

Members of the Low-Density Lipoprotein Receptor-Related Proteins Provide a Differential Molecular Signature Between Parental and CD133(+) DAOY Medulloblastoma Cells

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Members of the low-density lipoprotein receptor-related protein (LRP) family are involved in metabolic stress and resistance phenotypes of cancer cells. New breakthroughs in brain cancer therapy have exploited that molecular signature and proved that efficient delivery of therapeutic agents involve LRP-mediated mechanisms. We performed gene expression profiling of CD133, a cell surface cancer stem cell marker, and of LRP in response to *in vitro* nutrient deprivation. We found that CD133 was selectively induced in serum-starved DAOY medulloblastoma cells but not in U87MG glioblastoma cells. Such CD133 induction was correlated to increases in LRP-1 and LRP-1b gene and protein expression. When a specific CD133(+) DAOY cell population was sorted from parental DAOY, we found increases in LRP-5 and LRP-8. Uptake of α_2 -macroglobulin, a specific LRP-1/1b ligand, was increased in serum-starved parental DAOY cells but not in CD133(+) DAOY cells, and receptor-associated protein (RAP), which binds to all cell surface LRPs, was able to compete for that uptake. Conversely, RAP binding was increased in serum-starved parental DAOY but α_2 -macroglobulin was unable to compete for such uptake. Strategies aiming at targeting cancer stem cell metabolic adaptative responses, such as that through LRP differential expression within the brain tissue microenvironmental niche, can now be envisioned. © 2010 Wiley-Liss, Inc.

Key words: cancer stem cells; metabolic stress adaptation; resistance phenotype

INTRODUCTION

Cellular stress factors regulate the “angiogenic switch” in brain tumors allowing the passage from low invasive and poorly vascularized tumors to highly invasive and angiogenic tumors. Cells residing within a tumor microenvironment are thus likely to be frequently challenged by various stresses, of which hypoxia, nutrient deprivation, and proinflammatory conditions characterize the development of cancers [1]. *In vitro*, serum deprivation is commonly used to mimic cellular stress and to trigger the adaptative metabolic response of cancer cells. Among the molecular adaptative responses to serum deprivation, the increase in low-density lipoprotein receptor-related protein (LRP), which is structurally and functionally related to LDL receptor, is modulated by serum factors [2,3]. The important role of LRP in the regulation of lipid and lipoprotein metabolism in a variety of cells suggests that the increased level of LRP may be the consequence of the higher requirements of lipid and lipoprotein of serum-deprived cancer cells in relation to their energy production. While LRP cell surface receptor expression suggests the possibility of a protective role [4], no fundamental molecular evidence has yet

been provided to support any physiological relationship to cancer cell chemoresistance.

The central nervous system is protected by barriers which control the entry of compounds into the brain, thereby regulating brain homeostasis. Since the blood–brain barrier (BBB), formed by the endothelial cells of the brain capillaries, restricts access to brain cells of blood-borne compounds, this very tight regulation of the brain homeostasis results in the inability of some small and large therapeutic compounds to cross the BBB. Therefore, various strategies are being developed to enhance the amount and concentration of therapeutic compounds in the brain. Among all the approaches used for increasing brain delivery of therapeutics, the most accepted

Abbreviations: LRP, low-density lipoprotein receptor-related protein; BBB, blood–brain barrier; CSC, cancer stem cells; RAP, receptor-associated protein.

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method is the use of the physiological approach which takes advantage of the transcytosis capacity of specific receptors, such as members from the LRP family, expressed at the BBB [5,6]. LRP expression at the tumoral compartment still remains to be investigated. Recently, a small population of cancer stem cells (CSC) in adult and pediatric brain tumors has been identified [7,8]. These CSC, once isolated from tumor tissues, form neurospheres when cultured in vitro and possess the capacity for cell renewal. Based upon their high expression of the neural precursor cell surface marker CD133 (prominin-1), these CSC have been further hypothesized to bear properties such as resistance to apoptosis and resistance to both ionizing radiation [9] and drugs [10]. Interestingly, among primary human medulloblastoma and glioblastoma multiforme-derived cells, only cells expressing CD133 on their surfaces can initiate tumors in the brains of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [11]. In fact, injection of as few as 100 CD133(+) cells produced a tumor that could be serially transplanted and which was a phenocopy of the patient's original tumor, whereas injection of CD133(-) cells did not cause a tumor. Such evidence highlights the fact that in brain tumors, and in other malignancies, the tumor clone is phenotypically and functionally heterogeneous [12]. The original and intriguing observation that CD133 expression was turned off in GBM cells cultured in serum [13] further evidences crucial regulatory mechanisms possibly affecting the CSC phenotype.

CD133(+) GBM cells have been characterized as chemo-/radio-resistant tumor-initiating cells that would also be responsible for posttreatment recurrence [14]. In this study, we therefore asked whether differential LRP expression profiles may represent another phenotypic signature in the adaptative and resistance phenotype of CD133(+) medulloblastoma cells.

MATERIALS AND METHODS

Materials

Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON, Canada). Cell culture media were obtained from Life Technologies (Burlington, ON, Canada). The rabbit polyclonal antibody against CD133 was C24B9 from Cell Signaling (Danvers, MA). The receptor-associated protein (RAP) was from Oxford Biomedical Research (Oxford, MI). α_2 -Macroglobulin, activated by methylamine, was provided by Dr Salvatore V. Pizzo (Duke University Medical Center, USA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma.

Cell Culture

The U87MG glioblastoma cell line (passage 135) was purchased from American Type Culture Collection (Manassas, VA) and cultured in Eagle's minimum essential medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1 mM Na-pyruvate, at 37°C under a humidified atmosphere containing 95% air and 5% CO₂. The human DAOY medulloblastoma cell line (used for 8–10 passages once thawed) was purchased from American Type Culture Collection and was maintained in EMEM containing 10% (v/v) calf serum (CS) (HyClone Laboratories), and 1 mM Na-pyruvate. Serum deprivation assays were performed in cells cultured in their respective media to which no FBS or CS was added.

Magnetic Cell Sorting and Flow Cytometry

Confluent DAOY medulloblastoma parental cells were harvested with cell dissociation buffer (Hank's based; Invitrogen), centrifuged at 800g for 5 min and resuspended in 1× PBS with 0.5% BSA and 2 mM EDTA. Magnetic labeling with 100 μ L AC133 (CD133/1) microbeads per 10⁸ cells was performed for 30 min at 4°C using the Miltenyi Biotec CD133 Direct Cell Isolation kit. Magnetic separation was carried out using LS columns and a MACS separator (Miltenyi Biotec Inc., Auburn, CA) under a biological hood. CD133(+) fractions were eluted by removing the column from the magnetic field and using a sterile plunger. Aliquots of CD133(+)-sorted cells were evaluated for purity by flow cytometry with a FACS Calibur machine (BD Biosciences, Mississauga, ON). Fifty microliters of 293C3 (CD133-2)-phycoerythrin (fluorochrome-conjugated mouse monoclonal IgG2b; Miltenyi Biotec) was added for an additional 10 min at 4°C to evaluate the efficiency of magnetic separation by flow cytometry.

Total RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR

Total RNA was extracted from cell monolayers using TRIzol reagent (Life Technologies). For cDNA synthesis, 2 μ g total RNA was reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at -20°C prior to PCR. Gene expression was quantified by real-time quantitative PCR (Q-PCR) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Primers for CD133, LRP-1, LRP-1b, LRP-2, LRP-5, and LRP-8 (Qiagen, Valencia, CA) were all derived from human sequences. PCR conditions were optimized and were resolved on 1.8% agarose gels containing 1 μ g/mL ethidium bromide in order to confirm single amplicons. DNA amplification was carried out using an Icyler iQ5 (Bio-Rad, CA) and product detection was performed by measuring the binding of the fluorescent dye SYBR

Green I to double-stranded DNA. The relative quantities of target gene mRNA against an internal control, 18S ribosomal RNA, were measured by following a ΔC_T method. An amplification plot (fluorescence signal vs. cycle number) was drawn. The difference (ΔC_T) between the mean values in the triplicate samples of target genes and those of 18S ribosomal RNA were calculated by iQ5 Optical System Software version 2.0 (Bio-Rad, CA) and the relative quantified value (RQV) was expressed as $2^{-\Delta\Delta C_T}$.

Immunoblotting Procedures

Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with either CD133, LRP-1 (85 kDa) or GAPDH primary antibodies (1/1000 dilution) in TBST containing 3% BSA, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/2500 dilution) in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC, Canada).

Iodination of Proteins

LRP, RAP and α_2 -macroglobulin were radiolabeled with standard procedures using an Iodo-beads kit and D-Salt Dextran desalting columns from Pierce. A ratio of two iodo-beads per iodination was used for the labeling. Briefly, beads were washed twice with 1 mL of phosphate-buffered saline (PBS) on a Whatman filter and resuspended in 60 μ L of 0.1 M phosphate buffer solution, pH 6.5. Na^[125I] (1 mCi) from Perkin Elmer (Woodbridge, ON, Canada) was added to the bead suspension for 5 min at 20°C. Iodination of each protein was initiated by the addition of 100 μ g of protein (80–100 μ L) diluted in 0.1 M phosphate buffer solution, pH 6.5. After incubation for 10 min at 20°C, iodo-beads were removed and the supernatants were applied onto a desalting column prepacked with 5 mL of cross-linked dextran from Pierce. [^{125I}]-proteins were eluted with 5 mL of PBS. Fractions of 0.5 mL were collected and the radioactivity in 10 μ L from each fraction was measured. Fractions corresponding to [^{125I}]-proteins were pooled and dialyzed against Ringer/HEPES, pH 7.4.

Statistical Data Analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of

less than 0.05 were considered significant, and an asterisk (*) identifies such significance in these figures.

RESULTS

In Vitro Serum Deprivation Triggers Gene and Cell Surface Protein Expression of CD133 in DAOY Medulloblastoma Cells

CD133 cell surface expression is among the markers that enable the immunophenotyping of cancer stem cells. CD133 cell surface expression was therefore assessed in normal and in serum-starved DAOY medulloblastoma and U87MG glioblastoma cells by flow cytometry and by Q-PCR. DAOY and U87MG cells were cultured for 5 d in the presence of decreasing serum levels in their culture media and cells were harvested for CD133 cell surface labeling. While CD133 cell surface expression remained unaffected in U87MG cells, the level in DAOY cells increased significantly and dose-dependently in response to serum depletion (Figure 1A). CD44, used as another cell surface marker expressed in both cell lines, was unchanged (Figure 1B). Finally, DAOY cells were serum-starved (0% FBS) for 1, 2, 3, 4, and 5 d, total RNA was then isolated from cells at each time point, and CD133 gene expression found to increase in agreement with the cell surface expression of the CD133 protein (Figure 1C).

In Vitro Serum Deprivation Alters Gene Profiling Expression of Low-Density Lipoprotein Receptor-Related Protein (LRP) Family Members in DAOY Medulloblastoma Cells

The possible correlation between LRP levels and changes in CD133 gene expression was next assessed. Total RNA was extracted from control cells and from 4 d serum-starved DAOY cells and gene expression levels were evaluated by Q-PCR. The primer design enabled measurement of the specific expression levels of individual human LRP-1, LRP-1b, LRP-2, LRP-5, and LRP-8 genes. This was validated by visualization of a single cDNA amplicon product by semi-quantitative RT-PCR on an agarose gel (Figure 2A). When Q-PCR was then performed on control and serum-starved DAOY cells we observed that, within the LRP family, LRP-1, LRP-1b and LRP-5 gene expression were significantly induced in serum-starved cells (Figure 2B).

LRP-1 and CD133 Protein Expression Is Induced by Serum Deprivation

Cell lysates were used to assess the total protein expression of both CD133 and of the 85 kDa form of LRP-1 under full serum (10% FBS) or serum-deprived culture conditions. In agreement with the gene expression profiles, both LRP1 and CD133 protein expression were induced (Figure 3A). Scanning densitometry allowed us to identify the time of

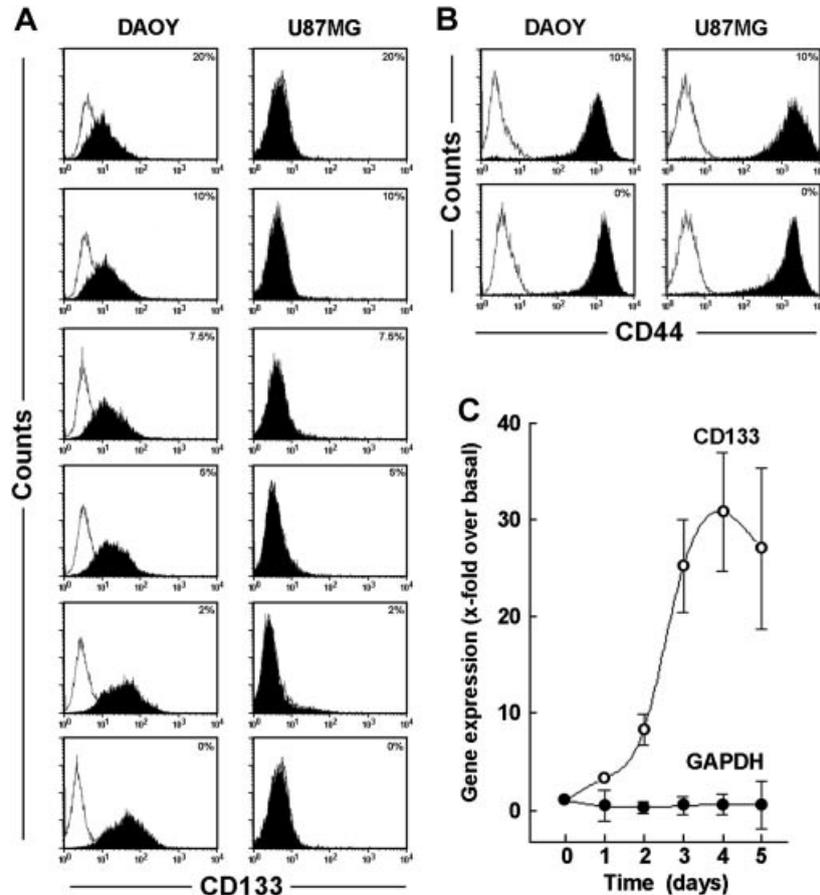


Figure 1. In vitro serum deprivation triggers gene and cell surface expression of CD133 in DAOY medulloblastoma cells. Subconfluent DAOY and U87MG cell monolayers were cultured for 5 d with either 20%, 10%, 7.5%, 5%, 2%, or 0% serum content. Evaluation of (A) CD133 cell surface and of (B) CD44 cell surface expression was then performed by flow cytometry on DAOY and U87MG cells (white,

control IgG isotype; black, CD133 or CD44 expression). (C) Total RNA was extracted from serum-starved DAOY cells at the indicated time of culture. CD133 (open circles) and GAPDH (closed circles) gene expression levels were then assessed by Q-PCR as described in the Materials and Methods Section. Data are representative of three independent experiments.

maximal expression for both proteins, which occurred at day 4 of serum depletion (Figure 3B).

Differential LRP Gene Expression Profile Between Parental and CD133-Enriched DAOY Cells

In an attempt to investigate whether CD133 cell surface expression is part of an adaptative phenotype of cancer stem cells, we used magnetic cell sorting (MACS) technology to isolate CD133(+) cells from the parental DAOY medulloblastoma cell population [15]. We found that the CD133(+) DAOY cell population represented ~0.2% of the total parental DAOY cells (Figure 4A, left panel). Sorting of the CD133(+) cells was then performed and we evaluated the cells as being ~52% CD133 positive (Figure 4A, right panel). The isolated subpopulation, with an enrichment of ~260-fold for CD133(+) DAOY cells, was put into culture, and total RNA was isolated from parental and CD133(+) DAOY cells in order to assess gene expression levels of CD133 and of the LRP family. We found that CD133 gene expression was increased by ~4-fold in the sorted

CD133(+) DAOY cells, in agreement with the increased CD133 cell surface expression (Figure 4A, right panel). In contrast to the serum-starved DAOY cells which had increased expression in LRP-1, LRP-1b, and LRP-5, we found that LRP-5 and LRP-8 were dominantly expressed while LRP-1 and LRP-1b were only modestly expressed in that CD133-enriched subpopulation (Figure 4B).

Functional Assessment of LRP Cell Surface Functions in Serum-Starved and in CD133-Enriched DAOY Cells

In order to distinguish the relative contributions of LRP-1/1b from the other cell surface LRPs, we measured the intracellular uptake of α_2 -macroglobulin, a specific LRP-1/1b ligand, and of RAP which binds to all cell surface LRP [16]. When α_2 -macroglobulin uptake was assessed in parental DAOY cells, it was found that serum starvation of cells resulted in a ~2.5-fold increase in uptake compared to parental cells cultured with serum (Figure 5A), in agreement with the 2- to 7-fold increases of LRP-1/1b gene expression. α_2 -Macroglobulin uptake was signifi-

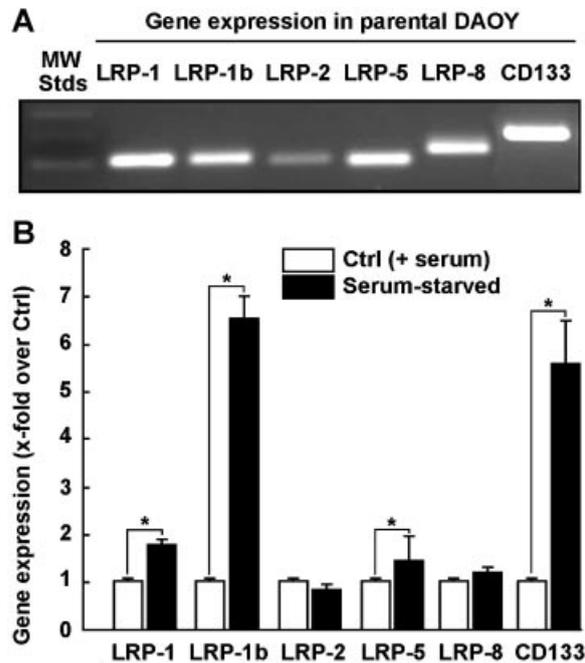


Figure 2. In vitro serum deprivation alters gene expression of low-density lipoprotein receptor-related protein (LRP) family members in DAOY medulloblastoma cells. Total RNA was extracted from 5 d serum-starved DAOY cells. (A) Validation was performed by semi-quantitative RT-PCR to confirm the presence of a single amplicon for LRP-1, -1b, -2, -5, -8, and CD133. (B) Relative gene expression levels were then assessed by Q-PCR in normal culture conditions (white bars; +serum) or serum-deprived culture conditions (black bars). Data are expressed as gene expression fold increase relative to normal culture conditions. Data are representative of three independent experiments. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in these figures.

cantly and similarly reduced by RAP for cells grown in both conditions. CD133(+) cells, which were characterized by modestly elevated expression of LRP-1/1b, did not internalize more α_2 -macroglobulin than their parental counterpart, but RAP still competed for α_2 -macroglobulin uptake (Figure 5A). Inversely, RAP uptake was also assessed and was increased in serum-starved parental DAOY (Figure 5B). RAP uptake was increased in CD133(+) cells in line with the observed massive LRP-5/8 increase in expression. A slight but significant decrease of RAP uptake by α_2 -macroglobulin was observed in control cells, while only a nonsignificant ($P > 0.05$) tendency was noted in serum-starved conditions, confirming the modest expression and contribution of LRP-1/1b. Again, RAP uptake was increased in both control and serum-starved CD133(+) cells but, this time, no decrease of RAP uptake by α_2 -macroglobulin was observed in these cells confirming the lack of LRP-1/1b enrichment in CD133(+) cells (Figure 5B).

DISCUSSION

The striking adaptation of CSC to restricted tumor microenvironment conditions, such as those imposed by hypoxia and nutrient shortage, have

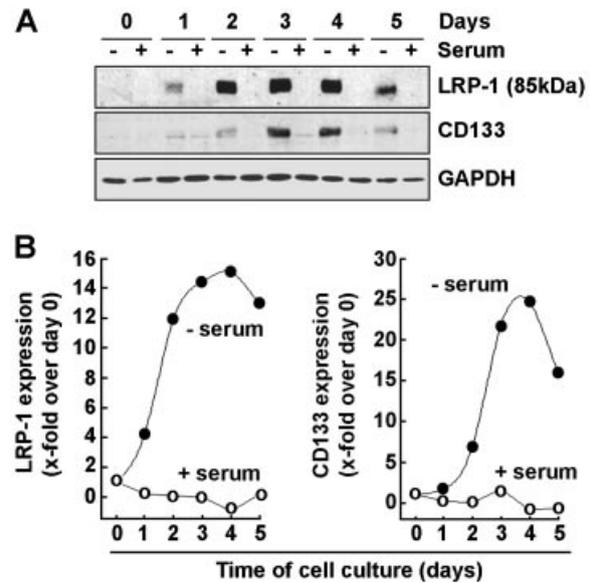


Figure 3. LRP-1 and CD133 protein expression is induced by serum deprivation. Subconfluent DAOY cell monolayers were cultured for up to 5 d in the presence or absence of 20% serum. (A) Cell lysates (20 μ g/well) were electrophoresed via SDS-PAGE and immunodetection was used to assess the levels of 85 kDa LRP-1, CD133 and GAPDH protein expression. (B) Quantification was performed by scanning densitometry of the autoradiograms. Data shown is representative of two independent experiments and represents the ratio of either LRP-1/GAPDH (left panel) or CD133/GAPDH (right panel).

recently been suggested to contribute to their radio- and chemoresistance phenotype [17]. The contribution of those microenvironmental restrictions also leads to significant metabolic orientations that trigger a fierce clonal competition giving an advantage to those cells that best adapt [18]. As such, studies on efficient tumor reformation from single cells have lead to the conclusion that not only CSC may restart experimental tumors [19]. Such apparently conflicting observations have recently been reconciled and it has been established that differences in the observed frequencies of CSC within tumors may reflect the various cancer types and hosts used to assay these cells [20]. In fact, cancer self-renewal indicate that there exists a minor subpopulation of stem cells endowed with an unlimited capacity for self-renewal, together with sufficient plasticity and metabolic adaptation capacity to generate multi-form progenies committed to different terminal fates. Recently, an isolated CD133(+) cell subpopulation from human brain tumors was shown to exhibit stem cell properties [11] and is thought to play a pivotal role in brain tumor initiation, growth, and recurrence [11,21]. While elevated CD133 expression level is associated with poor prognosis [22], a better understanding of the molecular signature of brain tumor-derived CSC will help develop new clinical approaches in order to improve the efficiency of current treatment.

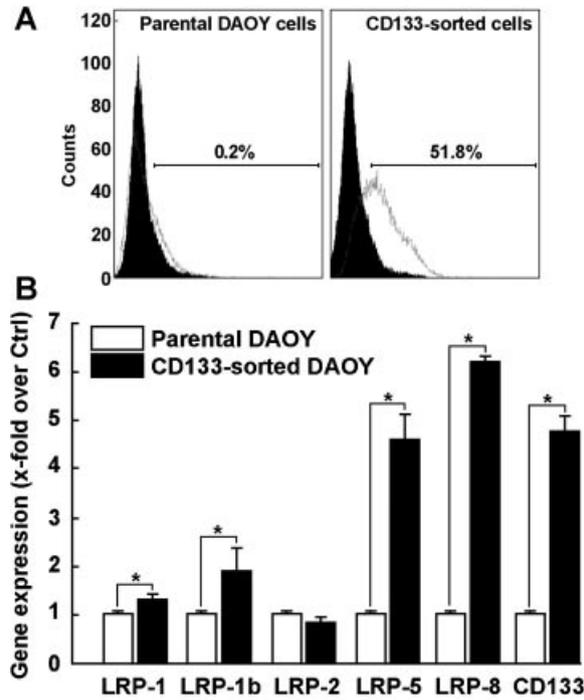


Figure 4. Differential LRP gene expression profile between parental and CD133-enriched DAOY cells. (A) CD133(+) DAOY cells were isolated from the parental DAOY cells as described in the Materials and Methods Section using MACS technology. Evaluation of CD133 cell surface expression was then performed by flow cytometry on parental DAOY and CD133(+) DAOY-sorted cells. (B) Total RNA was extracted from parental DAOY (white bars) and CD133(+) DAOY cells (black bars), and relative gene expression levels assessed by Q-PCR for LRP-1, -1b, -2, -5, -8, and CD133. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in these figures.

In the present study, we identified LRP members as molecular and biological features regulating the adaptive phenotype associated with CD133(+) medulloblastoma-derived cells. LRP is overexpressed in malignant astrocytomas, especially in glioblastomas [23]. LRP is a multiligand lipoprotein receptor which interacts with a broad range of secreted proteins and resident cell surface molecules including, but not restricted to, apolipoprotein E, α_2 -macroglobulin, tissue plasminogen activator-1, amyloid precursor protein, factor VIII, matrix metalloproteinase (MMP)-9, and lactoferrin, mediating their endocytosis or activating signaling pathways through multiple cytosolic adaptor and scaffold proteins. Interestingly, MMP-9 has been shown to bind LRP-1 [24], while the membrane type-1 (MT1)-MMP was demonstrated to proteolyse LRP in malignant cells [25]. In accordance with these observations, we have provided evidence for crucial roles for MMP-9 and for MT1-MMP, two major actors in cell invasion, metastasis and resistance to radiation, in CD133(+) neurosphere-derived DAOY medulloblastoma cells [26]. Interestingly, MMP-9 together with other pro-angiogenic growth factors was identified among those molecular markers that trigger the

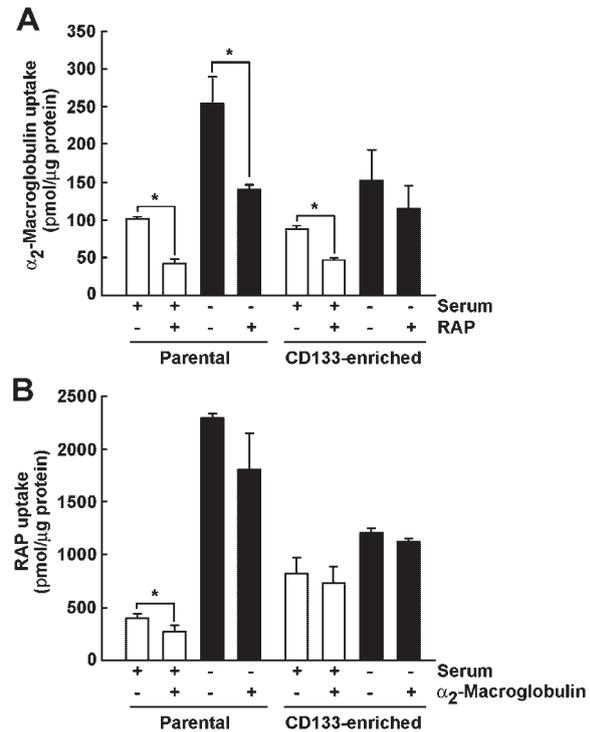


Figure 5. Functional assessment of cell surface LRP in serum-starved and in CD133-enriched DAOY cells. Parental and CD133(+) DAOY cells were cultured under serum culture conditions (white bars) or serum-deprived culture conditions (black bars) for 5 d. Cells were then subjected to (A) α_2 -macroglobulin uptake in the presence or absence of RAP, or (B) RAP uptake in the presence or absence of α_2 -macroglobulin competitor as described in the Materials and Methods Section. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance in these figures.

“angiogenic switch” during carcinogenesis [27,28]. Chronic growth factors deprivation, such as that achieved in vitro by removal of serum from cell culture media [29], may further play a role in the “angiogenic switch” enabling CSC to counteract the effects of cellular stress such as diminished cell proliferation or induction of apoptosis/autophagy mechanisms. Finally, decreases in LRP levels also correlated with enhanced functional MT1-MMP expression in advanced stages of Wilms tumors [30]. In light of the data showing a MT1-MMP/MMP-9 to LRP regulatory axis in pediatric kidney cancer and in pediatric brain cancer, we speculate that this molecular signature is characteristic of pediatric tumors.

It is still unclear whether CD133 expression plays a causative, contributing, or correlative role in both the generation and metabolic adaptation of the CSC population. Besides LRP-8, this study shows that a common metabolic upregulation of CD133, LRP-1, -1b, and -5 to serum deprivation is shared with the CD133-enriched cell population possibly establishing these LRP isoforms as part of the phenotypic signature of CSC. Lack of LRP-8 gene regulation in response to serum deprivation remains to be

investigated, but its significant overexpression in CD133(+) cells may ultimately be considered as a critical biomarker for CSC. Given that part of this metabolic adaptative phenotype is reflected by an increase in LRP expression (this study), and that LRP expression is correlated with tumor invasiveness, one can hypothesize that targeting LRP functions and/or expression within the vascular niche may ultimately alter the invasive phenotype of CSC. New possibilities for abrogating the tumor-promoting and metabolic adaptative functions of CSC through LRP targeting may ultimately be envisioned. In fact the identification of LRP as a cognate receptor for a peptide sequence of T7 phage protein p17 offers a new ligand–receptor combination for cell delivery of therapeutic agents [31]. A new neurotherapeutic platform based on the Angiopep-2 peptide, a sequence derived from ligands that bind to LRP-1 located at the BBB, demonstrated efficient transport across the BBB into brain parenchyma [5,32,33]. Interestingly, this potential therapeutic platform showed promising applications in targeting *in vivo* brain tumors, as conjugation of paclitaxel with the Angiopep-2 peptide vector could increase efficacy of anticancer agents in the treatment of brain cancer [34]. Whether this platform targets any LRP-induced phenotype, such as that we report here in CD133(+) CSC *in vivo*, is currently under investigation.

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