Gain of glucose-independent growth upon metastasis of breast cancer cells to the brain

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ABSTRACT

Breast cancer brain metastasis is resistant to therapy and a particularly poor prognostic feature in patient survival. Altered metabolism is a common feature of cancer cells but little is known as to what metabolic changes benefit breast cancer brain metastases. We found that brain-metastatic breast cancer cells evolved the ability to survive and proliferate independent of glucose due to enhanced gluconeogenesis and oxidations of glutamine and branched chain amino acids, which together sustain the non-oxidative pentose pathway for purine synthesis. Silencing expression of fructose-1,6-bisphosphatases (FBPs) in brain metastatic cells reduced their viability and improved the survival of metastasis-bearing immunocompetent hosts. Clinically, we showed that brain metastases from human breast cancer patients expressed higher levels of FBP and glycogen than the corresponding primary tumors. Together, our findings identify a critical metabolic condition required to sustain brain metastasis, and suggest that targeting gluconeogenesis may help eradicate this deadly feature in advanced breast cancer patients.
INTRODUCTION

Cancer cells consume excess nutrients and energy as compared to their non-malignant counterparts due to altered metabolism (1, 2). Enhanced glucose metabolism accompanied by fermentation (aerobic glycolysis), commonly known as the Warburg effect, is exhibited almost universally by cancer cells (3, 4). Amino acids, such as glutamine, can also be utilized for energy production (5). Enhanced oxidation of branched chain amino acids (BCAA), valine, leucine and isoleucine, can occur in late stage cancers (6-8). The driving force of altered metabolism in cancer cells is multifactorial. One factor is the intrinsic high demand of malignant tumor cells for biosynthetic intermediates, such as amino acids for protein synthesis, nucleic acids for DNA and RNA syntheses and fatty acids for membrane structures (4). Another complementary driving force is the extrinsic tissue environmental pressure, such as the presence of hypoxia or low levels of glucose (9). These pressures exist in the microenvironment to which metastatic cancer cells migrate, which could be quite different from the primary site in regard to nutrient and growth factor availability (10, 11).

The microenvironments of different tissues are diverse. Metastatic tumor cells extravasate from their primary site and reach multiple organs, but can only proliferate in specific sites (10). The fate of such metastatic cells is in large part determined by the compatibility between these cells and the microenvironment of the host tissue. Studies, for example, have shown that cancer-associated stromal cells can be reprogrammed to favor metabolizing lactate secreted by cancer cells (11, 12).

Breast cancer frequently metastasizes to the brain parenchyma. The microenvironment of the brain plays a key role in the development of therapeutic resistance of brain metastases (13). Distribution of energetic substrates and nutrients in the body is not uniform. Interstitial glucose levels of most organs are lower than that of the blood (14, 15). Glucose level in the brain interstitial space is lower than that of the blood (16-23). Brain interstitial space contains high levels of glutamine (24) and BCAAs (25,
26). Glutamine and BCAAs can serve as energy substrates (5). Their abundance may contribute to the survival of cancer cells growing in the brain. While expression levels of glycolytic enzymes were found to increase in cancer cells growing in the brain (27, 28); however, enhanced glucose uptake is not a feature of breast cancer brain metastasis (29-32), suggesting glucose may not be the only primary energy substrate for brain metastasis. The role of non-glucose carbon sources in the survival/growth of brain metastatic breast cancer cells is unknown. At the early phase of brain metastasis development, cancer cells may have to survive and grow in the low glucose brain interstitial space before achieving sufficient prosurvival modifications on the microenvironment and necessary metabolic reprogramming within cancer cells.

To determine the impact of the brain microenvironment on the metabolism of breast cancer brain metastases, we compared the brain metastatic breast cancer cell line, MDA-MB-231Br3 (derived from MDA-MB-231 cells by three rounds of selection from a brain metastasis subsequent to orthotopic implantation of MDA-MB-231 cells into the brain of nude mice (33)), with its parental cell line MDA-MB-231. We compared the ability of these cells to utilize three types of carbon substrate for survival and growth: glucose, glutamine and BCAAs. We questioned whether a substantial shift in the utilization of these carbon sources could occur. We found that, unlike parental cancer cells, brain-metastatic cells could proliferate in the absence of glucose by acquiring the ability to carry out gluconeogenesis and enhanced oxidation of BCAAs and glutamine, and exhibited upregulation of fructose-1,6-bisphosphatase. We also validated the findings using breast cancer cells directly derived from a human brain metastasis (MDA-MB-361), as well as clinical specimens of brain metastasis. Finally, we determined the role of fructose-1,6-bisphosphatase in tumor growth and survival of immunocompetent mice bearing orthotopic breast cancer brain metastasis formed by the 4T1 cells.

MATERIALS AND METHODS
Reagents and chemicals

The glucose/glutamine/glutamate/BCAAs free MEM was made by Life Technologies (Carlsbad, CA). Glucose, glutamine, $^{13}$C-glutamine, alpha-ketoglutarate, valine, leucine, isoleucine, epigallocatechin gallate (EGCG), $\alpha$-ketoglutaric acid sodium, fetal calf serum, rabbit polyclonal antibodies against beta actin, GLUD1, GLUD2, BCKDK, FBP1, FBP2, PEPCK1 and PEPCK2 were from Sigma-Aldrich (St. Louis, MO). Anti-pan-cytokeratin antibody was from Abcam (Cambridge, MA). Universal control shRNA, shRNA-FBP2, shRNA-BCKDH-E1 were from Sigma-Aldrich. The vector to express the negative control and target shRNA was from Genescript (Cat# SD1211, Piscataway, NJ). $^{14}$C-leucine and hydroxide haymine were purchased from Thermal Fisher Scientific (Waltham, MA). FBP2 expression plasmid was purchased from Addgene. MTT assay kit was purchased from Invitrogen. MDA-MB-231, MDA-MB-361, and 4T1 cell lines were originally purchased from American Type of Cell Culture (ATCC) (Manassas, VA). All cell lines are authenticated by short tandem repeat (STR) sequencing two months before usage and matched with 100% accuracy to the ATCC database. MDA-MB-231Br3 cell line was established by three round of in vivo selection as described previously (33), and was from the stock of Dr. Isaiah J. Fidler's laboratory at the MD Anderson Cancer Center.

Target sequences of shRNAs are: For FBP2 (human) at exon 5, GATCCGCAAACAGTGTGCT; at 3'-UTR, GCCACAGGCGATTCTATGG and CTGCTTACGAGGTTTGG; for BCKDH-E1 (human) at exon 5, GGAACGCCACTTCGTCACT; for FBP1/2 (mouse) at exon 3, GATGAGCCTTCTGAGAAGG.

Injection of tumor cells into the mammary gland fat pad

MDA-MB-231Br3 or MDA-MB-231 cells were injected (5,000 cells/100 µl/injection) into the fat pad of mammary glands of female nude mice (34). Twelve weeks later, the brains of tumor bearing mice were harvested, fixed in cold 4% paraformaldehyde. Paraffin embedded tissues were exhausted by serial sectioning and slides were stained with hematoxylin/eosin for histological analysis. For 4T1
cells, 50,000 cells were implanted in each animal, and animals were terminated at times when tumor reached 1.0 cm³ in size.

Orthotopic model of brain metastasis

Female wild type BALB/c mice (6-8 weeks old) were used to produce metastatic brain tumors. Luciferase expressing 4T1 cells, control-shRNA 4T1 cells, and FBP-shRNA 4T1 cells were injected (10,000 cells/100 µl/injection) into the internal carotid artery as previously described (35). Animals were imaged 10 minutes after D-luciferin injection to ensure consistent photon flux using an IVIS 100 in vivo imaging system (Caliper Life Sciences, Alameda, CA).

Antibody production

Peptides, CYRIGHHSTSDSS and CYRIGHHpSTSDSS were used for productions of rabbit polyclonal antibodies against the total BCKDH-E1 and pSer293-BCKDH-E1 respectively (36). The antibodies were produced by Genscript USA Inc. (Piscataway, NJ). Recombinant BCKDH-E1 and BCKD were purchased from Globozymes (Carlsbad, CA).

Transfections

All cell transfections were carried out using 2 µg DNA (or shRNA)/ml on cells at 70% confluence cultured in one well of a 6-well plate. Transfection reagent Genejuice was used according to the protocol provided by the manufacture (Roche).

Cell culture and Cell survival assay

Glucose free DMEM supplemented with formulary essential/non-essential amino acids including or excluding branched-chain amino acids (BCAAs) were customized by Invitrogen (CA, USA). Fetal bovine serum (FBS) was dialyzed in glucose-free or glucose/BCAA-free medium using a dialysis bag
with a cutoff molecular weight of 2K (Thermo Scientific, Rockford, IL). Glucose containing medium was made by adding glucose into medium at a final concentration of 5mM. For cell survival assay, cells were collected at indicated time points and stained with trypan blue. Viability counting was counted by Countess® Automated Cell Counter (Invitrogen, CA, USA).

**Immunohistochemistry**

Paraffin-embedded clinical specimens of breast cancer brain metastases were from MDACC tissue bank with the approval of Institutional Review Board. Immunohistochemical staining was carried out according to protocols provided by the manufactures of the antibodies.

**Periodic Acid-Schiff (PAS) and PAS-Diastase (PAS-D) staining**

PAS and PAS-D staining was performed according to the protocol provided by the manufacture of the reagents (Sigma-Aldrich). Glycogen content was quantified using the imaging analysis software NIS-Elements (Nikon), and normalized to values of PAS-D (set as 1.0).

**Mitochondria Extraction:**

Mitochondrial samples were isolated using mitochondrial isolation kit purchased from Thermo scientific Inc (Rockford, IL, USA) following the protocol provided by the manufacture.

**Western Blot assay**

Standard Western blot protocol was used to determine the expression levels of BCKDH-E1, pSer293-BCKDH-E1, BCKDHK, FBP1, FBP2, Caspase 3, LC3, Cox II, Cox IV, PEPCK1, PEPCK 2, PARP, GLUD1, GLUD2, and beta actin.

**Glycolysis/fermentation assay**
Glycolytic activity of cancer cells was determined by measuring glucose consumption and lactate production. Cell culture medium was sampled at 200 µl at three time points with 3hr intervals. The glucose and lactic acid concentrations of the cell culture medium were measured using a Dual-Channel Biochemistry Analyzer-2700D (YSI Life Sciences). Cell numbers were counted with a Beckman Coulter analyzer to normalize the glucose and lactic acid concentrations.

**HPLC measurement of amino acids in cell culture medium**

The levels of amino acids, glutamine, glutamate, valine, leucine and isoleucine were measured by HPLC at the co-facility of Medical Genetics Laboratories of Baylor College of Medicine, Houston, TX.

**14C-Leucine oxidation assay**

To determine the activity of BCAA oxidation, 14C-leucine was added into the cell culture medium at a concentration of 2.0 µM. To absorb CO₂, a filter paper pad of 2.5cm diameter soaked with 1ml hydroxide of haymine was inserted into the ventilation cap of the T-75 cell culture flask. After 8hr of culturing, the filter paper pads were removed and inserted into scintillation counter vials containing 4ml scintillation liquid. The radioactivity of 14C was measured by a Liquid Scintillation Analyzer, Tri-Carb 2810TR (PerkinElmer).

**Extraction of metabolites from cells**

Cells were cultured in glucose-free medium for 12 h before medium was replaced by glucose-containing for 12 h or glucose-free medium containing 2.0µM 13C-glutamine for 6 h. Metabolites were extracted according to the protocol described previously(37).

**Targeted mass spectrometry**
\( ^{13}\)C labeled carbohydrates were measured by LC-MS as described previously (38). \( ^{13}\)C labeled xanthine, hypoxanthine, thymine, and cytosine were determined by LC-MS as described previously (37).

Statistics

Student two-tailed \( t \)-test was used to compare the values (mean±SD) of control and experimental groups. Chi-square test was used in analyzing results of glycogen staining and immunohistochemical staining. Log-rank test was used to analyze the Kaplan-Meier survival curves. \( P \leq 0.05 \) is considered as significant difference.

RESULTS

MDA-MB-231Br3 cells proliferate in the absence of glucose

To compare the ability of metastasis to the brain between the MDA-MB-231Br3 cells and the parental MDA-MB-231 cells, we implanted 5,000 viable MDA-MB-231Br3 cells or MDA-MB-231 cells orthotopically into the mammary gland fat pad of immunodeficient female mice (\( n=5 \) in each group). After three months, the mice were sacrificed and the brains harvested for histological examination. Micro-metastases were observed in 3 of 5 the brains of MDA-MB-231Br3 cells injected mice (Figure 1A) and in none of the mice injected with MDA-MB-231 cells.

We then questioned whether the survival of the metastatic cells could be due to altered metabolism. We first investigated the effects of cell-autonomous glucose metabolism. To test the role of glucose in the survival of the parental MDA-MB-231 and MDA-MB-231Br3 cells, we first determined the activity of aerobic glycolysis of these cells by measuring glucose consumption and lactate production by cells cultured in media containing 5mM glucose. As shown in Figure 1B and 1C, the MDA-MB-
231Br3 cells consumed less glucose and produced less lactate than the parental cells. To test the dependency of these cells on glucose for survival, we removed glucose from the cell culture medium. More than 95% of the parental MDA-MB-231 cells died within 72h of cell culture, whereas the brain-metastatic MDA-MB-231Br3 cells remained viable and proliferative (Figure 1D-1F). These data suggest that a significant metabolic shift from glucose-dependent to -independent viability occurred in the brain metastatic cells. To probe the cause of cell death of MDA-MB-231 cells cultured in glucose free medium, we performed Western blot analyses on markers of apoptosis, caspase 3, and of autophagy, cleaved microtubule-associated protein 1A/1B-light chain 3 (LC3). As shown in Figure 1G, only the procaspase 3 (30kDa) but not the cleaved form of caspase 3 (20kDa) was detected in MDA-MB-231 and MDA-MB-231Br3 cells starved from glucose; however, a significant shift of LC3 from LC3I to LC3II occurred in MDA-MB-231 cells but not in MDA-MB-231Br3 cells (Figure 1H), indicating autophagy was associated with the death of parental cells in the absence of glucose.

**Glutamine and BCAAs are needed for the glucose independent survival of MDA-MB-231Br3 cells**

In addition to glucose, amino acids can also serve as energetic substrates. Considering the high levels of glutamine and BCAAs in the interstitial space of the brain (24, 25), we hypothesized that the catabolism of these two classes of amino acids might play an important role in the survival of brain metastatic cells. Glutamine can be metabolized to glutamate by glutaminase, and glutamate can be further oxidized by glutamate dehydrogenases (GLUD1 and GLUD2) (39) to the citric acid cycle intermediate α-ketoglutarate (α-KG) in the mitochondria. We therefore measured the changes of extracellular levels of glutamine and glutamate of parental MDA-MB-231 and brain metastatic MDA-MB-231Br3 cells cultured for 24hr in glucose free medium. We found that in the absence of glucose, the MDA-MB-231Br3 cells consumed more glutamine and produced more glutamate than the parental cells (Figure 2A, 2B), indicating that the brain metastatic cells had an enhanced glutamine
metabolism. Next, we determined the expression levels of GLUD1 and GLUD2 in isolated mitochondria by Western blot analysis. As shown in Figure 2C, the MDA-MB-231Br3 cells expressed a higher amount of GLUD1 and GLUD2 than the MDA-MB-231 cells, supporting that the brain metastatic cells have enhanced glutamine oxidation.

The BCAAs (valine, leucine, and isoleucine) also exist in abundance in the brain. To examine their role in the survival of brain metastatic cancer cells, we determined the consumption of BCAAs by MDA-MB-231 and MDA-MB-231Br3 cells cultured in the glucose free cell culture media. As shown in figure 2D to 2F, the MDA-MB-231Br3 cells consumed an increased amount of BCAAs than the MDA-MB-231 cells during 48 hr of cell culture. These data suggest that the brain metastatic cells, but not the parental cells, consumed BCAAs in the absence of glucose. To measure the BCAA-oxidizing abilities of these two cell types, we added $^{14}$C-Leucine to the culture media and measured $^{14}$CO$_2$ production after 8 h. As shown in Figure 2G, the brain metastatic cells exhibited a significantly higher BCAA-oxidation rate than the parental cells. We then compared the levels of the rate limiting enzymes of BCAA oxidation in the parental and brain metastatic cells: the branched-chain ketoacid dehydrogenase E1 subunit (BCKDH-E1); the inactivated form of BCKDH-E1, pSer293-BCDKH-E1 (phosphorylated at serine 293 of the E1 subunit); and the BCKDH-inactivating kinase, the branched chain ketoacid dehydrogenase kinase (BDK)(40). The total amounts of BCKDH-E1 and BDK were found to be equal in these two types of cells, but the brain metastatic cells have a significantly lower level of pSer293-BCKDH-E1 (Figure 2H), supporting that the brain metastatic cells have higher BCAAs oxidation capacity. To determine the relationship between pro-survival roles of BCAAs and glutamine, we performed cell death rescue experiments. The MDA-MB-231Br3 cells were first starved from glucose/BCAAs/glutamine for 6hr, and then supplemented with increasing amounts of glutamine (1-4mM) with/without BCAAs (800µM). Cell viabilities were measured at the 12hr after glutamine
addition. As shown in Figure 2I, BCAAs and glutamine synergistically increased the survival of MDA-MB-231Br3 cells. These data suggest that the brain metastatic cells own enhanced ability of utilizing glutamine and BCAAs to survive under low glucose condition.

**Gluconeogenic fructose-1,6-bisphosphatase 2 is up-regulated in MDA-MB-231Br3 cells**

Although the brain metastatic cells grow and survive in the absence of glucose, it remains unclear which carbon sources constitute the basic building blocks for proliferation, especially ribose production in the absence of glucose. One possibility is that the brain metastatic cancer cells have acquired the ability to carry out gluconeogenesis. There are two critical families of enzymes within the gluconeogenesis pathway: phosphoenylpyruvate carboxykinases (PEPCK1 and PEPCK2), which convert oxaloacetate to phosphoenylpyruvate (PEP); and fructose-1, 6-bisphosphatases (FBP1 and FBP2), which convert fructose-1,6-biphosphate to fructose-6-phosphate. We compared the expression levels of these two enzymes in the parental and brain metastatic cancer cells. As shown in Figure 3A, in MDA-MB-231Br3 cells, PEPCK2 expression was unchanged, FBP1 and PEPCK1 were significantly down-regulated, and FBP2 was significantly up-regulated, suggesting a shift favoring mitochondrial PEPCK2 for PEP production and FBP2 for fructose-6-phosphate production in the brain metastatic cells. We then tested the survival of MDA-MB-231Br3 cells after knockdown of FBP2 by shRNA. Knocking down FBP2 resulted in a significant amount of apoptotic cell death (as indicated by the increase of cleaved PARP) of the MDA-MB-231Br3 cells cultured in glucose free medium (Figure 3B), which was quantified by cell counting (Figure 3C), suggesting that gluconeogenesis is critical for the survival of the brain metastatic cells. To demonstrate that this phenotype was not due to an off-target effect of the shRNA, we expressed an exogenous FBP2 that does not contain the 3’-UTR sequence in MDA-MB-231Br3 cells treated with shRNAs targeting the 3’-
UTR of endogenous FBP2 (Figure 3D). It was found that the exogenous FBP2 significantly rescued cell death caused by FBP2 knock down (Figure 3E).

**MDA-MB-361 cells exhibit glucose independent phenotypes similar to the MDA-MB-231Br3 cells**

MDA-MB-231Br3 cells were derived by *in vivo* selection of orthotopically established brain metastases in the brain of nude mice (33). To determine whether glucose independent survival is generalizable to spontaneous breast cancer brain metastatic cells, we test the glucose dependence of another breast cancer cell line isolated from a human brain metastasis, the MDB-MB-361 cells (34). Like the MDA-MB-231Br3 cells, the MDA-MB-361 cells are completely viable in the absence of glucose (Figure 4A). Similar to the MDA-MB-231Br3, the MDA-MB-361 cells have higher levels of total BCKDH-E1 and lower levels of pSer293-BCDKH-E1 as compared to the MDA-MB-231 cells; however, the BDK level is higher than that of the MDA-MB-231 parental cells (Figure 4B). In addition, the MDA-MB-361 cells have significantly higher levels of PEPCK1, PEPCK2, FBP1, FBP2, GLUD1 and GLUD2 (Figure 4B), indicating that they have an even stronger gluconeogenic capacity than that of the MDA-MB-231Br3 cells. These data suggest that the brain metastatic breast cancer cells derived from metastases of mouse brain and the human brain all have gained, although to a different degree, the ability to survive and grow independent of glucose and adopted similar metabolic alterations, such as enhanced abilities of gluconeogenesis and oxidation of glutamine and BCAAs.

**Metabolic characterization of brain-metastatic breast cancer cells upon glucose deprivation**

Knowing the brain metastatic cells, MDA-MB-231Br3 and MDA-MB-361 cells, exhibit enhanced ability of metabolizing glutamine and BCAAs, we thought to determine the contribution of glucose, glutamine, and BCAAs to the growth of these cells in comparison with the MDA-MB-231 cells. Cells were cultured in medium devoid of one type of the aforementioned nutrients. Cell numbers were
counted every 48 hr after medium shift to each type of nutrient restrict media. As shown in figure 5A-5D, in the full medium or in the medium devoid of glutamine/BCAAs, all types of cells could grow (Figure 5A and 5B); in the absence of glucose only, the brain metastatic cells grew and the MDA-MB231 cells did not (Figure 5C). Although the brain metastatic MDA-MB-231Br3 and MDA-MB-361 cells are different in origin, compared to the MDA-MB-231 cells, they again exhibited a similar phenotype of glucose independent growth (Figure 5C). To validate whether the brain metastatic cancer cells carried out gluconeogenesis in the absence of glucose using amino acids as substrates, we first cultured MDA-MB-231, MDA-MB-231Br3 and MDA-MB-361 cells in glucose free medium for 6 h, then added $^{13}$C-glutamine into the glucose free cell culture medium and cultured cells for another 12 h before extraction of metabolites. The $^{13}$C labeled metabolites were measured by tandem mass spectrometry (LC-MS) (37, 38, 41, 42) with a focus on tricarboxylic acid cycle (TCA), gluconeogenesis, pentose phosphate pathway (PPP), and metabolisms of purine and pyrimidine. Levels of intracellular intermediate metabolites fluctuate with metabolic activities of upstream and downstream pathways as well as with cell proliferative status, thus increase or decrease of a metabolite does not necessary represent the activity of the metabolic pathway in which the metabolite is produced. The activity of a metabolic pathway can be reflected by several key metabolites within the pathway. Some relevant key intermediate metabolites that were consistently labeled by $^{13}$C in the brain metastatic cells, in comparison with the parental MDA-MB-231 cells, are presented in Figure 5D-5N (the values of these metabolite are also provided as Supplemental Table 1). The metabolomic results show that, in the absence of glucose, all three types of cells were able to conduct TCA cycle as well as partial gluconeogenesis as reflected by the production of $^{13}$C labeled α-ketoglutarate, fumarate, pyruvate (Figure 5D- 5E). Interestingly, all cells are able to produce lactate from glutamine (Figure 5G), suggesting aerobic fermentation (conversion of pyruvate to lactate) is independent of glucose availability. Importantly, the MDA-MB-231 cells are inactive in gluconeogenesis as
evidenced by the levels of labeled phosphoenopyruvate (PEP) (Figure 5H) and glyceraldehydesphosphate (GAP) (Figure 5I), defective in PPP as indicated by the levels of ribose phosphate (Figure 5J), and defective in purine synthesis as evidenced by the levels of labeled xanthine (Figure 5K), and hypoxanthine (Figure 5L). Pyrimidines, such as thymine and cytosine, whose synthesis does not depend on ribose, were labeled in all cells (Figure 5M and 5N). The major differences between MDA-MB-231 cells and the brain metastatic cells, and the similarities between the two types of brain metastatic cells, all reside at the enhanced gluconeogenesis, which sustains the non-oxidative pentose phosphate pathway (NOX-PPP) and the purine synthesis. These data indicate that, in the absence of external glucose, brain metastatic cancer cells are able to perform de novo purine synthesis for proliferation, which is sustained by the NOX-PPP.

**FBP2 is upregulated in breast cancer brain metastasis**

To further characterize the role of FBP2 in breast cancer brain metastasis, we measured the expression of FBP in clinical specimens of breast cancer brain metastases (n=5) and their matched cancer tissues from the primary sites (n=2), and the tumors formed by injection of MDA-MB-231 in the mammary gland fat pad and in the brain of nude mice, by immunohistochemistry. As shown in Figure 6, the brain metastases, but not the correspondent primary tumors, were positive for FPB2, suggesting that upregulation of FBP2 might be an adaptive event. When the status of FBP2 expression is compared between the brain metastases and unmatched primary breast cancer samples (n=34), it was found that there were 11 out of the 34 primary cancers contain more than 2.0% FBP2 positive cells, and the difference between the brain metastasis and these unmatched primary cancers is significant (P<0.001) (Figure 6L). Considering the evidence of enhanced gluconeogenic activity in brain metastasis, we further tested whether the brain metastases store glycogen. We used periodic acid-Schiff staining (PAS) to determine glycogen levels in the tissues of
human breast cancer brain metastases (n=5) and primary breast cancer tissue samples (n=34) on a tissue microarray. We found that the human brain metastases contained a significantly higher amount (P <0.0001) of PAS positive cancer cells than that of the primary cancers (Figure S2). These data indicate that the brain metastases produce and store glycogen.

**Knocking down FBPs prolonged survival of immunocompetent mice bearing orthotopic breast cancer brain metastasis**

To further determine and evaluate the generalizability of the role of FBP in the development of breast cancer brain metastasis, we used another breast cancer cell line, the murine 4T1 cells, which was derived from a spontaneous mouse mammary tumor of a BALB/C mouse (43). We first compared the expression of FBPs in the 4T1 tumors formed in the mammary fat pad (MFP) and in the orthotopic brain metastases (produced by intra-carotid artery injection of the 4T1 cells). As shown in Figure 7A, FBP1 and FBP2 were weakly expressed in the MFP tumors, but significantly upregulated in the brain metastases. We then stably simultaneously knocked down FBP1 and FBP2 in the mouse breast cancer 4T1 cells (expressing the fire-fly luciferase for *in vivo* imaging) (Figure 7B), and orthotopically implanted the intact 4T1 cells, and the 4T1 cells stably expressing either the control shRNA or the FBPs shRNA into the brains of female BALB/c mice (10,000 cells/mouse). We monitored the tumor growth by luciferase based in vivo live imaging and the survival of these mice. As shown in figure 7C and 7D, knocking down FBPs significantly inhibited the growth of brain metastasis and prolonged the survival of animals bearing brain metastasis, while the survival rates of mice bearing primary tumors are not different (Figure 7E, mice were terminated at times when tumor size reached 1.0cm³). These data support that FBP dependent *de novo* gluconeogenesis is important for the development of breast cancer brain metastasis within the brain.

**DISCUSSION**
Mutual adaptation between metastatic cancer cells and the microenvironment of a host organ have to be achieved for a successful establishment of cancer metastasis (10). Alterations at levels of gene expression and activities of signaling pathways in cells of both cancer metastasis and host organ have been well documented (44-46). Our data revealed that breast cancer brain metastatic cells can utilize gluconeogenesis and oxidation of branched chain amino acids for growth independent of glucose, which is important for the growth of breast cancer brain metastasis in vivo.

The brain interstitial space is a unique metabolic microenvironment. Because of the pre-metabolizing functions of astroglial cells that bridge blood vessels with neurons, the interstitial compartment of the brain is characterized with low levels of glucose (16, 22) and high levels of glutamate (24) and branched chain alpha-ketoacids (the first intermediate metabolite of BCAA oxidation)(26). Passing through the blood-brain-barrier, glucose is mainly taken up by astroglial cells and pre-processed in the astroglia for neuronal energy needs (47), which results in an interstitial glucose level that is lower than the glucose level in the blood (16-23). The elevated expression of mRNA of genes involved in glycolysis in brain metastatic cells (27) might be a compensatory response to the low levels of glucose in the interstitial space of the brain. The high rate of glutamate synthesis in the brain requires an amino group donor that is readily transaminated. At least two third of the amino groups of brain glutamate are derived from BCAAs (24). The constant large scale uptake of BCAAs by the brain is sustained by large neutral amino acid transporters, which are highly expressed in the endothelial cells of brain vessels (48). Brian is one of the high glucose consuming organs in the body. Astroglial cells produce glutamine for neurons via transfer of an amino group from BCAA to glutamate, which is derived from α-ketoglutarate from the TCA cycle, and the resulting branched chain α-ketoacid byproducts are released to the interstitial space, where they can be taken up by neurons for glutamine metabolism by deamination (24). The contribution of BCAAs to the survival of cancer brain metastasis is further supported by the fact that higher sensitivity was found by tracing $^{11}$C-BCAA for
brain metastasis imaging than using the glucose analogue tracer $^{18}$FDG (29-32, 49-52), suggesting that brain metastatic cells take up a high amount of BCAAs. After extravasation from the brain blood vasculature, brain metastatic cancer cells face a challenge to survival and grow in a low glucose environment. This environmental pressure persists before the metastatic microenvironment has been sufficiently altered in favor of providing a maximum nutritional support. Gaining the ability of utilizing amino acids for survival and growth by the brain metastatic cells provides further evidence that the organ microenvironment plays a critical role in the development of cancer metastasis. The two types of brain metastatic cancer cells (MDA-MB-231Br3 and MDA-MB-361) examined in this study are different in origin. However, these brain metastatic cells exhibited the following common features: survival and growth independent of glucose, increased expression of enzymes for glutamine and BCAAs oxidation, up-regulation of FBP(s), and enhanced gluconeogenesis. The data that up-regulation of FBP2 in the clinical specimens of brain metastasis and the growth inhibitory effect of knocking down FBPs on the orthotopic brain metastasis formed by 4T1 cells, strongly support that activation of FBP based gluconeogenesis is critical for the development of breast cancer brain metastasis.

Gluconeogenic activity is not normally presented in cells that are not originated from liver, kidney, intestine or muscle (53). The glycogenic feature of brain metastatic cells enables another pro-survival ability to these cells, glucose storage, which helps cancer cells to resist external metabolic stresses. Gaining gluconeogenic/glycogenic ability and increasing amino acid oxidation provides brain metastatic cells with survival and proliferation power independent of external glucose. While the molecular mechanisms by which the brain metastatic cancer cells achieve the adaptive metabolic switches warrant further investigations, this study suggests that targeting amino-acid-dependent gluconeogenesis may be a novel approach for the treatment of fatal brain metastasis.
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References


32. Manohar K, Bhattacharya A, Mittal BR. Low positive yield from routine inclusion of the brain in whole-body 18F-FDG PET/CT imaging for noncerebral malignancies: results from a large population study. Nuclear medicine


FIGURE LEGENDS

Figure 1. Characterization of MDA-MB-231Br3 cells.

(A) MDA-MB-231Br3 cells implanted into the mammary glands of female nude mice formed spontaneous micro-brain metastases (arrows).

(B) and (C) MDA-MB-231Br3 cells consume less glucose and produce less lactate than the MDA-MB-231 cells. (n=3, p<0.05).

(D) MDA-MB-231Br3 cells, but not MDA-MB-231 cells remained proliferative in the absence of glucose. (n=3 at each time point, p<0.05).

(E) Removal of glucose from cell culture medium caused massive cell death in MDA-MB-231 cells but not the MDA-MB-231Br3 cells within 72 hr of cell culture. Trypan blue staining was used to identify dead cells (arrow heads). Live cells in MDA-MB-231 cells are indicated by arrows.

(F) Quantification of results shown in F. (n=3, p<0.05).

(G) and (H) Western blot analyses of caspase 3 and LC3 of MDA-MB-231 cells cultured in the presence/absence of glucose.

Figure 2. Roles of Glutamine and BCAAs in the Survival of MDA-MB-231Br3 Cells.

(A) And (B) MDA-MB-231Br3 cells consumed a higher amount of glutamine and produced higher amount of glutamate than MDA-MB-231 cells cultured in glucose free medium. (n=3 at each time point, p<0.05).

(C) The mitochondria of MDA-MB-231Br3 cells contain a higher level of GLUD1 and GLUD2 than that of MDA-MB-231 cells. (mitochondrial proteins, Cox II and Cox IV, were used as loading controls).

(D) to (F) MDA-MB-231Br3 cells consumed more leucine, valine and isoleucine than
MDA-MB-231 cells cultured in glucose free condition. (n=3 at each time point, p<0.05).

(G) MDA-MB-231Br3 cells produced a significantly higher level of $^{14}$CO$_2$ from $^{14}$C-leucine than MDA-MB-231 cells. (CPM, count per minute, n=3, p<0.05).

(H) Western blot analysis of BCKDH-E1 (E1), BCKDH-E2, BDK, pSer293-BCKDH-E1 (pE1) in MDA-MB-231 and MDA-MB-231Br3 cells. Actin was used as loading controls.

(I) BCAA and glutamine are synergistically rescued MDA-MB-231Br3 cells from cell death caused by starvations of glucose/glutamine/BCAAs. Trypan blue uptake was used to identify dead cells (n=3 at each concentration, p<0.05).

**Figure 3.** Role of Gluconeogenic Enzymes in the Survival of Breast Cancer Brain Metastatic Cells.

(A) Western blot analyses of PEPCK1, PEPCK2, FBP1, FBP2 in MDA-MB-231 and MDA-MB-231Br3 cells. Actin was used as a loading control.

(B) Western blot analysis of FBP2 and PARP in MDA-MB-231Br3 cells treated by FBP2 shRNA. Actin was used as loading control.

(C) Knocking down FBP2 by shRNA caused a significant amount of cell death in MDA-MB-231Br3 cells culture in glucose free condition.

(D) and (E) Knocking down FBP2 induced MDA-MB-231Br3 cell death was rescued by re-expression of exogenous FBP2.

Trypan blue uptake was used to identify dead cells (n=3 at each time point, p<0.05).

**Figure 4.** Characterization of a Breast Cancer Brain Metastatic Cell Line, MDA-MB-361, Isolated from Human Tissues.

(A) Cell viability assay of MDA-MB-361 cells cultured in glucose free medium for 72 hr without refreshment of medium.(n=3 at each time point, p>0.05).
(B) Western blot analysis of BCKDH-E1, pBCKDH-E1, BDK, PEPCK1, PEPCK2, FBP1 and FBP2, GLUD1 and GLUD2. Actin was used as loading control.

Figure 5. Metabolic Characterization of Brain Metastatic Cells.

(A) to (C), Growth curve of cells cultured in medium supplemented with/without glucose, glutamine, or BCAAs. (n=3 at each time point).

(D) to (N) MS intensity values of indicated $^{13}$C-labeled metabolites for each cell line (231, MDA-MB-231; Br3, MDA-MB-231Br3; 361, MDA-MB-361) cultured in the absence of glucose and presence of $^{13}$C-glutamine. Y axis = mess intensity value/1.0×10$^6$ cells.

(raw counts are provided in Supplemental Table 1).

Figure 6. Immunohistochemical Analysis of FBP2 in Breast Cancer Brain Metastasis

(A) and (B), (C) and (D) Paired samples of human primary tumor (P) and brain metastasis (M).

(E) Primary tumor formed by MDA-MB-231 cells.

(F) Orthotopic brain metastasis formed by MDA-MB-231 cells.

(G) to (I) Human breast cancer brain metastasis.

(J) Representative immunohistochemical staining without primary antibody as negative control for FBP in brain metastasis.

(K) Representative immunohistochemical staining of cytokeratin expression as positive control identifying cancer cells.

(L) Quantification of FBP2 positive cells in brain metastases and unmatched primary cancers.

(Samples containing ≥2.0% FBP2 positive cancer cells were considered FBP2 positive. A chi-
squared with two-tailed test was used to compare the two groups, which generated a P value < 0.001).

(Arrows indicate cells strongly express FBP2; bar=100µm)

**Figure 7.** Knocking Down FBPs in 4T1 Cells Prolonged the Survival of Mice Bearing Orthotopic Brain Metastasis Formed by 4T1 Cells.

(A) Compared with the 4T1 tumors in the mammary fat pad (MFP), FBP1 and FBP2 are upregulated in the orthotopic brain metastases (BM) of 4T1 cells. (bar=100 µm).

(B) Western blot analysis of FBP1/2 in 4T1 cells stably transfected with shRNA against FBP1/2. Actin was used as loading control.

(C) Live imaging of the development of orthotopic brain metastasis formed by intact control 4T1 cells (no transfection), shRNA control cells (transfected with the control shRNA), and FBP shRNA cells (transfected with the shRNA against FBPs). (FD, found dead).

(D) Kaplan-Meier survival curve of mice bearing orthotopic brain metastasis. (P value was calculated by the log-rank test, which indicates the statistic significance of the difference between the intact control group and the shRNA group, n=8 in each group).

(E) Kaplan-Meier survival curve of mice bearing primary tumors. (Mice were terminated at times when tumor reached 1.0cm³ in size. P value was calculated by the log-rank test, which indicates the statistic significance of the difference between the intact control group and the shRNA group, n=8 in each group).
Figure 1

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Figure 1
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**Figure 2**

(A) Glutamine consumed (μmoles/1.0x10^6 cells) over time.

(B) Glutamate produced (mmoles/1.0x10^6 cells) over time.

(C) Western blot images showing GLUD 1, GLUD 2, COX II, and COX IV.

(D) Leucine concentration (μM) over time.

(E) Valine concentration (μM) over time.

(F) Isoleucine concentration (μM) over time.

(G) CPM/6.0x10^6 cells.

(H) Western blot images showing E1, E2, BDK, pSer-E1, and Actin.

(I) Cell viability (%) vs. Glutamine (mM) with or without BCAAs.
Figure 3
Figure 4
Figure 5

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Figure 6
Figure 7

(A) MFP | BM | MFP | BM

(FBP1: FBP1 | FBP1: BM | FBP2: FBP2 | FBP2: BM)

(B) Con-shRNA  FBP-shRNA

(FBP1: FBP1 | FBP2: FBP2 | Actin: Actin)

(C) Days

Control-shRNA  FBP-shRNA

(D) Percentage of survival (%)

Days

(E) Percentage of survival (%)

Days

P=0.79

P=0.001

P=0.950

P=0.572

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Gain of glucose-independent growth upon metastasis of breast cancer cells to the brain

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