Folate Deficiency Promotes the Selection of Bcr-Abl expressing B-cell Progenitors

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ABSTRACT

Dietary folate deficiency is associated with the functional decline of cells, tissues, and organs and the development of acute lymphoblastic leukemias in children. In mice models, leukemogenesis is initiated by Bcr-Abl oncogenic translocations promoted as a result of folate deficiency. Conventionally, cancer is thought to be the result of accumulations of oncogenic mutations over time. Through the application of the Adaptive Oncogenesis model we hypothesize that folate deficiency is likely to reduce the fitness of stem and progenitor cell populations. This reduction in fitness may lead to increase selection for oncogenic mutations that can partially alleviate folate deficiency fitness defects, thereby promoting the initiation of cancers. I tested this hypothesis by mimicking folate deficiency in B-progenitors by using methotrexate (MTX) to target DHFR (dihydrofolate reductase) an enzyme that converts dihydrofolate (i.e. DHF, a folic acid derivative) into tetrahydrofolate (THF) in the folate pathway. I found that *in vitro,* Bcr-Abl expressing B-progenitors offer partial protection in response to MTX. Furthermore, within a competitive environment, the decline in the fitness of folate deficient B-progenitors promote selection for Bcr-Abl expressing B-progenitors as a result of folate-dependent alterations of the fitness landscape. Expression of the Bcr-Abl oncogene provides a greater competitive advantage compared to control BaF3 Parentals and BaF3 vectors, but only in the presence of methotrexate. These studies establish the development of a cost effective, high-throughput *in vitro* system to assay for various required components that control folate metabolism and nucleotide synthesis in B-progenitors. Future research will focus on figuring out the mechanisms by which Bcr-Abl B-progenitors are advantageous in a folate deficient environment.

INTRODUCTION

Maintaining a healthy diet is important for the prevention of cancer. Lack of maintenance through the consumption of excessive alcohol, fats, salts, charred and cured foods are potentially capable of aggravating onocogenesis later in life (Dreosti 1998; Crott et al. 2002 and Kim et al. 1996). Dietary factors represented by antioxidants, fiber and calcium and are potentially capable of reducing the risk of cancer incidence (Dreosti 1998; Jacobs 1993 and Kim et al. 1999). For instance, increased levels of calcium can offer protection against colon cancers by possible precipitation of carcinogenic secondary bile and colonic epithelium proliferation suppression (Crott et al. 2002; Dreosti 1998 and Giovanucci et al. 1993). Dietary deficiencies created by the consumption of cancer-causing dietary factors and lack of consumption of protective dietary factors can increase cancer incidence (Doll 1992, Key et al. 2004; Mullick et al 2004 and Park et al. 2012).

A strong correlation exists between folate dietary deficiencies and the risk of developing colon (Giovanucci et al. 1993), cervical dysplasia, lung (Jang et al. 2005), pancreatic, esophageal, stomach and breast cancer (Kim 1999 and Mason 1994). Folate is also a protective dietary factor against the risk of developing childhood leukemia (Mori et al. 2002 and Wagner 1995). (ALL) Acute lymphoblastic leukemia, characterized by a B-cell precursor phenotype and gross chromosomal abnormalities, several of which have been shown to originate *in utero,* are thought to be the result of folate deficiency (Mori et al. 2002 and Lightfoot et al. 2010). The role folate deficiency plays in enhancing onocogenesis has not yet been determined. However, understanding the mechanisms behind the biochemical pathway of folate uptake within the body is important for elucidating the effects of dietary folate deficiency (Jang et al. 2005) (Figure 1). Folate is necessary for 1-carbon metabolism, a series of 1-carbon transfers responsible for

nucleotide synthesis and biological methylation (Jang et al. 2005 and Choi et al. 2002) (Figure 2). Folate metabolism is compartmentalized within the cytosol and mitochondria (Choi et al. 2002; Gonen et al. 2012 and Tibbetts et al. 2010) (Figure 3). In the cytosol, folate is an important co-factor for DNA synthesis, integrity and repair by serving as a coenzyme for purine synthesis (Jang et al. 2005 and Tibbetts et al. 2010). In the mitochondria, folate is used for the biosynthesis of formate, which in turn is necessary for cytoplasmic one-carbon reactions and mitochondrial glycine biosynthesis (Jang et al. 2005).

In vitro folate deficiency is studied via inhibition of the folate pathway through the use of anti-folates. Methotrexate (MTX), a common antifolate, was developed in 1948. However, MTX is also an anti-cancer drug used to treat acute leukemia, non-Hodgkin's lymphoma, breast carcinoma, and rheumatoid arthritis (Bleyer 1978). MTX is used as an anti-cancer drug due to the inhibition effects on dihydrofolate reductase (DHRF) which reduces cellular proliferation and consequently, results in the cellular death of cancerous cells (Bleyer 1978). In fact, low oral doses of MTX given to patients with metastatic breast cancer have demonstrated antitumor activity (Colleoni et al. 2002). On the other hand, there are documented cases that suggest patients treated with MTX for rheumatoid arthritis demonstrated increases in melanomas (Buchbinder et al. 2008). The incidence of malignancies in patients treated with MTX are thought to be the result of natural resistance or acquired resistance (e.g. reduced uptake, enhancing efflux, alterations in membrane lipids) of certain tumorous and normal cells to MTX (Bleyer 1978 and Gottesman 2002). One example of acquired resistance for MTX has been attributed to the synthesis of more dihydrofolate reductase to overcome the effects of MTX (Hryniuk and Bertino; 1969; Bleyer 1978 and Hill and Schimke 1985). For example, methotrexate resistant cancerous cell lines are associated with an increase in the number of

dihydrofolate reductase gene copies and thus, a higher level of DHFR (Alt et al. 1977 and Sager 1985). However, the use of MTX as an anti-cancer drug depends largely on stopping the cancerous cells from proliferating by inhibiting one of the many pathways cancers use to expand their numbers. Yet, treatment is not always effective because resistance, lack of cell site specific targeting, dose and duration of treatment have not necessarily been optimized for all cancers. Additionally, for the purpose of this experiment MTX was used because of the inhibiting effect on DHFR in the folate pathway.

Dietary folate is converted by the liver into dihydrofolate (DHF) which is then converted into tetrahydrofolate (THF). DHFR is necessary for the conversion of DHF into THF, a methyl group shuttle required for the de novo synthesis of purines (Figure 3). Folate deficiency can affect epigenetic phenomena such as uracil misincorporation, DNA methylation, protein methylation, mitochondrial DNA deletion and critical gene expression such as hypomethylation within the tumor suppressor gene p53 (Jang et al. 2005 and Kim et al. 1997) (Figure 4). Conventionally, the correlation between folate deficiency and cancer incidence has been primarily attributed to the mounting mutational load and epigenetic changes over time, which eventually may give rise to oncogenic mutations leading to cancer (Degregori 2012; Hanahan et al. 2010 and Kim et al. 1996) (Figure 5). But we find this hypothesis unlikely, and instead propose the Adaptive Oncogenesis Model.

The Adaptive Oncogenesis model is based upon Darwinian principals and proposes that folate deficiency alters the adaptive landscape by impinging on cellular proliferation, survival, metabolism, thereby reducing the fitness of stem cell progenitor pools within their niches (DeGregori 2012 and Henry et al. 2011) (Box of Definitions). Thus, cells harboring advantageous mutations which can overcome fitness defects will be selected for and dominate

the niche (DeGregori 2012 and Henry et al. 2011) (Figure 6). Therefore, in the context of folate deficiency, selection for cells with oncogenic mutations that overcome folate deficiency may occur (Figure 6).

The association between acute lymphoblastic leukemia, a cancer of the blood, and folate deficiency is not well understood. However, the chromosomal translocation between chromosome 9 and 22 that generate oncogenic fusions genes such as Bcr-Abl are known to occur in more committed progenitors of hematopoietic stem cells (HSCs), namely, B cell progenitors (impaired and normal) (Bilousova et al. 2005 and Passegue et al. 2003). Hematopoietic stem cells reside in the bone marrow and give rise to lymphocytes and other blood cells. Hematopoietic stem cells will give rise to the common lymphoid progenitor, which will, in turn, give rise to B-progenitors. The cell line I studied was a B-progenitor line named BaF3. BaF3 cells are immortal which means they can divide indefinitely *in vitro* and are not transformed which means they maintain their ability for contact inhibition of growth. Furthermore, they are IL-3 dependent and thus able to recapitulate the normal cytokine dependence of hematopoietic progenitors.

Bcr-Abl is formed from a head to tail translocation fusion of the Abelson murine leukemia oncogene homolog 1 found on chromosome 9 with the breakpoint cluster region protein (BCR)/renal carcinoma antigen NY-REN-2 on chromosome 22 (Lugo et al. 1990). Bcr-Abl encodes for a cytoplasmic and nuclear protein tyrosine kinase involved in processes of cell differentiation, cell division, cell adhesion and stress response (Lugo et al. 1990). Furthermore, tyrosine kinase allows cells to proliferate without being regulated by cytokines; consequently, unregulated proliferation of cells can contribute to tumorigenesis (Lugo et al. 1990). Bcr-Abl is an oncogene of interest because the Bcr-Abl gene is found within chronic myelogenous

leukemias (CML) and acute lymphoblastic leukemias (ALL) (Bose et al. 1998). Further research into the mechanism by which Bcr-Abl leads to leukemia is needed, especially, as an association exists between folate deficiency and the Bcr-Abl gene fusion that leads to leukemia.

The second oncogene I studied was Myc. The proto-oncogene Myc is normally found on chromosome 8 but a translocation between chromosomes 8 and 14 causes Myc to become constitutively expressed (Grandori et. al. 2000). Constitutive expression of the regulatory gene Myc causes the upregulation of the genes that Myc regulates. MYC is be found in several cancers and the upregulation of the genes Myc regulates result in increased cell proliferation, growth, apoptosis, tumorigenesis and inhibited differentiation (Grandori et al. 2000). Myc served as the only other oncogene due to the limited time frame of the project.

Based on the Adaptive Onocogenesis theory, an organismal insult such as folate deficiency should reduce the fitness of normal cells leading to an increase in selection for more fit oncogenic clones such as those expressing Bcr-abl or Myc (Henry et al. 2011 and Marusyk et al. 2008). Previous experiments have determined that B-cell progenitors isolated from mice on folate deficient diets have severe metabolic defects from decreased nucleotide synthesis indicating a decline in cellular fitness. More importantly, the selection for Bcr-Abl expressing B-progenitors in folate deficient backgrounds significantly increases *in vivo* (Henry et. al, unpublished data). This selection is thought to occur as a result of a dramatic competitive advantage of Bcr-Abl B-progenitor cells over normal cells in a folate deficient environment. The selection for Bcr-Abl may represent the ability of Bcr-Abl to regulate defective metabolisms induced by a folate deficient environment (Henry et. al, unpublished data). However, observation of selection has yet to be observed *in vitro* and determination of these mechanisms has yet to

occur *in vivo* or *in vitro*. Thus, this project focuses on whether folate deficiency selects for Bcr-Abl expressing B-progenitors *in vitro* using cultured BaF cells.

Folate deficiency can be mimicked *in vitro* by using drugs that target the folate pathway or starving the cell medium for folic acid. However, cultured cell are highly addicted to the high levels of folic acid in the medium so that starvation prevents an accurate analysis of the results of folate deficiency. Furthermore, little is known about the dNTP (i.e. nucleotide) metabolism and the multiple allosteric mechanisms involved in this metabolism and so by starving cells of folic acid we can't be confident starvation is the variable which is inducing apoptosis, but with MTX use we can be confident MTX is the factor inhibiting the folate pathway and subsequently affecting dNTP metabolism. Another problem is that the cultures cells are unable to reduce folic acid to (THF), like the human liver, so that establishing a control becomes difficult. Mimicking folate deficiency in cell culture is challenging; however, others have begun to study folic acid supplementation *in vitro*. The use of MTX as a way to mimic folate deficiency *in vitro* provides an opportunity to demonstrate the effect of MTX on the cells because they are dose dependent (i.e. cell cycle arrest is illustrated at lower doses and apoptosis at the higher doses) and allows us to be convinced that MTX is inhibiting the folate pathway.

For this experiment folate deficiency mimicking conditions were simulated via inhibition of DHFR in folate pathway with methotrexate in B-progenitors. Folate deficiency in Baf3 cells might increase cellular apoptosis by preventing cells from synthesizing purine and pyrimidine for DNA synthesis. Cell viability can be measured using a Propidium Iodide (PI) exclusion, which stains dead cells by intercalating within DNA. A lack of nucleotides, including adenosine for ATP may lead to a decrease in metabolic efficiency which can be measured using internal ATP levels. A combination of PI Exclusion, Competition assays and ATP levels in cells will be used

to determine how Bcr-Abl and Myc expression in B-cell progenitors may overcome specific fitness defects of an inhibited folate pathway. These studies were designed to improve our understanding of how the expression of an oncogene in B-progenitors circumvents or overcomes fitness defects in B-progenitors in a folate deficient environment. Studies of folate deficiency in B-progenitors enable identification of pathways or molecules that are adversely affected as a result of folate deficiency *in vitro* prior to determining their *in vivo* relevance. Essentially, the role of folate in B-progenitor fitness was studied by developing a cost effective, high-throughput *in vitro* system to assay for various components required for efficient B-lymphopoiesis.

METHODS

In Vitro B-progenitor cultures

Cell lines used include Ba/F3 cells, BaF3 Vector including Ba/F3 expressing the human protein BCR-ABL^{p210} (Ba/F3-p210) and BaF3 Myc. Ba/F3 is a murine interleukin-3 dependent bone marrow derived pro-B cell line. Ba/F3 vector cells are indistinguishable from the parental Ba/F3 cells except for an inserted viral vector with junk DNA and express GFP. GFP expressing p210BCR/ABL cells were derived from the IL-3-dependent murine hematopoietic cell line, Ba/F3, which was transected with a vector containing p210BCR/ABL cDNA and is known to cause chronic myeloid leukemia. GFP expressing Ba/F3 Myc cells were derived from a Ba/F3 cells transected with a vector containing Myc cDNA. Cells were maintained in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (HI-FBS), WEHI murine IL-3 (R and D Systems, Minneapolis, MN) and penicillin-streptomycin (P/S) on petri dishes at 37°C incubator and split every one to two days based on cell density.

Methotrexate

Cells were exposed to Methotrexate (MTX) (Sigma-ALDRICH Catalogue No. M9929) from a range of 0.004 ug/ml - 50 ug/ml and kept at 10 mg/ml stock in dimethyl sulfoxide.

PI Exclusion

Prior to plating, viable cell counting was performed by enumerating cells which exclude trypan blue dye using a hemocytometer. 1 x 10^6 cells were plated on a 12 well suspension culture plate sterile with lid at 37ºC for 24 hours. A range of MTX concentration from 0.004 ug/ml to 50.0 ug/ml MTX was used with a no drug control (Greiner-Bio-One CellStar Monroe, NC). After exposure to MTX, cells were washed with 1X PBS and stained with 100x Propidium Iodide (PI)

on 96-well round bottom plates. Cell Cycle analysis and live cell counts were conducted on Guava easy Cyte 8HT Benchtop Flow Cytometer (EMD Millipore Corporation, Billerica, MA).

Competition Assay

4.5 x 10^5 Ba/F3 Cells were plated with 5 x 10^4 cells (Ba/F3 Bcr-Abl or Ba/F3 Myc or Ba/F3 Vector) for 24 and 48 hours at both 0.02 ug/ml MTX and 0.002 ug/ml MTX concentration, along with a no drug control on a sterile 12 well suspension culture plate (Greiner-Bio-One CellStar Monroe, NC). After exposure to MTX, cells were washed with 1X PBS and stained with 100x PI on 96-well round bottom plates. Viable cell cycle analysis was conducted on the Guava easy Cyte 8HT Benchtop Flow Cytometer.

ATP assay

CellTiter-Glo® Assay was performed for 24 and 48 hour drug treatments, 5 x 10^4 cells cells were plated in replicates of three into 96-walled multiwell plates with control well plates containing medium without cells to obtain a value for background luminescence. Cells were incubated with CellTiter-Glo assay reagent (Promega Madison, WI) for 10 min and luminescence was measured using a microplate Luminometer.

Statistical Analysis

Statistical analyses were performed with using Prism 5 software from GraphPad. All error shown is Standard Error of the Mean (SEM). Statistical analyses were performed using two-tailed Student's t-test. Significance was established for p values < 0.05.

RESULTS

Bcr-Abl provides partial protection in the presence of methotrexate. To establish an *in vitro* system to delineate the role of folate deficiency on B-progenitor fitness, we first began by running dose response experiments via PI exclusion to determine cell viability. Literature values for methotrexate dose are stated at 1 ug/ml, but B-progenitors are sensitive to much lower doses (Nielsen et al. 2007). After several experiments, 0.02 ug/ml was a dose at which the effects of oncogenes on drug sensitivity and B-progenitors themselves (with and without oncogene expression) could be determined experimentally. We predicted that BaF3 cells, BaF3 Vectors, BaF3 Bcr-Abl and BaF3 Myc will experience a decline in proliferation in response to the drug. According to our hypothesis, the extent of proliferation decline is a measure for how MTX impacts the fitness of the different cell lines. All cell lines will experience a negative decline, but differences in the negative decline could indicate selection for Bcr-Abl or Myc cells. As hypothesized, the decline in proliferation was seen in all cell lines in response to the drug (Figure 7). It is likely that methotrexate affects the cell cycle because MTX is known to inhibit the folate pathway via DHFR, which affects purine and pyrimidine synthesis (e.g. folate is critical for making TTP via thymidylate synthase). Lack of nucleotide synthesis would prevent cells from proliferating and could lead to apoptosis. Furthermore, as we predicted, BaF3 Bcr-Abl shows a lower fold decrease in cell numbers than the controls (e.g. parental BaF3 and BaF3 vectors), indicating that the Bcr-Abl mutation is evolutionary advantages under folate deficiency conditions *in vitro* (Figure 8). Myc shows a lower fold change than parentals BaF3s, which is a slight indication that Myc expressing B-progenitors may also increase cellular fitness by providing partial protection from an MTX-induced folate deficiency (Figure 8). Importantly, this experiment shows that folate deficiency's effects on B-cell progenitors and the selective

advantage of cells that express Bcr-Abl can be observed in the less complex context of *in vitro* culture.

Fitness of Bcr-Abl B-progenitors is maintained in a folate pathway inhibited background. A competition assay was conducted to determine whether Bcr-Abl and Myc expressing B-progenitors are adaptive in a folate inhibited background. At intervals of 24 and 48 hours, within a competitive environment at a 10:1 ratio of BaF3 Parentals to BaF3 Bcr-Abl using 0.02 ug/ml and 0.002 ug/ml of methotrexate, BaF3 Bcr-Abl is adaptive (Figure 9A: not shown for 24 hours). The GFP positive and GFP negative populations (e.g. Bcr-Abl and BaF3 Parentals within one culture; respectively) are not statistically different from one another given that methotrexate is added to the environment (Figure 9A). Contrasting to the no drug environment the two populations are statistically different (Figure 9A). This trend is further indicated by a fold change (e.g. the number of Bcr-Abl expressing BaF3 relative to parental BaF3) that is lower for the drug environment verses the no drug environment (Figure 9B). Consequently, this indicates Bcr-Abl is selected for within a folate deficient environment, but not in an environment where the effects of the methotrexate are not felt a relatively healthy environment as hypothesized previously. BaF3 Vector did not provide a selective advantage compared to BaF3 cells in the presence of MTX (Figure 10A). BaF3 vector expressing B-progenitors were unable to surpass original 1:10 plated ratios (e.g. BaF3 vectors to Parentals BaF3), as indicated by the significant statistical difference between GFP positive percent to parental GFP negative percent (Figure 10A). This conclusion is further supported by a fold change (e.g. the number of BaF3 vectors relative to parental BaF3) that did not differ between the no MTX population and the MTX population (Figure 10B). Accordingly, this indicates BaF3 vector provides is not selected for within a folate deficient environment and a “healthy” environment (e.g. no methotrexate) when competing

against parental BaF3. Furthermore, Myc expressing B-progenitors are not adaptive in a folate deficient environment compared to BaF3 parentals (Figure 11A). For example, analysis of GFP percent expression illustrates that like BaF3 vector BaF3 Myc is unable to surpass original 1:10 plated ratios representation as determined by the significant p-value demonstrating a difference in GFP percent between BaF3 Myc and parental BaF3 (Figure 11A.) This conclusion is further supported by a fold change (e.g. the number of BaF3 Myc relative to parental BaF3) that is similar with and without methotrexate (Figure 11B). BaF3 Myc provides no selective advantage to Parental BaF3, in contrast to, BaF3 Bcr-Abl (Figure 11).

Bcr-Abl maintains ATP synthesis in a folate pathway inhibited background. Given that Bcr-Abl is advantageous in a folate pathway inhibited background, we asked whether oncogene expressing cells would lead to maintenance of ATP synthesis in the presence of the drug. Using luminescence as an indicator of ATP synthesis, Bcr-Abl and Myc showed higher levels of ATP in response to methotrexate compared to BaF3 parentals and BaF3 Vectors both of which decreased in ATP synthesis activity over 24 hours (Figure 12). However, based on statistical analysis only BaF3 Myc showed a statistical difference in ATP levels. Importantly, this supports the previous findings that BaF3 Bcr-Abl expressing B-progenitors are adaptive in an inhibited folate pathway background. Myc expressing B-progenitors may be advantageous compared to our controls, but the restoration of nucleotide synthesis appears insufficient. Indeed, while ATP levels are maintained, MTX still effectively inhibits the proliferation of Bcr-Abl and Myc expressing cells, indicating that the drug effects are far from fully overcome. The ATP assay only investigates one nucleotide and more general effects of MTX on metabolism still need to be determined.

DISCUSSION

Data presented here demonstrates that in an inhibited folate pathway environment, Bcr-Abl expressing B-progenitors are at a competitive advantage compared to control BaF3 parentals and vector expressing cells. An explanation for this increased selective advantage as determined via the competition assay is that folate deficiency results in reduced purine synthesis in B-progenitors. This in turn provides selective pressure for the expression of oncogenes like Bcr-Abl, which may use their ability to restore receptor-mediated kinase signaling via an unknown pathway to circumvent the effects of folate deficiency. For example, signaling downstream of Bcr-Abl should lead to increased expression of numerous genes important for nucleotide synthesis, providing partial protection from dietary or drug induced inhibition of nucleotide synthesis. Nonetheless, the folate deficiency phenotype is complex. Folate deficiency-associated fitness reductions in B progenitors as well as mechanisms for adaptation conferred by Bcr-Abl expression and Myc expression involve other factors besides signaling and require further experiments.

The inhibited folate pathway *in vitro* reduces the fitness pool of progenitors, resulting in selection for oncogene expressing B-progenitors populations; thus, increasing the frequency at which these oncogene expressing cell are adaptive within the folate deficient environment. Similarly *in vivo,* in an animal subjected to long-term folate deficiency; increased genetic diversity due to an altered fitness landscape could contribute to oncogenesis. An altered fitness landscape includes increasing the frequency of oncogenic events and/or decreasing progenitor fitness (the latter consistent with the Adaptive Oncogenesis Model). As a result, folate deficiency alters the fitness landscape, providing contexts conducive for selection *in vitro.*

Notably, not all oncogenically initiated cells will be equally competitive in a folate inhibited pathway background as evidenced by Bcr-Abl and Myc expressing B-progenitors in the competition experiments. The Bcr-Abl oncogene proved to be advantageous in a folate inhibited pathway background and adaptive onocogenesis is supported in the context of this oncogene. Further tests are needed to make a conclusive argument. However, the data suggest that Bcr-Abl expressing B-progenitors are more advantageous than Myc expressing B-progenitors. An explanation for this would be that Bcr-Abl expressing cells alleviate the defects of a folate deficient environment by controlling more of the upstream signaling pathways than Myc expressing B-progenitors. Consequently, Bcr-Abl expressing cells are able to circumvent the effects of folate deficiency to a greater degree and are selected for because they are adaptive. While Myc expressing B-progenitors are only able to circumvent the effects of folate deficiency through the use of the downstream signaling and are advantageous only to a lesser extent than Bcr-Abl expressing B-progenitors.

Most importantly, our data suggest that without changing the adaptive landscape via folate deficiency there are no contexts conductive for somatic evolution and thus no selection for the oncogenic expressing cells. Oncogenic expressing cells are not advantageous in a non-inhibited folate environment as shown by the statistically significant difference in GFP positive percentage and GFP negative percentage in the no drug population; for example, Bcr-Abl is not selected for in the absence of methotrexate, but further experiments with other oncogenic cell lines are needed (Figure 7-12). Consequently, this is consistent with the idea that oncogenic mutations need to provide a positive fitness gain (relative to other cells within the niche) in order to be fixed and trigger clonal expansion (DeGregori 2011; DeGregori 2012 and Hanahan et al. 2000). Lack of clonal expansion by Bcr-Abl expressing cells within the no drug competitive

environment is best explained as a result of highly effective competition among healthy young BaF3 parentals serving to prevent somatic evolution. Furthermore, this mechanism behaves as a tumor suppressor preserving the environment in order to maximize fitness for the reproductive success of the parental BaF3 over Bcr-Abl expressing B-progenitors (DeGregori 2011; DeGregori 2012 and Marusyk et al. 2008).

Stem populations are highly adapted to their niches in an unchanged "healthy" adaptive landscape, minimizing selective pressure for adaptive changes (Henry et al. 2010 and Marusyk et al. 2008). Cellular competition in a healthy adaptive landscape appears to be oncogene-suppressive as evidenced in the competition experiment with the difference in cell number of BaF3 normal cells to BaF3 Myc in the no drug population (Figure 7-12). When cellular fitness is reduced as a result of alterations in the microenvironment, however, a progenitor cell population will no longer possess maximal fitness and certain oncogenic mutations will have an increased chance of being adaptive and hence advantageous (Marusyk et al. 2008). Results presented here provide support for this model. We propose that in a healthy adaptive landscape BaF3 B-progenitors are optimally fit with maintenance of appropriate signaling via IL-3. In this context, the activation of downstream effectors by Bcr-Abl and Myc expression will lead to excessive signaling beyond the levels needed for optimal fitness. In a folate deficient background, however, receptor signaling and/or other mechanisms are deficient. Thus, Bcr-Abl is adaptive, in part, by the ability of Bcr-Abl to increase the expression of genes involved in nucleotide metabolism, via activation of transcription factors like E2F which help to prevent apoptosis and cell cycle arrest (DeGregori et al. 1997 and Parada et al. 2001). Fitness reductions in folate deficient progenitor pools create an environment of selection. The fitness of a cell becomes the critical criteria for survival. In the short term oncogenically expressing cells alleviate folate deficiency defects.

However, in the long term selection for oncogenically expressing cells threaten normal cell populations by multiplying at an extensive rate eventually outcompeting normal cells. Although there are many aspects which contribute to increasing cancer incidence, data presented here implicate oncogenic adaptation within a changed adaptive landscape in the context of folate deficiency and support the link between folate deficiency and onocogenesis.

FIGURES

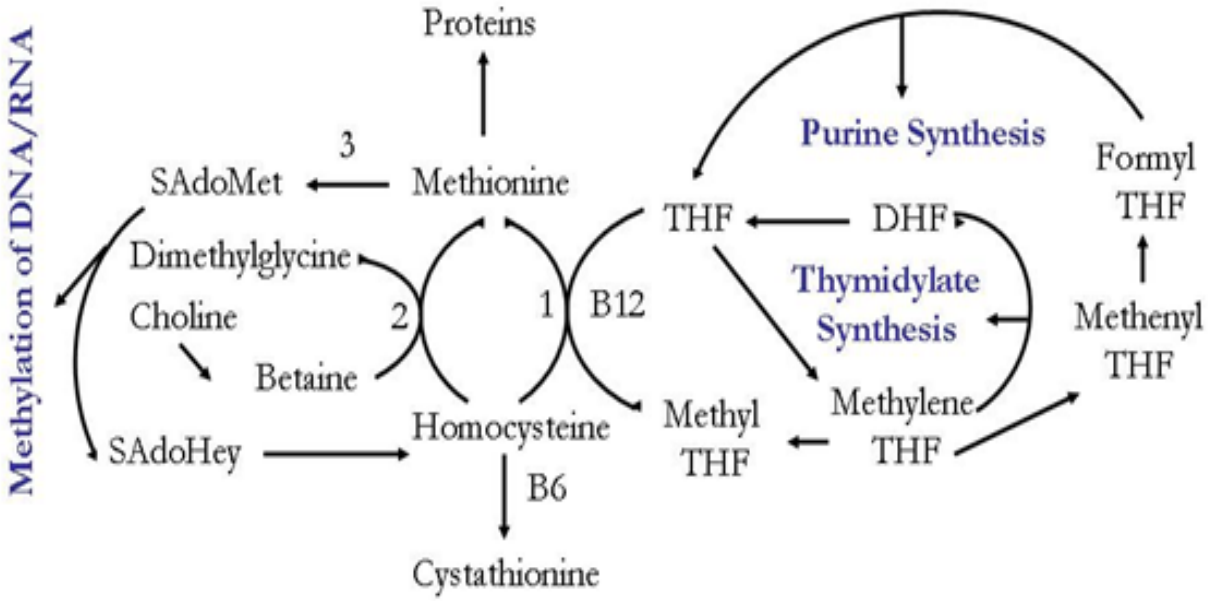

Figure 1: Folate in nucleic acid metabolism. THF, Tetrahydrofolate, DHF: dihydrofolate, SAdoMet, S-adenosylmethionine; SAdoHcy, S-adenosylhomocysteine; 1, methyltetrahydrofolate: homocysteine methytransferase; 2, betaine:homocysteine methyltransferase; 3, methionine adenosyl transferase; 4, methylenetetrahydrofolate reductase. Adapted from Jang, H., Mason, J. B., & Choi, S. W. (2005). Genetic and epigenetic interactions between folate and aging in carcinogenesis. *The Journal of nutrition*, *135*(12), 2967S-2971S.

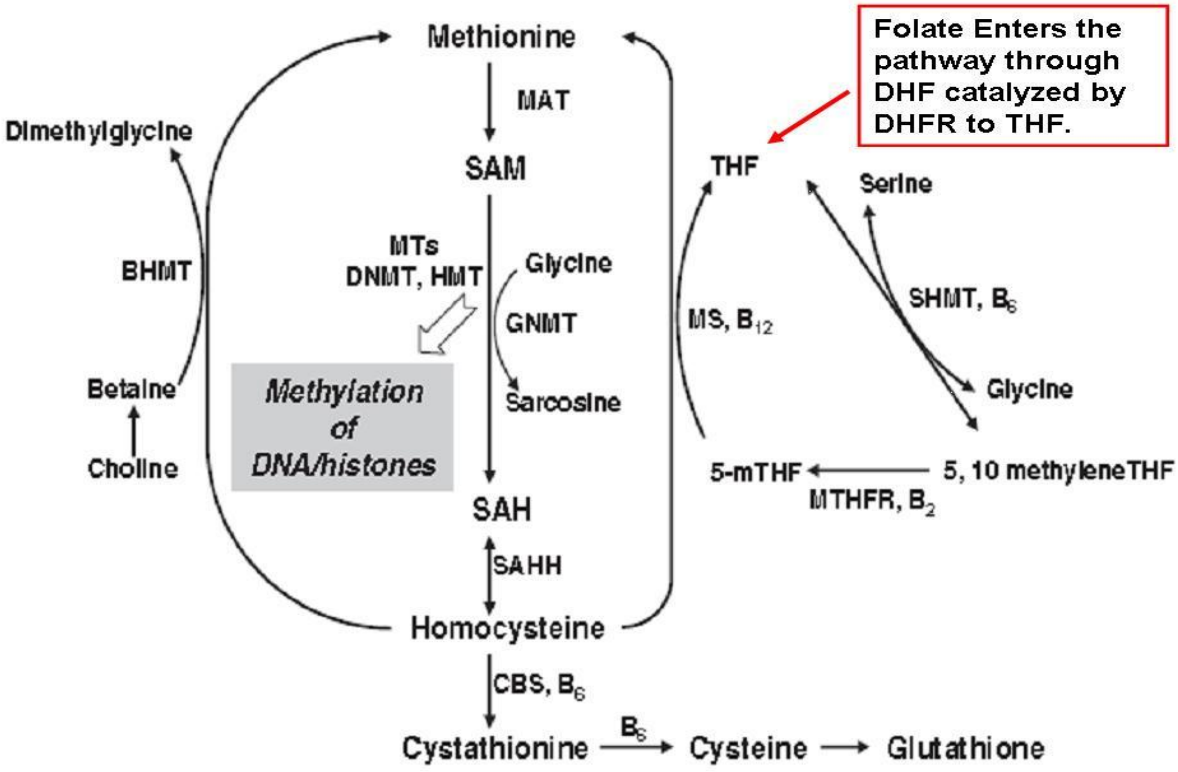

Figure 2: One-carbon metabolism. S-adenosylmethionine (SAM) is the unique methyl donor for many biological methylation adenosylhomocysteine(SAH) is an inhibitor of methyltransferases such as DNA methyltransferases (DNMT) and histone methyltransferases. In one-carbon metabolism vitamins B2, B6, B12 and folate are coenzymes, while methionine, choline, betaine and serine are methyl donors. THF, tetrahydrofolate; 5-mTHF, 5-methyltetrahydrofolate; MT, methyltransferases; HMT, histone methyltransferases; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; SHMT, serine hydroxymethyltransferase; GNMT, glycine *N*-methyltransferase; CBS, cystathionine-b-synthase; MAT, methionine adenosyltransferase; SAHH, S-adenosylhomocysteine hydrolase; BHMT, Betaine homocysteine methyltransferase; B2, vitamin B2; B6, vitamin B6; B12, vitamin B12. DHFR, dihydrofolate reductase and DHF, dihydrofolate. Reprinted from Park, L. K., Friso, S., & Choi, S. W. (2012). Symposium 4: Vitamins, infectious and chronic disease during adulthood and aging Nutritional influences on epigenetics and age-related disease. *Proc Nutr Soc*, *71*, 75-83.

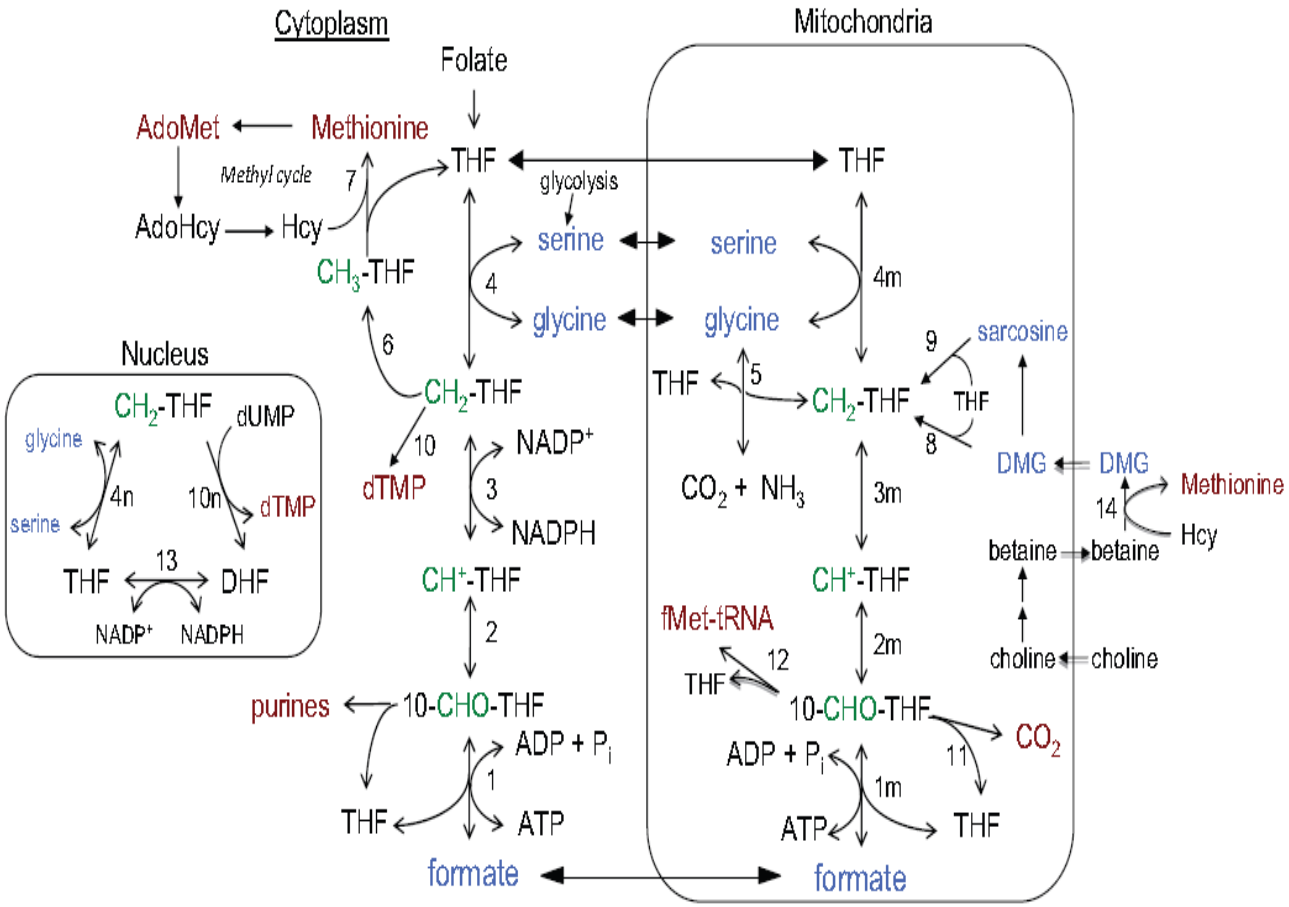

Figure 3: Compartmentalization of mammalian one-carbon metabolism. End products of one-carbon metabolism are in red. One-carbon donors are in blue. Activated one-carbon units carried by tetrahydrofolate (THF) are in green. Reactions 1–4 are in both the cytoplasmic and mitochondrial (m) compartments. Reactions 4 and 10 are also present in the nucleus (n). Reactions 1, 2, and 3: 10-formyl-THF synthetase, 5,10-methenyl-THF (CH+-THF) cyclohydrolase, and 5,10-methylene-THF (CH2-THF) dehydrogenase, respectively, are catalyzed by trifunctional C1-THF synthase in the cytoplasm (MTHFD1). In mammalian mitochondria, reaction 1m is catalyzed by monofunctional MTHFD1L and reactions 2m and 3m by bifunctional MTHFD2 or MTHFD2L. The other reactions are catalyzed by the following: 4, 4n, and 4m, serine hydroxymethyltransferase; 5, glycine cleavage system; 6, 5,10-methylene-THF reductase; 7, methionine synthase; 8, dimethylglycine (DMG) dehydrogenase; 9, sarcosine dehydrogenase; 10 and 10n, thymidylate synthase; 11, 10-formyl-THF dehydrogenase (only the mitochondrial activity of this enzyme is shown, but it has been reported in both compartments in mammals); 12, methionyl-tRNA formyltransferase; 13, dihydrofolate (DHF) reductase; 14, betaine-homocysteine methyltransferase. AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; Hcy, homocysteine.Reprinted from: Tibbetts, A. S., & Appling, D. R. (2010). Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annual review of nutrition*, *30*, 57-81.

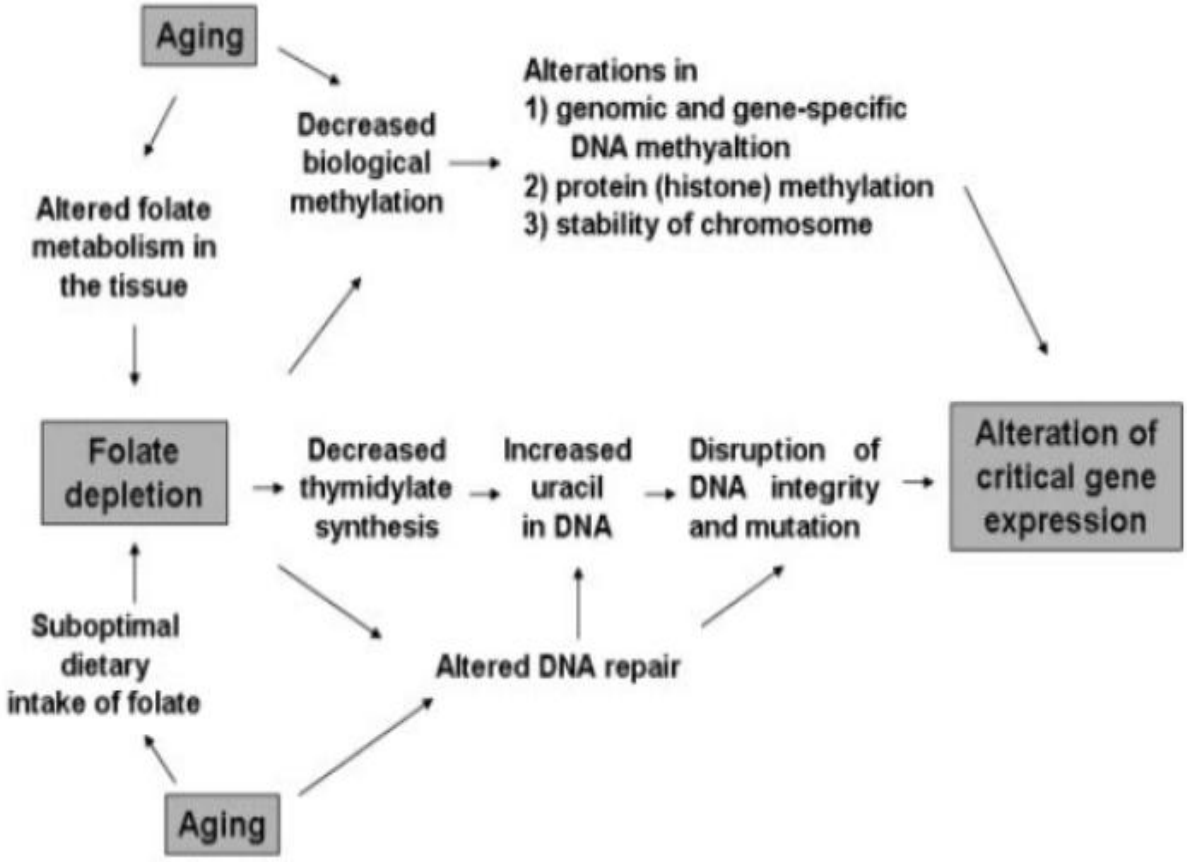

Figure 4: Scheme of candidate mechanisms for the interactions between folate and aging in carcinogenesis. Reprinted from Jang, H., Mason, J. B., & Choi, S. W. (2005). Genetic and epigenetic interactions between folate and aging in carcinogenesis. *The Journal of nutrition*, *135*(12), 2967S-2971S.

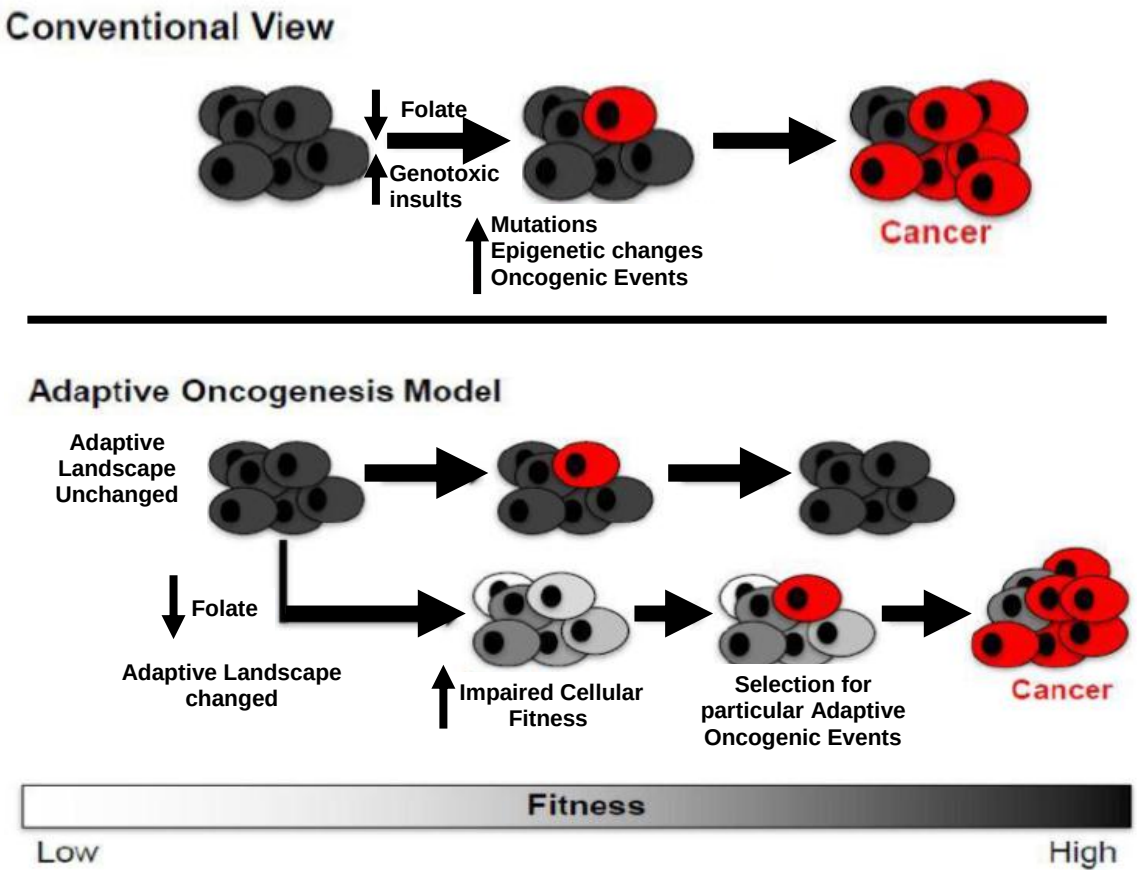

Figure 5: Conventional and Adaptive Oncogenesis Models for Tumorigenesis. *Conventional View* (top): Aging primarily contributes to increases cancers by facilitating the accumulation of oncogenic mutations (red cells), including activating mutations in oncogenes or genetic/epigenetic inactivation of tumor suppressor genes. *Adaptive Oncogenesis Model* (bottom): The ability of an oncogene to induce cancer is context specific. In a healthy population, the ability of cells to effectively compete for niche space is high to the optimal progenitor fitness. Thus, this competition is inherently tumor suppressive. However, if cellular fitness decreases as a result of aging Or environmental insults, the acquisition of an oncogenic mutation could be adaptive due to its ability to correct to circumvent defective cellular function. In this context, these cells would be selected for leading to carcinogenesis(oncogenically mutated and cancer cells are shown in red). Adapted from Henry, C. J., Marusyk, A., & DeGregori, J. (2011). Aging-associated changes in hematopoiesis and leukemogenesis: what's the connection?. *Aging (Albany NY)*, *3*(6), 643.

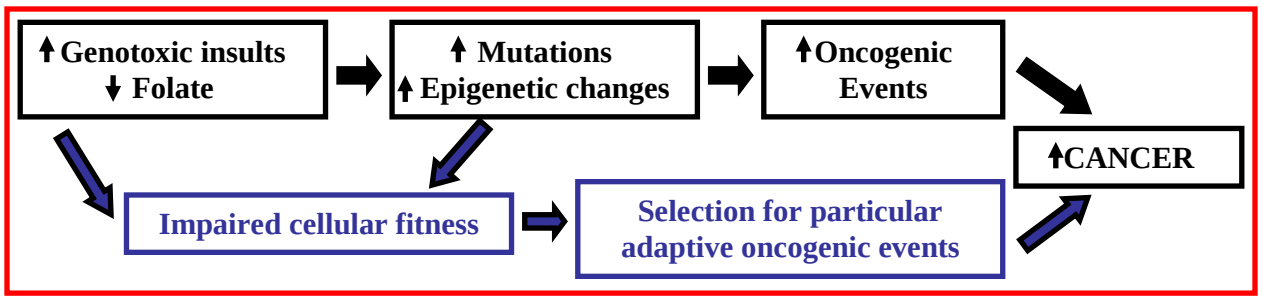

Figure 6: Adaptive Oncogenesis in the context of Folate Deficiency. Conventionally, the correlation between folate deficiency and an increasing cancer incidence has been primarily attributed to the mounting mutational load and epigenetic changes over time, which eventually may give rise to oncogenic mutations leading to cancer (black type). Alternatively, the Adaptive Onocogenesis model proposes that folate deficiency decreases cellular fitness (cellular proliferation/ survival/metabolism/etc.) resulting in competition within the niche. Thus, cells harboring advantageous mutations which can overcome fitness defects will be selected for and dominate the niche (blue part of figure).

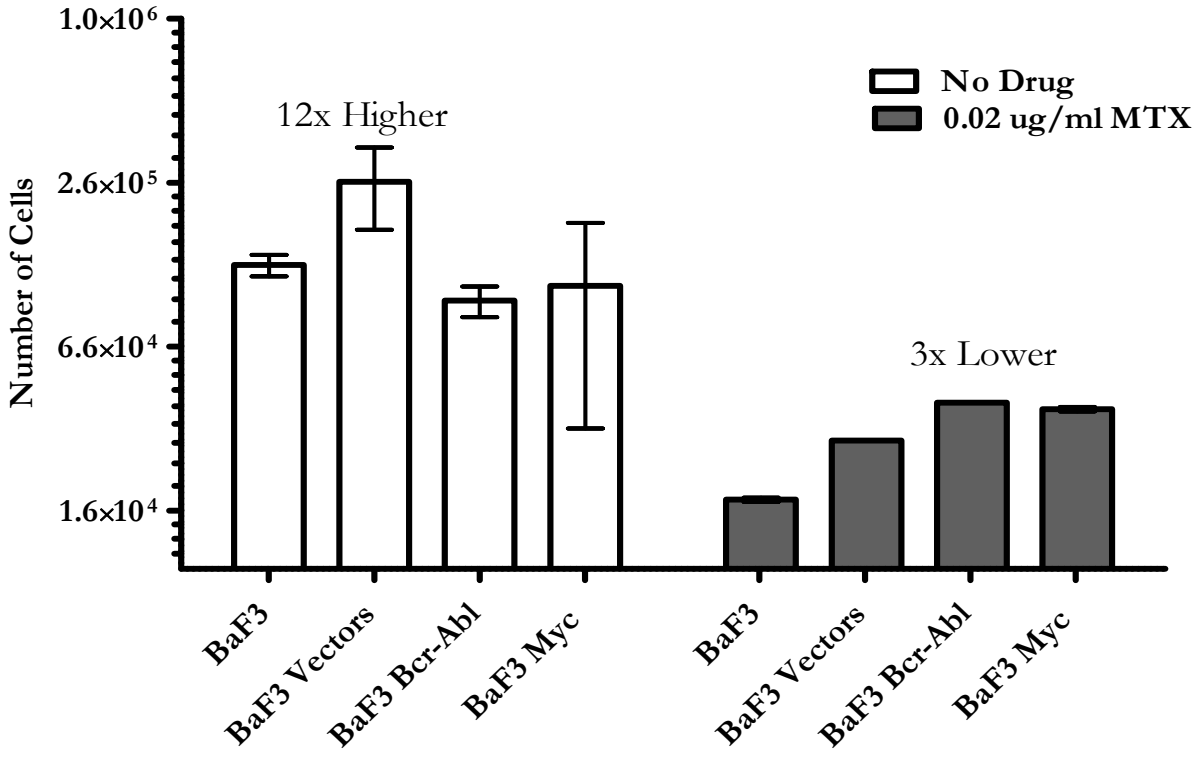

Figure 7. Oncogenitically initiated cells proliferate in the presence of Methrotrexate. 1 x 10^6 cells were plated for 24 hours at 0.02 ug/ml MTX and a no drug control on a 12 well suspension culture plate at 37ºC. After exposure to MTX, cells were washed with 1X PBS and stained with Propidium iodide 100x (PI).

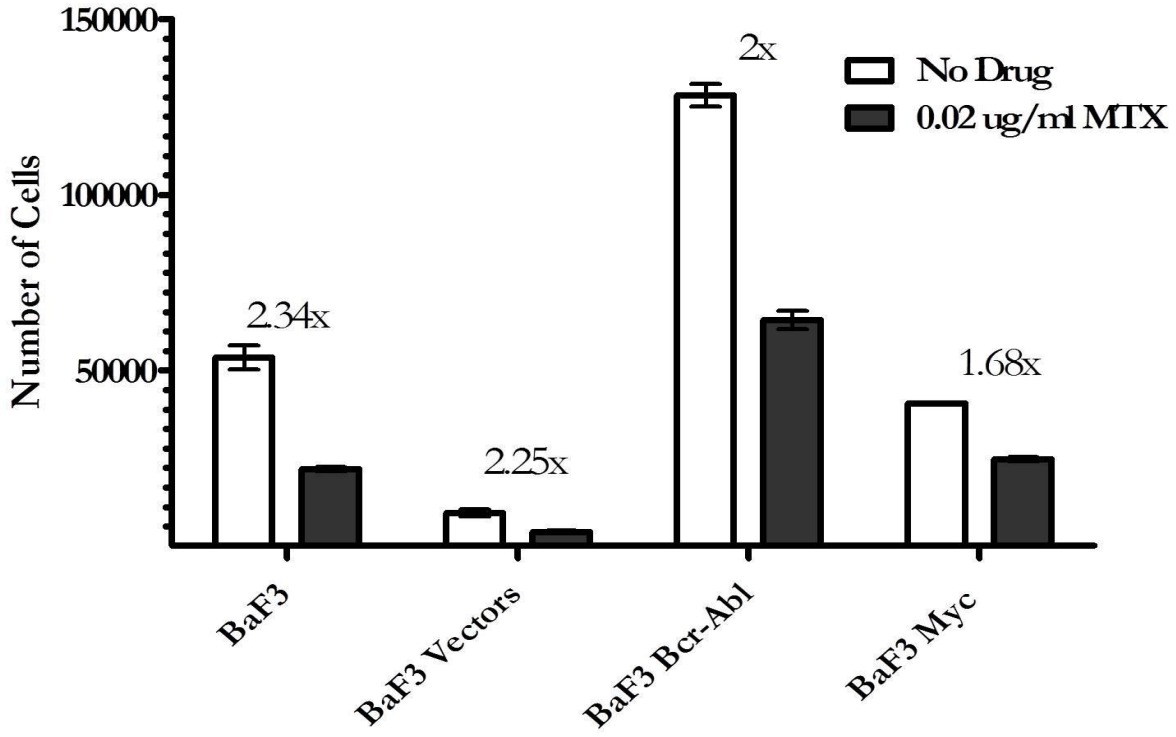

Figure 8. Bcr-Abl and Myc expressing B-progenitors demonstrate lower decrease in viable cell populations compared to controls. 1 x 106 cells were plated for 24 hours with MTX from 0.02 ug/ml with a no drug control on a 12 well suspension culture plate at 37ºC. After exposure to MTX, cells were washed with 1X PBS and stained with Propidium iodide 100x (PI).

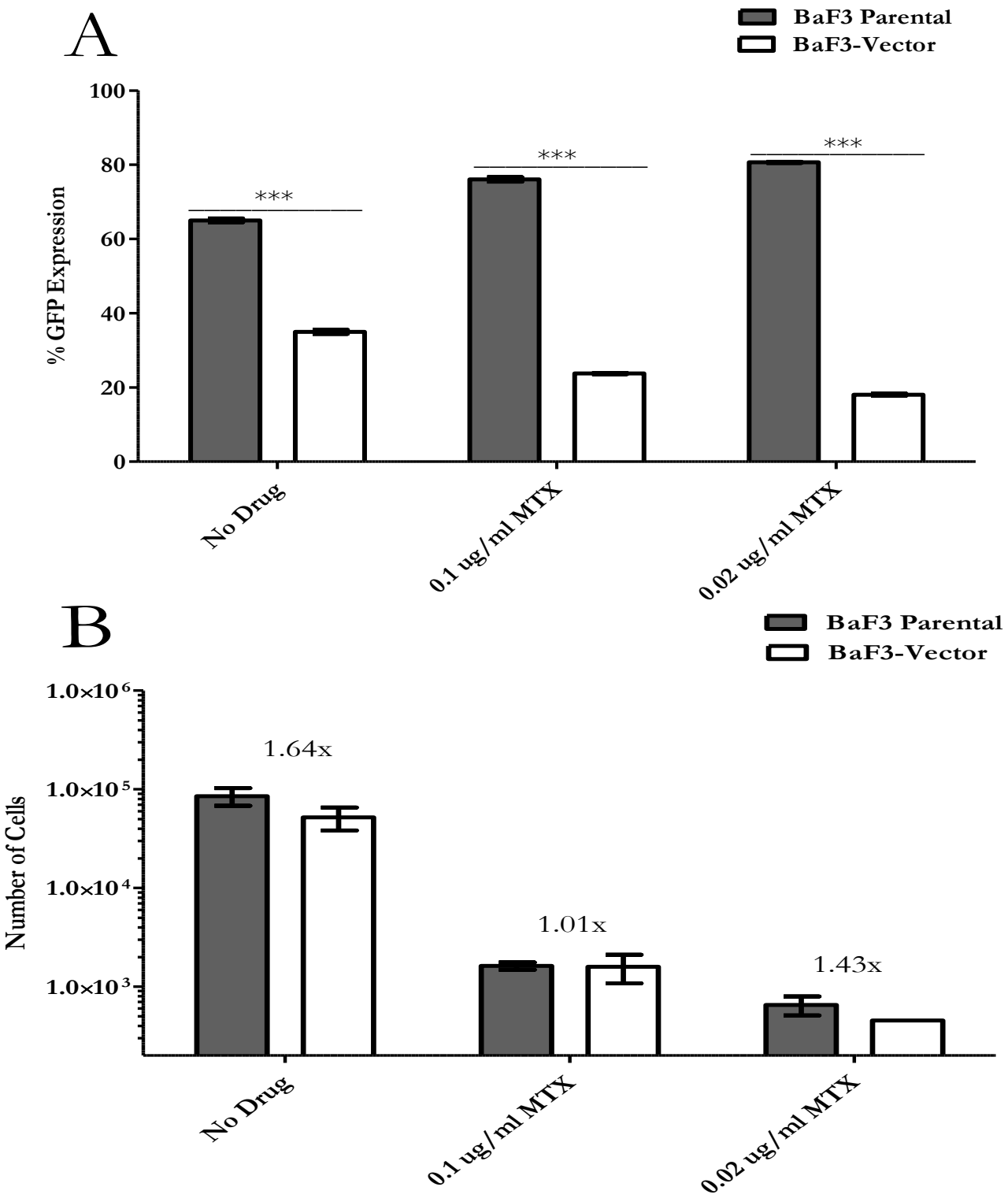

Figure 9. BaF3 Parentals with BaF3 Vector Competition over 48 hours. 4.5 x 105 Ba/F3 Cells were plated with 5 x 104 cells Ba/F3 Vector for 48 hours at No Drug, 0.1 ug/ml MTX and 0.002 ug/ml MTX. After exposure to MTX, cells were washed with 1X PBS and stained with Propidium iodide 100x. The percentage GFP Expression for BaF3 (GFP -/Gray bars) and BaF3 Vector (GFP+/white bars). An unpaired Student's exact t test was used for statistical analyses. ns, not significant (indicates $P > 0.05$); *P value between 0.05 and 0.01; **P value between 0.01 and 0.001; ***$P < 0.001$. (B) Number of Cells based on GFP Expression.

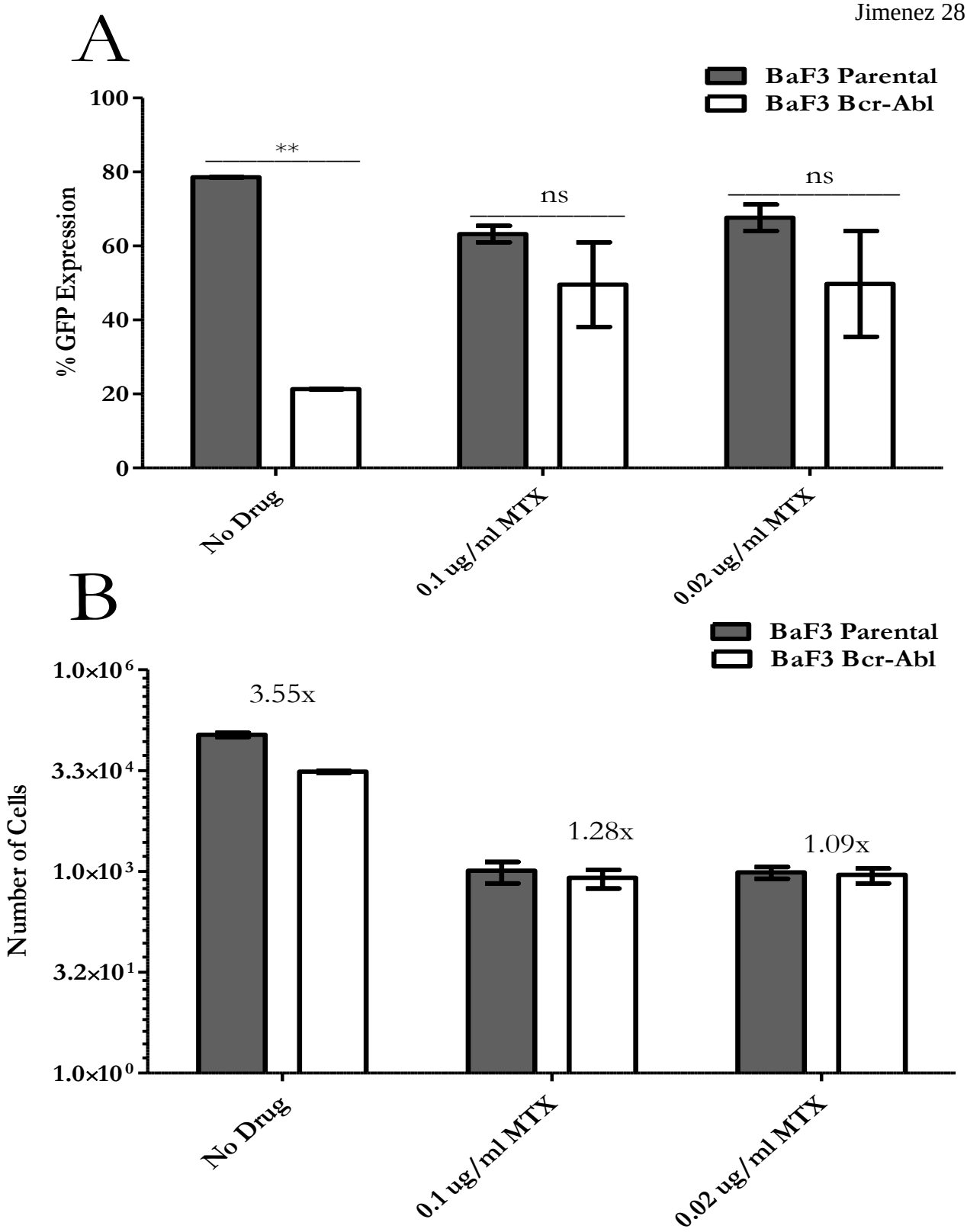

Figure 10. BaF3 Parentals with BaF3 Bcr-Abl Competition over 48 hours. 4.5 x 105 Ba/F3 Cells were plated with 5 x 104 cells Ba/F3 Vector for 48 hours at No Drug, 0.1 ug/ml MTX and 0.002 ug/ml MTX. After exposure to MTX, cells were washed with 1X PBS and stained with Propidium iodide 100x. (A) The percentage GFP Expression for BaF3 (GFP -/Gray bars) and BaF3 Bcr-Abl (GFP+/white bars). An unpaired Student's exact t test was used for statistical analyses. ns, not significant (indicates $P > 0.05$); *P value between 0.05 and 0.01; **P value between 0.01 and 0.001; ***$P < 0.001$.(B) Number of Cells based on GFP Expression.

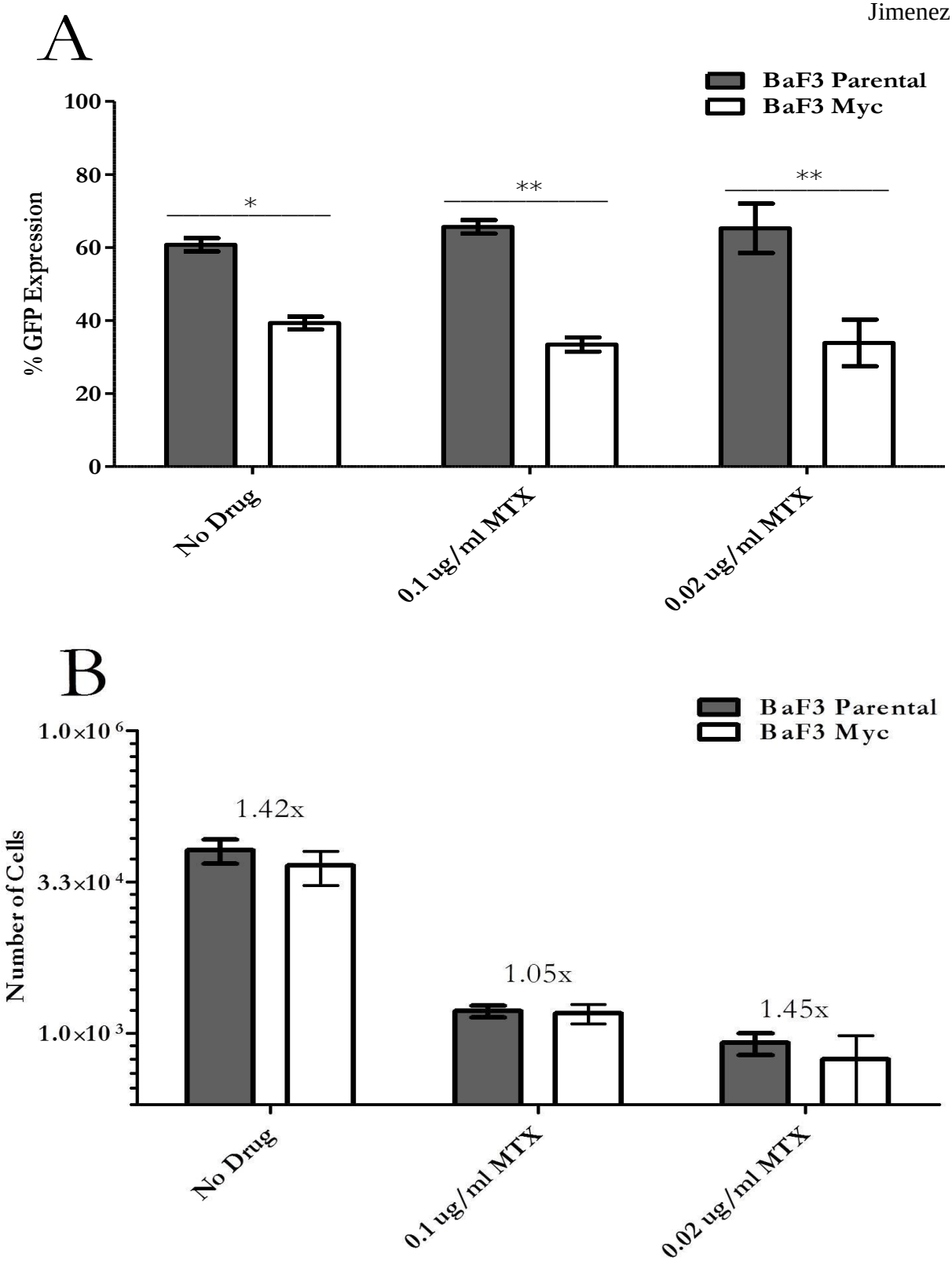

Figure 11. BaF3 Parentals with BaF3 Myc Competition over 48 hours. 4.5 x 105 Ba/F3 Cells were plated with 5 x 104 cells Ba/F3 MYC for 48 hours at 0.1 ug/ml MTX and 0.002 ug/ml MTX. After exposure to MTX, cells were washed with 1X PBS and stained with Propidium iodide 100x. (A) The percentage GFP Expression for BaF3 (GFP -/Gray bars) and BaF Myc (GFP+/white bars). An unpaired Student's exact t test was used for statistical analyses. ns, not significant (indicates P > 0.05); *P value between 0.05 and 0.01; **P value between 0.01 and 0.001; ***P <0.001. (B) Number of Cells based on GFP Expression.

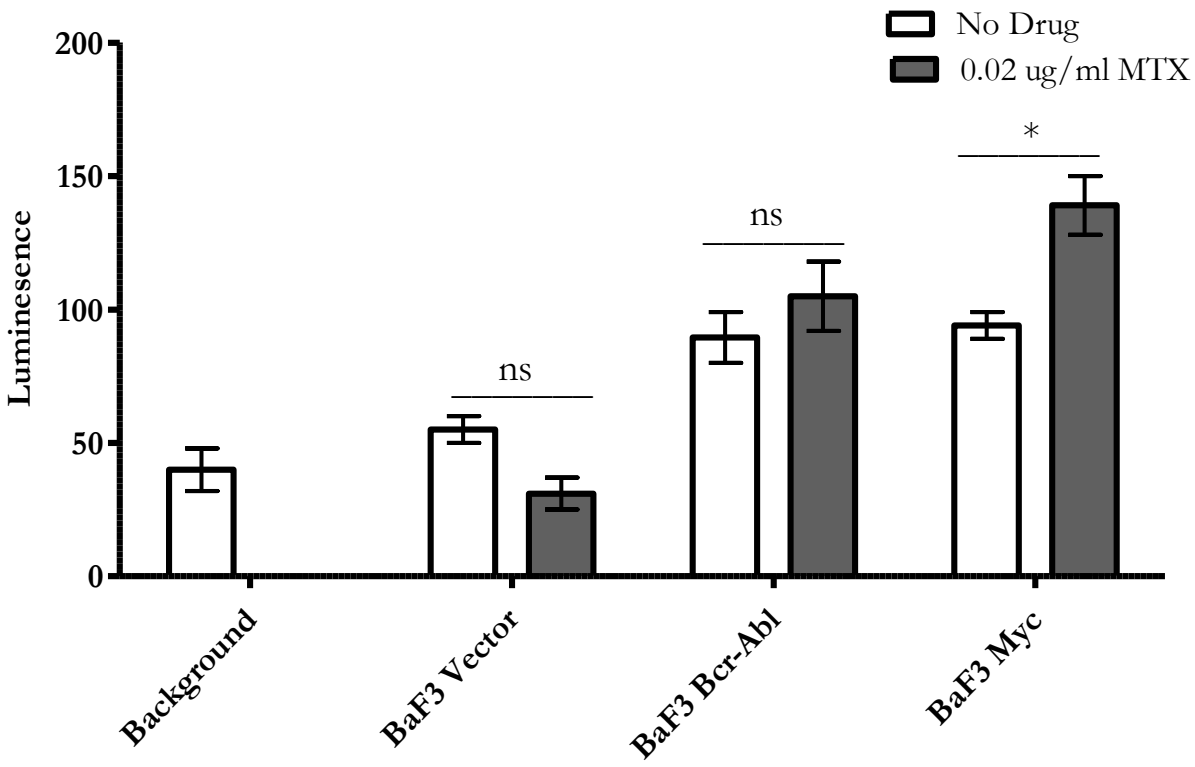

Figure 12. ATP synthesis after 24 hours of 0.02 ug/ml methorexate addition. 5 x 10^4 cells were plated in replicates of three into 96-walled multiwell plates with control well plates containing medium without cells to obtain a value for background luminescence.

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