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Argininosuccinate Synthetase 1 (ASS1) Is a Common Metabolic Marker of Chemosensitivity for Targeted Arginine- and Glutamine-Starvation Therapy

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Short Title: Arginine-Auxotrophic Cells Are Sensitive to Glutamine-Deprivation Strategies

Conflict of Interest: None

Highlights

- **Arginine-auxotrophic cancer cells are also sensitive to glutamine starvation.**
- **Induction of ASS1 expression is involved in arginine- and glutamine-starvation.**
- **Modulating ASS1 expression alters sensitivity to arginine and glutamine-starvation.**
- **The HIF-1α/c-Myc axis regulates ASS1 under Arginine- and Glutamine-starvation.**
- **ASS1 is a common target for arginine- and glutamine-starvation cancer therapy.**

Abstract

Argininosuccinate synthetase 1 (ASS1) is the rate-limiting enzyme that catalyzes the biosynthesis of arginine (Arg). Many malignant human tumors are auxotrophic for Arg because ASS1 is silenced. ASS1 has been established as a sensor of Arg auxotrophic response and a chemosensitivity marker for Arg starvation therapy. Here, we report that ASS1 is also a sensor for glutamine (Gln)-deprivation response, and that upregulation of ASS1 expression is associated with resistance to Gln-starvation treatments. Knockdown of ASS1 expression resulted in increased sensitivity to both Arg- and Gln- starvation, whereas increased ASS1 expression by ectopic transfection is associated with resistance to both Arg- and Gln-starvation. The addition of permeable fumarate, a metabolite that bridges the tricarboxylic acid and urea cycles, resulted in downregulation of ASS1 expression and increased sensitivity to both Arg- and Gln-deprivation treatments. Mechanistically, the Gln-deprivation response, like the arginine-auxotrophic response, downregulates HIF-1α resulting in de-silencing of ASS1. Our results demonstrate that ASS1 is a common biosensor for Arg and Gln deprivation response and a shared target for Arg- and Gln-starvation therapies which have been in several current clinical trials.

Keywords: Glutamine-starvation therapy, Arginine-starvation therapy, ASS1, HIF-1alpha and c-Myc, ADI-PEG20

1. Introduction

Argininosuccinate synthetase 1 (ASS1) which catalyzes the reaction of L-citrulline and aspartate to argininosuccinate, is the rate-limiting enzyme for the biosynthesis of arginine (Arg). Malignant melanoma, hepatocellular carcinoma, mesothelioma, and prostate cancer are virtually ASS1-silenced, whereas about 40% to 60% of breast cancers, small-cell lung cancers, and leukemias are ASS1-negative. These tumors use extracellular Arg for their survival. Pegylated recombinant arginine deiminase (ADI-PEG20), which digests Arg into citrulline and ammonia [18, 38], and human recombinant arginase (peg-rhArg1 also known as PEG-BCT-100) which digests Arg into ornithine and urea [57] (Fig. 1), have been in clinical trials for treating ASS1-silencing tumors. ASS1 has already been established a marker for sensitivity of Arg-starvation therapy [18, 19, 25, 48] because mutations of *ASS1* are very rare [16].

Glutamine (Gln) is an important nutrient for cancer cell growth by supporting macromolecular biosynthesis, maintenance of bioenergetics and redox-regulation. Gln is also a nitrogen source for nucleosides, amino acids and hexosamines [24, 33, 54, 55]. Enhanced Gln metabolism has been observed in many human malignancies [4]. Most cancer cells are addicted to Gln as they require extracellular Gln for survival [24, 54]. These make Gln as an important target in recent cancer therapy.

Oncogenic transformation is often associated with metabolic rewiring [12, 16]. This rewiring provides metabolic adaptation of growth advantage for cancer cells, but it may also render these cells vulnerable to targeted therapy. Given that most cancer cells avidly require Gln for growth and that multiple pathways can feed the Gln requirements (Fig. 1, also see Discussion), it is plausible that cancer cells may have certain metabolic abnormalities that are vulnerable to Gln-depletion therapy. Targeted Gln therapy has been in current clinical trials, however, it is unclear whether specific subsets of tumors are particularly effective to this therapy and whether any metabolic abnormality are associated with these tumors. We report here that tumor cells with reduced ASS1 expression which are generally auxotrophic to Arg deprivation,

also exhibit elevated sensitivity to Gln-deprivation treatment. Our results thus identify ASS1 as a common marker for Arg- and Gln-deprivation sensitivity. This study unifies Gln and Arg metabolisms as a relevant target for therapy using combination Gln- and Arg-starvation strategies.

2. Materials and Methods

2.1. Cell lines and cultures conditions

Melanoma cell lines A2048, A375, and WM2664 [50], immortalized human skin fibroblast line BJ-l [2], and human triple-negative breast cancer cell lines (TNBC) (BT20, BT549, Hs578T, MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-453, MDA-MB-468, HCC70, HCC38, and HCC1838) [21] were obtained from American Type Culture Collection (ATCC). ASS1-transfected A375 and 2058 cells were described previously [31]. All cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere. For Arg-free and Gln-free cultures, 10% dialyzed FBS was used. Alternatively, Arg-free medium was prepared by treating the complete medium (DMEM + 10 FBS) with 10 μM ADI-PEG20 for 48 hr at 37°C. In experiments when ADI-PEG20 was used, ADI-PEG20 was directly added into cultured cells grown in the complete medium. We found that the kinetics of HIF-1α degradation induced by Arg(-) medium or by ADI-PEG20 were similar (t½ = ~ 20 min) [48].

2.2. Reagents and antibodies

Reagents were obtained from the following sources: ADI-PEG20 (specific activity, 5 – 10 IU/mg) from Polaris Pharmaceuticals, Inc.; 6-diazo-5-oxo-L-norleucine (DON), azaserine, dimethyl fumarate, dimethyl ketoglutarate, and dimethyl succinate from Sigma-Aldrich; mouse anti-

human ASS1 monoclonal antibody from Polaris Pharmaceuticals; rabbit anti-HIF-1α, rabbit anti-c-Myc (N262), and rabbit anti-kidney type glutaminase 1 (GLS1) from BD Bioscience; and rabbit anti-α-tubulin antibodies from Sigma-Aldrich.

2.3. Lentiviral and siRNA transfection

Human *GLS* 1 shRNAs (NM_014905.3-2247s21c1, clone #4, NM_014905.3-1610s21c1, clone #5) in lentiviral vectors were from Sigma-Aldrich. Preparation of lentiviral recombinants followed the procedure previously described [49]. siRNA for *ASS1* (5′-GCAAUGACCUGAUGGAGUAdTdT) was synthesized by Sigma-Aldrich, The siRNA was transfected using lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions**.**

2.4. Western blot analysis, chromatin immunoprecipitation, transcriptional regulation using reporter assay, and cytotoxicity assay

Procedures of immunoblotting and chromatin immunoprecipitation (ChIP) using primer sequences for probing HIF-1α and c-Myc bindings to the *ASS1* promoter were conducted as described previously [49, 50]. Transcriptional regulation assays using reporter recombinants pGL3-AS-85 and mutant mE-box have been described previously [48] .

Cytotoxicity assay using MTT followed the procedure previously described [31]. The IC_{50} values were obtained from a non-linear regression analysis of concentration–effect curves by GraphPad Prism Software and were represented by the mean ± SD of three independent experiments. The IC_{50} values were plotted in reference to that of A2058, which was set at 100%. Statistical significance was determined set at $P < 0.05$.

3. Results

3.1 Reduced ASS1 expression is associated with increased sensitivity to Arg- and Gln-deprivation in melanoma cells

Nearly 100% melanomas express very low ASS1 levels. Malignant melanoma has been in multiple clinical trials using ADI-PEG20 [6, 17, 36, 50] We chose three human melanoma cell lines (A2058, A375, and WM2664) and a non-transformed immortalized human skin cell line (BJ-1) as the reference. Western blots showed that BJ-1 and WM2664 cells expressed higher levels of ASS1 ($ASS1^{high}$) than did A2058 and A375 cells ($ASS1^{low}$) (Fig. 2A). We cultured these cells in Arg-free or in Gln-free media for up to three days. As anticipated, the $ASS1^{low}$ cell lines were more sensitive than were their $ASS1^{high}$ counterparts to the killing by Arg-starvation (Fig. 2B). Similar results were obtained when these cells were cultured in Gln-free medium (Fig. 2C). These results demonstrate that reduced ASS1 expression is associated with elevated sensitivity to the killing by Arg-starvation and Gln-starvation.

We previously demonstrated that ASS1 induction in $ASS1^{low}$ cells but not in $ASS1^{high}$ cells is associated with rapid downregulation of HIF-1α which functions as a negative transcriptional regulator. It is also frequently associated with upregulation of c-Myc expression, which functions as a positive transcriptional regulator of ASS1 [48-50]. This was found in A2058 cells but not in A375 cells because of an impairment in upstream signaling for c-Myc upregulation [49, 50]. Consistent with these findings, we found that downregulation of HIF-1α in all the four cell lines treated with ADI-PEG20 (Figs. 2D & 2E). Also consistent with our previous findings, we found that levels of c-Myc and ASS1 expression in A2058 cells, but not in A375 (Figs. 2D & 2E). However, increases of ASS1 expression in the $ASS1^{high}$ cell lines (BJ-1 and WM2664) were marginal, perhaps because these cells already expressed relatively higher levels of ASS1 and as we have previously demonstrated that Arg and ASS1 levels are

homeostatically regulated [50]. Similar results were observed in these cell lines correspondingly cultured in Gln-free medium, except reduction of c-Myc was found in WM2664 cells. The insensitivity of ASS1 induction by Arg-free (Fig. 2D) and Gln-free (Fig. 2E) conditions in these ASS1^{high} cell lines was correlated with their relative resistance to Arg-free and Gln-free treatments as shown in Figs. 2B and 2C, respectively.

We performed a chromatin immunoprecipitation (ChIP) assay to determine the interactions of HIF-1α and c-Myc with the E-box at the *ASS1* promoter. We found that like the Arg-free medium treatment, Gln-deprivation also reduced HIF-1α binding to the *ASS1* promoter, although the magnitude of reduction was not as great as that in the Arg-deprivation (Fig. 2F, upper panel). Likewise, Gln-deprivation induced c-Myc binding to the ASS1 promoter, but the increment was not as great as that with Arg-deprivation-treated cells (Fig. 2F, lower panel).

To substantiate that transcriptional regulation of ASS1 by Gln-depletion, we performed transient transfection assay using reporter constructs containing wild-type of ASS1-E-Box sequence (pGL-AS-85) vs mutant E-box-containing reporter [48]. Elevated induction of reporter expression by Gln-depletion was evident in A2058 (Fig. 2G) and in MDA-MB-231 (Fig. 2I), using ADI-PEG20 or Arg-depletion treatments for comparisons. Induction of ASS1 in WM2664 cells by these treatment was also observed, but the magnitudes of induction were correspondingly reduced (Fig. 2H) as compared with those in other two cell lines. These results demonstrate that the mechanism of the Gln-starvation response is generally similar to that of the Arg-starvation response.

3.2. Inverse correlation between ASS1 expression and sensitivity to Arg- and Gln-starvation in TNBC cell lines

We next investigated the TNBC cell lines because breast cancer is a heterogeneous disease and levels of ASS1 expression are variable [15], providing an excellent model for investigating the relationships between Arg-deprivation and Gln-deprivation responses. We randomly chose

11 TNBC cell lines, six (BT20, MDA-MB-157, MDA-MB-436, MDA-MB-453, MDA-MB-468, and HCC-70) had detectable ASS1 expression by Western blotting ($ASS1^{high}$) and the other five (BT549, Hs578T, MDA-MB-231, HCC38, and HCC1806) were $ASS1^{low}$. All $ASS1^{high}$ cell lines also expressed detectable GLS1 and all $ASS1^{low}$ lines, with the exception of BT549 and MDA-MB-231, also showed reduced GLS1 expression, suggesting a correlation between ASS1 and GLS1 expression in these TNBC cells (Fig. 3A). However, no correlation was found between epidermal growth factor receptor and ASS1/GLS1 (data not shown).

We then determined the expression of ASS1 in these TNBC cells and their sensitivity to Arg- and Gln-starvation conditions. We found that the sensitivity to the killing between Arg-starvation and Gln-starvation among these cell lines was strongly correlated [Pearson analysis, $r(11)=0.664$, $p=0.025$] (Fig. 3C). Moreover, the sensitivity of these cells to Arg- and Gln-deprivation was negatively correlated with ASS1 expression levels (comparing between Figs. 3B and 3C), $ASS1^{high}$-cell lines (BT20, MDA-MB-453, MDA-MB-469, and HCC70) were more resistant to killing by Arg- and Gln-deprivation than $ASS1^{low}$ cell lines (BT-549, Hs578T, MDA-MB-231, HCC38, and HCC1806). However, there are exceptions specifically when some cell lines were compared between each other. For example, MDA-MB-231 (line #5) which expresses reduced levels of ASS1 as compared with that in MDA-MB-453 (#7) (Figs. 3A&B), yet both cell lines exhibit similar levels of sensitivities to Arg- and Gln-depletions (Fig. 3C). This may not be suppressing, given the inter-cellular heterogeneity and the complexity of cell killing mechanisms by Arg-deprivation and Gln-deprivation treatments.

To investigate the roles of HIF-1α and c-Myc in the regulation of ASS1 by Arg- and Gln-deprivations in these TNBC cells, we randomly selected six cell lines, two $ASS1^{high}$ (MDA-MB-436 and MDA-MB-157) and four $ASS1^{low}$ (Hs578T, HCC1806, MDA-

MB-231, and BT-549). We treated these cells with ADI-PEG20 or grew them in Gln-free medium. The expression of HIF-1α, c-Myc, and ASS1 was determined by Western blottings. Gross examination of Western blots between ADI-PEG20 (Fig. 3D) and Gln-free treatment (Fig. 3E) revealed very similar gene expression patterns among these six TNBC cell lines.

HIF-1α expression levels were generally low except in Hs578T cells (Figs. 3D and 3E). Moreover, the expression of HIF-1α in these cells was downregulated under both Arg-deprivation and Gln-free conditions. Induction of c-Myc and ASS1 expression was observed in Hst578T, HCC1808, and MDA-MB-231 cells, but not in MDA-MB-436, and MDA-MB-157 cells, under both treatments. Expression of ASS1 in BT549 was undetectable with or without treatment. The concordance between downregulation of HIF-1α and upregulation of ASS1 and c-Myc expression under Arg- and Gln-deprivation conditions (Fig. 3F), again, supports the roles of HIF-1α and c-Myc in the regulation of ASS1 expression by Gln-deprivation.

3.3. Elevated fumarate, but not α-ketoglutarate and succinate, upregulates HIF-1α resulting in downregulation of ASS1 and sensitization of cells to both Arg- and Gln-deprivation

Previous studies have demonstrated that germline mutations in the *fumarate hydratase* (*FH*) gene predispose individuals to leiomyomas and renal cell cancer (RCC). The encoded enzyme catalyzes fumarate-to-malate conversion in the TCA cycle (Fig. 1). RCC is characterized by fumarate accumulation and HIF-1α overexpression [39], because fumarate functions as a competitor of prolyl hydroxylase, an enzyme that hydroxylates HIF-1α for proteasome-mediated HIF-1α degradation [23]. The accumulated fumarate in FH-deficient cancer cells promotes the

conjugation between fumarate and glutathione (GSH), resulting in enhanced reactive oxygen species (ROS) production which can be inhibited by the antioxidant, N-acetylcysteine (NAC) [42, 58].

Since HIF-1α is a negative regulator of *ASS1* expression [48, 49], we hypothesized that increasing cellular fumarate levels may suppress ASS1 expression *via* HIF-1α accumulation, thereby sensitization to Arg- and Gln-deprivation treatments. To test this hypothesis, we treated A2058 cells and MDA-MB-231 cells with cell-permeable dimethyl fumarate (DMF), using dimethyl ketoglutarate (DMK) and dimethyl succinate (DMS) for comparisons (see Fig. 1 for the relationship of these metabolites). We found that DMF significantly (greater than 20-fold) upregulated HIF-1α but downregulated c-Myc/ASS1 and the effects were blunted by NAC (Fig. 4A), whereas induction of HIF-1α by DMK and DMS was marginal (less than 2-fold at most) in A2058 cells using equimolar concentrations (Fig. 4B). High levels of HIF-1α induction but suppression of c-Myc/ASS1 by DMF can also be seen in MDA-MB-231 (Fig. 4C). Consistent with these findings, we demonstrated that DMF, but not DMK and DMS, was toxic to cultured A2058 and A375 cells (Figs. 4D and 4E). These results demonstrated that DMF can significantly regulate the expression of HIF-1α/c-Myc/ASS1.

To determine whether downregulation of ASS1 by DMF sensitizes cells to Arg- and Gln-starvation challenges, we cultured two $ASS1^{low}$ cell lines, A2058 and A375, in Arg-free and Gln-free media, with or without DMF. We found that DMF induced additive cell killing capacities in both A2058 (Figs. 4F) and A375 (Fig. 4G) cells. No additive cell killing by DMK and DMS under these cultured conditions (data not shown), consistent with the non-toxic properties of DMK and DMS. These results further support the roles of ASS1 expression in conferring Arg- and Gln-sensitivity.

3.4. Sensitivity to Gln-deprivation can be modulated by ASS1 expression

We also used genetic approaches of modulating cellular ASS1 expression levels. First, we used two each of previously established *ASS1*-transfected cell lines in A375 and A2058 backgrounds, containing 5 to 10 copies of ectopic *ASS1* [30]. We found that these ASS1-overexpressing cells increased cell survival when cultured under both Arg-deprivation and Gln-deprivation conditions (Figs. 5A and 5B). Next, we performed ASS1 shRNA knockdown experiments in two $ASS1^{high}$-TNBC cell lines (MDA-MB-436 and MDA-MB-468). As shown in Fig. 5C, knockdown of ASS1 slightly increased HIF-1α expression levels. Strikingly, knockdown of ASS1 in these cells resulted in increased sensitivity to both Gln- and Arg-deprivation treatments (Fig. 5D). These results demonstrated that cellular sensitivity to Gln-deprivation treatment is also influenced by ASS1 expression levels.

3.5. Intervention of Gln metabolism alters cell sensitivity to Arg starvation

To further support the the link between Gln and Arg metabolism in terms of sensitivity to Gln- and Arg-starvation, we determined whether intervention of Gln metabolism would also alter cell sensitivity to Arg deprivation. While multiple pathways participate in the metabolic flux from Gln to Arg (Fig. 1, denoted by (i), (ii), and (iii)), however, all these pathways require the initial conversion of Gln to Glu by GLS (Fig. 1). GLS has two isoforms, GLS1 and GLS2, encoded by separate genes in humans. GLS1 is a kidney enzyme, whereas GLS2 is a liver-type enzyme [22, 43]. GLS1 is more closely relevant to Gln metabolism and its elevated expression is associated with Gln addiction in transformed cells [20, 30, 54]. Fig. 6A shows that knockdown of GLS1 in A2058 cells with two GLS1 shRNA resulted in downregulation of HIF-1α but upegulation of c-Myc and ASS1, much like cells treated with Gln-deprivation or ADI-PEG20. Reduced GLS1 expression enhanced cellular sensitivity to ADI-PEG20 (Fig. 6B). Similar results were obtained in WM-2664 cells (Figs. 6C and 6D). These results further support the cross-talk between Gln metabolism and Arg starvation sensitivity.

3.6. Reduced ASS1 expression show preferential sensitivity to anti-Gln drugs

Finally, we used two GLS1 inhibitors, DON and azerserine [9, 27, 35, 37, 51]. A2058, A375, BJ-1, and WM2664 cells were treated with 40 μM each of DON or azerserine for 48 hr. Figs. 7A and 7C show that these inhibitors downregulate HIF-1α in all cell lines except BJ-1, which expresses undetectable HIF-1α, and upregulation of ASS1 in A2058 and WM2664 cells by Western blots. BJ-1 and WM2664, which have higher expression levels of ASS1 than the other two $ASS1^{low}$ cell lines (A2058 and A375), were less sensitive to treatments by these inhibitors (Figs. 7B and 7D). These results demonstrated that Gln-inhibitors preferentially kill $ASS1^{low}$ cells. Taken together, our results demonstrate that reduced ASS1 expression levels serve as a common indicator for cell sensitivity to Arg- and Gln-depletion treatments.

4. Discussion

ASS1-silencing is a metabolic dysregulation responsible for Arg-auxotropy in many ongoing clinical investigations of Arg-starvation therapy. In the present study, we demonstrate that ASS1 is also a biosensor for Gln-deprivation stress and that its expression levels also affect sensitivity to Gln-depleting treatment, thus providing previously unrecognized finding of using Gln depleting strategies for targeting Arg-auxotrophic tumors. While ADI-PEG20 and peg-rhArg1 have shown activities in targeting Arg-auxotrophic tumors, however, ADI-PEG20 is a bacterial enzyme with allergic reactions and the induction of neutralizing antibodies is almost inevitable in patients [36, 56]. Peg-rhArg1 is a human enzyme that has reduced antigenicity, however, its catalytic activity for Arg depletion is substantially lower than that of ADI-PEG20 [14]. Thus, our current results provide preclinical evidence for targeting ASS1-negative cancers with Gln-starvation strategy, either alone or in combination with Arg-starvation.

We demonstrated that two anti-Gln drugs, DON and azaserine, exhibit preferential killing to $ASS1^{low}$ cells. Although clinical studies showed that these drugs have unwanted toxicities preventing them from clinical uses [1], several allosteric inhibitors of GLS1 have been developed in the recent years. These include bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) [41], compound 968 [52], and CB839 [21]. These GLS1 inhibitors have shown anti-proliferative activities in cultured cells and in animal tumor xenografts. CB839 has been in several clinical studies (*Clinicaltrials.gov*, NCT02071862). Other anti-Gln drugs such as inhibitors of Gln transporters and GDH [10] are also under clinical development. The identification of ASS1 as a biosensor of Gln-deprivation stress would facilitate the clinical development of anti-Gln approaches in treating a subset of human malignancies that require Arg for growth.

How can ASS1 serve as a common sensor for both Arg- and Gln-starvations? There are multiple pathways that communicate between Gln and Arg metabolism (Fig. 1), *i.e.,* (*i*) *via* α-KG intermediate which is the precursor of fumarate in the TCA cycle, and fumarate is then

converted to Arg by the reversible reaction catalyzed by ASL [59]; (*ii*) *via* the GOT transaminase pathway that produces aspartate, which is the co-substrate of ASS1 for the production of Arg; and (*iii*) *via* prroline-5-carboxylate (P5C) which is converted to ornithine by ornithine aminotransferase (OAT) and then to citrulline by ornithine transcarbomylase, and citrulline is the co-substrate of ASS1. It has been reported that proliferating cells consume more Gln than do senescent cells, but the first pathway which drives Glu into α-KG is not the rate-determining step in proliferating cells [34, 46], rather, by preferentially utilizing the GOT transaminase pathway (pathway ii) [11]. Another study using specific inhibitors also suggested that transaminase pathway is the major route through which Gln-derived carbon for entering the TCA cycle. Although Gln has been reported to be the main precursor for the biosynthesis of citrulline from which Arg is synthesized *via* ASS1 in the third (iii) pathway [8, 29], this remains controversial because reports also demonstrated that citrulline may originate from proline by proline oxidase (PO) [32, 47] (Fig. 1). Regardless, ASS1 is at the midpoint of the two transaminase pathways, this may explain how ASS1 can sense the availabilities of both Gln and Arg from opposite directions.

Mechanistically, previous reports showed that ASS1-silencing in Arg-auxotrophic tumors is regulated epigenetically by DNA methylation at the *ASS1* promoter [3, 26, 44, 45]. We demonstrated that HIF-1α is a suppressor for ASS1 expression by binding to the E-box at the *ASS1* promoter [48]. These results were also demonstrated in multiple cell lines here (Fig. 3F). We also demonstrated that upregulation of HIF-1α by ectopic DMF results in downregulation of ASS1, and that downregulation of HIF-1α by knockdown of GLS1 results in upregulation of ASS1. These results further support the negative role of HIF-1α in the regulation of ASS1. Mechanism by which Arg- and Gln-depletion induces HIF-1α downregulation remains to be investigated.

Arg-depletion induces HIF-1α degradation, allowing the positive E-box regulator such as c-Myc to turn on ASS1 expression [48]. This is also demonstrated here under Gln-deprivation

conditions by ChIP (Fig. 2F). c-Myc is known to regulate Gln catabolism by enhancing the expression of GLS1 [20, 53], Gln synthetase (GS) [7] and Gln transporters (SLC1A5 and SLC7A5) [13], rendering cancer cells addicted to Gln [5, 53]. Furthermore, knockdown of GLS1 which blocks anaplerosis also upregulates ASS1 *via* c-Myc upregulation, indicating that ASS1 can bi-directionally sense Gln- and Arg- status (Fig. 8). We previously demonstrated that the upstream signal of Arg starvation-induced c-Myc expression is regulated by a ROS-related signal involving the Gas6/Axl→Ras→PI3K/AKT→GSK3β cascade [50]. It is unclear whether this cascade is also involved in Gln-starvation-induced c-Myc. While upregulated c-Myc induces ASS1 expression but elevated ASS1 can reversely suppress c-Myc expression by a yet-to-be determined mechanism [50] (Fig. 8). Moreover, increased Arg resulting from overexpression of ASS1 may increase orinithine accumulation through the reaction catalyzed by arginase (Fig. 1) [28]. This may explain why ASS1-transfected cells exhibit increased resistance to Gln-deprivation treatment (Fig. 5). These may also explain why $ASS1^{high}$ cells are not sensitive to Arg- and Gln-deprivation treatments, and that Arg-auxotrophic cells are preferentially sensitive to Arg- and Gln-starvation.

We do not believe that the induction of rapid HIF-1α downregulation by Arg- and Gln-depletion is related to the general amino acid starvation response which triggers elF2α phosphorylation resulting in suppression of global protein synthesis. We found that induction of elf2α phosphorylation occurs about 20 hr after Arg-deprivation, much later than the time frame by which HIF-1α degradation occurs (t½ ~ 15 min) [48]. Likewise, downregulation of mTORC1 signaling under Arg-starvation occurs about 16 hr after the treatment, ruling out the possible involvement of mTORC1. However, recent reports demonstrated that cancer cells may use alternative pathways, such as enhanced nucleotide biosynthesis [46] and B55α-EDD-p53 signaling [40] in response to Gln-depleting conditions. Whether these pathways are also involved in the Arg-auxotrophic response remains to be investigated.

In conclusion, we have demonstrated ASS1 as a common sensor for Arg- and Gln-deprivation responses. We have also provided the mechanistic insights that HIF-1α/c-Myc axis is involved in these responses. Our results have important clinical implications for improving the treatment efficacy of Arg-auxotrophic tumors.

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Figure Legends

Figure 1. Pathways linking glutamine and arginine metabolism. Abbreviations: AS, argininosuccinate; ASS1, argininosuccinate synthetase 1; ASL, argininosuccinate lyase; GOT1, glutamic-oxaloacetic transaminase 1; FH, fumarate hydratase; GLS, glutaminase; GS, glutamine synthetase; GDH, glutamine dehydrogenase; NOS, nitric oxide synthetase; OAT, Ornithine aminotransferase; OTC, ornithine transcarbamylase; P5C, pyrroline 5-carboxylate; PO, Proline oxidase. (i) – (iii) refer to three pathways that link Gln to Arg.

Figure 2. **Expression of ASS1 and sensitivity of melanoma cells to Arg-deprivation and Gln-deprivation treatments**. (A) Western blots showing ASS1 expression in melanoma cell lines. (B) Sensitivity of cell lines to Arg-free cultured conditions, (C) sensitivity of cell lines to Gln-free cultured conditions; (D) expression of HIF-1α, c-Myc, ASS1, and tubulin in four cell lines to ADI-PEG20 (0.5 μg/ml) or (E) in Gln-free medium for the time intervals as indicated. (F) ChIP assays showing HIF-1α (upper panel) and c-Myc (bottom panel) bindings to the ASS1 promoter in A2058 cells treated with Arg- or Gln-free media as indicated for one hr. (G-I) Transient transfections of reporter recombinants containing wild-type ASS1 promoter (E-box) (pGL3-AS-85) or mutant E-box (mE-Box) in A2058, WM2664, and MDA-MB-231, respectively. (n = 2).

Figure 3. Expression of ASS1 and cellular sensitivity grown in Arg- and Gln-free media of 11 TNBC lines. (A) Western blots showing ASS1 and GLS1 expression. (B) Densitometry of ASS1 expression in cell lines, as correspondingly shown in (A). (C) Sensitivity of TNBC cells cultured in the regular medium and in Arg- or Gln-free media, (D and E) Expression of ASS1, HIF-1α, and c-Myc in 6 TNBC cells treated with ADI-PEG20 and Gln-free media, respectively.

(F) Response of HIF-1α, c-Myc, and ASS1 to Arg-depletion (Arg(-)) or Gln-depletion (Gln(-)) treatments in ASS1-low and ASS1-high cell lines as indicated. Data were derived from Western Blots shown in Figs. 2D, 2E, 3D, and 3E). " ↑ " refers to upregulation; " ↓ ", downregulation, and " - ", no response. (A, C, D and E) (n = 2, each).

Figure 4. **Effects of metabolites on expression of ASS1 and cellular sensitivity to various cultured conditions.** (A) Effects of DMF and NAC on expression of HIF-1α, c-Myc, and ASS1 in A2058 cells. (B) Effects of NAC, DMK and DMS on expression of HIF-1α, c-Myc, and ASS1. (C) Similar experiment as shown in (A) was performed in MDA-MB-231 cells. (D) Effects of DMS, DMK, and DMF (150 μM each) for 48 hr on the growth of A2058 cultured in the regular medium. (E) Similar experiments as shown in (D) were performed in A375 (Fig. 4E). (F & G) DMF induces additive cell killing in the presence of glutamine –free and arginine-free media in A2058 cells (F) and in A375 cells (G). " * ", significant (P <0.01, student's t-test); " * " significance (p<0.001, student's t-test). (A-B, D-G) (n = 2, each), (C) (n = 1).

Figure 5. Modulations of ASS1 expression on sensitivity to Gln- and Arg-free culture conditions. (A) Sensitivity of A2058 cells and their ASS1-transfected cells to Gln- and Arg-free-cultured conditions, (B) Similar as (A) except A375 cells were used; (C) Western blots showing expression of ASS1, HIF-1α, and c-Myc in two TNBC cell lines treated with *ASS1* siRNA, (D) Cellular sensitivity of two ASS1-knockdown TNBC cell lines to Gln- and Arg-free media (n = 2).

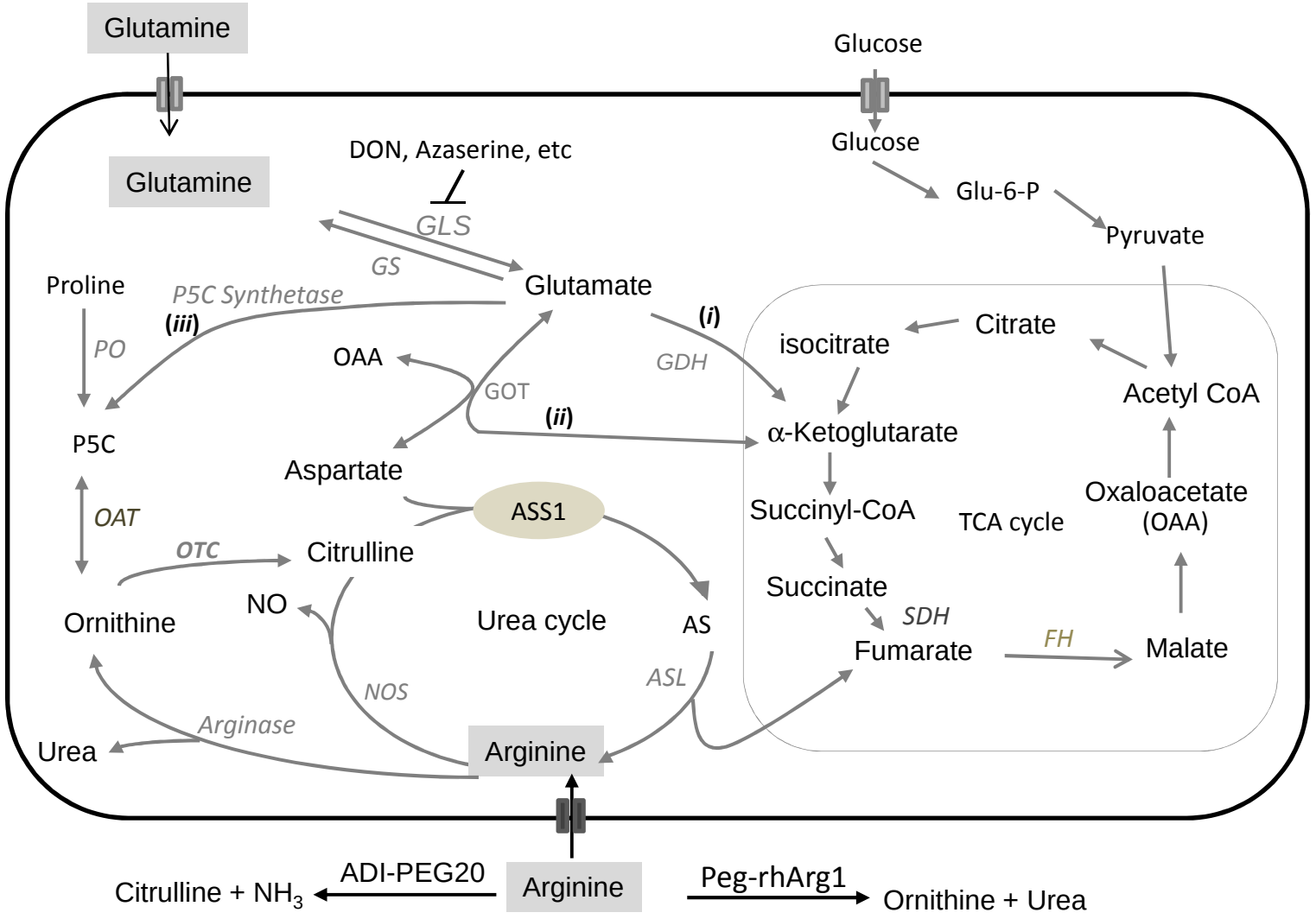
Figure 6. Effects of GLS1 knockdown on cellular sensitivity to ADI-PEG20. Western blots of protein expression in A2058 cells (A) and in WM2664 cells (C) transfected with two GLS1

shRNA, effects of GLS1 knockdown on the sensitivity of A2058 cells (B) and WM2664 cells (D) to different concentrations of ADI-PEG20 in 48 hr treatments. (A and C), (n = 2); (B and D) (n = 3).

Figure 7. Effects of the anti-Gln drugs, Don and azaserine, on protein expression and cellular sensitivity on four cell lines. (A) and (C), Western blots of HIF-1α, c-Myc, ASS1, and tubulin in DON- and azaserine-treated cells for 24 hr. (B) and (D), cellular sensitivities of four cell lines treated with Don and azaserine for 48 hr. (A and C) (n = 3), (B &D) (n= 2).

Figure 8. Schematic depicting the regulatory network among Gln, Arg, HIF-1α, c-Myc, and ASS1. Gln or Arg depletion induce upregulation of c-Myc (↑) and downregulation of HIF-1α (↓), resulting in upregulation of ASS1. Elevated ASS1 downregulates c-Myc and HIF-1α to maintain Arg homeostasis. Double headed arrows denote cross-talk linking Gln-ASS-Arg as described in the text.

Fig. 1


Glutamine
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OAA
GOT
(ii)
(i)
GDH
Citrate
isocitrate
Acetyl CoA
α-Ketoglutarate
Aspartate
ASS1
Succinyl-CoA
TCA cycle
Oxaloacetate
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OAT
OTC
Citrulline
Succinate
SDH
NO
Ornithine
Urea cycle
AS
Fumarate
FH
Malate
ASL
NOS
Arginase
Urea
Arginine
Citrulline + NH3
ADI-PEG20
Arginine
Peg-rhArg1
Ornithine + Urea

Fig. 2

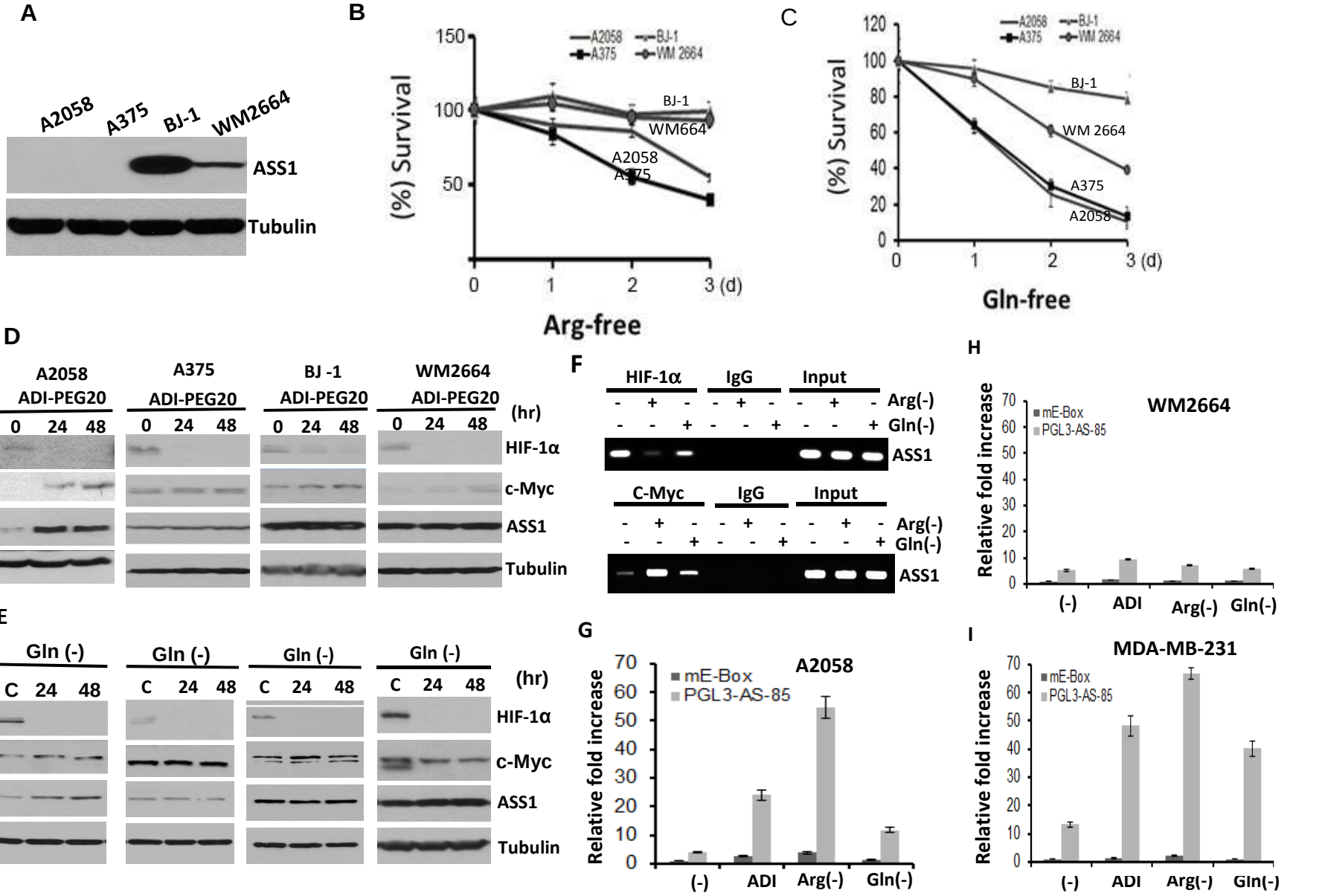
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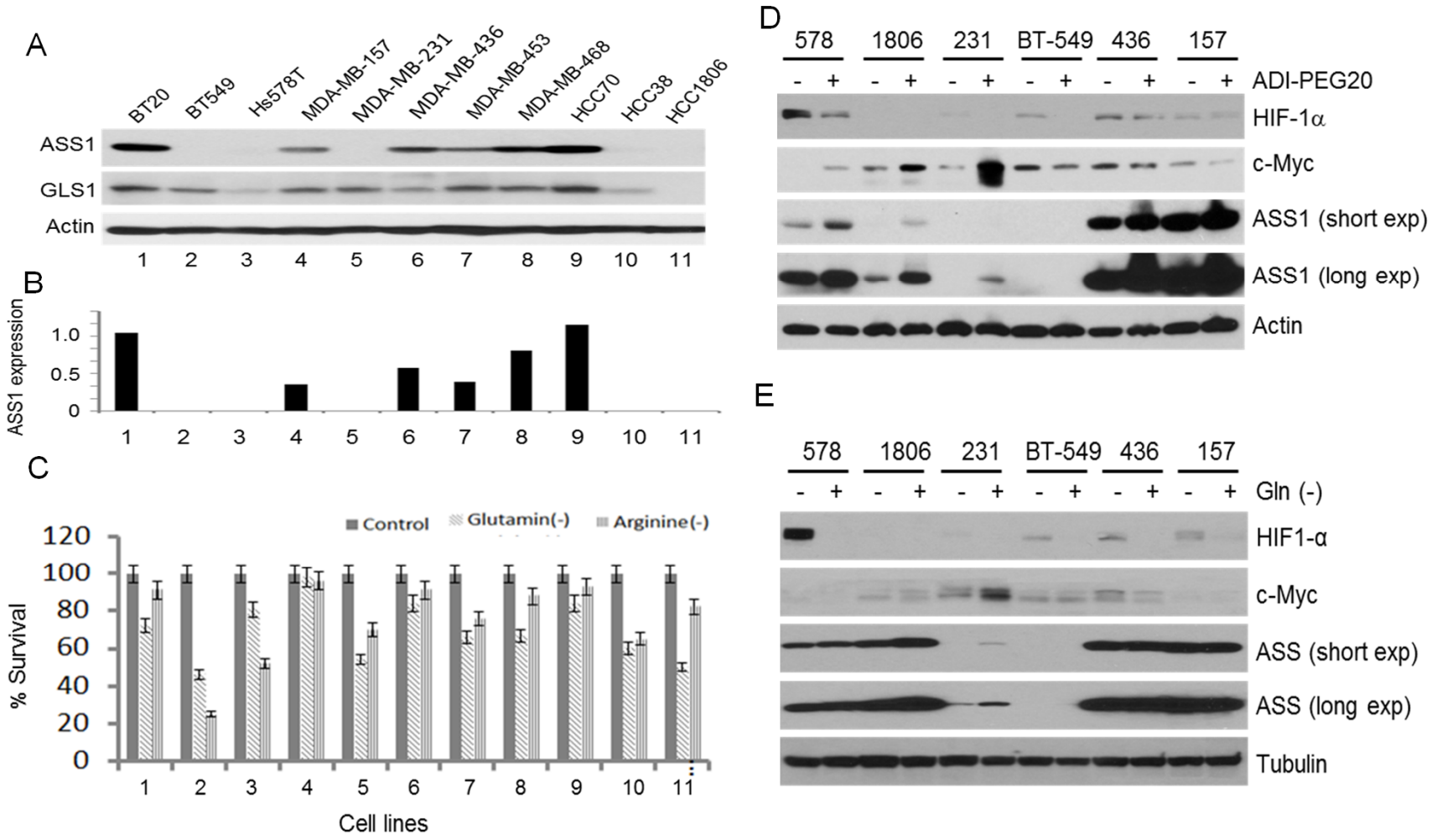
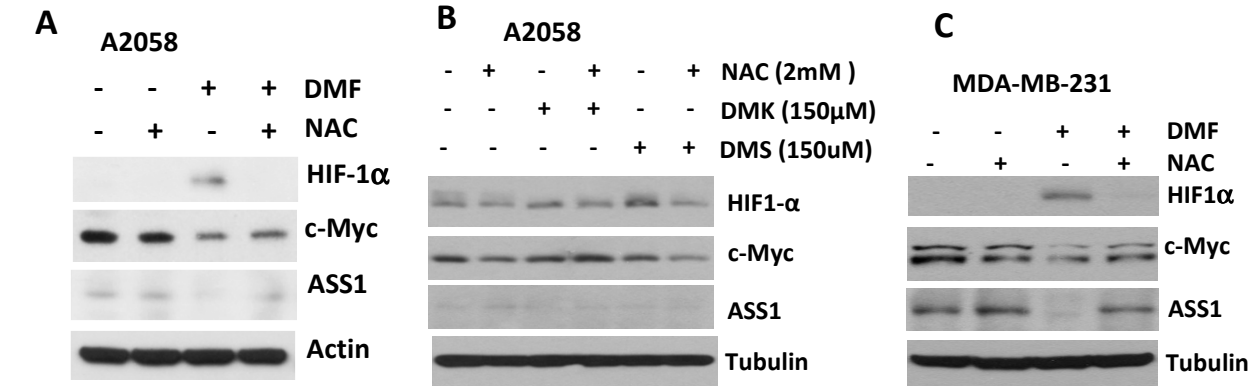
Fig. 3

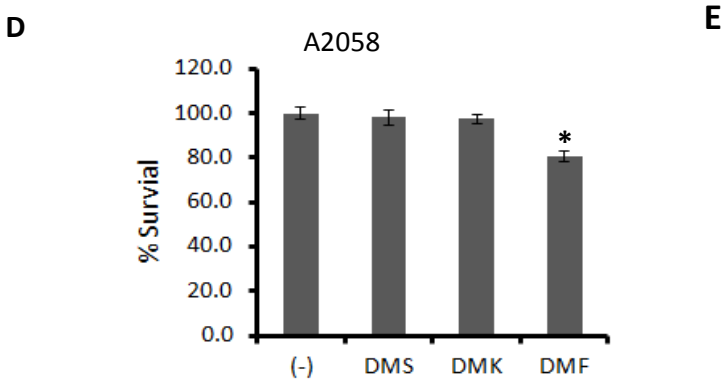


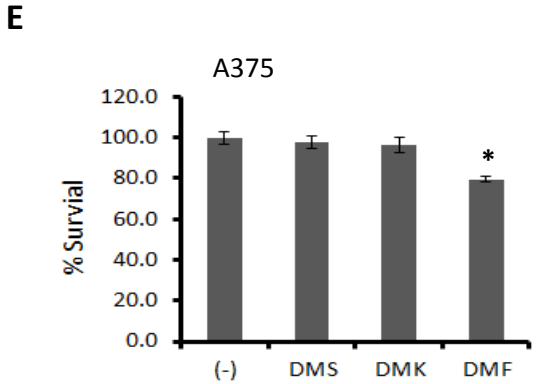
F

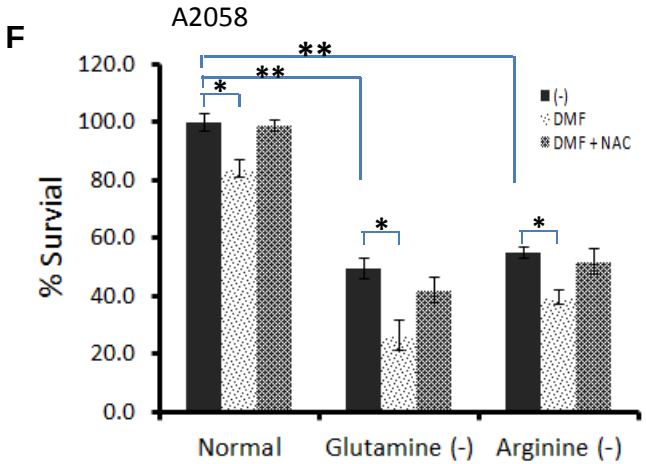
	ASS1-low cell lines				ASS1-high cell lines			
	A2058	Hs578T	1806	231	BJ-1	WM2664	MB-436	MB-157
	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)	Arg(-)/Gln(-/-)
HIF-1α	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓
c-Myc	↑/↑	↑/↑	↑/↑	↑/↑	↑/↑	↑/↓	↓/↓	↓/↓
ASS1	↑/↑	↑/↑	↑/↑	↑/↑	-/-	-/-	-/-	-/-

Fig. 4


A
A2058
- - + + DMF
- + - + NAC
HIF-1α
c-Myc
ASS1
Actin
B
A2058
- + - + - + NAC (2mM)
- - + + - - DMK (150μM)
- - - - + + DMS (150uM)
HIF1-α
c-Myc
ASS1
Tubulin
C
MDA-MB-231
- - + + DMF
- + - + NAC
HIF1α
c-Myc
ASS1
Tubulin


D
A2058
% Survial
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
(-)
DMS
DMK
DMF


E
A375
% Survial
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
(-)
DMS
DMK
DMF


F
A2058
% Survial
120.0
100.0
80.0
60.0
40.0
20.0
0.0
**
**
*
*
*
(-)
DMF
DMF + NAC
Normal
Glutamine (-)
Arginine (-)

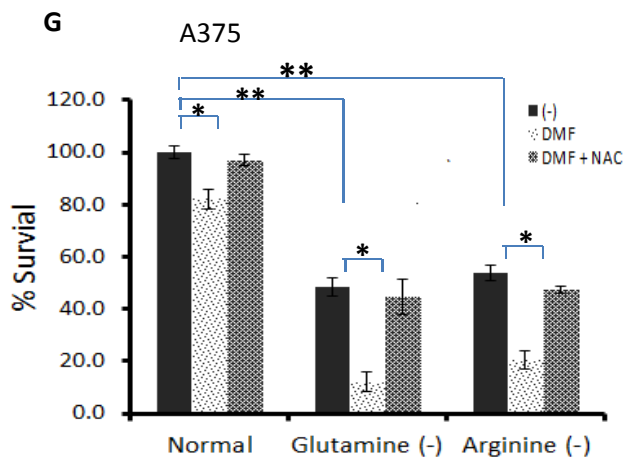

G
A375
% Survial
120.0
100.0
80.0
60.0
40.0
20.0
0.0
**
**
*
*
*
(-)
DMF
DMF + NAC
Normal
Glutamine (-)
Arginine (-)

Fig. 5

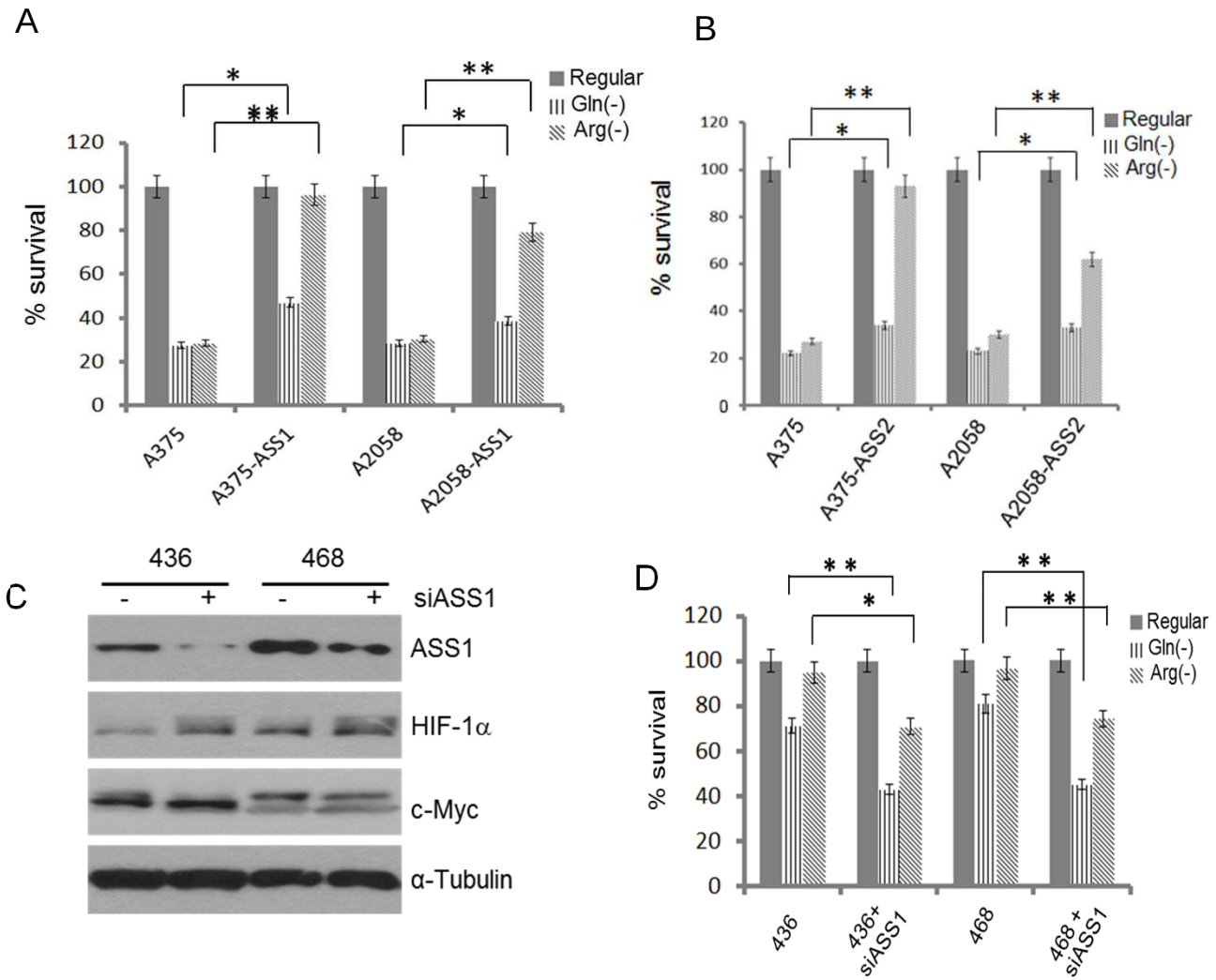

A
% survival
Regular
Gln(-)
Arg(-)
A375
A375-ASS1
A2058
A2058-ASS1
B
% survival
Regular
Gln(-)
Arg(-)
A375
A375-ASS2
A2058
A2058-ASS2
C
436
468
siASS1
ASS1
HIF-1α
c-Myc
α-Tubulin
D
% survival
Regular
Gln(-)
Arg(-)
436
436+ siASS1
468
468 + siASS1

Fig. 6

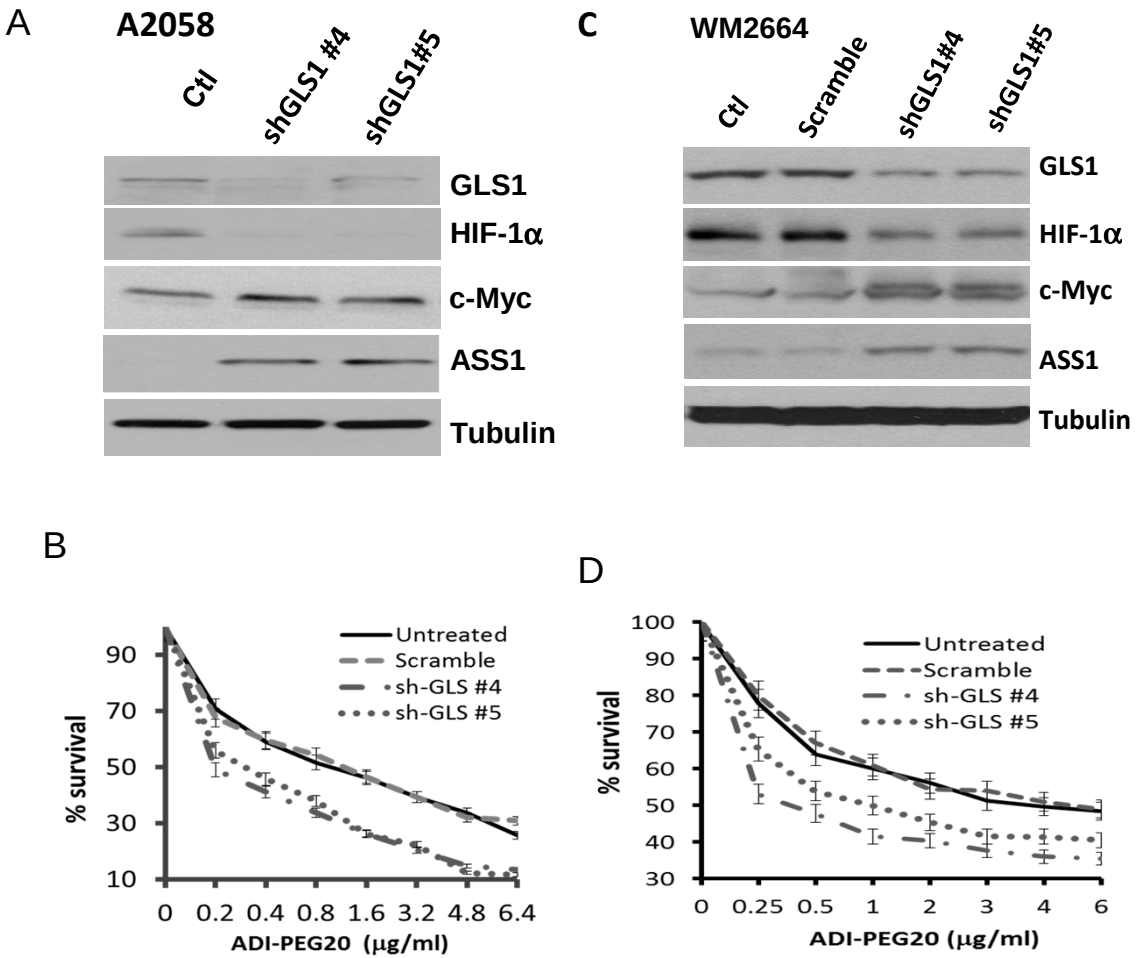
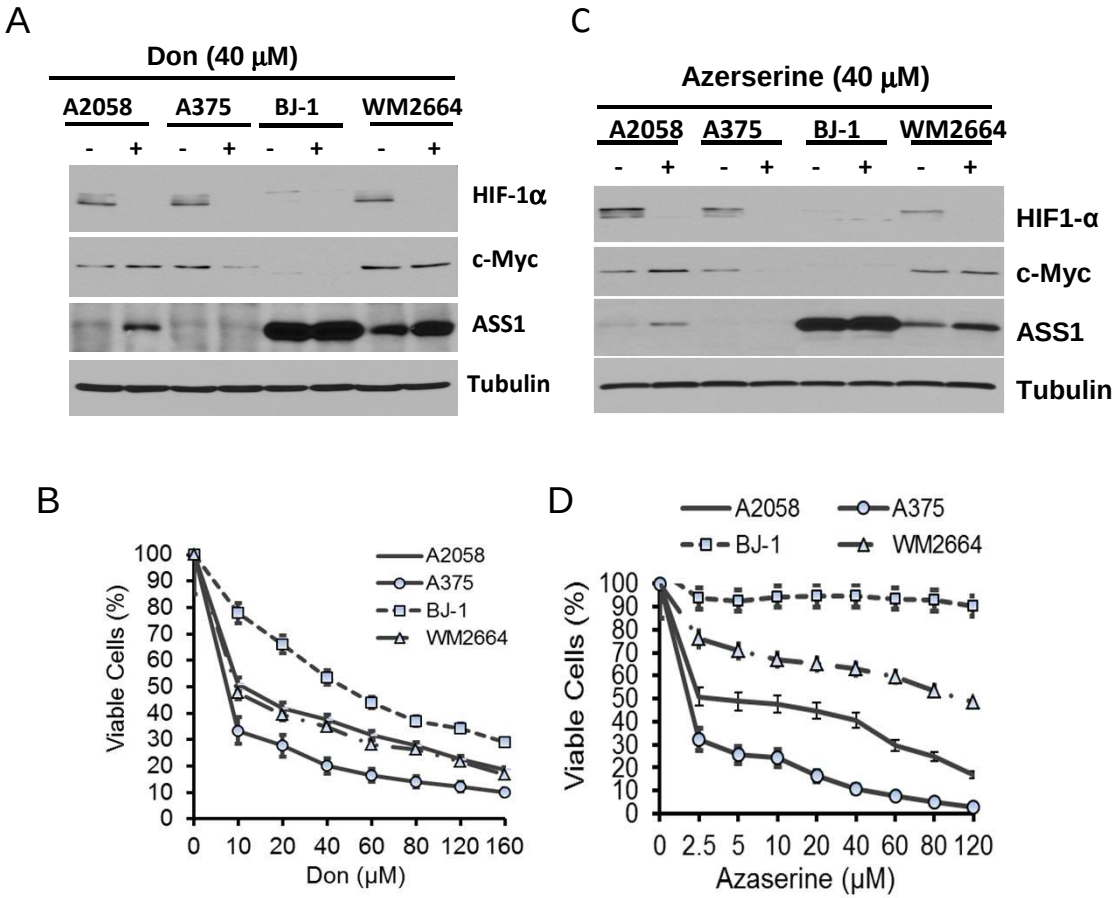

A
A2058
Ctl
shGLS1 #4
shGLS1#5
GLS1
HIF-1α
c-Myc
ASS1
Tubulin
C
WM2664
Ctl
Scramble
shGLS1#4
shGLS1#5
GLS1
HIF-1α
c-Myc
ASS1
Tubulin
B
% survival
Untreated
Scramble
sh-GLS #4
sh-GLS #5
ADI-PEG20 (μg/ml)
D
% survival
Untreated
Scramble
sh-GLS #4
sh-GLS #5
ADI-PEG20 (μg/ml)

Fig. 7


A
Don (40 μM)
A2058
A375
BJ-1
WM2664
- + - + - + - +
HIF-1α
c-Myc
ASS1
Tubulin
C
Azerserine (40 μM)
A2058
A375
BJ-1
WM2664
- + - + - + - +
HIF1-α
c-Myc
ASS1
Tubulin
B
Viable Cells (%)
100 90 80 70 60 50 40 30 20 10 0
A2058
A375
BJ-1
WM2664
0 10 20 40 60 80 120 160
Don (μM)
D
A2058
A375
BJ-1
WM2664
Viable Cells (%)
100 90 80 70 60 50 40 30 20 10 0
0 2.5 5 10 20 40 60 80 120
Azaserine (μM)

F

Fig. 8