FOLATE METABOLISM

Methionine synthase – unmasking a new foe in folate metabolism

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Summary

Folates are necessary for cellular growth and division. 5-methyl-tetrahydrofolate (5-methyl-THF) is the predominant circulating folate species that fuels folate metabolism in the physiological folate environment. A pair of studies by Ghergurovich, Xu and Wang et al. [1] and Sullivan and Darnell et al. [2] unmask the essentiality of methionine synthase (*MTR*) activity in powering the folate cycle to replenish the tetrahydrofolate pool from 5-methyl-THF; this generates nucleotides for supporting tumor growth. Dietary manipulation alters folate availability and holds implications for anti-cancer therapy response.

Main text

Folate, also known as vitamin B9, is an essential nutrient obtained from diet as animals, including humans, do not possess the biochemical capacity to synthesize folate *de novo*. Deficiency in folate interferes with growth and development, and may result in neural tube and congenital heart defects during fetal development or megaloblastic anemia in adults [3]. Folate metabolism regulates the bioavailability of various folate species and forms a crucial part of one-carbon metabolism. The one-carbon metabolism encompasses a set of juxtaposed metabolic networks – folate cycle, methionine cycle, and trans-sulphuration pathway – that are mediated by folate intermediates for the transfer one-carbon units. The carbon units feed into diverse cellular processes that include the biosynthesis of purines and thymidine, regulation of redox, as well as the regeneration of methionine. These biosynthetic processes, in turn, support cellular proliferation, embryonic development and tissue homeostasis.

Owing to its essential role in nucleic acid synthesis, the inhibition of folate metabolism impairs cellular proliferation; this knowledge has provided the basis for the use of anti-folates as therapeutic agents in malignancies. The antagonism of folate metabolism and its downstream nucleotide pathways has formed the cornerstone for chemotherapy for many decades. Folates broadly refer to a family of chemically related compounds that includes tetrahydrofolate (THF), 5-methyl-THF, 5,10-methylene-THF, and 5-formyl-THF (or folic acid). Of these, 5-methyl-THF is the predominant circulating species in human blood that is converted to THF by methionine synthase (encoded by 5-methyltetrahydrofolate-homocysteine methyltransferase, *MTR*) in the cell, while synthetically-derived folic acid found in food supplements must first be reduced to dihydrofolate (DHF), and then to THF by dihydrofolate reductase (*DHFR*) before it enters the folate cycle (Fig. 1). In a series of steps, THF acquires a single carbon group from serine, catalyzed by serine hydroxymethyltransferase (*SHMT*), and becomes reduced to 5,10-methylene THF, which ultimately feeds into nucleotide production. Of note, most natural folate species in diet and in circulation are present in the reduced form, typically 5-methyl-THF [4]. In the context of cancer, deciphering the rate-limiting steps of folate metabolism may yield insights into the control of cancer cell proliferation in relation to the physiological sources of folates, as well as helping to explain the less-than-ideal response to anti-folate therapies in clinical settings and suggesting approaches to overcome this limitation.

In this issue of Nature Metabolism, a pair of studies by Ghergurovich, Xu and Wang et al. and Sullivan and Darnell et al. converge on the essentiality of methionine synthase activity under physiological folate conditions for tumor growth through maintaining THF pools in cancer cells [1,2]. The production of THF is coupled to the methionine cycle, whereby MTR catalyzes the methyl transfer from 5-methyl-THF to homocysteine (Fig. 1). This regenerates methionine and yields THF. Methionine produces S-adenosyl methionine (SAM), which is a key substrate for cellular methylation reactions that regulate epigenetic processes in cancer. However, whether methionine synthase is responsible for driving methionine flux and dependency as its namesake suggests, or serves to replenish the THF pool, remains debatable. In order to probe the contribution of methionine from remethylation of homocysteine under physiological conditions, Ghergurovich, Xu and Wang et al. conducted a series of elegant flux tracing experiments through [U-^{13}C]methionine labeling in cultured cells and in tissues of [U-^{13}C]methionine-infused mice bearing pancreatic ductal adenocarcinoma allograft tumors. Remethylated methionine catalyzed by MTR only accounted for about 3% of total cellular methionine pool *in vitro* and approximately 10% in tumors, non-liver tissues and serum, thus indicating that MTR has a limited role in contributing to methionine pool.

To probe the physiological role of methionine synthase, genetic deletion of *MTR* (ΔMTR) was performed in colorectal, liver, and pancreatic cancer cell lines, followed by their proliferation assessment either in the presence of 5-methyl-THF or folic acid, commonly supplemented in cell culture medium. Indeed, in the presence of 5-methyl-THF only, ΔMTR cells were restricted in their proliferative capacity due to their inability to generate THF. However, when folic acid was supplemented, cancer cells bypassed the requirement for MTR and their proliferation was restored. In animals, where 5-methyl-THF is the predominant folate species, the growth of ΔMTR tumors were inhibited. Metabolomics analyses revealed elevated levels of 5-methyl-THF coupled with depletion of other folate species such as 5,10-methylene-THF and 10-formyl-THF, reminiscent of phenomenon known as 'methyl-trapping' arising from the loss of MTR activity. Such a similar phenomenon has been described in individuals harboring *MTR* gene mutation that resulted in methylcobalamin deficiency G disorder [5]. Consistent with 'methyl-trapping', Ghergurovich, Xu and Wang et al. demonstrated that the loss of *MTR* was accompanied by an accumulation of intermediates of purine and pyrimidine synthesis with corresponding decrease in nucleotide species. Through

supplementation of leucovorin (folinic acid) – a drug approved for treating folate deficiency – the growth of ΔMTR tumors could be rescued, indicating that methionine synthase has a major role in contributing to folate metabolism.

Independently, Sullivan and Darnell et al. reasoned that standard cell culture conditions contain folic acid, which is a non-physiological source for most tissues. From the perspective of anti-folate cancer therapy, this can confound the interpretation of therapy response or mask the influence of folate cycle enzymes playing key physiological functions *in vivo*. In proposing the understated importance of methionine synthase, which couples the folate and methionine cycles, the authors argued that 5-methyl-THF is the major source of folate for tumors that replenishes the all-important THF pool. Experimental cell culture conditions, which utilize folic acid supplementation to generate THF through DHFR, artificially bypass the otherwise essential function MTR. In line with findings from Ghergurovich, Xu and Wang et al., lung cancer cells bearing ΔMTR were unable to grow in 5-methyl-THF, but regained their proliferative capacity when folic acid was supplemented. Levels of adenosine and guanosine nucleotides were markedly decreased in ΔMTR cells cultured in 5-methyl-THF relative to folic acid. In a series of metabolite rescue experiments, supplementation of pyrimidine salvage precursors (uridine and thymidine) and purine salvage precursor (hypoxanthine) were able to rescue proliferation of ΔMTR cells grown in 5-methyl-THF. This recovery, however was short-lived, as hypoxanthine alone could not fully rescue dTMP synthesis due to a depleted overall pool of folate precursors. These results reinforced the essentiality of MTR for *de novo* nucleotide synthesis in cancer.

DHFR is a target of the chemotherapy agent, methotrexate. Numerous cell-based biological studies have previously demonstrated that methotrexate ablates cancer cells in culture. Clinically, however, its efficacy can be hampered by the lack of response or the acquisition of resistance [6]. This raises questions on the physiological source of folate that replenishes the THF pool, and thereby contributing to anti-folate resistance *in vivo*. If folic acid is the major source of folate in cell culture, this predicts that methotrexate would have an impact on cultured cancer cells through its reduction of the THF pool. To address this, Sullivan and Darnell et al. leveraged on a multiplexed proliferation assay with a library of 489 barcoded human cancer cell lines (termed PRISM)[7], and demonstrated that most cancer lines have similar proliferative capabilities in either folic acid or 5-methyl-THF supplemented

media, regardless of variability in *MTR* expression levels. Strikingly, however, at a population level and in response to therapeutic stress, cells across various cancer types were indeed more resistant to methotrexate when cultured with 5-methyl-THF, further underscoring the broad contribution of methionine synthase in different cancers. Why the differential gene expression of *MTR* did not predict methotrexate response is somewhat surprising. Quite possibly, the flux of folate cycle is determined by MTR activity or that of other enzymes, including the methionine cycle, in totality. Such findings cautioned against studies that relied solely on cell culture-based interpretations of methotrexate sensitivity, where cells are commonly cultured in the presence of folic acid, thereby dampening their physiological reliance on methionine synthase.

Dietary nutrition is associated with cancer. Ample evidence suggest correlation, but causative mechanistic links between dietary nutrition and tumor metabolism have often been challenging to ascertain. For instance, studies that sought to investigate the role of ketogenic diet in cancer have often provided conflicting outcomes on its pro- or anti-tumorigenic effects [8]. Perturbations to dietary composition change whole-body metabolism. The systemic nutrient availability, in turn, influences metabolite levels within the tumor-microenvironment. A randomized clinical trial previously suggested that high blood folate levels are associated with increased risk of prostate cancer [9]. Paradoxically, other epidemiological studies reported an inverse relationship between folate intake and cancer risk [10]. The inconclusive verdict on how folates influence cancer acquisition or progression highlighted a need to more carefully dissect the contributions of one-carbon metabolism enzymes in the context of cancer. To delineate the influence of dietary folate and essentiality of *MTR* in tumorigenesis, Sullivan and Darnell et al. fed xenograft tumor-bearing mice with a diet containing folic acid levels consistent with typical human diet. At the physiological level, 'baseline' folic acid levels permitted the growth of wild-type cancer cells, but failed to support tumorigenesis in ΔMTR counterparts. Remarkably, however, increasing circulating folic acid level through its supplementation in drinking water of mice rescued the growth of ΔMTR tumors. This observation lends support to the notion that folic acid can bypass methionine synthase activity and contribute directly to the THF pool, which is then channeled towards nucleotide synthesis (Fig. 1).

Recognizing how dietary interventions can influence metabolic pathways for the control of disease progression or improve therapeutic response is an area of untapped potential. Methionine-restricted diet inhibited *de novo* methionine synthesis and was postulated to lower methionine synthase activity; this sensitized murine models of sarcomas to radiation via disrupting flux through one-carbon and nucleotide metabolism [11]. Similar methionine restriction is argued to be effective in targeting cancer stem cells, which are addicted to exogenous methionine [12]. Key findings from Sullivan and Darnell et al. suggest that the benefits of inhibiting methionine synthase, for which a highly specific inhibitor is not yet available, as a singular approach may not be a panacea since folic acid from dietary intake may still provide the required THF pool to sustain *de novo* nucleotide biosynthesis. Dietary interventions such as a low-folate diet or the use of a DHFR inhibitor, in combination with MTR inhibition, may potentially exert a more potent anti-tumor activity. This underscores the need to develop clinically-viable inhibitors of MTR, even as we note that several such chemical structures had been previously described [13,14]. While dietary methionine restriction is achievable in humans [11], there is presently little evidence to suggest that long-term dietary folate restriction is feasible. As methionine-restriction similarly impacts one-carbon and nucleotide metabolism pathways, further studies on how dietary methionine-restriction can synergize with an MTR inhibitor may be warranted.

The findings by both groups now position methionine synthase as a central regulator of the juxtaposed folate and methionine cycles in the physiological tumor biology context, along with mediating pleiotropic effects on the bioavailability of various nucleotide precursors and amino acids that feed into biosynthetic pathways. Nonetheless, the manner by which MTR more strongly influences purine rather than thymidine synthesis remains unclear. Ghergurovich, Xu and Wang et al. noted a depletion of 5,10-methylene-THF, a precursor for thymidine synthesis, when MTR was deleted; yet, thymidine levels were maintained in these cells. In comparison, purine synthesis was significantly diminished, even though 10-formyl-THF, a precursor of purine synthesis, was only modestly affected. This raises questions on whether accumulation of 5-methyl-THF could have exerted a more far-reaching effect than is currently appreciated on nucleotide metabolism. One possibility involves the inhibition of AICAR transformylase, necessary for conversion of AICAR to FAICAR, and finally IMP for purine synthesis, as a consequence of 5-methyl-THF accumulation [15]. A better

understanding of how 5-methyl-THF, which bears structural similarities to other folate species, exerts its inhibitory effect may aid in further target identification of nucleotide metabolism.

The careful consideration for the physiological context of folate species availability has unearthed methionine synthase as a central folate cycle enzyme for maintaining tumor THF pools. In addition to its potential importance as a drug target, this newfound appreciation paves the way for designing innovative cancer treatment modalities that incorporates dietary manipulation with anti-folate therapy, which together, may result in more a durable response.

Figure 1: Methionine synthase (MTR) replenishes the tetrahydrofolate (THF) pool to drive nucleotide synthesis in tumors under physiological folate condition. (A) Circulating 5-methyl-THF is the major physiological folate, which is converted to THF by MTR activity. THF is essential for dTMP and purine synthesis, through a series of one-carbon units transfer, to support the proliferation of cancer cells. MTR couples the folate and methionine cycles, but exert a more dominant role in powering the folate cycle in the context of cancer. In cancer cell cultures, the supplementation with folic acid, not naturally present in diet, creates a 'MTR bypass' and directly contributes towards the THF pool through the dihydrofolate reductase (DHFR) enzyme; this masks the essentiality of MTR function in the folate cycle for driving cancer cell proliferation. **(B)** Low folate/methionine diet, in combination with the inhibition of MTR, may potently reduce the availability of the THF pool, resulting in a more severe dampening of nucleotide synthesis.

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Competing interests

The authors declare no competing interests.