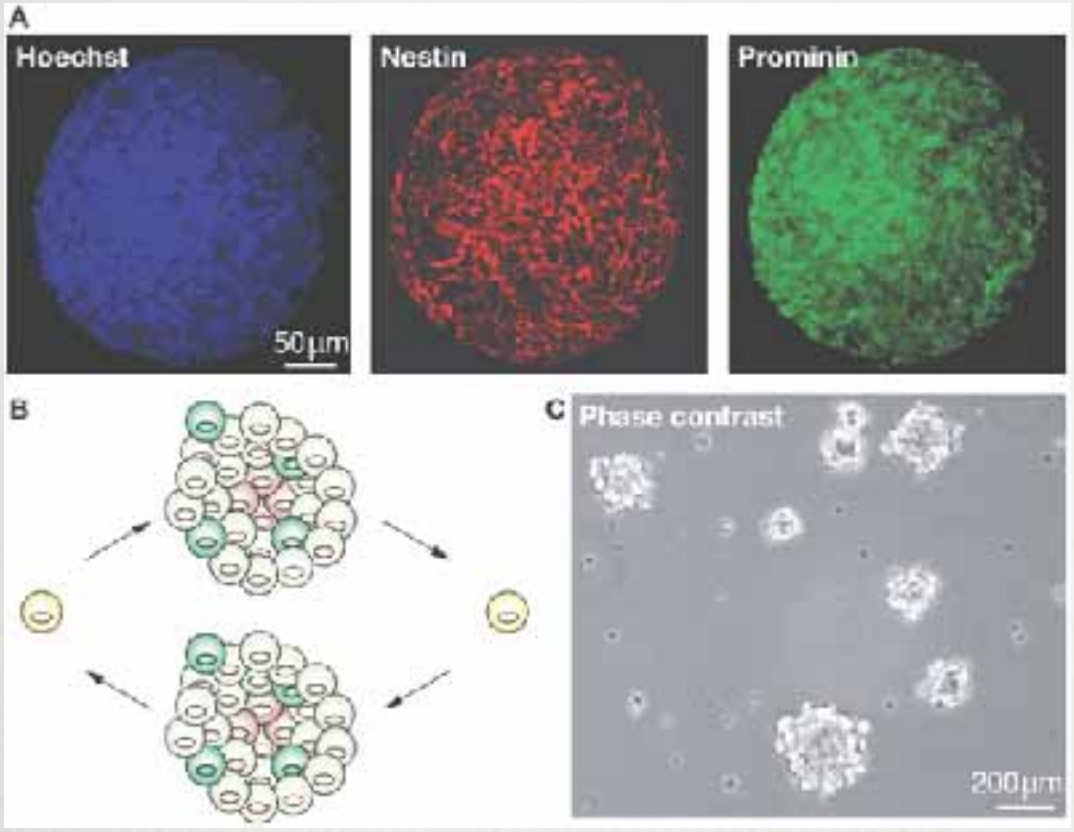
ISTITUTO SCIENTIFICO UNIVERSITARIO
SAN RAFFAELE

The cancer stem cell hypothesis



Das S *et al.* (2008) Cancer stem cells and glioma
Nat Clin Pract Neurol 10.1038/ncpneuro0642

Neurospheres and the neurosphere assay



Dirks P et al. (2006) Cancer stem cells and glioma
Nat Clin Pract Neurol 10.1038/ncpneuro0302

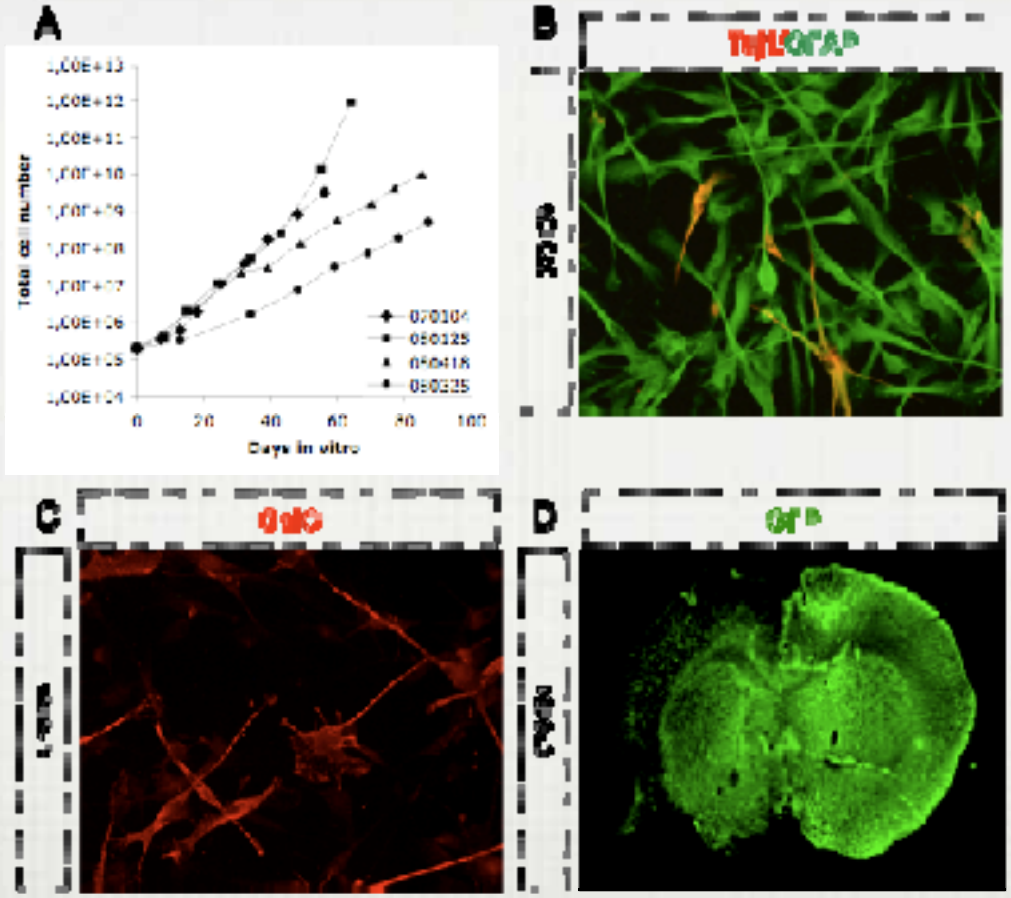
CSCs can be defined as:

1) self-renewing cells;

2) cells that give rise to the variety of differentiated cells found in the malignancies (multipotency);

3) cells able to generate a phenocopy of the orignal malignancy in immunocompromised mice (tumorigenic ability).

Self renewal, tumorigenicity and differentiation ability of GBM CSCs



NEURONAL/GLIAL MARKERS

OLIGODENDROGLIAL MARKER

TUMORIGENICITY BY INTRACRANIAL TRANSPLANTATION IN NUDE MICE

BY MAZZOLENI S. ET AL. SUBMITTED

EXPRESSION OF NEURAL STEM CELL-ASSOCIATED MOLECULES AND OF TRANSCRIPTION FACTORS BY GBM CSCs AND FBS TUMOR CELLS.

Cell Line	Molecule						
	c-Myc	Nestin	Nanog	S100A4	S100A6	Sall4	SOX2
080125 CSCs	**2**	n.d.	**2**	**2**	**2**	**5**	**7**
080125 FBS	**2**	n.d.	**4**	**2**	**2**	**8**	**3**
080418 CSCs	**2**	**35**	**2**	**2**	**4**	**4**	**38**
080418 FBS	**4**	**15**	**2**	1	**2**	**3**	**20**
080325 CSCs	1	n.d.	**3**	**4**	**2**	**3**	**15**
080325 FBS	1	n.d.	**4**	1	1	n.d.	8

Data are represented as MRFI that is the ratio between the mean of intensity of fluorescence of the cells stained with the selected mAb and that of the negative control; bold value means MRFI ≥ 2.

Expression of MHC and APM molecules and NKG2DLs in GBM-derived CSCs and FBS tumor cells

- **The expression of:**

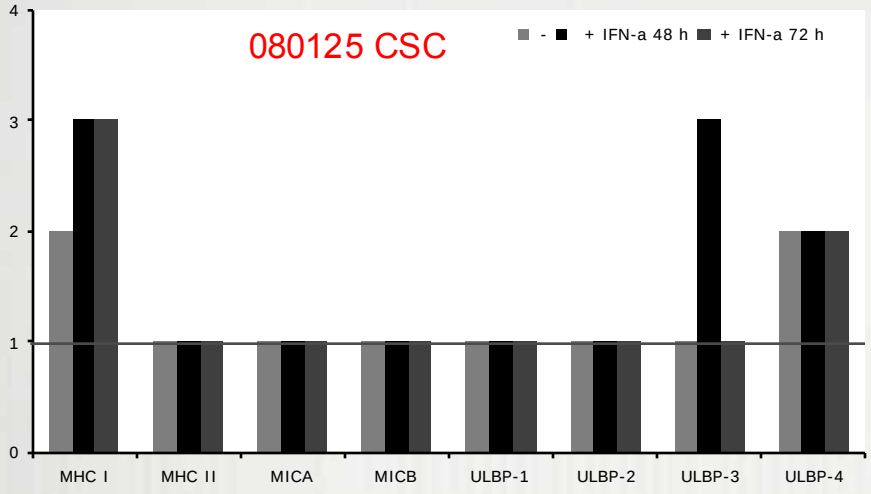
- **MHC class I and II;**

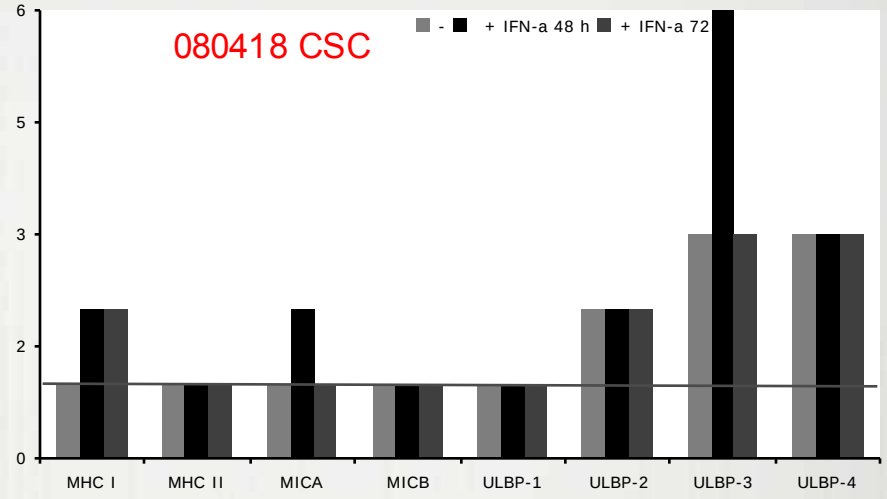
- **Antigen processing machinery (APM), using 21 different mAbs directed against HLA molecules, their heavy chains, β2-microglobulin immunoproteasome, constitutive proteasome subunits, chaperon molecules, TAPs etc.;**

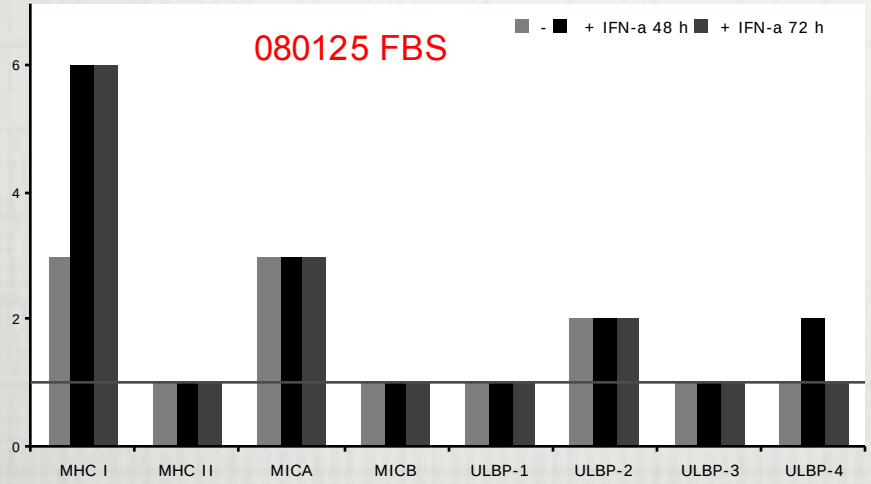
- **NKG2DLs;**

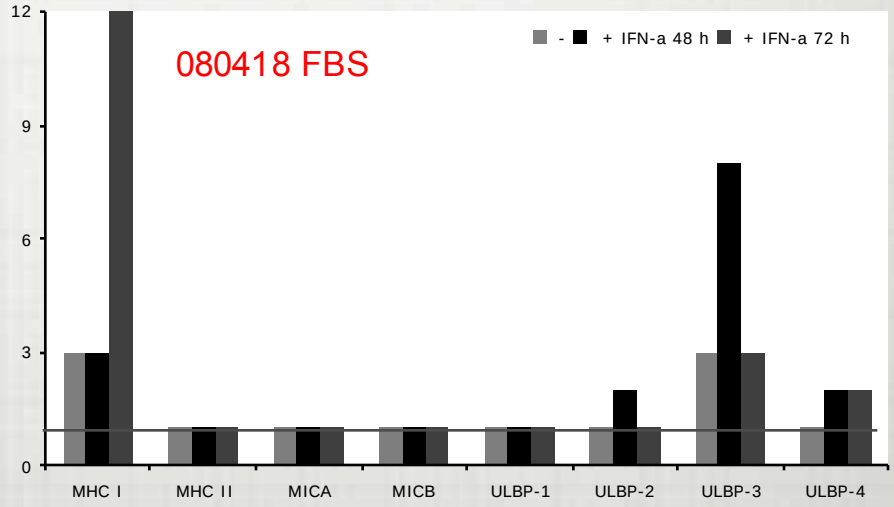
- **has been tested in 11 different GBM CSCs and, for 5 of them, in their paired tumor cells grown in the presence of FBS (FBS tumor cells).**

Expression and modulation of MHC molecules and NKG2DLs in GBM CSCs vs FBS tumor cells



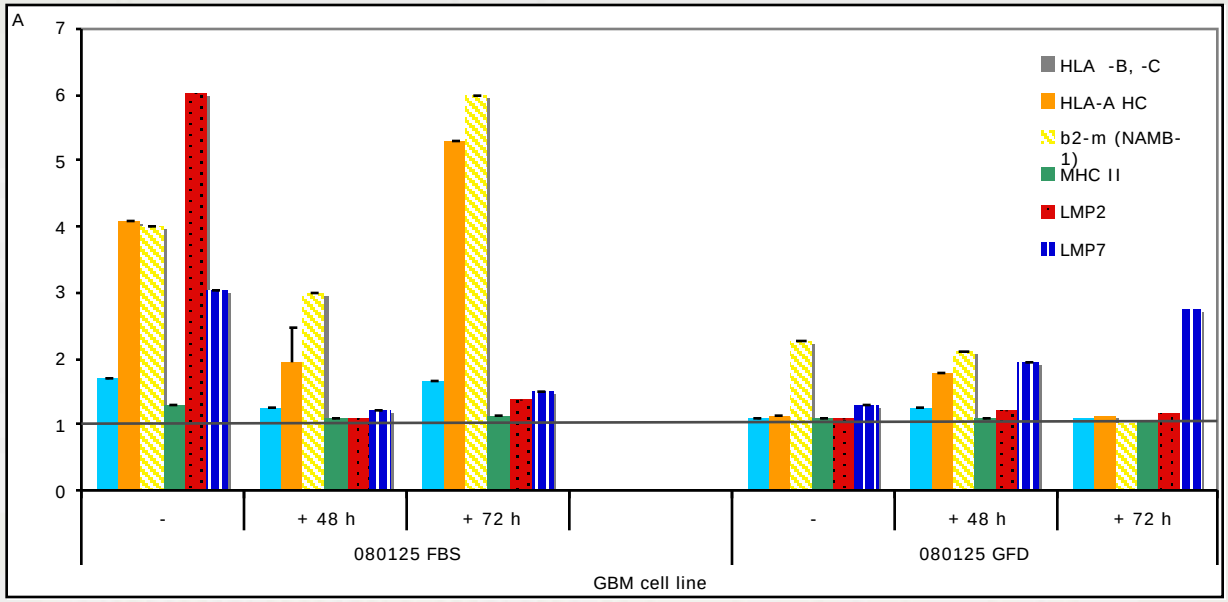




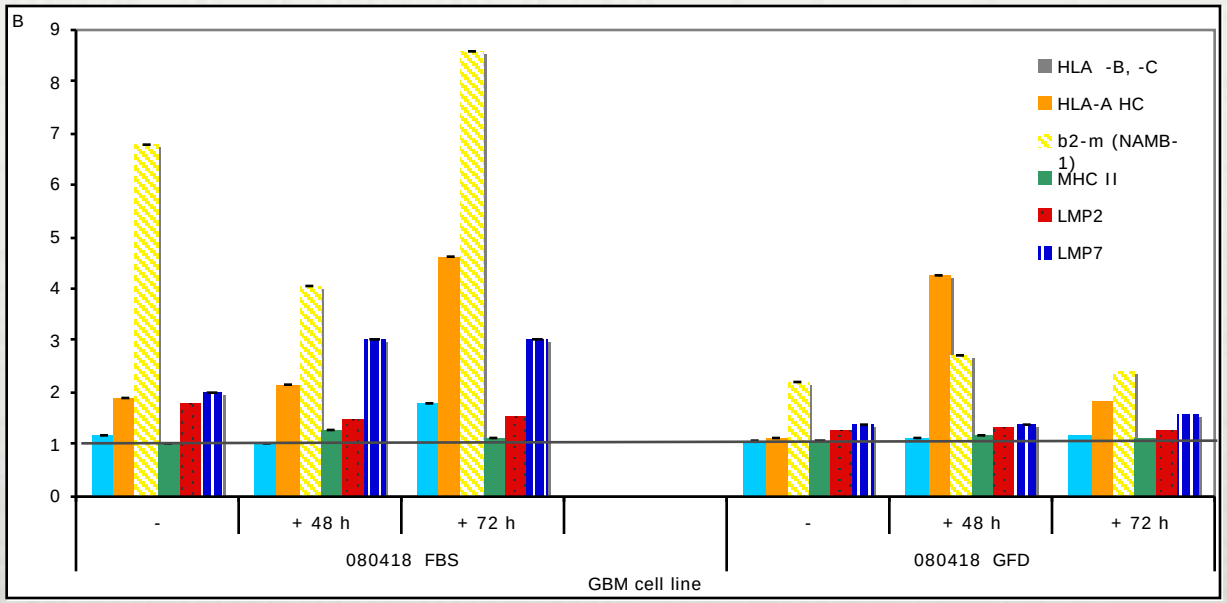


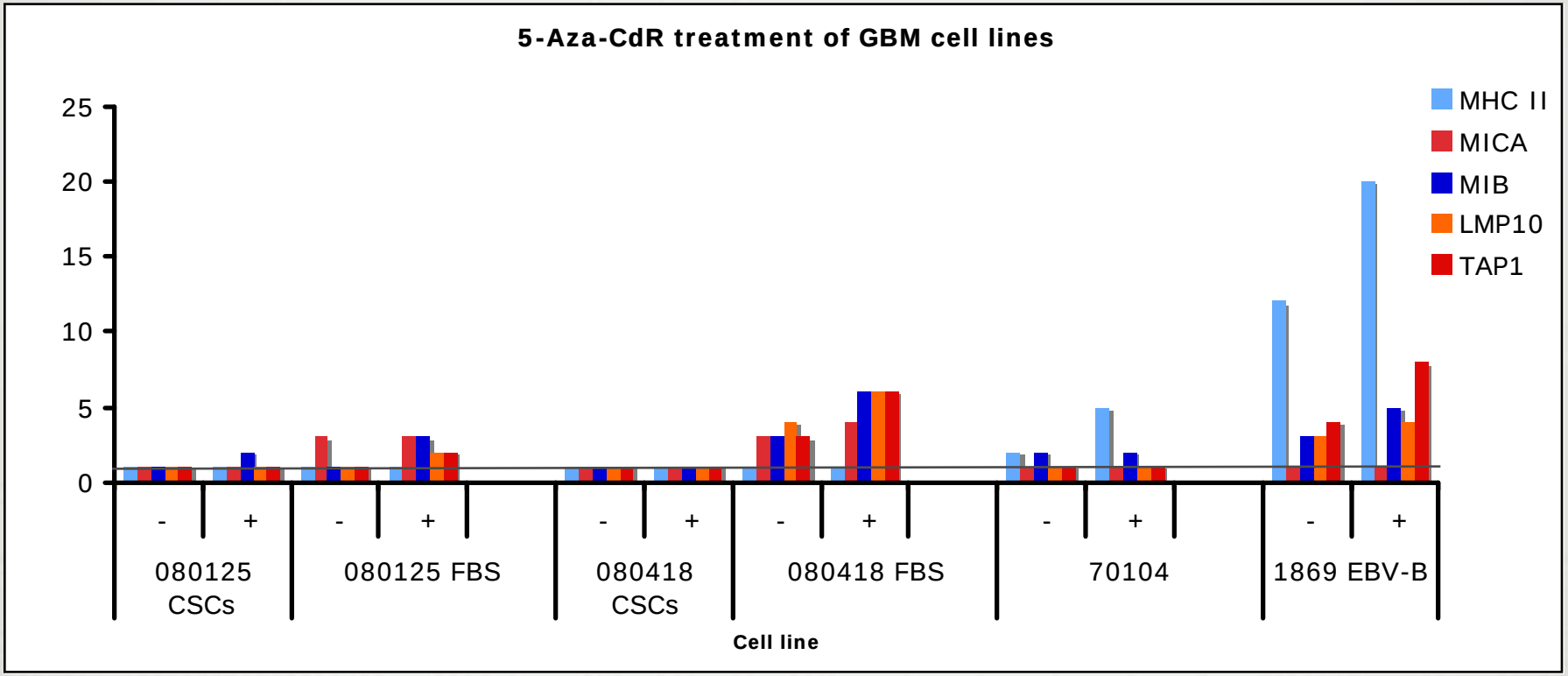
APM expression and modulation in CSCs vs FBS tumor cells

080125



080418

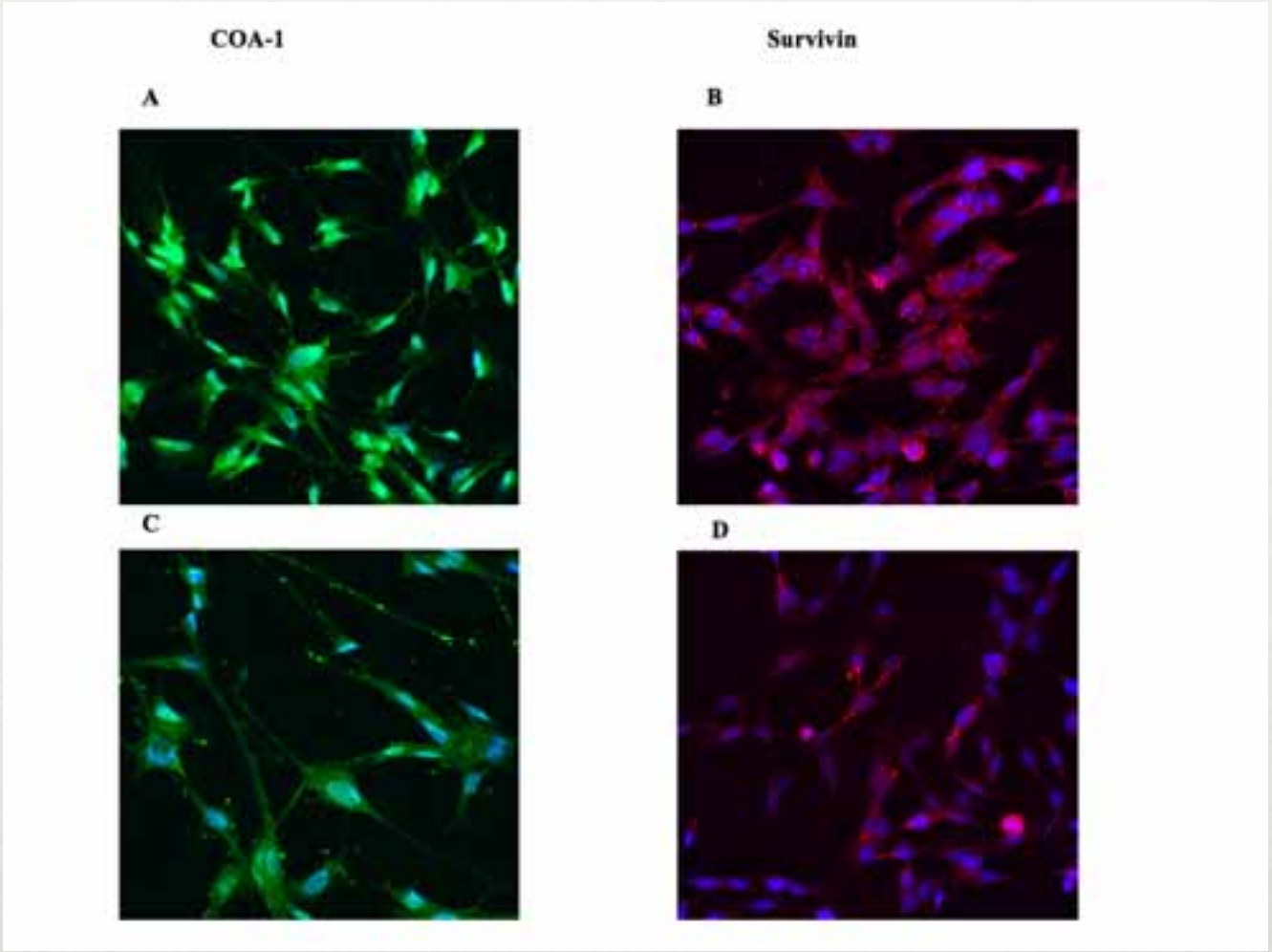




THE CELL LINES WERE TREATED in vitro WITH 10 nM OF 5-AZA-CdRFOR 4 DAYS. THE EXPRESSION OF MHC, APM MOLECULES AND OF NKG2DLs WAS EVALUATED BY CYTOFLUORIMETRIC ANALYSIS;
DATA ARE REPRESENTED AS MRFI.

TAA expression in GBM CSCs

Survivin, COA-1 AND SOX2 WERE COMMONLY EXPRESSED IN BOTH CSCs AND FBS TUMOR CELLS;
NO EXPRESSION OF MAGE, NY-ESO-1, GP100 AND IL-13Rα2 WAS FOUND.



080125

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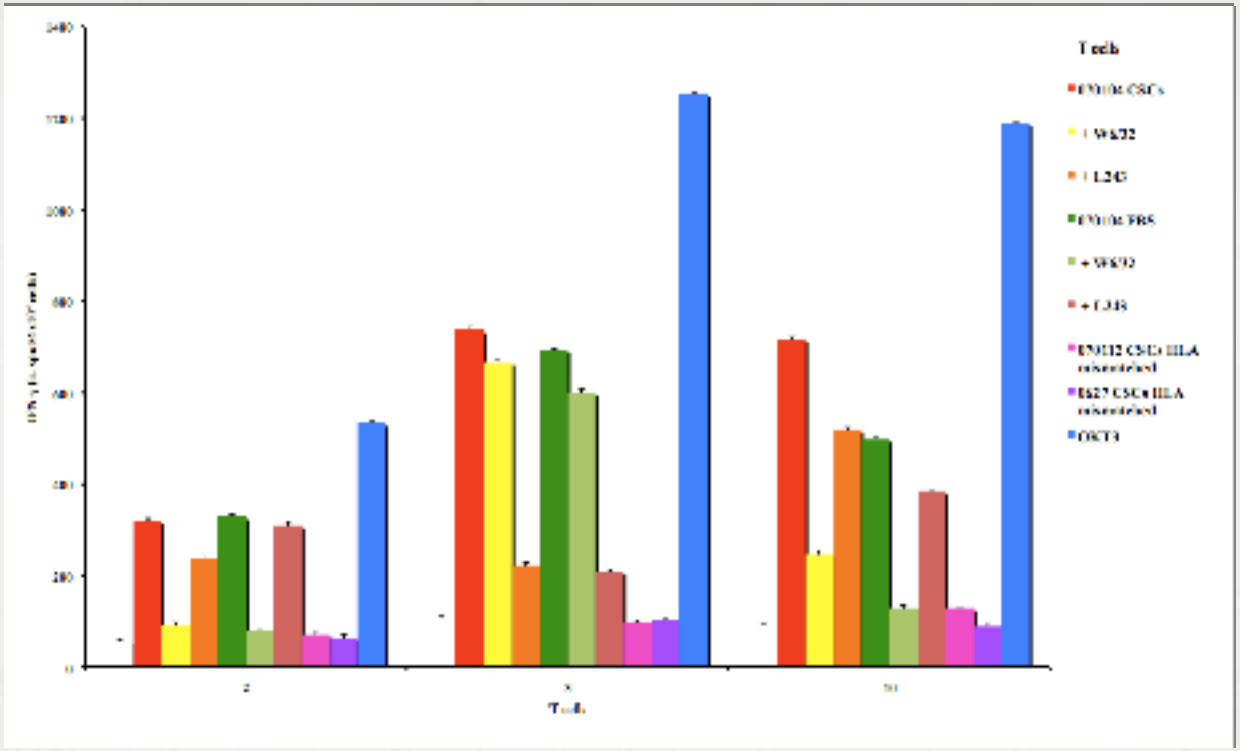
071210

080418

CSCs can elicit autologous t cell-mediated immune responses

PT. # 070104



B

T cells	CD4 / CD8	Target Cells					
		Ly alone	070104 CSCs	+ W6/32	+ L243	0627 CSCs HLA mismatched	OKT3
2	5 / 95 [a]	1 [b]	20	9	17	7	56
3	96 / 4	1	2	1	1	1	8
10	20 / 76	2	13	N.D.	N.D.	1	24

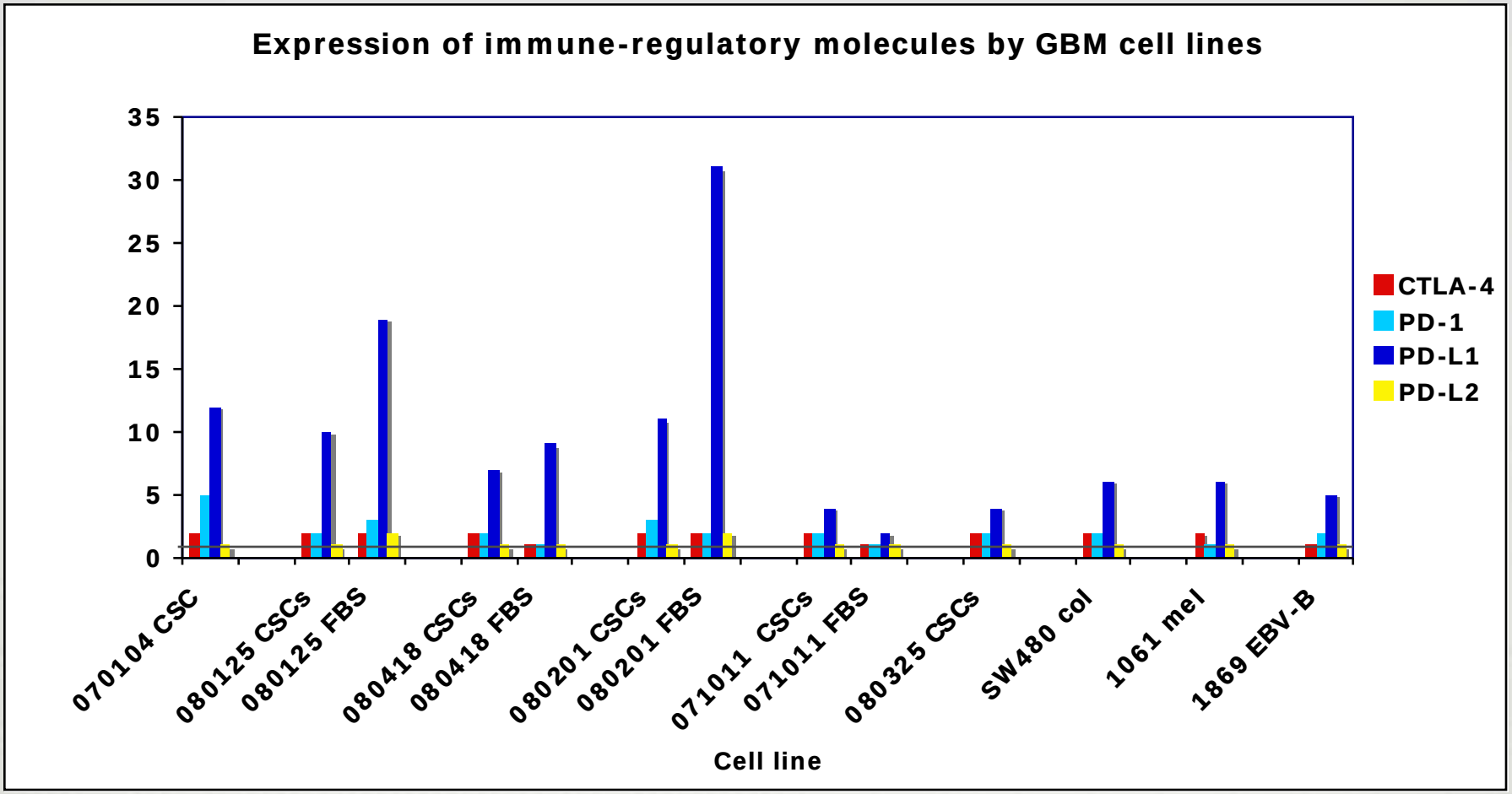
[a] Results represent the percentage of CD4 and CD8 positive T cells.
[b] Results represent the percentage of CD107a positive T cells.

Reactivity against CSC or FBS tumor cell lines in autologous setting by T lymphocytes isolated from 4 GBM patients

Patient #	T cell line #	Autologous CSC recognition	Autologous FBS tumor cell recognition	MHC-restriction [a]	Cytokine release	TH type subset
070104	2	+++ [b]	N. A.	MHC I	IFN-γ	TH1
	3	+++	N.A.	MHC II	IFN-γ	TH1
	10	+++	N. A.	MHC I	IFN-γ	TH1
080325	1	+	+++	MHC II	IL-5	TH2
	2	+	++	MHC I	IFN-γ	TH1
	4	+	++	MHC II	IL-5	TH2
080125	4	+	++	MHC II	IL-5	TH2
080418	1	+	+	-	IL-5	TH2

+: ÅÜ0 < 100 spots/ 4x10^4 T cells; ++: ÅÜ00 < 200 spots/ 4x10 ^{4} T cells; +++: ÅÜ00 < 800 spots/ 4x10^4 T cells;

N.A.: FBS tumor cells are not available;

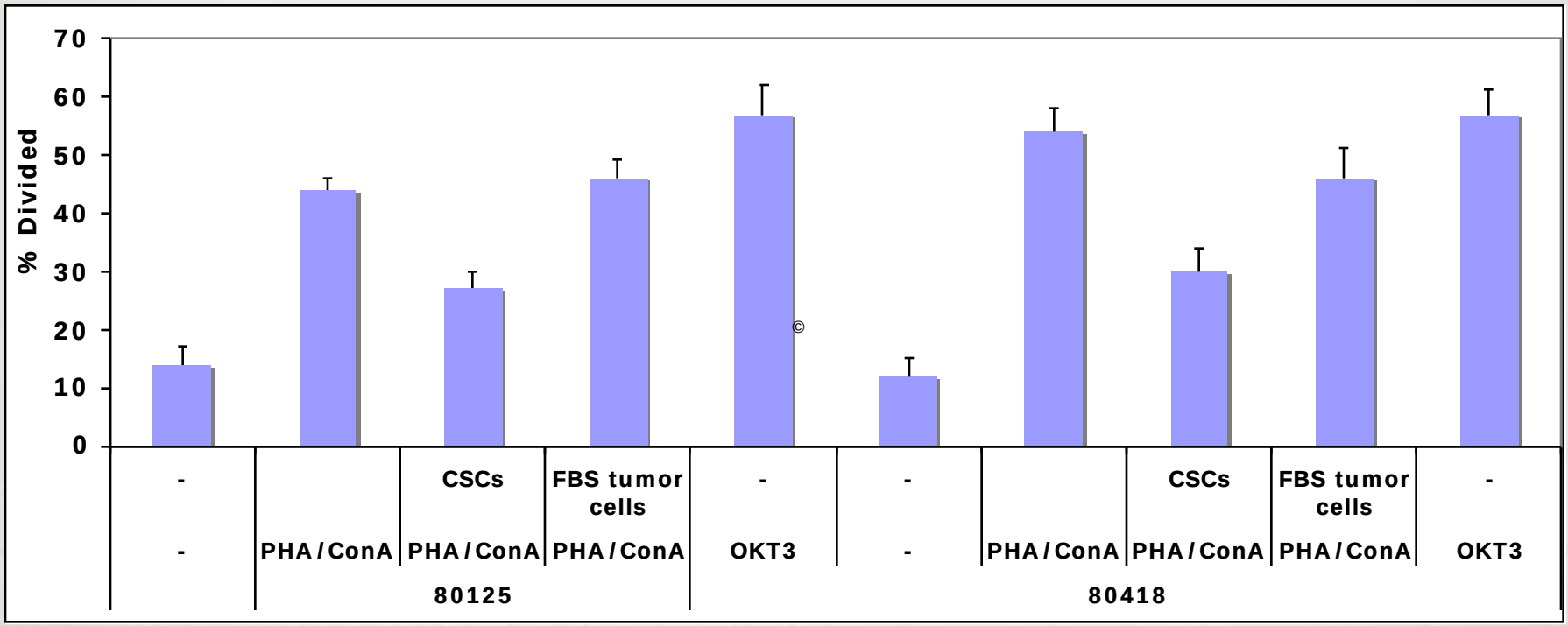


THE EXPRESSION OF IMMUNE-REGULATORY MOLECULES WAS EVALUATED BY IF AND CYTOFLUORIMETRIC ANALYSIS;

DATA ARE REPRESENTED AS MRFI;

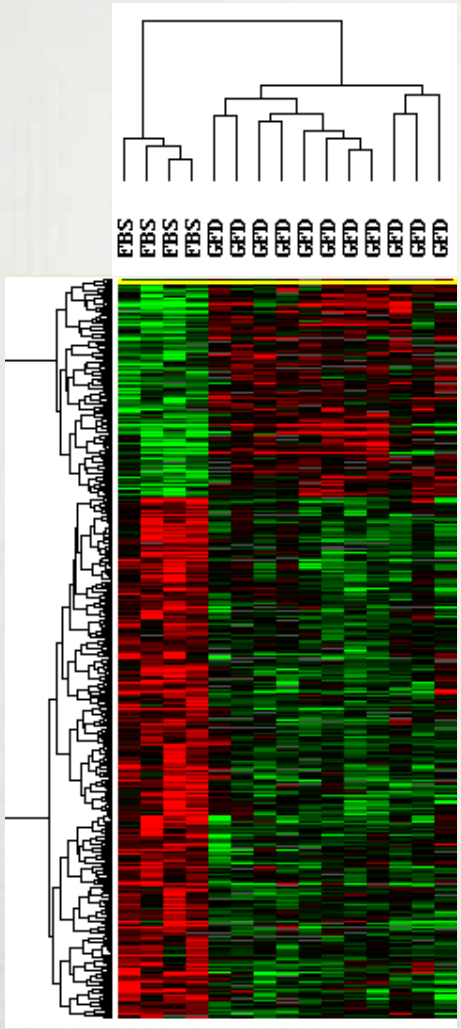
B7-1 AND B7-2 WERE NOT DETECTED ON THESE CELL LINES.

INHIBITION OF THE PROLIFERATIVE ACTIVITY OF ALLOGENEIC T LYMPHOCYTES BY GBM CSCs AND NOT FBS CELLS



PBMc were stimulated in vitro with mitogens with or without GBM CScs or FBS tumor cells; after 72 and 120 hrs the proliferative ability of of CD3+ gated cells was analyzed by CFSE staining and cytofluorimetric analysis.
this experiment has been repeated twice.
The proliferative index and the divison index were also calculated.

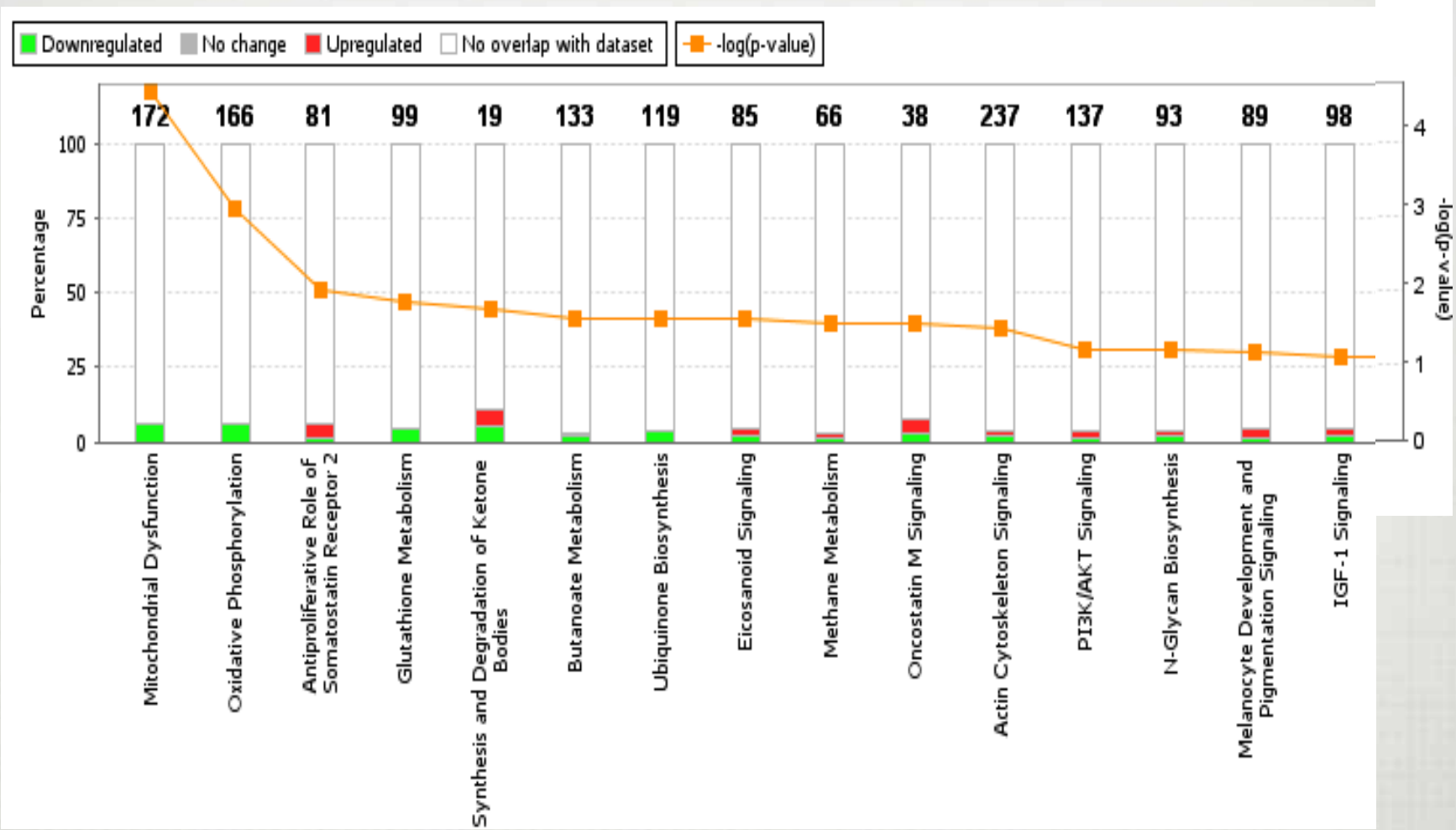
Whole transcriptome analysis of CSCs vs. FBS tumor cells



GFD vs FBS comparison:
Fold>1.5, p<0.005, permutation p<0.017
469 genes, 393 gene pass 80% present filter.

See gene data list analysis output file and attached

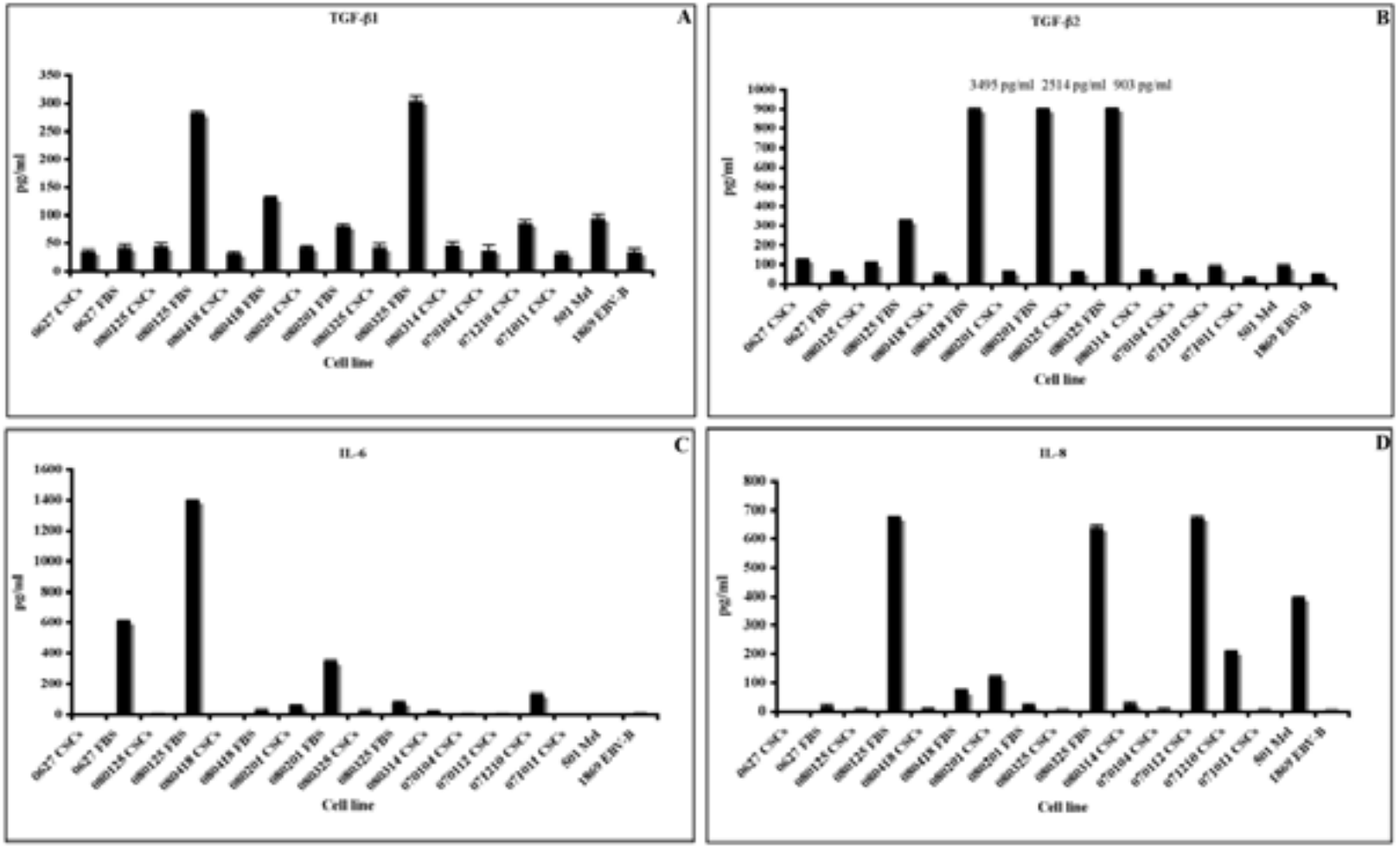
Genes involved in canonical pathways



Immune related Gene profile signature

- **Genes with immunological function were differentially expressed in CSCs vs FBS tumor cells.**
- In particular the proteosome maturation protein and the proteasome activator subunit 1 were down-modulated (-1,8 and -2.4 fold change, respectively) in CSCs compared to FBS tumor cells correlating with alterations we found at protein level of APM molecule (i.e. MB1) expression.
- Genes related to IFN signaling, such as IFN regulatory factor binding 2 protein (2.7 fold), Tax1 (3,2 fold), IFNGR1(1,7 fold) and the TNF receptor-associated factor 2 (TRAF2) (1,8 fold) were also under expressed in CSCs in comparison with FBS tumor cells.
- Notably, IL-6 and IL-8 were also found down-modulated in CSCs vs FBS tumor cells and these findings correlating with protein secretion levels we have detected in the supernatants of these cell lines
- No differential gene expression of IFN-α, IFN-β, TNFR and IL-1 was detected between CSCs and FBS tumor cells.
- Conversely, genes involved in JAK-STAT signal pathway were found up-regulated (2 fold) in CSCs compared to FBS tumor cells, in line with the previously reported evidence that members of this protein family were aberrantly activated in a variety of tumors including GBM (Bromberg, 2002; Brantley et a., 2008).

Secretion of suppressive or pro-inflammatory cytokines by CSCs vs. FBS tumor cells



CYTOKINE DETECTION IN THE SUPERNATANTS WAS DETECTED BY ELISA OR SEARCHLight ASSAYS; NO DETECTION IN THE SUPS OF IL-10, IL-13 AND TNF-A WAS OBSERVED.

Immunobiological differences between CSC and FBS tumor cell lines.

	Immune-related molecules/activity							
	MHC I	MHC II	NKG2DLs	APM	IFN modulation [a]	5-Aza-(CdR)[b]	T cell-mediated recognition	Supressive activity
CSCs	+	-/+	+/-	+	+	+/-	+	++
FBS	++	-/+	+/-	++	++	++	++	+/-
	Stem cell-associated molecule and/or TAA							
	Nestin	S100A4	S100A6	SOX2	Survivin	COA-1	GP100	NY-ESO-1
CSCs	+++	++	++	+++	+++	+++	-	-
FBS	+	+	+	++	+++	+++	-	-

a: modulation of the expression of MHC, NKG2D and APM molecules following *in vitro* treatment of the cells with either IFN-α or-γ;

b: modulation of the expression of MHC, NKG2D and APM molecules following *in vitro* treatment of the cells with the demethylating agent 5-Aza.-(CdR).

CSCs

Conclusions

- A Low immunogenic profile was found in both CSCs and FBS tumor cells isolated from GBM patients, with higher defective APC pattern in CSCs;
- the immune profile can be rescued, though more efficiently in FBS tumor cells, by treatment with IFNs or with 5-Aza-CdR of GBM cell lines;
- T cell-mediated immune responses can be isolated from GBM patients, though mostly TH2-mediated subset;
- Differential gene signature, including immune related genes, was detected in CSCs vs FBS tumor cells; in some cases we could confirm these results at the protein levels (ELISA; SEarchLight).

Future directions

- To identify the TAA recognized by anti-GBM CSC T lymphocytes;
- To carry out functional experiments based on gene profile data to possibly identify CSC-associated markers and/or TAA;
- to investigate whether the ability of CSCs to elicit T cell-mediated immune responses can be increased by usage of antagonist mAbs directed against negative immune regulatory molecules (anti-CTLA-4 or -PD-L1);
- to translate all the obtained information to design CSC-specific immunotherapy protocols for GBM patients.

ACKNOWLEDGEMENT

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Dept. Of Surgery, Immunology and Pathology, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

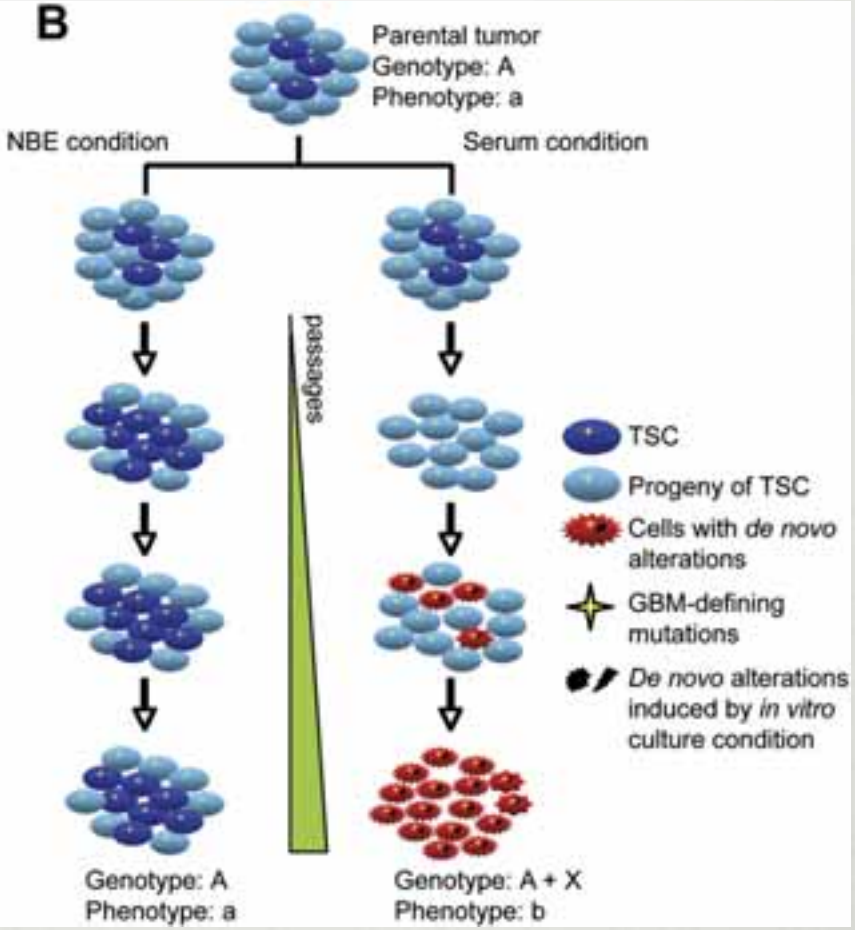
Soldano Ferrone

A HYPOTHETICAL MODEL OF THE RELATIONSHIP BETWEEN PRIMARY TUMOR-DERIVED TUMOR STEM CELLS AND GBM TUMOR CELL LINES

A

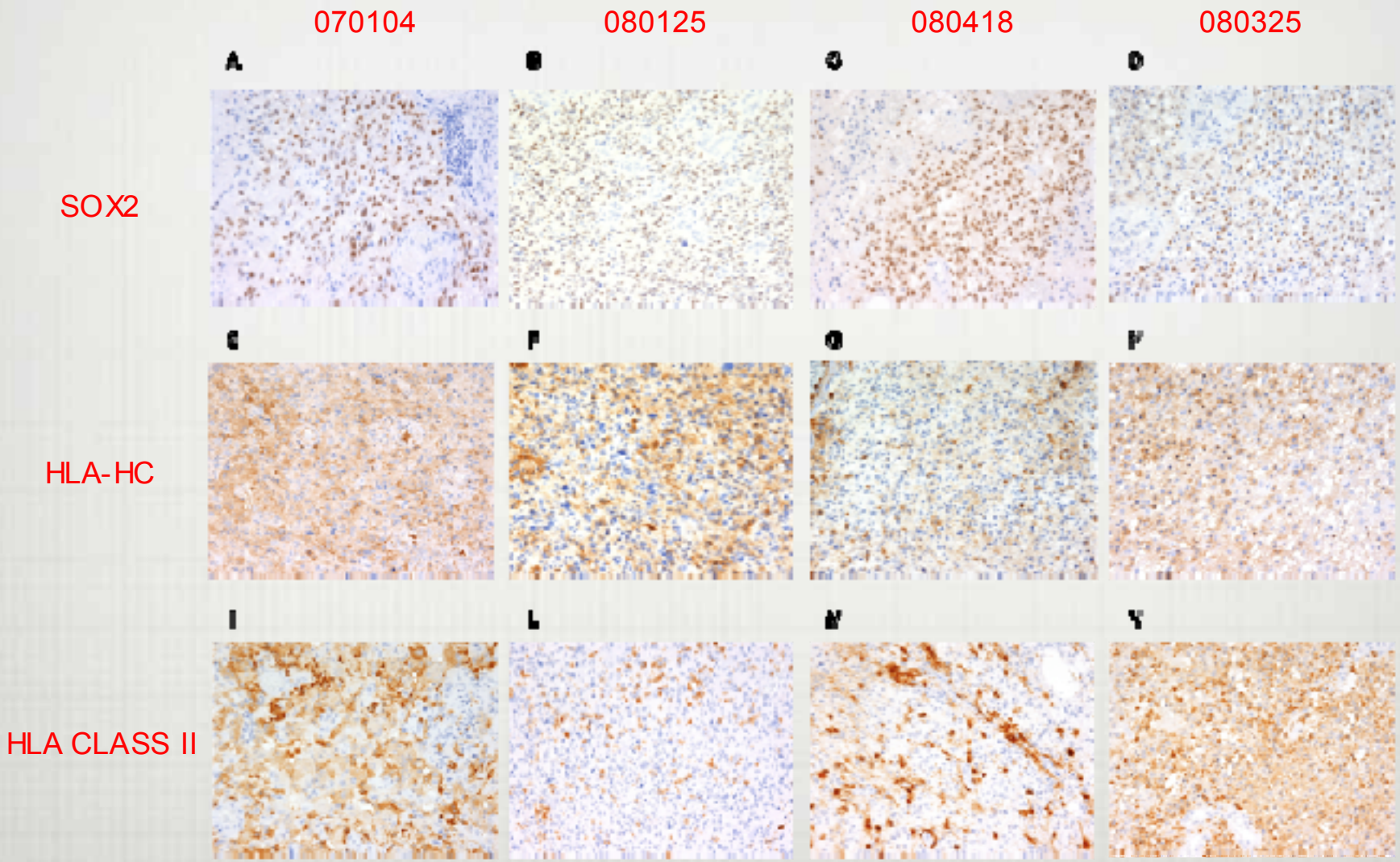
	NBE-cultured GBM cells	Serum-cultured GBM cells
Proliferation	Constant	Limited growth, reaches plateau, followed by exponential growth
Clonogenicity Tumorigenicity	Clonogenic and tumorigenic regardless of passages	Not at early passages
Differentiation potential	Induce to become glial and neuronal lineages	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



LEE J. ET AL., CANCER CELL 2006

in vivo Expression of MHC and APM molecules in GBM lesions



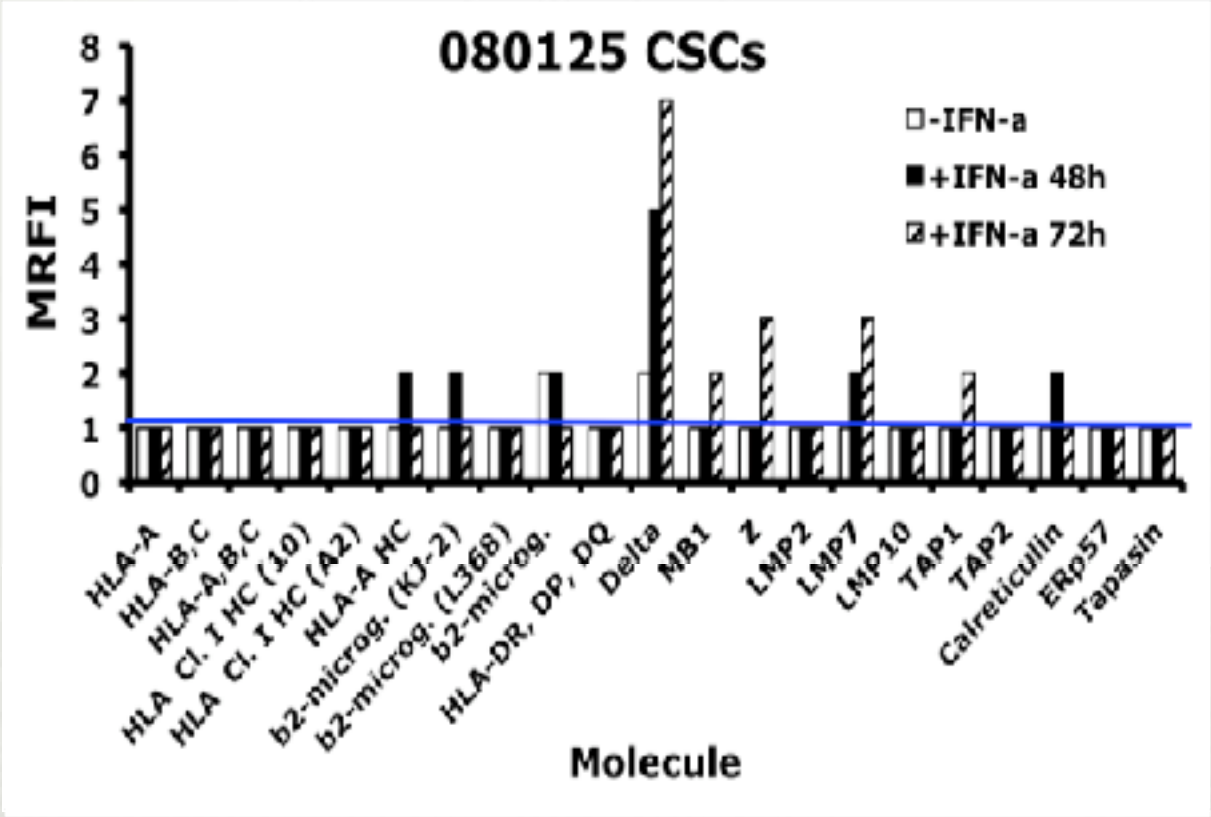
TUMORS CONTAINING CANCER STEM CELLS

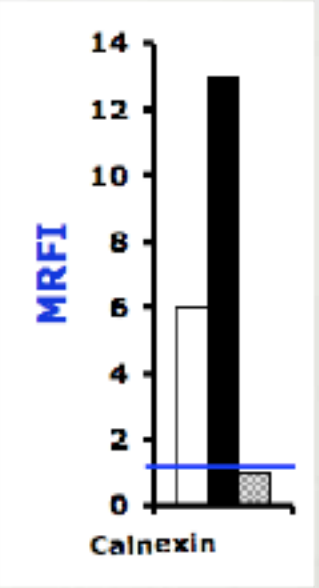
Acute myeloid leukemia (AML)	$CD34^+ CD38^-$ (Bonnet D et al, 1997; Hope KJ et al, 2004)
Chronic myeloid leukemia (CML)	$BCR\text{-}ABL\ CD34^+ CD38^-$ (Barret et al, 2003)
BREAST CANCER	$CD44^+ CD24^{-/low}$ (AL-Hajj M et al, 2003)
COLON CANCER	$AC133^+$ / $ESA^+ CD44^+ CD166^+$ (Ricci -Vitiani et, 2007; O'Brien et al, 2007; Li C et al, 2007)
PANCREATIC CANCER	$CD44^+ CD24^+ ESA^+$ (Prince ME, et al, 2007)
MELANOMA	$CD20^+$ (Fang D et al, 2005)
PROSTATE	$CD44^+$ [redacted] $1^+ CD133^+$ (Collins AT et al, 2005)
LIVER CANCER	$CD90^+ CD44^+$ (Yang ZF et al, 2008)
GLIOMA	$AC133^+$ and/ or $EGFR^+$ (Sinh SK et al, 2004; Galli R et al, 2004; Liu G et al 2006)

Aims

- To characterize the immune profile of CSCs isolated from GBM patients;
- to analyze the immunobiological functions of GBM CSCs compared to their autologous differentiated tumor cells (grown *in vitro* in the presence of FBS and, referred as FBS tumor cells);
- to define whether CSCs can represent suitable targets for immune based therapeutic interventions for GBM patients.

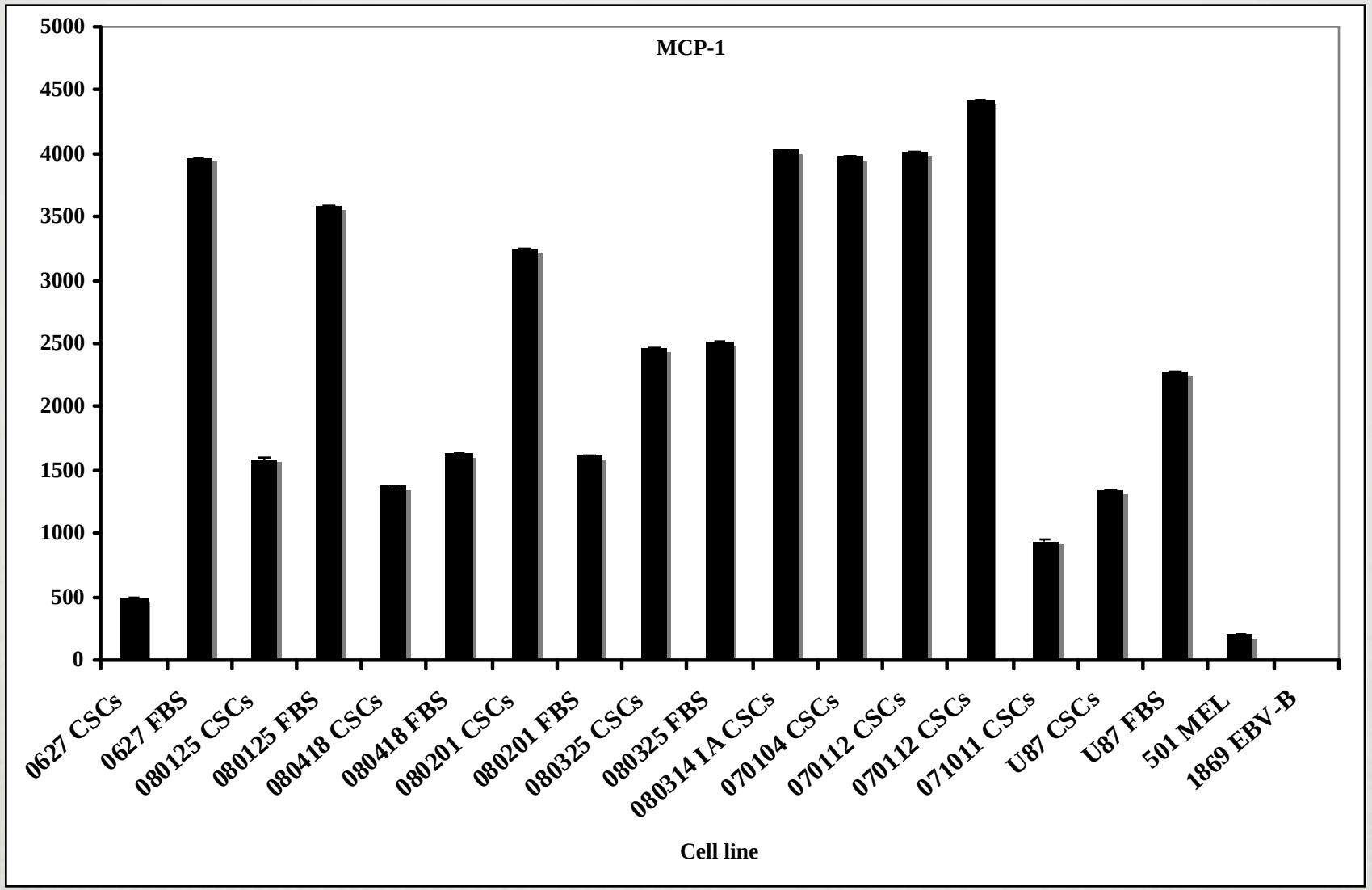
IFN-α modulation of the expression of antigen processing molecules by CSCs





MRFI: ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with isotype-matched control mouse immunoglobulins.

Pro-angiogenic factor release by both GBM CSCs and FBS tumor cells



VEGF, FGF-b AND ANGIOPOIETIN 2 WERE ALSO FOUND IN THE SUPS, WHILE NO SECRETION OF MPIF1 AND EOTAXIN WAS FOUND.