

Identification of Cell Surface Glycoprotein Markers for Glioblastoma-Derived Stem-Like Cells Using a Lectin Microarray and LC–MS/MS Approach

Jintang He,[†] Yashu Liu,[†] Xiaolei Xie,[†] Thant Zhu,[‡] Mary Soules,[‡] Francesco DiMeco,^{||,⊥}
 Angelo L. Vescovi,[#] Xing Fan,^{*,‡,§} and David M. Lubman^{*,†}

Department of Surgery, University of Michigan Medical Center, Ann Arbor, Michigan 48109, Department of Neurosurgery, University of Michigan Medical Center, Ann Arbor, Michigan 48109, Department of Cell and Developmental Biology, University of Michigan Medical Center, Ann Arbor, Michigan 48109, Neurological Surgery, Johns Hopkins University, Baltimore, Maryland 21205, Department of Neurosurgery, Istituto Nazionale Neurologico C. Besta, Milan, Italy 20133, Department of Biotechnology and Biosciences, University of Milan Bicocca, Milan, Italy 20126

Received January 6, 2010

Despite progress in the treatment of glioblastoma, more than 95% of patients suffering from this disease still die within 2 years. Recent findings support the belief that cancer stem-like cells are responsible for tumor formation and ongoing growth. Here a method combining lectin microarray and LC–MS/MS was used to discover the cell surface glycoprotein markers of a glioblastoma-derived stem-like cell line. Lectin microarray analysis of cell surface glycans showed that two galactose-specific lectins *Trichosanthes kirilowii* agglutinin (TKA) and Peanut agglutinin (PNA) could distinguish the stem-like glioblastoma neurosphere culture from a traditional adherent glioblastoma cell line. Agarose-bound TKA and PNA were used to capture the glycoproteins from the two cell cultures, which were analyzed by LC–MS/MS. The glycoproteins were quantified by spectral counting, resulting in the identification of 12 and 11 potential glycoprotein markers from the TKA and PNA captured fractions respectively. Almost all of these proteins were membrane proteins. Differential expression was verified by Western blotting analysis of 6 interesting proteins, including the up-regulated Receptor-type tyrosine-protein phosphatase zeta, Tenascin-C, Chondroitin sulfate proteoglycan NG2, Podocalyxin-like protein 1 and CD90, and the down-regulated CD44. An improved understanding of these proteins may be important for earlier diagnosis and better therapeutic targeting of glioblastoma.

Keywords: glycoprotein • biomarker • lectin microarray • glioblastoma • stem-like cells • LC–MS/MS

Introduction

Glioblastoma (GBM) is the most common malignant brain tumor in adults. Even with advanced technology in surgery, *in vivo* imaging, chemotherapy and radiotherapy, the overall survival rate of GBM patients was only 17.7% at 1 year, and 3.3% at 2 years.^{1,2} Therefore, new strategies to treat GBM are urgently needed. There is emerging evidence showing that cancer stem cells within tumors are responsible for tumor formation and ongoing growth.³ Cancer stem cells have the

ability to self-renew and drive tumorigenesis. The term cancer stem cells reflects the “stem-like” properties of these cells.⁴ Such stem-like cells have been found in various solid tumors such as breast, colon, head and neck, pancreas, and liver. Their existence has also been demonstrated in brain tumors, including GBM.^{5–8} We have demonstrated recently that Notch pathway blockade depletes cancer stem-like cells in medulloblastoma and GBM.^{9–11} Therefore, new therapeutic methods targeting cancer stem-like cells may bring hope for GBM patients. Identification of markers of GBM stem cells will be indispensable, since this may lead to earlier diagnosis and improved therapeutic targeting of the disease. Most of the known markers of cancer stem-like cells in solid tumors are cell surface glycoproteins.⁴ The discovery of unique glycan expression patterns on the surface of cancer stem-like cells is an important step to identify novel surface glycoprotein markers of these cells.

The lectin microarray is a powerful tool to analyze the glycan structures of glycoproteins.^{12–15} Recently, the technology has been employed to study diverse cell processes by profiling cell surface glycans of live cells.^{16–19} Using lectin microarray, Chen

* To whom correspondence should be addressed. David M. Lubman, Department of Surgery, The University of Michigan Medical Center, 1150 West Medical Center Drive, Building MSRB1, Rm A510B, Ann Arbor, MI 48109-0656. E-mail: dmlubman@umich.edu. Xing Fan, Department of Neurosurgery, Department of Cell and Developmental Biology, 109 Zina Pitcher Place, BSRB 5018, Ann Arbor, MI 48109. E-mail: xingf@umich.edu.

[†] Department of Surgery, University of Michigan Medical Center.

[‡] Department of Neurosurgery, University of Michigan Medical Center.

^{||} Johns Hopkins University.

[⊥] Istituto Nazionale Neurologico C. Besta.

[#] University of Milan Bicocca.

[§] Department of Cell and Developmental Biology, University of Michigan Medical Center.

Table 1. Carbohydrate Specificities of the 16 Lectins Used for Lectin Microarray^a

lectin	abbreviation	carbohydrate specificity	source
Concanavalin A	Con A	Branched and terminal α -linked Man	Vector Laboratories, Inc.
<i>Ulex europaeus</i> lectin I	UEA-1	α -linked Fuc	EY Laboratories, Inc.
<i>Sambucus nigra</i> lectin II	SNA-2	GalNAc β > Gal β	EY Laboratories, Inc.
Wheat germ agglutinin	WGA	GlcNAc β	EY Laboratories, Inc.
<i>Sambucus nigra</i> lectin	SNA	Neu5Ac α (2-6)Gal	Vector Laboratories, Inc.
<i>Lycopersicon esculentum</i> (Tomato) lectin	LEL	GlcNAc (prefers trimer and tetramer)	Vector Laboratories, Inc.
<i>Helix pomatia</i> agglutinin	HPA	GalNAc α (Terminal)	EY Laboratories, Inc.
<i>Phaseolus vulgaris</i> leucoagglutinin	PHA-L	Gal β (1-4)GlcNAc β (1-6)Man	EY Laboratories, Inc.
<i>Maackia amurensis</i> lectin II	MAL-2	Neu5Ac α (2-3)	Vector Laboratories, Inc.
<i>Aleuria aurentia</i> lectin	AAL	Terminal α -linked Fuc	Vector Laboratories, Inc.
<i>Lens culinaris</i> agglutinin	LCA	α -linked mannose, Fuc α (1-6)GlcNAc	Vector Laboratories, Inc.
<i>Solanum tuberosum</i> lectin	STL	GlcNAc β (1-4)GlcNAc	Vector Laboratories, Inc.
<i>Bauhinia purpurea</i> lectin	BPL	Gal β (1-3)GalNAc, GalNAc	Vector Laboratories, Inc.
<i>Trichosanthes kirilowii</i>	TKA	Gal β	EY Laboratories, Inc.
Peanut agglutinin	PNA	Gal β (terminal)	Vector Laboratories, Inc.
Soybean agglutinin	SBA	Terminal α - or β -linked GalNAc	Vector Laboratories, Inc.

^a Man, mannose; Fuc, fucose; GalNAc, *N*-acetyl-D-galactosamine; Gal, galactose; GlcNAc, *N*-acetyl-D-glucosamine; Neu5Ac, *N*-acetylneuraminic acid (a member of the sialic acid family).

et al.¹⁷ evaluated glycan expression patterns on the surface of several human breast cancer cell lines and observed significant differences that related to cancer metastasis. Tao et al.¹⁹ analyzed the cell surface glycan signature of a human breast cancer cell line MCF7 and found that the *Lycopersicon esculentum* lectin (LEL) may be used as a biomarker of this cell line. The investigation of cell surface glycan changes and the discovery of specific glycan-binding lectins facilitate the identification of cell surface glycoprotein markers. In a recent study, Lee et al.²⁰ found that the *Griffonia simplicifolia* lectin II (GS II) selectively bound to rat fibroblast cells but not to rat endothelial cells, and therefore, they captured glycoproteins from fibroblast cells using GS II and identified several glycoprotein markers by LC-MS/MS. This work provided a useful strategy that combined lectin microarray and mass spectrometry to identify cell surface glycoprotein biomarkers.

In our work, high-sensitive fluorescence-assisted lectin microarrays were used to discover the cell surface glycan expression patterns of a human GBM stem-like cell line and a traditional adherent GBM cell line as a control. Two lectins *Trichosanthes kirilowii* agglutinin (TKA) and Peanut agglutinin (PNA) which can distinguish the two cell lines were used to capture the glycoproteins by affinity chromatography. The LC-MS/MS analysis and label-free quantification resulted in the identification of 12 and 11 potential glycoprotein markers from the TKA and PNA captured fractions, respectively. Western blotting analysis of 6 selected proteins confirmed the differential expression. Future investigations of these proteins may be helpful for identifying cancer stem-like cells from GBM.

Materials and Methods

Cell Culture. The human GBM-derived stem-like cell line HSR-GBM1 was established by Vescovi and colleagues and maintained in serum-free Neurocult medium (Stem Cell Technologies) supplemented with 20 ng/mL of both epidermal growth factor (EGF) and fibroblast growth factor.^{6,21} Human GBM cell line U373 was maintained in DMEM supplemented with 10% fetal bovine serum. Cells growing exponentially were harvested and washed twice with PBS.

Lectin Microarray. Sixteen lectins were used in this study. The carbohydrate specificities of these lectins were listed in Table 1. Each lectin was dissolved in PBS buffer (0.01 M

phosphate, 0.15 M NaCl, pH 7.4) to a concentration of 1 mg/mL and printed on SuperAmine slides (Arrayit, Sunnyvale, CA) using a piezoelectric noncontact printer (Nano plotter; GeSiM, GmbH, Germany). Three spots per lectin were printed in each block and 12 blocks were printed per slide. The total volume of each spot was 2.5 nL, which resulted from spotting of 500 pL for 5 times. The slides were incubated in a humidity-controlled incubator (>45% humidity) overnight to allow lectin immobilization. After incubation, the slides were blocked with 1% BSA/PBS for 1 h and washed three times with PBST (0.1% Tween 20 in PBS). U373 and HSR-GBM1 cells were labeled with 10 μ M CFSE cell-tracing dye (Invitrogen, Carlsbad, CA) for 15 min in darkness. Each lectin blot on the slide was seeded with 2×10^5 CFSE-labeled cells in PBS with 0.5 mM CaCl₂ and 1% BSA. Cells were incubated with lectin microarrays at room temperature for 40 min in darkness. After being washed with PBS for 5 min, the slides were air-dried and scanned with a microarray scanner (Genepix 4000A; Axon). Genepix 6.0 was used to analyze the images.

Protein Extraction. Cells were washed once with PBS and then suspended in 1 mL of lysis buffer (1% octyl- β -D-glucopyranoside, 20 mM Tris-HCl, pH7.4, 150 mM NaCl, and 1% protease inhibitor mixture (Sigma-Aldrich)). The cells were homogenized with 30 strokes in a Dounce glass homogenizer with a tight-fitting pestle. The cell lysate was centrifuged at 40 000g for 30 min at 4 °C. The supernatant was collected and the protein concentration was determined by the Bradford method.²²

Capture of Glycoproteins by Lectin Affinity. A Pierce centrifuge column was packed with 1.5 mL of agarose-bound PNA or TKA. The column was first washed with 3 vol of binding buffer (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM MnCl₂). Cell lysate containing 800 μ g of protein was diluted four times with binding buffer, and passed through the column twice. The column was washed with 6 mL of binding buffer to remove the nonspecific binding proteins. Finally, the captured glycoproteins were released with 3 mL of elution buffer (200 mM D-galactose in binding buffer, pH 7.4). This step was repeated twice, and the eluted fractions were pooled. For mass spectrometric analysis, the samples were concentrated with Microcon YM-10 centrifugal filter devices, reduced with 5 mM disulfide bond reducing agent Tris(2-

Glycoprotein Markers for Glioblastoma Stem-Like Cells

carboxyethyl)phosphine (TCEP) for 30 min, alkylated with 25 mM iodoacetamide for 20 min, and digested with trypsin and PNGase F (Sigma-Aldrich).

Mass Spectrometry. The resulting tryptic peptides were analyzed by LC–MS/MS using an LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was performed on a Paradigm MG4 micropump system (Michrom Biosciences, Inc., Auburn, CA) equipped with a C18 separation column (0.1 mm × 150 mm, C18 AQ particles, 5 μm, 120 Å, Michrom Biosciences, Inc., Auburn, CA). Peptides were separated with a linear gradient of acetonitrile/water containing 0.1% formic acid at a flow rate of 300 nL/min. A 90 min linear gradient from 5 to 40% acetonitrile was used. The MS instrument was operated in positive ion mode. The ESI spray voltage was set at 2.5 kV, and the capillary voltage at 30 V. The ion activation was achieved by utilizing helium at a normalized collision energy of 35%. The data were acquired in data-dependent mode using the Xcaliber software. For each cycle of one full mass scan (range of m/z 400–2000), the three most intense ions in the spectrum were selected for tandem MS analysis, unless they appeared in the dynamic or mass exclusion lists.

Western Blotting. Western blotting was performed essentially as described before.²³ Briefly, 20 μg of proteins from HSR-GBM1, U373, U87, and T98G GBM cells were separated by 4–20% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad). U87 and T98G cell lysates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were blocked for 2 h, and then incubated with various primary antibodies for 4 h or overnight. Anti-NG2 and anti-CD44 were obtained from Millipore (Billerica, MA); anti-PODXL and anti-beta actin were from Abcam (Cambridge, MA); anti-CD90 and anti-Tenascin-C were from Abnova (Taipei, China); anti-Receptor-type tyrosine-protein phosphatase zeta (PTPRZ1) was from Sigma-Aldrich (St. Louis, MO). After being washed three times, the membranes were incubated with peroxidase-conjugated IgG (H + L) for 1 h, washed three times, and detected by Immobilon Western Chemiluminescent HRP Substrate Kit (Millipore, Billerica, MA).

Data Analysis. All MS/MS spectra were searched against the IPI database (IPI.human.v3.39). The search was performed using SEQUEST algorithm version 27 incorporated in Bioworks software version 3.1 SR1 (Thermo Finnigan). The search parameters were as follows: (1) Fixed modification, Carbamidomethyl of C; (2) variable modification, oxidation of M; (3) allowing two missed cleavages; (4) peptide ion mass tolerance 1.50 Da; (5) fragment ion mass tolerance 0.0 Da; (6) peptide charges +1, +2, and +3. The identified peptides were processed by the Trans-Proteomic Pipeline (TPP).²⁴ This software includes both the PeptideProphet and ProteinProphet programs. The database search results were first validated using the PeptideProphet software, and then the peptides were assigned for protein identification using the ProteinProphet software. In this study, both the PeptideProphet probability score and the ProteinProphet probability score were set to be higher than 0.9. This resulted in an overall false positive rate below 1%.²⁵

We performed a comparative analysis between two cell lines using the spectral counting method.²⁶ Spectral counting is an isotope label-free quantitation method to measure relative abundance between proteins in a complex protein mixture. The spectral count of individual proteins in both samples is acquired after TPP analysis. Spectral count fold-change was calculated as the ratio of the average spectral count for the

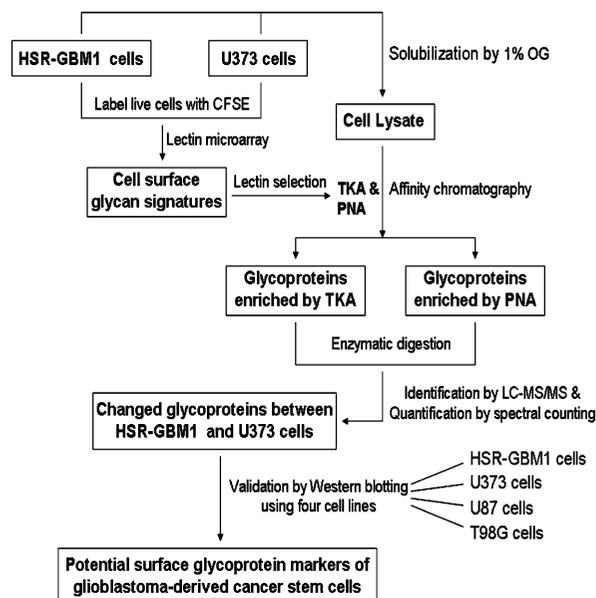


Figure 1. Workflow of lectin microarray and LC–MS/MS based identification of cell surface glycoprotein markers. The HSR-GBM1 and U373 live cells were first labeled with a fluorescent dye CFSE, and then their cell surface glycan signatures were profiled by lectin microarray. According to the binding patterns of the two cell lines to the arrays, two lectins TKA and PNA were selected for subsequent enrichment of glycoproteins by affinity chromatography. The glycoproteins were identified by LC–MS/MS and quantified by spectral counting. Finally, the changed glycoproteins between the two cell lines were further validated by Western blotting using four cell lines including the human GBM-derived stem-like cell line HSR-GBM1 and three traditional adherent human GBM cell lines U373, U87, and T98G.

target protein. A 2-fold change was considered as a significant difference between the two samples.

Results and Discussion

In the present work, we employed a method combining lectin microarray, lectin affinity chromatography, and LC–MS/MS to identify the cell surface glycoprotein markers for a GBM-derived stem-like cell line. A diagram of the workflow of this method is shown in Figure 1. The cell surface glycan expression patterns of the stem-like cell line and a control cell line were first explored by fluorescence-assisted lectin microarray, and then two lectins TKA and PNA were selected to capture the glycoproteins of the two cell lines by affinity chromatography. Subsequently, the glycoproteins were identified by LC–MS/MS and quantified by the spectral counting method. Finally, the differentially expressed glycoproteins were further verified by Western blotting analysis.

Illustration of Cell Surface Glycan Signatures by Lectin Microarray. Lectin microarrays consisting of 16 lectins were used to profile the surface glycan signature of the HSR-GBM1 neurosphere. As shown in Table 1, the carbohydrate specificities of the 16 lectins range extensively. The HSR-GBM1 cell line is a GBM neurosphere cell line derived from human glioblastoma, and it displayed self-renewal and multilineage differentiation features of neural stem cells.⁶ To investigate the specific glycan patterns on the surface of this undifferentiated cell line, a widely used traditional adherent GBM cell line U373 was chosen as a control. Figure 2A shows the binding patterns of fluorescently labeled live cells to the lectin microarrays. Note

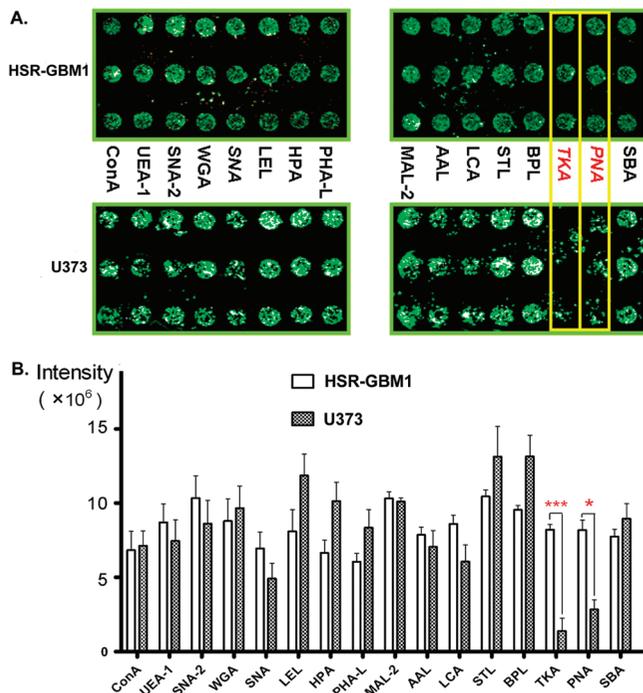


Figure 2. Lectin microarray. (A) A typical lectin microarray image depicts the binding patterns of two cell lines to the arrays of 16 lectins. Note that TKA and PNA lectins captured many more HSR-GBM1 cells than U373 cells. Each lectin was spotted in triplicate. (B) Fluorescent intensities of the 16 lectins. Spot intensities were the average of three values. * $p < 0.05$; *** $p < 0.001$.

that the lectins TKA and PNA had much stronger binding capacity to HSR-GBM1 cells than to U373 cells. Both of the two lectins showed significant differences in fluorescent intensities (Figure 2B). This indicates TKA and PNA may be used to distinguish the two cell lines.

Interestingly, both TKA and PNA have binding specificities to β -galactose (Table 1), which implies much higher expression of galactosylated glycoproteins on the surface of the GBM-derived stem-like cells as compared with traditional adherent GBM cells. In a previous study, the galactosylated glycoproteins were found to be expressed at higher level in glioma compared with normal brains.²⁷ Our findings suggest that the increase of galactosylation in glioma brain tissues might be contributed by cancer stem-like cells.

As shown in Table 1, the lectin *Sambucus nigra* lectin II (SNA-2) also has binding specificity to β -galactose, but it primarily binds to β -GalNAc. Therefore, we did not observe a significant difference in fluorescent intensities for SNA-2 (Figure 2).

Preparation and Identification of Membrane Glycoproteins.

The binding patterns of TKA and PNA (Figure 2A) suggest that their binding partners on the cell surface were potential markers to distinguish the two cell lines. To discover these glycoprotein markers, we applied lectin affinity chromatography to capture glycoproteins from the HSR-GBM1 and U373 cells. A nonionic detergent, octyl- β -D-glucopyranoside, was used to lyse cells. This class of detergents can improve the solubility of membrane proteins.^{28,29} Agarose-bound TKA and PNA were packed into a column to capture the glycoproteins. The eluted glycoproteins from three independent enrichment experiments were digested and analyzed by

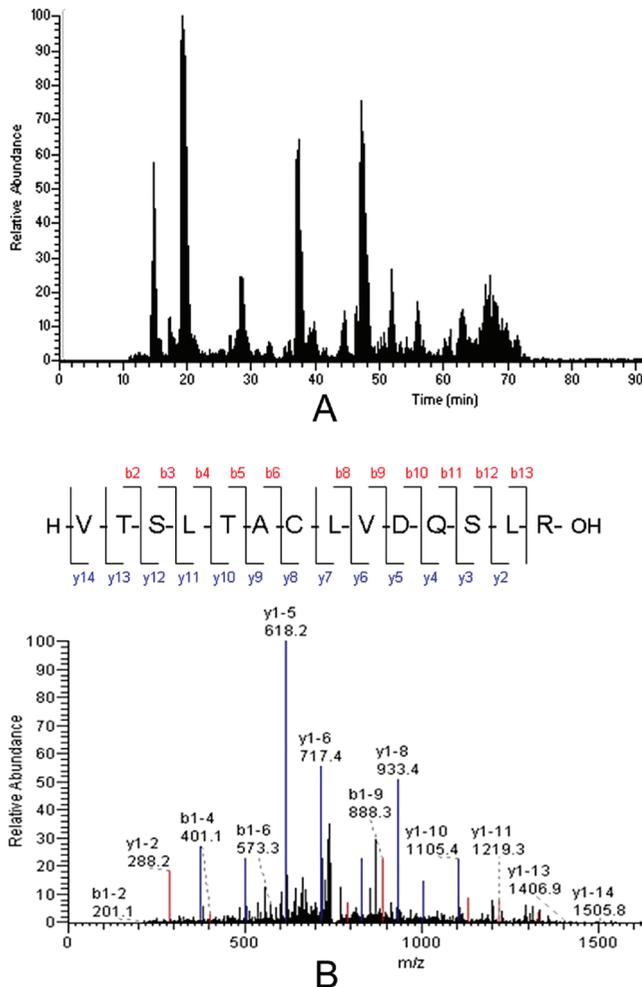


Figure 3. LC-MS/MS analysis. (A) A representative base peak chromatogram of TKA captured fraction from HSR-GBM1 cells. (B) MS/MS spectrum of a peptide. The sequence of the peptide was identified as VTSLTACLVDQSLR, which was from CD90 antigen.

LC-MS/MS using an LTQ mass spectrometer. As an example, Figure 3A shows a representative base peak chromatogram of TKA captured fraction from HSR-GBM1 cells, and Figure 3B is a typical MS/MS spectrum.

The LTQ analysis resulted in the identification of 43 glycoproteins from TKA enriched fractions (Supplementary Table S1) and 48 glycoproteins from PNA enriched fractions (Supplementary Table S2), wherein 24 glycoproteins overlapped by the two lectins. Consequently, a total of 67 glycoproteins were identified from the two cell lines after PNA and TKA enrichment. The detailed information of these glycoproteins is listed in Supplementary Tables S3–S6. The difference observed in protein identification from TKA and PNA enriched fractions may result from the minor difference in the carbohydrate specificities of the two lectins. Although TKA and PNA have similar binding specificities to Gal β , PNA can only bind to terminal Gal β , preferentially to terminal Gal β (1-3)GalNAc α , while TKA binds both terminal Gal β and internal Gal β (1-3)GalNAc α .

The subcellular locations of the 67 glycoproteins were annotated according to the UniProtKB database. A majority of these glycoproteins were localized to the plasma membrane, ER, and extracellular (Figure 4). Approximately 54% of these glycoproteins were plasma membrane proteins.

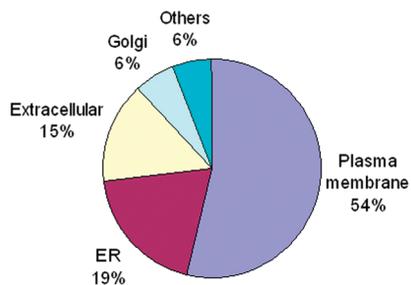


Figure 4. Subcellular location of glycoproteins identified from HSR-GBM1 and U373 cells after TKA and PNA enrichment.

The number of glycoproteins identified after TKA and PNA enrichment in this work was relatively low compared to previous studies using other lectins to capture glycoproteins from cells.^{29–31} To confirm that the difference was caused by different lectins rather than our working system, we captured glycoproteins from the HSR-GBM1 and U373 cells using Concanavalin A (Con A) and analyzed these proteins by LC–MS/MS, resulting in the identification of 83 and 100 glycoproteins from the two cell lines, respectively (Supplementary Tables S7 and S8). The numbers are comparable to that from similar studies.^{30,31} The glycoproteins bound by either TKA or PNA were less than half of those bound by Con A. This indicates that both TKA and PNA have a more narrow specificity than Con A.

A concern of the method used in this work was the reproducibility. We used R^2 values from linear regression for spectral counts to investigate the reproducibility of three replicate experiments. Here 33 and 40 glycoproteins were identified from HSR-GBM1 cells after TKA and PNA enrichment, respectively (Supplementary Tables S3 and S5), and the average R^2 values were around 0.7–0.8 for the three independent experiments of the two lectins, which are comparable to the values in some similar studies.^{32,33} This indicates that the experiments from cell lysis to glycoprotein enrichment, and to LTQ analysis were reproducible in the present work. Scatter plots for each TKA or PNA experiment pairs of HSR-GBM1 cells are available in Supplementary Figure S1. Consistent with the lectin microarray analysis (Figure 2), less glycoproteins were identified from U373 cells after TKA and PNA enrichment (only 23 and 10 glycoproteins were identified, respectively, see Supplementary Tables S4 and S6), and most of the spectral

counts for individual proteins were less than 5. Therefore, the U373 data sets were not suitable to evaluate the reproducibility.

Differentially Expressed Glycoproteins Analyzed by the Label-Free Spectral Counting Method. The glycoproteins identified by LC–MS/MS were analyzed by the spectral counting method to quantify the relative protein abundance.^{26,34} As a result, 12 differentially expressed glycoproteins between HSR-GBM1 and U373 cells were identified from the TKA enriched fractions (Table 2), and 11 differentially expressed glycoproteins were identified from the PNA enriched fractions (Table 3), wherein 5 proteins overlapped. As shown in Tables 2 and 3, a majority of these proteins were membrane proteins, and a large part of them were CD antigens. Note that most of these glycoproteins were highly expressed in HSR-GBM1 cells. This is consistent with the results of lectin microarray, where both TKA and PNA showed higher binding capacity to HSR-GBM1 cells than to U373 cells (Figure 2). Figure 5 shows heat maps based on the spectral counts of differentially expressed glycoproteins enriched by TKA (Figure 5A) and PNA (Figure 5B) from HSR-GBM1 and U373 cells. In this figure, protein abundance increased from red to blue color. The differential expression of these proteins was remarkable between the two cell lines. Hierarchical clustering revealed good correlation between each experimental replicate.

Western Blotting Analysis of Selected Proteins. To make the previous results more confident, we applied Western blotting analysis to evaluate the changes of a suite of differentially expressed glycoproteins (Figure 6). To verify that these changes were universal between the HSR-GBM1 glioblastoma-derived stem-like cells and the GBM cells from traditional cultured adherent cell lines, we selected four cell lines including the HSR-GBM1 neurosphere line and three human GBM cell lines U373, U87, and T98G. Five glycoproteins including Podocalyxin-like protein 1 (PODXL), Chondroitin sulfate proteoglycan NG2 (NG2), Tenascin-C (TNC), Receptor-type tyrosine-protein phosphatase zeta (PTPRZ1), and CD90 were expressed at much higher levels in the HSR-GBM1 neurosphere cell line than in the three adherent GBM cell lines. CD44 antigen was undetectable in the HSR-GBM1 neurosphere cell line, while it was highly expressed in all of the other three cell lines (Figure 6). The trends in expression of all of the six proteins shown in Figure 6 were consistent with the previous MS results shown in Tables 2 and 3.

Noteworthy, PTPRZ1 was detected only in the HSR-GBM1 neurosphere cells, as shown in Figure 6. Similar to our results,

Table 2. Differentially Expressed Glycoproteins from TKA Captured Fractions

accession	protein name	gene	location	ratio ^a	<i>p</i> -value ^b
IPI00022462	CD71 antigen	TFRC	membrane	12.89	0.0011
IPI00748312	Receptor-type tyrosine-protein phosphatase zeta	PTPRZ1	membrane	NA ^c	0.0019
IPI00027230	Endoplasmic	HSP90B1	ER	2.78	0.0020
IPI00299116	Podocalyxin-like protein 1	PODXL	membrane	NA	0.0033
IPI00027493	CD98 antigen	SLC3A2	membrane	0.15	0.0058
IPI00022892	CD90 antigen	THY1	membrane	NA	0.0074
IPI00297160	CD44 antigen	CD44	membrane	0	0.011
IPI00022463	Serotransferrin	TF	extracellular	NA	0.019
IPI00031008	Tenascin-C	TNC	extracellular	NA	0.023
IPI00217563	CD29 antigen	ITGB1	membrane	0	0.039
IPI00019275	CD276 antigen	CD276	membrane	NA	0.045
IPI00019157	Chondroitin sulfate proteoglycan NG2	CSPG4	membrane	NA	0.045

^a The ratio of the spectral count of a target protein identified from HSR-GBM1 cells to that identified from U373 cells. ^b *P*-value: statistical significance of differentially expressed proteins between HSR-GBM1 and U373 cells. The values were generated by Student's *t*-test for three independent experiments. ^c NA means the protein was only identified from HSR-GBM1 cells.

Table 3. Differentially Expressed Glycoproteins from PNA Captured Fractions

accession	protein name	gene	location	ratio ^a	p-value ^b
IPI00299116	Podocalyxin-like protein 1	PODXL	membrane	NA ^c	0.00005
IPI00748312	Receptor-type tyrosine-protein phosphatase zeta	PTPRZ1	membrane	NA	0.00027
IPI00031131	Adipocyte plasma membrane-associated protein	APMAP	membrane	NA	0.00089
IPI00005707	CD280 antigen	MRC2	membrane	NA	0.00096
IPI00215998	CD63 antigen	CD63	membrane	NA	0.0015
IPI00022892	CD90 antigen	THY1	membrane	NA	0.0016
IPI00019906	CD147 antigen	BSG	membrane	NA	0.0023
IPI00019157	Chondroitin sulfate proteoglycan NG2	CSPG4	membrane	NA	0.0027
IPI00025874	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1	RPN1	ER	NA	0.0036
IPI00022462	CD71 antigen	TFRC	membrane	6.10	0.021
IPI00004656	Beta-2-microglobulin	B2M	extracellular	0	0.029

^a The ratio of the spectral count of a target protein identified from HSR-GBM1 cells to that identified from U373 cells. ^b P-value: statistical significance of differentially expressed proteins between HSR-GBM1 and U373 cells. The values were generated by Student's *t*-test for three independent experiments. ^c NA means the protein was only identified from HSR-GBM1 cells.

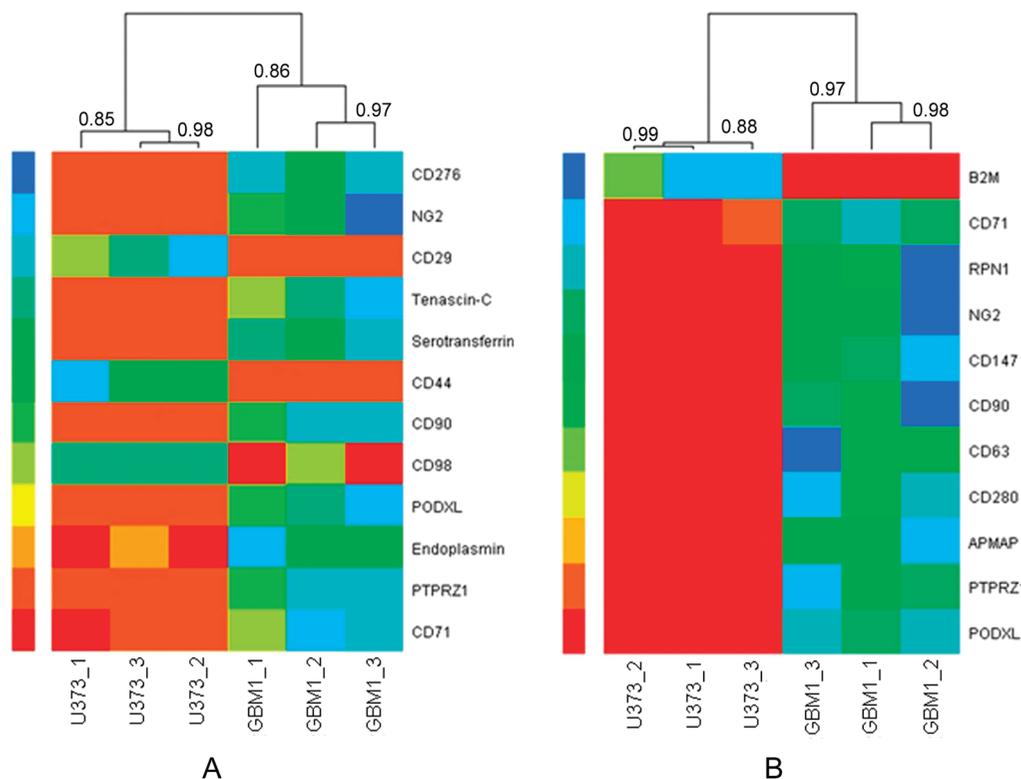


Figure 5. Heat maps with dendrograms of hierarchical clustering for triplicate experiments. The heat maps were based on the spectral counts of differentially expressed glycoproteins identified from TKA (A) and PNA (B) enriched fractions of HSR-GBM1 and U373 cells. Each column indicates an independent experiment of each cell line, and each row indicates a glycoprotein identified by LC-MS/MS. The number of spectral count increased from red color to blue color. The Pearson correlation coefficients were indicated at each branching point. Both of the heat maps were generated with R language. GBM1 represents HSR-GBM1.

PTPRZ1 was highly expressed in the undifferentiated embryonic stem cells compared with differentiated cells.³⁵ PTPRZ1 was also found to be overexpressed in human glioblastomas compared to normal brains, and a knockdown study of this protein established its function in regulating cell motility.³⁶ Therefore, the overexpression of PTPRZ1 in cancer stem-like cells may play important roles in cell migration and cancer metastasis.

PTPRZ1 has been found to interact with a glycoprotein Tenascin-C in vivo.^{37,38} Tenascin-C was originally discovered as a glioma-mesenchymal extracellular matrix antigen that is ubiquitously expressed in glioblastomas but not in normal brains.³⁹ In a recent work, this protein has been identified as

a novel target gene for Notch signaling in GBM.⁴⁰ Tenascin-C contributes to the generation of a stem cell niche, which is important to the development of neural stem cells.^{41,42} In the present work, the overexpression of Tenascin-C in the HSR-GBM1 neurosphere cell line compared to the other three adherent GBM cell lines (Figure 6) indicates that the protein is mainly expressed in the stem-like cells in GBM. Tenascin-C has previously been shown to interact with Chondroitin sulfate proteoglycan NG2,⁴³ which was also highly expressed in the HSR-GBM1 cells (Figure 6). NG2 has been widely used as a marker of multipotent neural progenitor cells.⁴⁴⁻⁴⁶

The interactions among PTPRZ1, Tenascin-C, and NG2 may play important roles in the development and metastasis of

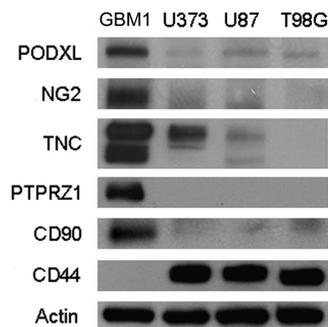


Figure 6. Western blotting analysis of selected proteins. Twenty micrograms of proteins from HSR-GBM1, U373, U87 and T98G cells was separated by 4–20% SDS-PAGE and transferred to PVDF membranes. The blots were probed with antibodies against Podocalyxin-like protein 1 (PODXL), Chondroitin sulfate proteoglycan NG2 (NG2), Tenascin-C (TNC), Receptor-type tyrosine-protein phosphatase zeta (PTPRZ1), CD90, CD44, and Beta-actin. GBM1 represents HSR-GBM1.

GBM. These glycoproteins may be used as markers for GBM stem-like cells, and they may be targeted for drug treatment of GBM patients. An anti-Tenascin monoclonal antibody has been studied for clinical treatment of malignant brain tumors.⁴⁷ PTPRZ1 and NG2 may also be used for the similar clinical studies.

The changes of three other glycoproteins Podocalyxin-like protein 1, CD90, and CD44 were also confirmed by Western blotting analysis. Podocalyxin-like protein 1 belongs to the CD34 family, and it has been used as a marker of embryonic and hematopoietic stem cells.^{48,49} CD90 has been identified as a marker of cancer stem cells in human liver cancer.⁵⁰ CD44 is the receptor for hyaluronic acid, and it has been used to fractionate cancer stem-like cells from several kinds of solid tumors including breast,⁵¹ head and neck,⁵² pancreatic,⁵³ and colorectal⁵⁴ cancers. However, this protein was not detectable in the GBM-derived stem-like cells in our work (Figure 6).

Conclusions

We employed a method combining lectin microarray and LC–MS/MS to discover the cell surface glycoprotein markers of a GBM-derived cancer stem-like cell line. The cell surface glycan expression patterns of a GBM-derived stem-like neurosphere line HSR-GBM1 and a traditional adherent GBM cell line U373 were first explored by lectin microarrays consisting of 16 lectins. Two galactose-specific lectins TKA and PNA showed much stronger binding capacity to HSR-GBM1 neurosphere cells than to U373 cells, indicating higher expression of galactosylated glycoproteins on the surface of GBM stem-like cells. Agarose-bound TKA and PNA were then used to capture the glycoproteins of the two cell lines by affinity chromatography, followed by protein identification by LC–MS/MS and quantification by spectral counting. Consequently, 12 differentially expressed glycoproteins between HSR-GBM1 and U373 cells were identified from the TKA enriched fractions, and 11 were identified from the PNA enriched fractions. Of these proteins, 5 were overlapped, making it a total of 18 differentially expressed glycoproteins. The changes of six interesting glycoproteins were further verified by Western blotting analysis using four cell lines including the HSR-GBM1 neurosphere line and three adherent GBM cell lines U373, U87, and T98G. These proteins include the overexpressed Receptor-type tyrosine-protein phosphatase zeta, Tenascin-C, Chondroitin sulfate

proteoglycan NG2, Podocalyxin-like protein 1 and CD90, and the down-regulated CD44. A better understanding of these proteins may bring an exciting opportunity for identifying markers for cancer stem-like cells in GBM.

Acknowledgment. This work was funded under the National Institute of Health Grant No. R01 49500 (D.M.L.) and the National Cancer Institute Grant No. R21 CA134623 (D.M.L.). We would like to acknowledge grant support to Dr. Fan from Accelerate Brain Cancer Cure Project Award, American Brain Tumor Association Translational Grant, and Voices Against Brain Cancer Research Grant.

Supporting Information Available: Glycoproteins identified from TKA-captured fractions (Table S1) and PNA-captured fractions (Table S2). Detailed information of the glycoproteins identified from HSR-GBM1 and U373 cells after TKA, PNA or Con A enrichment (Tables S3–S8). Scatter plots for each Con A experiment pairs (Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Ohgaki, H.; Dessen, P.; Jourde, B.; Horstmann, S.; Nishikawa, T.; Di Patre, P. L.; Burkhard, C.; Schuler, D.; Probst-Hensch, N. M.; Maiorica, P. C.; Baeza, N.; Pisani, P.; Yonekawa, Y.; Yasargil, M. G.; Lutolf, U. M.; Kleihues, P. Genetic pathways to glioblastoma: a population-based study. *Cancer Res.* **2004**, *64* (19), 6892–6899.
- (2) Reardon, D. A.; Rich, J. N.; Friedman, H. S.; Bigner, D. D. Recent advances in the treatment of malignant astrocytoma. *J. Clin. Oncol.* **2006**, *24* (8), 1253–1265.
- (3) Reya, T.; Morrison, S. J.; Clarke, M. F.; Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **2001**, *414* (6859), 105–111.
- (4) Visvader, J. E.; Lindeman, G. J. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat. Rev. Cancer* **2008**, *8* (10), 755–768.
- (5) Ignatova, T. N.; Kukekov, V. G.; Laywell, E. D.; Suslov, O. N.; Vrionis, F. D.; Steindler, D. A. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* **2002**, *39* (3), 193–206.
- (6) Galli, R.; Binda, E.; Orfanelli, U.; Cipelletti, B.; Gritti, A.; De Vitis, S.; Fiocco, R.; Foroni, C.; Dimeco, F.; Vescovi, A. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res.* **2004**, *64* (19), 7011–7021.
- (7) Singh, S. K.; Hawkins, C.; Clarke, I. D.; Squire, J. A.; Bayani, J.; Hide, T.; Henkelman, R. M.; Cusimano, M. D.; Dirks, P. B. Identification of human brain tumour initiating cells. *Nature* **2004**, *432* (7015), 396–401.
- (8) Sanai, N.; Alvarez-Buylla, A.; Berger, M. S. Neural stem cells and the origin of gliomas. *N. Engl. J. Med.* **2005**, *353* (8), 811–822.
- (9) Fan, X.; Matsui, W.; Khaki, L.; Stearns, D.; Chun, J.; Li, Y. M.; Eberhart, C. G. Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. *Cancer Res.* **2006**, *66* (15), 7445–7452.
- (10) Fan, X.; Eberhart, C. G. Medulloblastoma stem cells. *J. Clin. Oncol.* **2008**, *26* (17), 2821–2827.
- (11) Fan, X.; Khaki, L.; Zhu, T. S.; Soules, M. E.; Talsma, C. E.; Gul, N.; Koh, C.; Zhang, J.; Li, Y. M.; Maciaczyk, J.; Nikkhah, G.; Dimeco, F.; Piccirillo, S.; Vescovi, A. L.; Eberhart, C. G. Notch pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. *Stem Cells* **2010**, *28*, 5–16.
- (12) Hirabayashi, J. Lectin-based structural glycomics: glycoproteomics and glycan profiling. *Glycoconjugate J.* **2004**, *21* (1–2), 35–40.
- (13) Kuno, A.; Uchiyama, N.; Koseki-Kuno, S.; Ebe, Y.; Takashima, S.; Yamada, M.; Hirabayashi, J. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat. Methods* **2005**, *2* (11), 851–856.
- (14) Pilobello, K. T.; Mahal, L. K. Lectin microarrays for glycoprotein analysis. *Methods Mol. Biol.* **2007**, *385*, 193–203.
- (15) Liu, Y.; He, J.; Li, C.; Benitez, R.; Fu, S.; Marrero, J.; Lubman, D. M. Identification and confirmation of biomarkers using an integrated platform for quantitative analysis of glycoproteins and their glycosylations. *J. Proteome Res.* **2010**, *9* (2), 798–805.
- (16) Zheng, T.; Peelen, D.; Smith, L. M. Lectin arrays for profiling cell surface carbohydrate expression. *J. Am. Chem. Soc.* **2005**, *127* (28), 9982–9983.

- (17) Chen, S.; Zheng, T.; Shortreed, M. R.; Alexander, C.; Smith, L. M. Analysis of cell surface carbohydrate expression patterns in normal and tumorigenic human breast cell lines using lectin arrays. *Anal. Chem.* **2007**, *79* (15), 5698–5702.
- (18) Lee, M. R.; Park, S.; Shin, I. Protein microarrays to study carbohydrate-recognition events. *Bioorg. Med. Chem. Lett.* **2006**, *16* (19), 5132–5135.
- (19) Tao, S. C.; Li, Y.; Zhou, J.; Qian, J.; Schnaar, R. L.; Zhang, Y.; Goldstein, I. J.; Zhu, H.; Schneck, J. P. Lectin microarrays identify cell-specific and functionally significant cell surface glycan markers. *Glycobiology* **2008**, *18* (10), 761–769.
- (20) Lee, J. E.; Mirza, S. P.; Didier, D. N.; Scalf, M.; Olivier, M.; Greene, A. S.; Smith, L. M. Identification of cell surface markers to differentiate rat endothelial and fibroblast cells using lectin arrays and LC-ESI-MS/MS. *Anal. Chem.* **2008**, *80* (21), 8269–8275.
- (21) Sun, P.; Xia, S.; Lal, B.; Eberhart, C. G.; Quinones-Hinojosa, A.; Maciaczyk, J.; Matsui, W.; Dimeco, F.; Piccirillo, S. M.; Vescovi, A. L.; Laterra, J. DNER an epigenetically modulated gene, regulates glioblastoma-derived neurosphere cell differentiation and tumor propagation. *Stem Cells* **2009**, *27* (7), 1473–1486.
- (22) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
- (23) He, J.; Liu, Y.; He, S.; Wang, Q.; Pu, H.; Ji, J. Proteomic analysis of a membrane skeleton fraction from human liver. *J. Proteome Res.* **2007**, *6* (9), 3509–3518.
- (24) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74* (20), 5383–5392.
- (25) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75* (17), 4646–4658.
- (26) Liu, H.; Sadygov, R. G.; Yates, J. R., III. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76* (14), 4193–4201.
- (27) Xu, S.; Zhu, X.; Zhang, S.; Yin, S.; Zhou, L.; Chen, C.; Gu, J. Overexpression of beta-1,4-galactosyltransferase I, II, and V in human astrocytoma. *J. Cancer Res. Clin. Oncol.* **2001**, *127* (8), 502–506.
- (28) Garner, A. E.; Smith, D. A.; Hooper, N. M. Visualization of detergent solubilization of membranes: implications for the isolation of rafts. *Biophys. J.* **2008**, *94* (4), 1326–1340.
- (29) McDonald, C. A.; Yang, J. Y.; Marathe, V.; Yen, T. Y.; Macher, B. A. Combining results from lectin affinity chromatography and glyco-capture approaches substantially improves the coverage of the glycoproteome. *Mol. Cell. Proteomics* **2009**, *8* (2), 287–301.
- (30) Wang, Y.; Ao, X.; Vuong, H.; Konanur, M.; Miller, F. R.; Goodison, S.; Lubman, D. M. Membrane glycoproteins associated with breast tumor cell progression identified by a lectin affinity approach. *J. Proteome Res.* **2008**, *7* (10), 4313–4325.
- (31) Alvarez-Manilla, G.; Warren, N. L.; Atwood, J. A.; Dalton, S.; Orlando, R.; Pierce, M. Glycoproteomic analysis of embryonic stem cells: identification of potential glyco-biomarkers using lectin affinity chromatography of glycopeptides. *J. Proteome Res.* [Online early access]. DOI: 10.1021/pr8007489. Published Online: Jun 22, 2009.
- (32) Niittyla, T.; Fuglsang, A. T.; Palmgren, M. G.; Frommer, W. B.; Schulze, W. X. Temporal analysis of sucrose-induced phosphorylation changes in plasma membrane proteins of Arabidopsis. *Mol. Cell. Proteomics* **2007**, *6* (10), 1711–1726.
- (33) Yu, H.; Wakim, B.; Li, M.; Halligan, B.; Tint, G. S.; Patel, S. B. Quantifying raft proteins in neonatal mouse brain by 'tube-gel' protein digestion label-free shotgun proteomics. *Proteome Sci.* **2007**, *5*, 17.
- (34) Zhang, B.; VerBerkmoes, N. C.; Langston, M. A.; Uberbacher, E.; Hettich, R. L.; Samatova, N. F. Detecting differential and correlated protein expression in label-free shotgun proteomics. *J. Proteome Res.* **2006**, *5* (11), 2909–2918.
- (35) Prokhorova, T. A.; Rigbolt, K. T.; Johansen, P. T.; Henningsen, J.; Kratchmarova, I.; Kassem, M.; Blagoev, B. Stable isotope labeling by amino acids in cell culture (SILAC) and quantitative comparison of the membrane proteomes of self-renewing and differentiating human embryonic stem cells. *Mol. Cell. Proteomics* **2009**, *8* (5), 959–970.
- (36) Muller, S.; Kunkel, P.; Lamszus, K.; Ulbricht, U.; Lorente, G. A.; Nelson, A. M.; von Schack, D.; Chin, D. J.; Lohr, S. C.; Westphal, M.; Melcher, T. A role for receptor tyrosine phosphatase zeta in glioma cell migration. *Oncogene* **2003**, *22* (43), 6661–6668.
- (37) Grumet, M.; Milev, P.; Sakurai, T.; Karthikeyan, L.; Bourdon, M.; Margolis, R. K.; Margolis, R. U. Interactions with tenascin and differential effects on cell adhesion of neurocan and phosphacan, two major chondroitin sulfate proteoglycans of nervous tissue. *J. Biol. Chem.* **1994**, *269* (16), 12142–12146.
- (38) Milev, P.; Friedlander, D. R.; Sakurai, T.; Karthikeyan, L.; Flad, M.; Margolis, R. K.; Grumet, M.; Margolis, R. U. Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *J. Cell Biol.* **1994**, *127* (6 Pt. 1), 1703–1715.
- (39) Bourdon, M. A.; Wikstrand, C. J.; Furthmayr, H.; Matthews, T. J.; Bigner, D. D. Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. *Cancer Res.* **1983**, *43* (6), 2796–2805.
- (40) Sivasankaran, B.; Degen, M.; Ghaffari, A.; Hegi, M. E.; Hamou, M. F.; Ionescu, M. C.; Zweifel, C.; Tolnay, M.; Wasner, M.; Mergenthaler, S.; Miserez, A. R.; Kiss, R.; Lino, M. M.; Merlo, A.; Chiquet-Ehrismann, R.; Boulay, J. L. Tenascin-C is a novel RB-PI3kappa-induced target gene for Notch signaling in gliomas. *Cancer Res.* **2009**, *69* (2), 458–465.
- (41) Garcion, E.; Hallagic, A.; Faissner, A.; French-Constant, C. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. *Development* **2004**, *131* (14), 3423–3432.
- (42) von Holst, A. Tenascin C in stem cell niches: redundant, permissive or instructive. *Cells Tissues Organs* **2008**, *188* (1–2), 170–177.
- (43) Burg, M. A.; Tillet, E.; Timpl, R.; Stallcup, W. B. Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. *J. Biol. Chem.* **1996**, *271* (42), 26110–26116.
- (44) Dawson, M. R.; Levine, J. M.; Reynolds, R. NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors. *J. Neurosci. Res.* **2000**, *61* (5), 471–479.
- (45) Levine, J. M.; Reynolds, R.; Fawcett, J. W. The oligodendrocyte precursor cell in health and disease. *Trends Neurosci.* **2001**, *24* (1), 39–47.
- (46) Sakariassen, P. O.; Immervoll, H.; Chekenya, M. Cancer stem cells as mediators of treatment resistance in brain tumors: status and controversies. *Neoplasia* **2007**, *9* (11), 882–892.
- (47) Akabani, G.; Reardon, D. A.; Coleman, R. E.; Wong, T. Z.; Metzler, S. D.; Bowsher, J. E.; Barboriak, D. P.; Provenzale, J. M.; Greer, K. L.; DeLong, D.; Friedman, H. S.; Friedman, A. H.; Zhao, X. G.; Pegram, C. N.; McLendon, R. E.; Bigner, D. D.; Zalutsky, M. R. Dosimetry and radiographic analysis of 131I-labeled anti-tenascin 81C6 murine monoclonal antibody in newly diagnosed patients with malignant gliomas: a phase II study. *J. Nucl. Med.* **2005**, *46* (6), 1042–1051.
- (48) Nielsen, J. S.; McNagny, K. M. Novel functions of the CD34 family. *J. Cell Sci.* **2008**, *121* (Pt. 22), 3683–3692.
- (49) Doyonnas, R.; Nielsen, J. S.; Chelliah, S.; Drew, E.; Hara, T.; Miyajima, A.; McNagny, K. M. Podocalyxin is a CD34-related marker of murine hematopoietic stem cells and embryonic erythroid cells. *Blood* **2005**, *105* (11), 4170–4178.
- (50) Yang, Z. F.; Ho, D. W.; Ng, M. N.; Lau, C. K.; Yu, W. C.; Ngai, P.; Chu, P. W.; Lam, C. T.; Poon, R. T.; Fan, S. T. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell* **2008**, *13* (2), 153–166.
- (51) Al-Hajj, M.; Wicha, M. S.; Benito-Hernandez, A.; Morrison, S. J.; Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (7), 3983–3988.
- (52) Prince, M. E.; Sivanandan, R.; Kaczorowski, A.; Wolf, G. T.; Kaplan, M. J.; Dalerba, P.; Weissman, I. L.; Clarke, M. F.; Ailles, L. E. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (3), 973–978.
- (53) Li, C.; Heidt, D. G.; Dalerba, P.; Burant, C. F.; Zhang, L.; Adsay, V.; Wicha, M.; Clarke, M. F.; Simeone, D. M. Identification of pancreatic cancer stem cells. *Cancer Res.* **2007**, *67* (3), 1030–1037.
- (54) Dalerba, P.; Dylla, S. J.; Park, I. K.; Liu, R.; Wang, X.; Cho, R. W.; Hoey, T.; Gurney, A.; Huang, E. H.; Simeone, D. M.; Shelton, A. A.; Parmiani, G.; Castelli, C.; Clarke, M. F. Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (24), 10158–10163.

PR100012P