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## 1,25-Dihydroxyvitamin D inhibits glutamine metabolism in *Harvey-ras* transformed MCF10A human breast epithelial cell

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### Abstract

Breast cancer is the second most common cancer among women in the US. The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), is proposed to inhibit cellular processes and to prevent breast cancer. The current studies investigated the effect of 1,25(OH)<sub>2</sub>D on glutamine metabolism during cancer progression employing *Harvey-ras* oncogene transformed MCF10A human breast epithelial cells (MCF10A-*ras*). Treatment with 1,25(OH)<sub>2</sub>D significantly reduced intracellular glutamine and glutamate levels measured by nuclear magnetic resonance (NMR) by 23 ± 2% each. Further, 1,25(OH)<sub>2</sub>D treatment reduced glutamine and glutamate flux, determined by [U-<sup>13</sup>C<sub>5</sub>] glutamine tracer kinetics, into the TCA cycle by 31 ± 0.2% and 17 ± 0.4%, respectively. The relative levels of mRNA and protein abundance of the major glutamine transporter, solute linked carrier family 1 member A5 (SLC1A5), was significantly decreased by 1,25(OH)<sub>2</sub>D treatment in both MCF10A-*ras* cells and MCF10A which overexpress *ErbB2* (*HER-2/neu*). Consistent with these results, glutamine uptake was reduced by 1,25(OH)<sub>2</sub>D treatment and the impact was eliminated with the SLC1A5 inhibitor L-γ-Glutamyl-p-nitroanilide (GPNA). A consensus sequence to the vitamin D responsive element (VDRE) was identified *in silico* in the SLC1A5 gene promoter, and site-directed mutagenesis analyses with reporter gene studies demonstrate a functional negative VDRE in the promoter of the SLC1A5 gene. siRNA-SLC1A5 transfection in MCF10A-*ras* cells significantly reduced SLC1A5 mRNA expression as well as decreased viable cell number similar to 1,25(OH)<sub>2</sub>D treatment. SLC1A5 knockdown also

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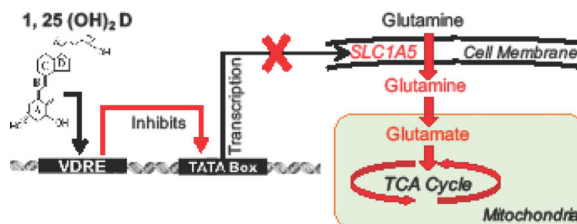
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induced an increase in apoptotic cells in MCF10A-*ras* cells. These results suggest 1,25(OH)<sub>2</sub>D alters glutamine metabolism in MCF10A-*ras* cells by inhibiting glutamine uptake and utilization, in part through down-regulation of SLC1A5 transcript abundance. Thus, 1,25(OH)<sub>2</sub>D down-regulation of the glutamine transporter, SLC1A5, may facilitate vitamin D prevention of breast cancer.

## Graphical abstract



## Keywords

1,25-dihydroxyvitamin D; *Harvey-ras* oncogene; breast cancer; prevention; glutamine metabolism; energy metabolism

## 1. Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer death among U.S. females. More than 230,000 women will be diagnosed with breast cancer in the U.S. this year, and over 40,000 will die from the disease (1). Genetics, environment, as well as dietary factors such as vitamin D are thought to play significant roles in breast cancer risk (2). For example, epidemiological evidence suggests that increased sun exposure as well as increased dietary vitamin D intake, are correlated with decreased breast cancer incidence (3–5). Further, low levels of circulating 25(OH)D, an indicator of vitamin D status, are associated with high breast cancer risk (6) and results from the Women's Health Initiative shows that women who consumed calcium and vitamin D supplements have lower incidence of breast carcinoma *in situ* (7). However, the underlying mechanism by which vitamin D contributes to breast cancer prevention is still not clear.

Cancer cells have been characterized with "the Warburg effect", a critical shift of glucose flux from mitochondrial oxidative phosphorylation towards aerobic glycolysis despite the availability of adequate oxygen (8). This phenomenon suggests that glucose is in part redirected into providing intermediates needed for growth rather than towards energy in the form of adenosine-triphosphate (ATP) (9). Glucose and glutamine are the two most catabolized molecules for the supply of carbon, nitrogen, free energy, and reducing equivalents that are necessary to support cell growth (10). Because glucose is not efficiently used for ATP production during cancer progression (Warburg effect), glutamine, the most abundant free amino acid in the human body, may be an alternative energy source (11). In addition to the importance of glutamine in glutathione synthesis, and in protein and nucleotide synthesis, glutamine can also enter the tricarboxylic acid (TCA) cycle and contribute to the synthesis of reducing equivalents for ATP production (12).

Certain cancer cells exhibit reduced cell survival rates in the absence of exogenous glutamine (13–15). In 1955, Dr. Harry Eagle first highlighted that L-glutamine is essential for the survival and growth of a mouse fibroblast cell line (strain L) and a human carcinoma cell line (strain HeLa) in vitro (16). In fact, a wide variety of human cancer cells have shown sensitivity to glutamine starvation (17,18). Glutamine is transported into cells through the neutral amino acid transporter family system, which includes sodium-dependent systems A, ASC, N and sodium-independent system L (19). One of the major high affinity transporters, solute carrier family 1 member 5 (SLC1A5), is over-expressed in many types of cancer cells, and SLC1A5 mediated glutamine transport is required for cell growth (20,21). Intracellular glutamine can be converted to glutamate by glutaminase (GLS), and further metabolized into  $\alpha$ -ketoglutarate by either deamination or transamination. The carbon backbones from glutamine therefore enter the TCA cycle to provide energy for cell growth (22). Understanding the regulation of glutamine metabolism during cancer progression may contribute to the development of future cancer therapeutic targets.

Mammary cancer development is a multistage process, which includes cellular mutagenesis for genes that regulate cell proliferation. The acquisition of multiple mutations in proto-oncogenes and tumor suppressor genes can result in uncontrolled cell proliferation and metastasis of the cells. The role of mutated *ras* genes in inducing malignant transformation is well documented (23–25). Mutations of the *ras* gene are found in a variety of tumor types and the activated *ras* gene can result in continuous stimulation of cellular proliferation and development of mammary cancer (26). In this study, MCF10A and *Harvey-ras* transfected MCF10A (MCF10A-*ras*) breast epithelial cells were used as a model of cells in early progression to cancer. Previous work from our laboratory shows greater glucose influx, increased glycolysis and lactate production in MCF10A-*ras* breast epithelial cells, similar to the Warburg effect (27). Importantly, 1,25(OH)<sub>2</sub>D inhibits the altered glucose metabolism in the MCF10A-*ras* cells, as well as further inhibits flux of glucose into the TCA cycle (28). Therefore, it is important to also determine if 1,25(OH)<sub>2</sub>D alters glutamine metabolism in cancer progression.

The purpose of the current study was to investigate the role of 1,25(OH)<sub>2</sub>D in regulating glutamine metabolism in mammary epithelial cells during cancer progression. Our hypothesis is that 1,25(OH)<sub>2</sub>D inhibits glutamine uptake and utilization in the cells, a process that is essential for cell growth and proliferation during mammary cancer progression. The results of these studies provide insights into the role of vitamin D in regulating cancer energy metabolism and mammary cancer prevention.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), Nutrient Mixture F-12 (DMEM/F12) media, horse serum, trypsin and penicillin/streptomycin, Annexin V (Alexa Fluor 488 conjugate) were obtained from Life Technologies (Rockville, MD). Cholera toxin was purchased from Calbiochem (Darmstadt, Germany) and 1,25(OH)<sub>2</sub>D was purchased from Biomol (Plymouth Meeting, PA). Protease inhibitor cocktail, trypan blue, insulin, epidermal growth factor, hydrocortisone, and L- $\gamma$ -glutamyl-p-nitroanilide, propidium iodide were purchased from

Sigma-Aldrich (St. Louis, MO). U<sup>13</sup>C<sub>5</sub>-L-glutamine was purchased from Cambridge Isotope labs (Woburn, MA). L-[<sup>3</sup>H] glutamine was obtained from PerkinElmer (Shelton, CT), and Ultima Gold™ Cocktail from PerkinElmer (Waltham, MA). Luciferase reporter plasmid with SLC1A5 promoter pEZX-PG02-SLC1A5 and promoterless pEZX-PG02-Negative control plasmid was purchased from GeneCopoeia (Rokville, MD). FlexiTube GeneSolution GS6510 for SLC1A5 (siRNA-SLC1A5) or AllStars Negative Control siRNA (siRNA-cont) was purchased from QIAGEN (Germantown, MD).

## 2.2. Cell culture

MCF10A, MCF10A-*ras*, MCF10A-*ErbB2*, and MCF10CA1a human breast epithelial cells were gifts from Dr. Michael Kinch, Purdue University. MCF10A, MCF10A-*ras*, and MCF10A-*ErbB2* cells were cultured in DMEM/F12 (1:1) or DMEM media supplemented with 5% horse serum, 10 mg/L insulin, 20 ug/L epidermal growth factor, 50 ug/L cholera toxin, 50 mg/L hydrocortisone, 100 units /ml penicillin, and 0.1 mg/mL streptomycin in a cell incubator at 37°C with 5% CO<sub>2</sub>. MCF10CA1a cells used in MTT assays and flow cytometry analyses were maintained in DMEM media supplemented with 5% horse serum and 100 units /ml penicillin. Cell culture media supplemented with fresh 1,25(OH)<sub>2</sub>D or vehicle at a concentration of 10 nM is changed every 24 hours during treatment period. The 10 nM 1,25(OH)<sub>2</sub>D treatment was delivered to cells in 100% ethanol at a final ethanol concentration <1%.

## 2.3. MTT cell viability assay

Cells were pretreated with either vehicle or 10 nM 1,25(OH)<sub>2</sub>D for 3 days while cultured in supplemented DMEM media (as described above) containing 5 mM glucose and 4 mM glutamine. Cell media was then replaced with supplemented DMEM media containing only glucose or glutamine for another 24 h before being analyzed for viable cell numbers. Relative numbers of viable cell levels were determined by the (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) MTT assay according to the manufacturer's recommendations.

## 2.4. Flow Cytometry

MCF10A-*ras* cells were cultured in same condition as for the MTT assay. From each sample, 1 × 10<sup>6</sup> cells were harvested with phosphate buffered saline in a single cell suspension. For cell cycle analysis, cells were fixed with ice cold ethanol, pretreated with 0.2 mg/ml Rnase A, and stained with 10 ug/ml propidium iodide. For cell apoptosis analysis, cells were resuspended calcium rich Annexin V binding buffer and stained with 5 ul Annexin V and 10 ug/ml propidium iodide. Flow cytometry analysis was performed with a Beckman Coulter FC500 flow cytometry equipped with a 488 nm laser. The results were analyzed using FlowJo (Tree Star, Inc. Ashland, OR). Results were expressed as percentage of total cells arrested in each phase in the cell cycle or apoptotic stages

## 2.5. Metabolomics

Cells were washed with ice cold calcium and magnesium free phosphate buffered saline (CMF-PBS) and harvested into doubly distilled water. Cell lysates were obtained by freezing

the cells in  $-80^{\circ}\text{C}$  freezer for 5 min, and thawing in  $37^{\circ}\text{C}$  water bath for 1 min. Cell debris was pelleted by centrifugation at 12,000 RPM for 2 min at  $4^{\circ}\text{C}$ . The supernatant was collected for metabolite profiling analysis using NMR spectroscopy and mass spectrometry (MS) as previously described (27,28). Metabolite levels were normalized to protein content.

## 2.6. Glutamine tracer kinetics

Two hours prior to cell harvest, media was changed to fresh media containing 1.25 mM of unlabeled and 1.25 mM  $^{13}\text{C}$  labeled L-glutamine. Both media and cell lysates were collected for analysis.  $[\text{U-}^{13}\text{C}_5]$  glutamine flux was determined by  $^{13}\text{C}$ -mass isotopomer distribution analysis of media metabolites using gas chromatography-mass spectrometry (GC-MS) (27,28). Flux calculations were based on tracer ratios in the form: mol  $^{13}\text{C}$ -isotopomer (M+n) per 100 mol  $^{12}\text{C}$  analyte (M+0), where n equals the number of  $^{13}\text{C}$ -labelled carbons in the analyte.

## 2.7. Glutamine uptake measurement

To study the effect of  $1,25(\text{OH})_2\text{D}$  on SLC1A5 mediated glutamine uptake, cells were pretreated with  $1,25(\text{OH})_2\text{D}$  for 4 days (13), and cell culture media was replaced with media containing L- $^3\text{H}$  glutamine with and without the SLC1A5 inhibitor, L- $\gamma$ -glutamyl-p-nitroanilide at the last 5 min of treatment. Glutamine uptake was terminated by adding ice cold CMF-PBS and lysing cells with 0.2%SDS /0.2N NaOH solution. The cell lysate was neutralized with 2N HCL and cell associated radioactivity determined. Results were normalized to cell number at the end of the four-day treatment period using the MTT assay.

## 2.8. ATP assay

Cells were plated in 96-well plates, and the cellular ATP concentration was determined employing a luminescent ATP detection assay kit (Abcam, Cambridge, MA). Results were normalized to cell number measured at the same time as the ATP concentration using the MTT assay in paired plates.

## 2.9. RNA isolation and analysis

RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Reverse transcription of total RNA was performed using MMLV reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed using the Brilliant III SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA). The mRNA abundances of enzymes involved in glutamine metabolism were determined from the threshold cycle (Ct) value. The mRNA expression was normalized to 18S expression and results were expressed as arbitrary units. Primers used are: SLC1A5 forward, 5'-TGAACATCCTGGGCTT GGTAGTGT-3' and reverse, 5'—AGCAGGCAGCACAGAATGTACTTG-3'; 18S forward, 5'-TTAGAGTGTTCAAAGCAGGCCCGA-3' and reverse, 5'-TCTTGGCAAATGCTTTCGCTCTGG-3'.

### 2.10. Western blotting

Cells were washed with cold CMF-PBS twice, and harvested with lysis buffer containing 1% Triton. Cell lysates were obtained by performing freeze and thaw cycles three times. Cells were centrifuged at 14,000 RPM for 15 min at 4 °C, and the supernatant was collected for Western blotting. Protein concentration was determined using the Bicinchoninic Acid Protein assay (Pierce, Rockford, IL, USA). Proteins were resolved by SDS-PAGE on 10% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA), transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and probed with a specific antibody against SLC1A5 (EMD Millipore, Billerica, MA) or an actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

### 2.11. Mutagenesis of the VDRE sequence in SLC1A5 promoter constructs

Mutations in the putative VDRE sequence were introduced into the pEZX-PG02-SLC1A5 promoter plasmid using the GeneArt site-directed mutagenesis system (Invitrogen, Carlsbad, CA). Primers used were: FW: 5'-CTAGGTGTGGCCTCAATTCTAATGACTTCCTCTGCC-3' and RV: 5'-GGCAGAGGAAGTCATTAGAATTGAGGCCACACCTAG-3'

### 2.12. Transient transfection and luciferase assay

MCF10A-*ras* cells were plated in 24-well plates at a density of  $1.4 \times 10^5$  cells per well. At 90% confluence, the cells were transfected using 2  $\mu$ l lipofectamine (Invitrogen, Carlsbad, CA) with 500 ng of the promoterless pEZX-PG02-Negative control plasmid or the pEZX-PG02-SLC1A5 promoter plasmid, and 10 ng of the pGL3-Basic-Firefly luciferase plasmid as a transfection efficiency control. 12 h after transfection, cells were treated with vehicle or 10 nM 1,25(OH)<sub>2</sub>D containing media for 72 h. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), which was normalized to the firefly luciferase activity.

### 2.13. siRNA transfection

MCF10A-*ras* cells were plated and allowed to attach overnight. At 70% confluence, the cells were transfected with either siRNA-SLC1A5 or siRNA-cont using Lipofectamine RNAiMAX and Opti-MEM Reduced Serum Medium (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. After transfection, cells were treated with media containing vehicle or 10 nM 1,25(OH)<sub>2</sub>D for 72 h for analysis.

### 2.14. Statistical analysis

Values are presented as mean  $\pm$  SEM. Results are expressed compared to the vehicle within the same cell line, by the Student's t-tests (LSD), or by analysis of variance (ANOVA), with  $P < 0.05$  considered statistically significant.

### 3. Results

#### 3.1. 1,25(OH)<sub>2</sub>D reduces the effect of glutamine deprivation on inducing further cell death in MCF10A-*ras* cells

To determine the importance of glutamine in MCF10A-*ras* cells, viable cell number was tested when cells were cultured for 24 h with or without glutamine in the cell culture media. Results showed a significant decrease in the number of viable cells in the glutamine-deprived group when compared with the glutamine-supplemented group in MCF10A-*ras* (Figure 1 B). Glutamine dependency was also observed in *HER2/neu/ErbB2* transformed MCF10A cells and metastatic MCF10CA1a cell lines (Figure 1 C, D), though not in the untransformed MCF10A cells (Figure 1 A). We also observed significantly lower dependency on glucose for optimal growth compared with glutamine in these cell lines. These results suggest the importance of glutamine in maintaining optimal MCF10A-*ras* cell growth.

The effect of 1,25(OH)<sub>2</sub>D on viable cell number was also assessed. Results showed that 1,25(OH)<sub>2</sub>D does not affect the number of viable cells in MCF10A cells under either glutamine supplemented or deprived condition (Figure 1 A). However, 1,25(OH)<sub>2</sub>D significantly decreased viable cell number of MCF10A-*ras*, MCF10A-*ErB2*, and MCF10CA1a cells in all three media conditions (Figure 1 B–D), which suggests the effect of 1,25(OH)<sub>2</sub>D in regulating viable cell number is specific to transformed cells.

To determine if the effect of 1,25(OH)<sub>2</sub>D and glutamine deprivation on viable cell number affects a specific phase of the cell cycle arrest or apoptosis, we used flow cytometry to assess the cell cycle profile in MCF10A-*ras* cells. In vehicle treated cells, glutamine deprivation significantly increased the percentage of cells in the sub-G1 phase by 5.4 fold, which suggested induced cell death as shown by flow cytometry (Figure 2 A, C, E). Treatment with 1,25(OH)<sub>2</sub>D alone also significantly induced cell death, indicated by a 2.2 fold increase of cells in sub-G1 phase compared to vehicle control, in glutamine supplemented group (Figure 2 A, B, E). However, in the 1,25(OH)<sub>2</sub>D pre-treated groups, glutamine deprivation did not affect the percentage of cells in the sub-G1 phase compared with its control group. In fact, in glutamine deprived groups, 1,25(OH)<sub>2</sub>D pre-treated group shows significantly decreased cell death compared to the vehicle treated group (Figure 2 A–E). Glutamine deprivation significantly decreased the number of cells arrested in the G0/G1 and G2/M phases in vehicle groups, whereas it had no effect on 1,25(OH)<sub>2</sub>D pre-treated groups (Figure 2 F, H). Overall, these results suggested that pretreatment of 1,25(OH)<sub>2</sub>D reduced the effect of glutamine deprivation on inducing further cell death in MCF10A-*ras* cells.

#### 3.2. 1,25(OH)<sub>2</sub>D alters glutamine metabolism

Because of the reduced dependence of cells on glutamine, we examined the impact of 1,25(OH)<sub>2</sub>D on glutamine metabolism first by assessing the intracellular levels of glutamine and its metabolite glutamate in MCF10A and MCF10A-*ras* cells. Results from metabolomics analysis showed that MCF10A-*ras* contain significantly higher intracellular glutamine and glutamate levels relative to MCF10A cells (Figure 3 A, B). 1,25(OH)<sub>2</sub>D treatment for 4 days reduced intracellular glutamine and glutamate levels in the MCF10A-

*ras* cells significantly (Figure 3 A, B). In MCF10A cells, 1,25(OH)<sub>2</sub>D treatment reduced only intracellular glutamine levels, but not glutamate levels (Figure 3 A, B). In addition, the intracellular levels of other glutamine related metabolites, proline, alanine, creatine, and glycine, were all higher in MCF10A-*ras* cells relative to MCF10A, and decreased significantly by 1,25(OH)<sub>2</sub>D in MCF10A-*ras* cells (Figure 3 C–F). In MCF10A cells, 1,25(OH)<sub>2</sub>D only decreased intracellular alanine levels, but not the other metabolites (Figure 3 C–F). As all four metabolites can be directly or indirectly converted from glutamine in the cell, these results further support the inhibitory role of 1,25(OH)<sub>2</sub>D on intracellular glutamine metabolism in MCF10A-*ras*. Cumulatively, these results suggest that 1,25(OH)<sub>2</sub>D reduces the accumulation of glutamine and glutamate in MCF10-*ras* cells and thus alters glutamine metabolism in this cell model.

### 3.3. 1,25(OH)<sub>2</sub>D inhibits glutamine flux into the TCA cycle

MCF10A-*ras* cells exhibited the Warburg effect, showing a critical shift of glucose flux away from mitochondrial oxidative phosphorylation, and towards aerobic glycolysis (27). Glycolysis provides less energy (ATP) than mitochondrial oxidative phosphorylation. This leads to the question whether 1,25(OH)<sub>2</sub>D alters ATP levels, particularly as no impaired mitochondrial function is observed in most cancer cells exhibiting the Warburg effect (9). As shown above, 1,25(OH)<sub>2</sub>D treatment significantly decreases intracellular glutamine concentration in MCF10A-*ras* cells. Therefore, it is of interest to know if 1,25(OH)<sub>2</sub>D plays a role in regulating the intracellular utilization of glutamine into the TCA cycle. The fraction of glutamine metabolized through the TCA cycle in MCF10A and MCF10A-*ras* was assessed using stable isotope tracer kinetics (U<sup>13</sup>C<sub>5</sub>-L-glutamine). Results show significantly higher glutamine and glutamate flux into the TCA cycle in MCF10A-*ras* cells relative to MCF10A (Figure 4A, B). Treatment with 1,25(OH)<sub>2</sub>D decreased fractional glutamine flux into the TCA cycle by 31 ± 0.2% and decreased fractional glutamate flux by 17 ± 0.4% in MCF10A-*ras* (Figure 4A, B). Intracellular glutamate is converted to α-ketoglutarate either by glutamate dehydrogenase or transaminases to be used in the TCA cycle. Fractional glutamine flux into α-ketoglutarate is decreased by 17 ± 0.6% with 1,25(OH)<sub>2</sub>D treatment in MCF10A-*ras* (Figure 4C). The inhibitory effect of 1,25(OH)<sub>2</sub>D treatment on inhibiting fractional glutamine and glutamate flux into the TCA cycle, as well as fractional glutamine flux into α-ketoglutarate is less potent in MCF10A cells (Figure 4 A–C). These results are consistent with the hypothesis that 1,25(OH)<sub>2</sub>D inhibits the glutamine use in the TCA cycle in MCF10A-*ras*. Interestingly, 1,25(OH)<sub>2</sub>D treatment had no effect on cellular ATP concentration in either cell line, despite inhibition of both glucose and glutamine flux into the TCA cycle (Figure 4 D).

### 3.4. 1,25(OH)<sub>2</sub>D inhibits the glutamine uptake partially through down-regulating glutamine transporter, SLC1A5

In order to determine the mechanism by which 1,25(OH)<sub>2</sub>D reduces glutamine accumulation, the impact on glutamine uptake was examined in MCF10A-*ras* cells. We measured the effect of 1,25(OH)<sub>2</sub>D on regulating glutamine uptake using L-[<sup>3</sup>H] glutamine. Results showed that glutamine uptake into MCF10A-*ras* cells was decreased significantly with 1,25(OH)<sub>2</sub>D treatment (Figure 5 A). These results suggest that 1,25(OH)<sub>2</sub>D may regulate glutamine metabolism through inhibiting glutamine uptake. In addition, a specific



competitive SLC1A5 inhibitor, L- $\gamma$ -glutamyl-p-nitroanilide (GPNA) (29), diminished the effect of 1,25(OH) $_2$ D on inhibiting glutamine uptake, which supports the role of SLC1A5 as a target for 1,25(OH) $_2$ D in regulating glutamine metabolism (Figure 5 A).

To determine if the expression of the SCL1A5 is altered by 1,25(OH) $_2$ D and because the SLC1A5 activity is primarily regulated at a transcriptional level (14,15), the impact of 1,25(OH) $_2$ D on the mRNA and protein expression of SLC1A5 was assessed in MCF10A, MCF10A-*ras* and MCF10A-*ErbB2* cells. Results show that 1,25(OH) $_2$ D treatment significantly decreased mRNA and protein expression of SLC1A5 in both transformed cell lines, but had no effect in MCF10A cells. These results suggest that the effect of 1,25(OH) $_2$ D on SLC1A5 inhibition is specific to transformed cell lines. (Figure 5 B–D).

To further understand the role of SLC1A5 in regulating cell viability, we employed siRNA to knockdown SLC1A5 expression. siRNA-SLC1A5 transfected MCF10A-*ras* cells have significantly decreased SLC1A5 mRNA expression as well as reduced viable cell number (Figure 5 E, F). Further, SLC1A5 knockdown significantly induced an increase in percent of apoptotic cells in MCF10A-*ras* cells from both cell cycle analysis (as illustrated by cells in sub-G1 phase) and cell apoptosis analysis (Figure 5 G, H). These results demonstrate that, that similar to the effect of 1,25(OH) $_2$ D and glutamine deprivation, SLC1A5 knockdown also inhibited viable cell number and induced apoptosis, which suggests the importance of SLC1A5 in maintaining cell survival.

### 3.5. 1,25(OH) $_2$ D regulation of human SLC1A5 gene promoter

Results from the current studies suggest that 1,25(OH) $_2$ D may inhibit SLC1A5 expression through direct transcriptional regulation, consequently it is important to determine the source of regulation of human SLC1A5 gene promoter activity by 1,25(OH) $_2$ D. To examine whether 1,25(OH) $_2$ D regulates the transcriptional activity of the human SLC1A5 promoter in MCF10A-*ras* cells, a sequence 1118 bp upstream of the transcription start site (TSS) and 157 bp downstream of TSS was designed and constructed into the pEZXP-G02-Luciferase reporter gene plasmid by GeneCopoeia. The cells were treated with vehicle or 10 nM 1,25(OH) $_2$ D for 72 hours after transfection. Results showed that the promoter was functional in inducing luciferase activity by 26 fold compared to the promoterless pEZXP-G02-Negative control plasmid, and 1,25(OH) $_2$ D reduced the transcriptional activity of the pEZXP-G02-SLC1A5 promoter plasmid by 30% compared to vehicle (Figure 5 I). These suggest the presence of (a) negative regulatory element(s) by 1,25(OH) $_2$ D in this region and that 1,25(OH) $_2$ D may directly inhibit SLC1A5 expression at the transcriptional level.

Analysis of the –1118/+157 region of the SLC1A5 promoter revealed a potential VDRE sequence (–766/ –752: AGGTGA ATG ACTTCC) by homology with the consensus VDRE sequences (30), supporting that 1,25(OH) $_2$ D may inhibit SLC1A5 gene expression at transcriptional level via vitamin D receptor (VDR). A mutational analysis was carried out to determine whether the suppression of the SLC1A5 promoter activity by 1,25(OH) $_2$ D was mediated through the potential negative VDRE. Site-directed mutagenesis was used to create mutants with base changes in the putative VDRE in the pEZXP-G02-SLC1A5 promoter construct. Wild-type (WT) SLC1A5 promoter and the mutant constructs were transfected into the MCF10A-*ras* cells, and 1,25(OH) $_2$ D responsiveness was assessed by measuring

luciferase reporter activity. 1,25(OH)<sub>2</sub>D significantly inhibited the activity of the wild-type promoter construct, but not the mutated promoter constructs (Figure 5 J). These results suggest that the putative VDRE in the SLC1A5 promoter is responsive to 1,25(OH)<sub>2</sub>D suppressor activity. Collectively, these results suggest that 1,25(OH)<sub>2</sub>D regulates glutamine metabolism and uptake in MCF10A-*ras* cells in part through down-regulating SLC1A5 expression transcriptionally.

#### 4. Discussion

Glutamine metabolism is essential for tumorigenesis in many types of cancer. Cancer cells alter their glutamine metabolic pathways as part of metabolic reprogramming to support rapid cell proliferation (31,32). Despite the essential role of glutamine in cancer, the importance and regulation of glutamine metabolism in mammary cancer is not thoroughly understood. Many cancer cells are dependent on glutamine for growth and proliferation, and glutamine starvation *in vitro* leads to rapid loss of cell viability in these cells (20,33–35). Most tumor cells consume glutamine at a much higher rate compare to normal cells such that an increased rate of glutamine metabolism is, therefore, suggested to be a feature of many cancer cells (16,36). Consistent with other studies, our results demonstrated that glutamine deprivation significantly decreased cell viability of MCF10A-*ras*, but not MCF10A. Importantly, the current studies show for the first time that 1,25(OH)<sub>2</sub>D significantly decreased intracellular levels of glutamine and glutamine, as well as other glutamine related metabolites, supporting the role of 1,25(OH)<sub>2</sub>D on inhibiting glutamine metabolism in MCF10A-*ras* cells.

As many cancer cells display higher glutamine consumption relative to normal cells, and show sensitivity to glutamine deprivation, inhibition of glutamine uptake is proposed to be a possible therapeutic opportunity (22,36). SLC1A5, a major glutamine transporter, is expressed in several human tissues, including placenta, lung, skeletal muscle, kidney, pancreas, as well as carcinoma cells and over-expressed in many glutamine dependent cancer cells (19,20). Further, the SLC1A5 expression level is related to the aggressiveness of the tumor in non-small cell lung cancer, tongue cancer, colorectal cancer, pancreatic ductal carcinoma and prostate adenocarcinomas (21,34,37–39). In a recently published paper by Shimizu et al., SLC1A5 expression was expressed in 66% of patients with non-small cell lung cancer. Its expression was closely correlated with disease stage and metastasis (38). The authors suggested SLC1A5 as a molecular marker for predicting poor prognosis after surgery in non-small cell lung adenocarcinoma patients. In surgically resected tongue cancer, the expression of SLC1A5 was also shown to be significantly associated with disease staging, lymph-node metastasis, lymphatic permeation, and cell proliferation (39). Tissue microarrays of cancer or adjacent normal tissue pairs from 90 colorectal cancer patients revealed that higher SLC1A5 expression levels were strongly associated with higher tumor stages (21). Similarly, in 97 pancreatic ductal carcinoma samples, higher SLC1A5 expression was associated with poorer prognosis (37). SLC1A5 was reported to be expressed at higher levels in prostate cancer patient samples in comparison to normal prostate samples by Wang et al., suggesting SLC1A5 as a putative therapeutic target in prostate cancer (34). The inhibition of SLC1A5 expression with antisense RNA specific to SLC1A5 reduced viable cell number in liver cancer cells, prostate cancer cells, non-small cell lung cancer

cells, melanoma, myeloid leukemia, as well as colon cancer cells (13,20,21,34–36). It is suggested that decreased cell viability due to SLC1A5 suppression was mainly through the activation of cellular apoptosis pathway, evidenced by the increased caspase-3 activity and poly-(ADPribose) polymerase (PPAR) cleavage (20). Despite the importance of SLC1A5 in regulating glutamine metabolism in cancer cells, the involvement of SLC1A5 in regulating glutamine uptake in mammary cancer has not been studied.

Results of the current study demonstrate that SLC1A5 is important in regulating glutamine uptake and maintaining cell viability in *Harvey-ras* transformed human breast epithelial cells. In order to explore a mechanism by which 1,25(OH)<sub>2</sub>D inhibits glutamine metabolism, we investigated the effect of 1,25(OH)<sub>2</sub>D on SLC1A5 expression and glutamine uptake. Consistent with these previous results showing the importance of the SLC1A5 transporter in glutamine metabolism in tumors, the results show that 1,25(OH)<sub>2</sub>D is able to regulate the expression of SLC1A5 transcriptionally, which is an essential step in altering glutamine metabolism in MCF10A-*ras* cells. A putative VDRE responsive to 1,25(OH)<sub>2</sub>D suppressor activity was also identified in the SLC1A5 promoter region.

## 5. Conclusion

Collectively, these results suggest 1,25(OH)<sub>2</sub>D alters glutamine metabolism in MCF10A-*ras* cells by inhibiting glutamine uptake and utilization, in part through transcriptional down-regulation of the expression of SLC1A5 (Figure 6). To our knowledge, this is the first study to demonstrate the effect of 1,25(OH)<sub>2</sub>D on SLC1A5 expression and glutamine metabolism. Therefore, regulation of glutamine metabolism may contribute to the impact of vitamin D on breast cancer prevention.

## Acknowledgments

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## Abbreviations

<b>1,25(OH)<sub>2</sub>D</b>	1,25-dihydroxyvitamin D
<b>SLC1A5</b>	solute carrier family 1 member 5
<b>GLS</b>	glutaminase
<b>GPNA</b>	L-γ-glutamyl-p-nitroanilide
<b>VDR</b>	vitamin D receptor
<b>VDRE</b>	vitamin D responsive element
<b>NMR</b>	nuclear magnetic resonance
<b>GC-MS</b>	gas chromatography-mass spectrometry

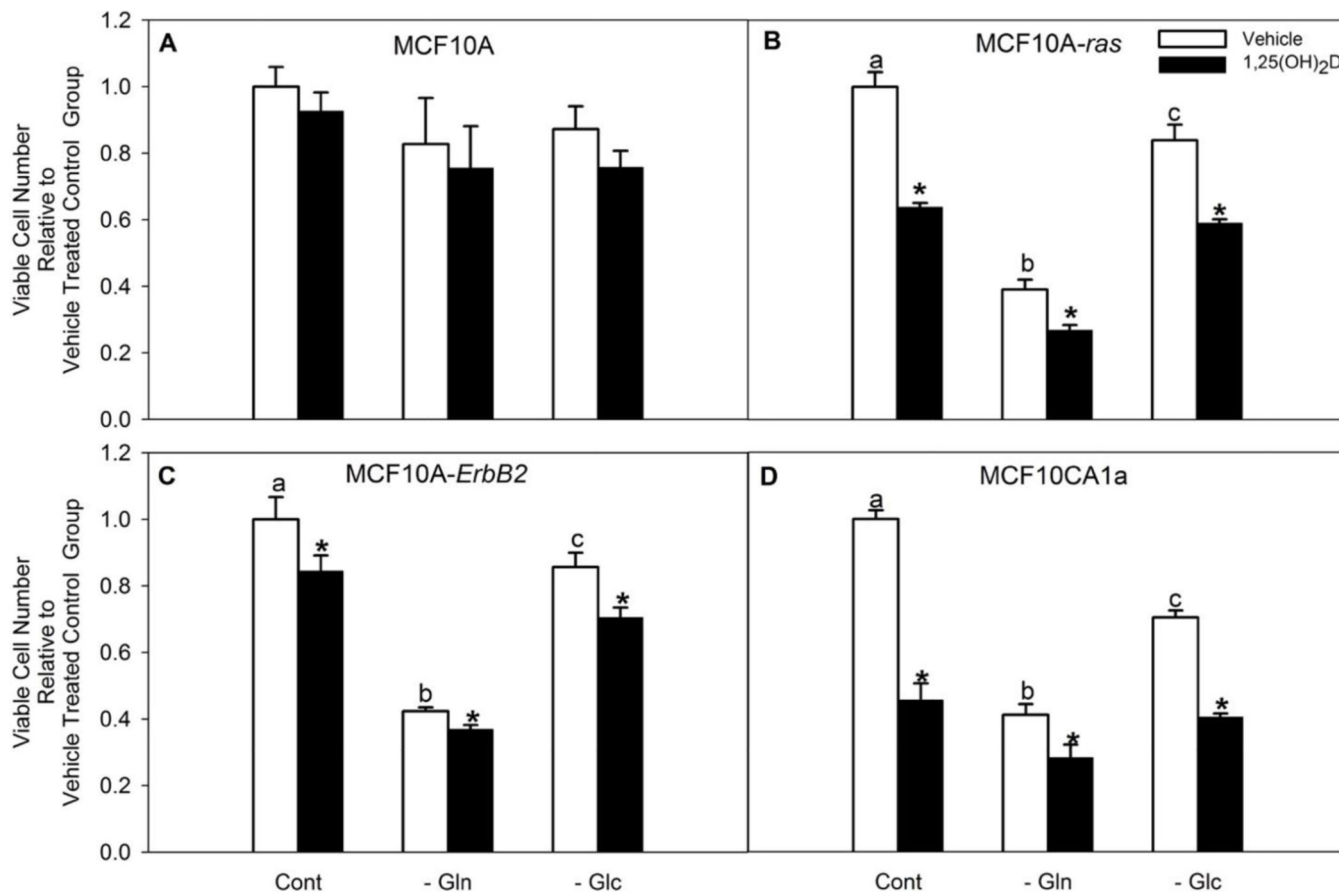
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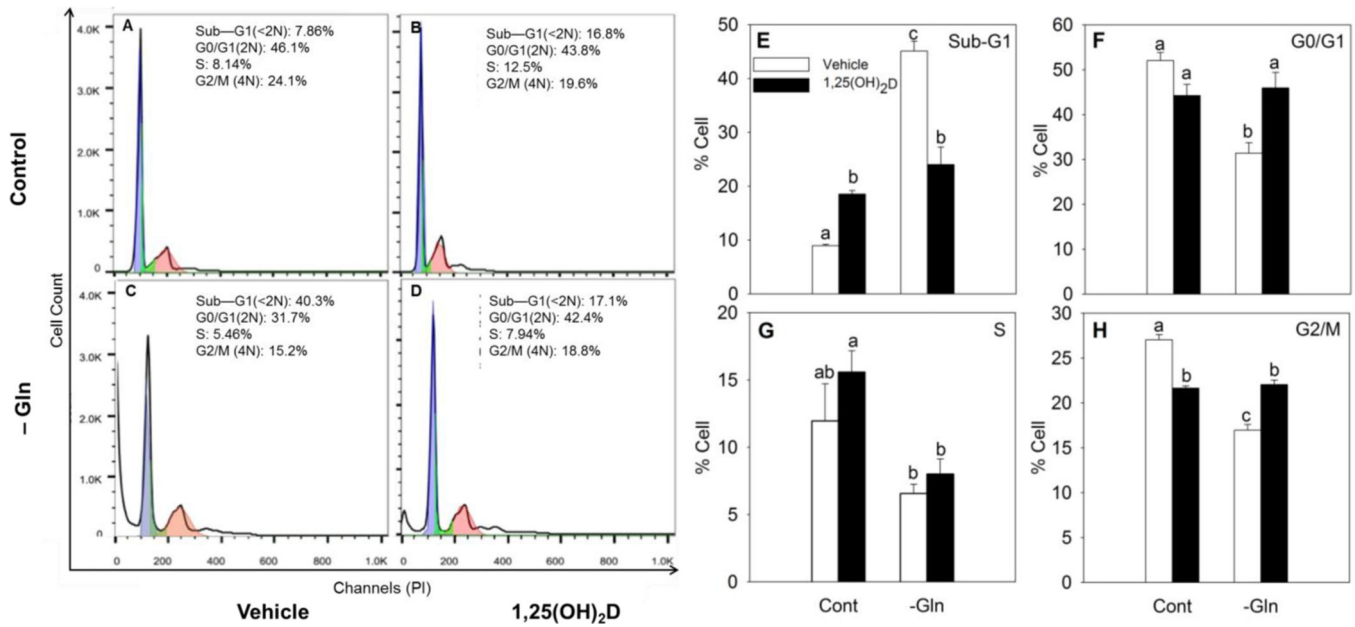
**Highlights**

- Glutamine addiction is reduced by 1,25(OH)<sub>2</sub>D in MCF10A-*ras* cells
- Intracellular glutamine and glutamate concentrations are reduced by 1,25(OH)<sub>2</sub>D
- Flux of glutamine into the TCA cycle is decreased by 1,25(OH)<sub>2</sub>D
- 1,25(OH)<sub>2</sub>D inhibits glutamine uptake through the glutamine transporter SLC1A5
- SLC1A5 is transcriptionally down-regulated through a functional VDRE



**Figure 1. Glutamine deprivation as well as 1,25(OH)<sub>2</sub>D significantly reduced MCF10A-ras cell number**

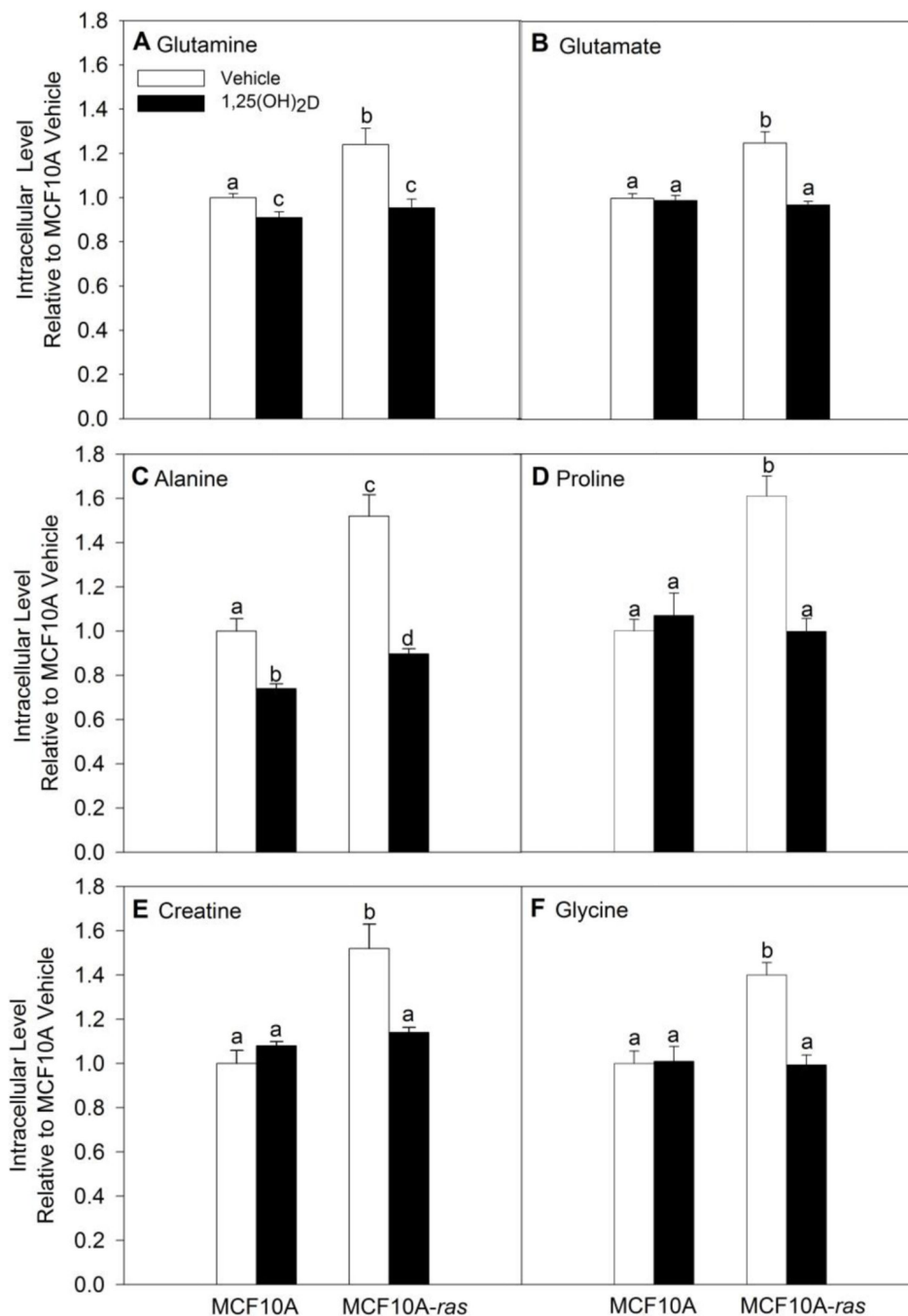
Cells were cultured with media containing 4 mM glutamine and 5 mM glucose for three days with either vehicle or 1,25(OH)<sub>2</sub>D treatment, followed by replacing media with no glutamine or glucose for another 24 h along with either vehicle or 1,25(OH)<sub>2</sub>D treatment. Viable cell number was assessed by MTT assay in (A) MCF10A, (B) MCF10A-ras, (C) MCF10A-ErbB2 and (D) MCF10CA1a cells. Values are presented as mean  $\pm$  SEM and n = 5 from two independent experiments. In each cell line, relative amount of vehicle treated cells in glutamine deprived (-Gln) or glucose deprived (-Glc) groups were compared to the number of vehicle treated cells kept in 4 mM glutamine and 5 mM glucose (Cont) groups. In each figure, vehicle treated groups with different letters are significantly different (p < 0.05) tested by ANOVA. An asterisk (\*) indicates a significant difference (p < 0.05) between each vehicle and 1,25(OH)<sub>2</sub>D treatment groups by the Student's *t*-test.



**Figure 2. 1,25(OH)<sub>2</sub>D reversed the effect of glutamine deprivation on inducing cell death in MCF10A-*ras* cells**

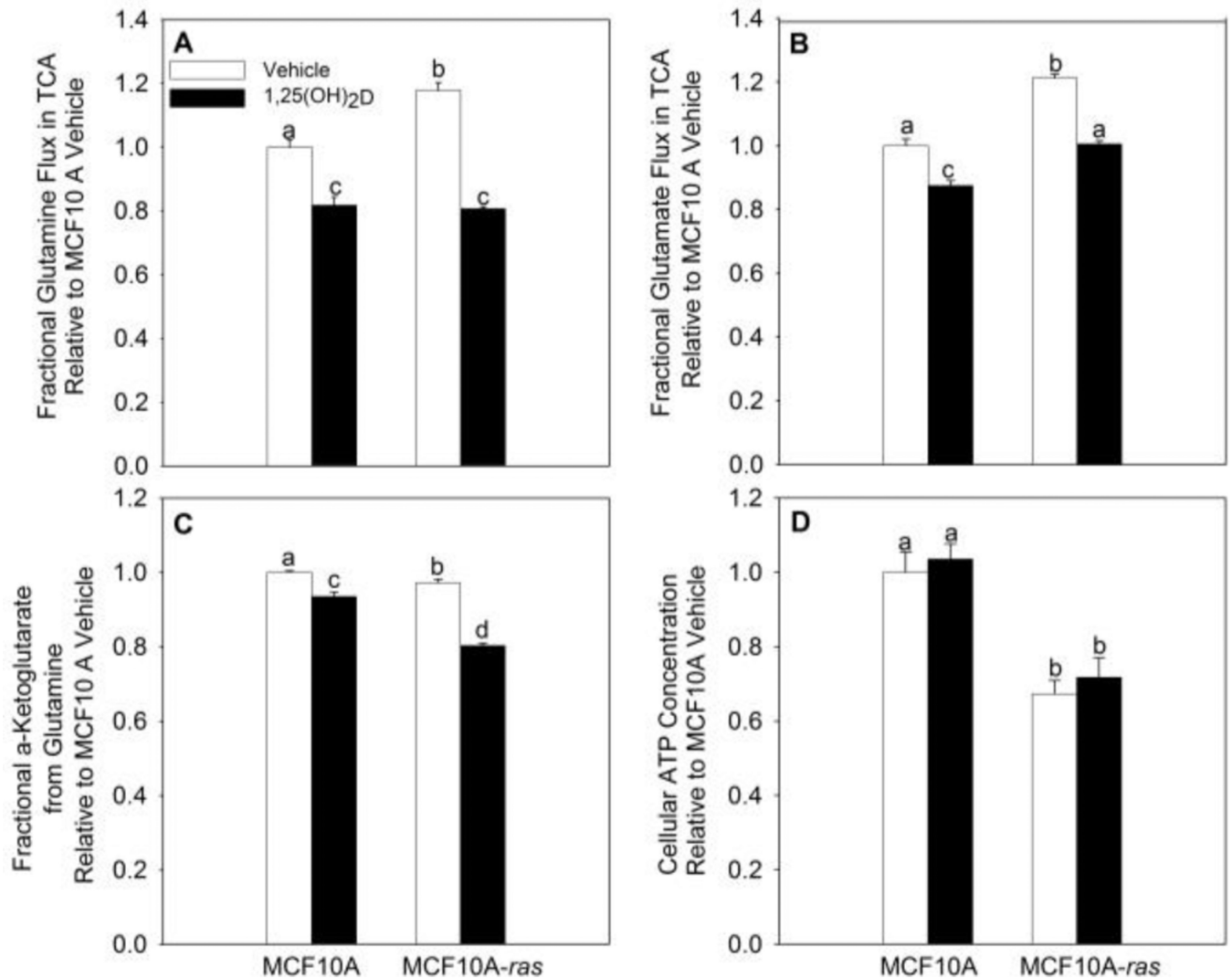
MCF10A-*ras* cells were cultured with media containing 4 mM glutamine and 5 mM glucose for three days with either vehicle or 1,25(OH)<sub>2</sub>D treatment, and media replaced with media containing no glutamine in addition to either vehicle or 1,25(OH)<sub>2</sub>D treatment for another 24 h. The percent of cells in each cell cycle phase was analyzed by flow cytometry following propidium iodide staining. (A–D) Representative cell cycle analysis results for cells in each treatment group. (E–H) Percentages of cells in each cell cycle phase. Values are presented as mean ± SEM and n = 4 from two independent experiments. In each figure, groups with different letters are significantly different (p < 0.05) tested by ANOVA.



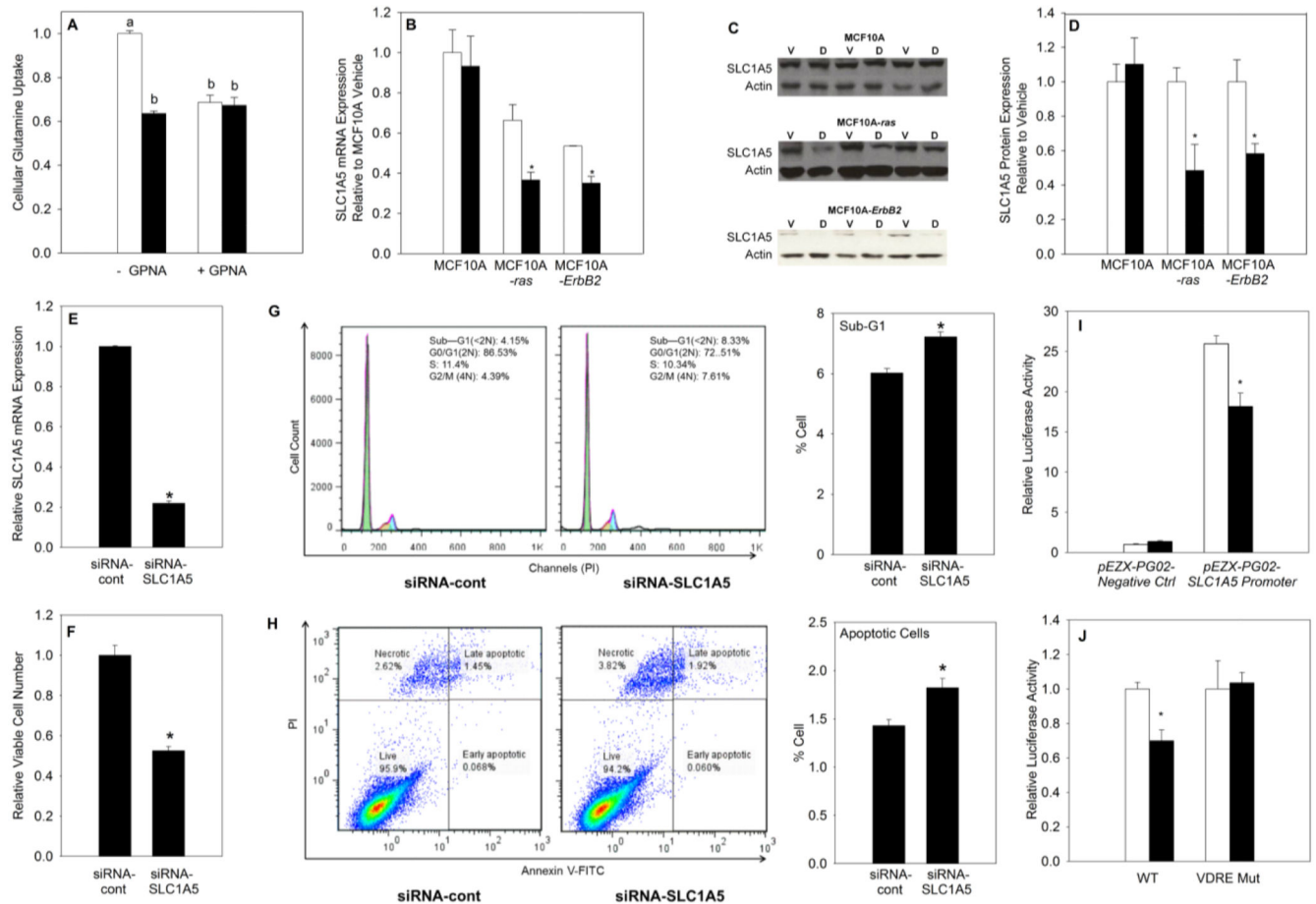


**Figure 3. 1,25(OH)<sub>2</sub>D decreased intracellular levels of glutamine and its related metabolites in MCF10A-ras cells**

MCF10A and MCF10A-ras cells were treated with vehicle or 1,25(OH)<sub>2</sub>D (10nM) for four days before metabolite profiling analysis using NMR spectroscopy and MS. Metabolite levels of intracellular (A) glutamine, (B) glutamate, (C) alanine, (D) proline, (E) creatine, and (F) glycine are normalized to protein content. Values are presented as mean  $\pm$  SEM (n=4) relative to MCF10A vehicle groups. Groups with different letters are significantly different ( $p < 0.05$ ) tested by ANOVA.



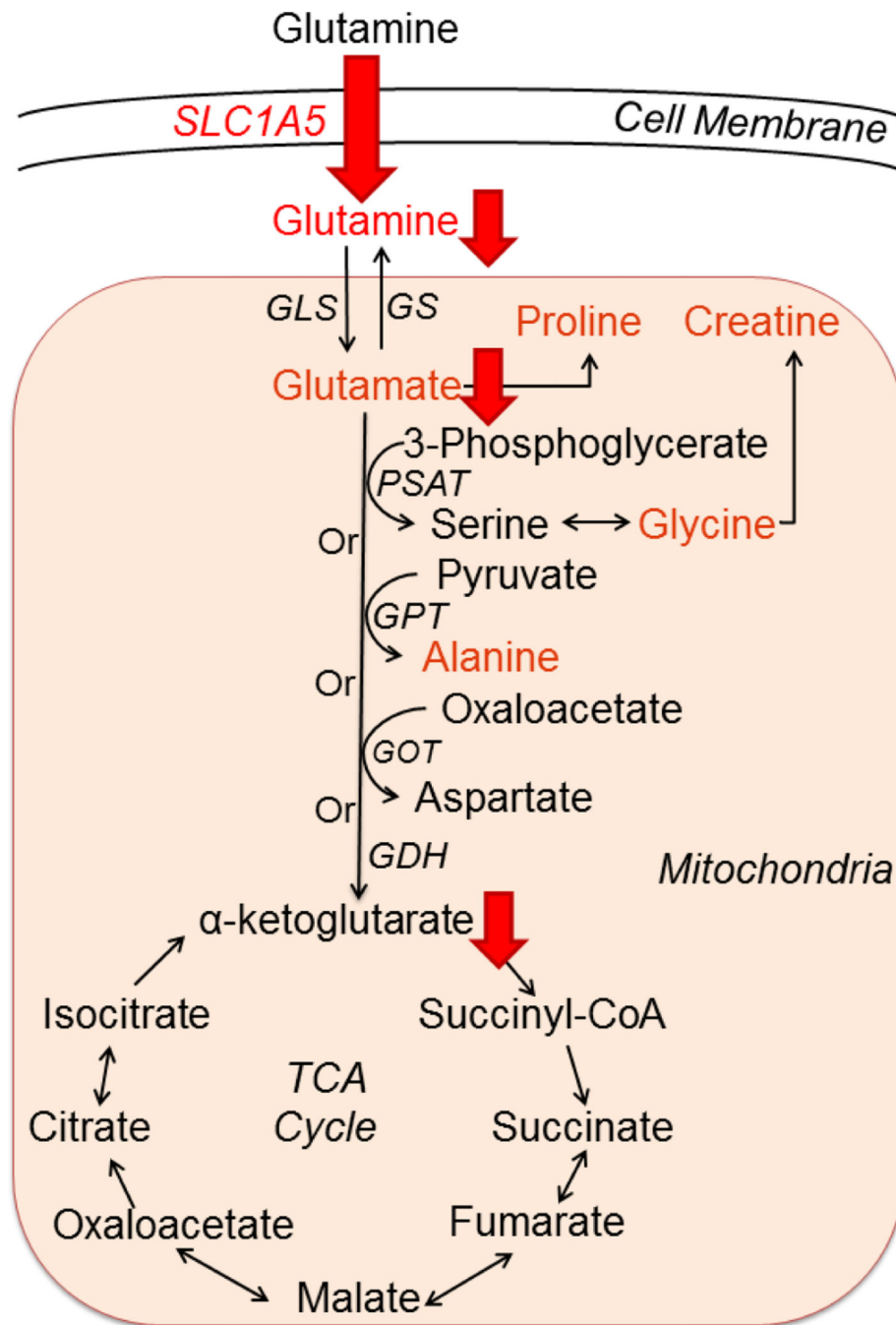
**Figure 4. 1,25(OH)<sub>2</sub>D decreased glutamine flux into the TCA cycle in MCF10A-ras**  
MCF10A and MCF10A-ras cells were treated with vehicle or 1,25(OH)<sub>2</sub>D (10nM) for four days. Two hours prior to cell harvest, media was changed to fresh media containing both 1.25 mM unlabeled and 1.25 mM <sup>13</sup>C labeled L-glutamine. Fractional (A) glutamine and (B) glutamate flux into the TCA cycle and (C) fractional glutamine flux into α-ketoglutarate were shown. (D) Cellular ATP concentration was also measured after 4-day vehicle or 1,25(OH)<sub>2</sub>D treatment with a luminescent ATP detection assay kit. Values are presented as mean ± SEM (n=4) relative to MCF10A vehicle groups. Groups with different letters are significantly different (p < 0.05) tested by ANOVA.



**Figure 5. 1,25(OH)<sub>2</sub>D inhibits glutamine uptake through down-regulation of the glutamine transporter SLC1A5 in MCF10A-ras cells**

Cells were treated with vehicle or 1,25(OH)<sub>2</sub>D (10 nM) for 4 days. (A) Glutamine uptake was determined by analyzing cell lysate radioactivity 5 min following replacing the media with media containing L-[<sup>3</sup>H] glutamine, or L- $\gamma$ -glutamyl-p-nitroanilide (GPNA). Results were normalized to cell number obtained from MTT assay. (B) SLC1A5 mRNA expression is shown relative to vehicle in each cell line. (C) Representative blots of SLC1A5 protein expression with vehicle (V) or 1,25(OH)<sub>2</sub>D (D) treatment by Western blot analysis. Actin was used as a loading control. (D) Quantification of SLC1A5 protein expression relative to vehicle in each cell line. Effect of siRNA-cont and siRNA-SLC1A5 transfection in MCF10A-ras cells on (E) SLC1A5 mRNA expression and (F) viable cell number. (G) Representative cell cycle analysis results and quantification of percentage of cells in sub-G1 phase in SLC1A5 knockdown MCF10A-ras cells. (H) Representative cell apoptosis analysis results and quantification of percentage of apoptotic (includes both early and late apoptotic) cells in SLC1A5 knockdown MCF10A-ras cells. (I) Relative luciferase activity measured after 72 h pEZX-PG02-Negative Ctrl or pEZX-PG02-SLC1A5 promoter plasmid transfection in MCF10A-ras cells. (J) Relative luciferase activity measured after 72 h wild-type (WT) or VDRE mutant (VDRE Mut) pEZX-PG02-SLC1A5 promoter plasmid transfection in MCF10A-ras cells. Values are presented as mean  $\pm$  SEM and n= at least 3

from two independent experiments. Groups with different letters are significantly different ( $p < 0.05$ ) tested by ANOVA. An asterisk (\*) indicates a significant difference ( $p < 0.05$ ) from vehicle treatments by the Student's *t*-tests.



**Figure 6. Summary of the inhibitory effects of 1,25(OH)<sub>2</sub>D on glutamine metabolism in MCF10A-*ras***

Intracellular glutamine and its related metabolites (in red font color) levels were decreased by 1,25(OH)<sub>2</sub>D in MCF10A-*ras* cells. 1,25(OH)<sub>2</sub>D inhibited fractional glutamine and glutamate flux into the TCA cycle, as well as glutamine flux into α-ketoglutarate shown by red downward arrows. 1,25(OH)<sub>2</sub>D significantly decreased SLC1A5 mediated glutamine uptake into the cells, as well as the mRNA and protein abundance of SLC1A5.

Abbreviations: GLS, glutaminase; GS, glutamine synthetase; PSAT, phosphoserine

aminotransferase; GPT, glutamate-pyruvate transaminase; GOT, glutamicoxaloacetic transaminase; GDH, glutamate dehydrogenase.

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