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A non-proliferative role of pyrimidine metabolism in cancer

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ABSTRACT

Background: Nucleotide metabolism is a critical pathway that generates purine and pyrimidine molecules for DNA replication, RNA synthesis, and cellular bioenergetics. Increased nucleotide metabolism supports uncontrolled growth of tumors and is a hallmark of cancer. Agents inhibiting synthesis and incorporation of nucleotides in DNA are widely used as chemotherapeutics to reduce tumor growth, cause DNA damage, and induce cell death. Thus, the research on nucleotide metabolism in cancer is primarily focused on its role in cell proliferation. However, in addition to proliferation, the role of pyrimine molecules is established as ligands for purinergic signals. However, so far, the role of the pyrimidines has not been discussed beyond cell growth.

Scope of the review: In this review we present the key evidence from recent pivotal studies supporting the notion of a non-proliferative role for pyrimidine metabolism (PyM) in cancer, with a special focus on its effect on differentiation in cancers from different origins.

Major conclusion: In leukemic cells, the pyrimidine catabolism induces terminal differentiation toward monocytic lineage to check the aberrant cell proliferation, whereas in some solid tumors (e.g., triple negative breast cancer and hepatocellular carcinoma), catalytic degradation of pyrimidines maintains the mesenchymal-like state driven by epithelial-to-mesenchymal transition (EMT). This review further broadens this concept to understand the effect of PyM on metastasis and, ultimately, delivers a rationale to investigate the involvement of the pyrimidines molecules as oncometabolites. Overall, understanding the non-proliferative role of PyM in cancer will lead to improvement of the existing antimetabolites and to development of new therapeutic options.

Keywords Cancer; Pyrimidine metabolism; Epithelial-to-mesenchymal transition; Chemoresistance

1. SYNTHESIZING PYRIMIDINES FOR EPITHELIAL-TO-MESENCHYMAL TRANSITION?

Pyrimidine metabolism (PyM) is a complex enzymatic network that integrates nucleoside salvage, de novo nucleotide synthesis, and catalytic degradation of pyrimidines. Cancer cells, unlike resting cells, rely on the de novo pathway to ensure a continuous supply of deoxyribonucleoside triphosphates (dNTPs) and, thereby, support the uncontrolled tumor growth [1,2]. PyM has been implicated in differentiation of leukemic cells, but not much has been conceptualized in context of the differentiation status of solid tumors [3]. Emerging literature has linked PyM genes to epithelial-to-mesenchymal transition (EMT), a genetic and molecular program that is associated with uncontrolled tumor growth [e.g., tumor growth factor-beta (TGF-β)] [27–29], bone morphogenetic protein (BMP) [30,31], and hypoxia [32] that activate EMT master transcription factors (TFs) like TWIST [33], SNAI1 [34,35], and ZEB1/2 [36,37]. Several micro-RNAs (miRNAs) also regulate EMT, some of which do so by forming a feedback-inhibition loop with EMT-TFs [37]. One of the most studied feedback loop is mediated by members of the miR-200 family and ZEB1/2 [38–40], but other similar mechanisms have been described to determine EMT-associated alterations in cancer [41–43]. The transcriptional activation of EMT is relayed to an array of diverse downstream effector pathways that are essential for

It is a type-3 EMT, which is functionally distinct from type-1 and type-2 EMT programs observed during embryogenesis and the wound-healing process respectively [7]. EMT is experimentally characterized by change in morphology and assayed by gain of functions such motility and invasiveness [8,9], aggressiveness [10–12], stemness [13–19], and chemoresistance [20–26]. However, in some cases EMT could be restricted to acquisition of a combination of these functional properties in absence of distinct morphological change. EMT associated with cancer is orchestrated by extracellular factors [e.g., tumor growth factor-beta (TGF-β)] [27–29], bone morphogenetic protein (BMP) [30,31], and hypoxia [32] that activate EMT master transcription factors (TFs) like TWIST [33], SNAI1 [34,35], and ZEB1/2 [36,37]. Several micro-RNAs (miRNAs) also regulate EMT, some of which do so by forming a feedback-inhibition loop with EMT-TFs [37]. One of the most studied feedback loop is mediated by members of the miR-200 family and ZEB1/2 [38–40], but other similar mechanisms have been described to determine EMT-associated alterations in cancer [41–43]. The transcriptional activation of EMT is relayed to an array of diverse downstream effector pathways that are essential for
the resultant functional and morphological mesenchymal-like phenotypes. Metabolic adjustments and role of metabolites, especially from the tricarboxylic acid cycle, have been established as one of the important downstream effectors of EMT [44–48]. EMT induction has also been associated with alteration in PyM [49]. However, the exact role of PyM in EMT is not comprehensively understood.

Thus, we revisit the PyM-mediated differentiation model in leukemia, systemically scrutinize the evidence to establish a role of PyM in EMT-driven changes in differentiation in carcinoma and weigh a possibility of a similar mechanism in non-epithelial cancers. We further assess pathological and clinical consequences of differentiation, such as metastasis and chemoresistance, in context of PyM. As a corollary to the established role of PyM enzymes in EMT, we finally aim to propose pyrimidine nucleotides as oncometabolites. Overall, we aim to initiate the follow-up studies that will explore the mechanism behind the PyM in the context of metastasis and chemoresistance and will result in improving therapies that target PyM pathways.

2. ONCOGENES UPRGULATE DE NOVO PYRIMIDINE SYNTHESIS AND THE CATABOLIC PATHWAY

2.1. Pathways of pyrimidine metabolism

PyM is a branch of nucleotide metabolism that produces nucleosides and deoxyribonucleotides of the pyrimidine bases (cytosine, thymine, and uracil). Together with purine metabolism, it generates the deoxyribonucleotide pool required for cell proliferation. PyM consists of three pathways, as follows: 1) salvaging of free nucleosides and bases, 2) the de novo synthesis from amino acids and ribose precursors, and 3) the catabolism of excess nucleoside and nucleotides (illustrated in Figure 1) [50]. Salvaging of bases and nucleosides is a key feature of the terminally differentiated or resting cells [51]. Cells assimilate free nucleosides and pyrimidine bases from the extracellular space and convert them into their respective ribonucleotides and deoxyribonucleotides. The salvage enzymes, such as thymidine kinase, can also convert thymidine from nucleotide catabolism to thymidylate [50].

The de novo synthesis of pyrimidines is a sequential synthesis of pyrimidine bases from amino acids and priming the resulting base to a ribose moiety from phosphoribosyl pyrophosphate (PRPP). In the first step of the de novo pyrimidine synthesis, the trifunctional protein carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydororotase (CAD) fixes cytosolic glutamine to dihydororotate. Dihydororotate is converted to orotate by the mitochondrial enzyme dihydororotase dehydrogenase (DHODH). The cytosolic enzyme uridine monophosphate synthase (UMPS) uses dihydorotate as a precursor for the synthesis of UMP. The ribose moiety for UMP is donated by PRPP. UMP is then sequentially converted to CTP. Deoxyribonucleotides are synthesized from ribonucleotides by action of the enzyme ribonucleotide reductase (RNR) that converts diphosphates of the ribonucleotides to respective deoxyribonucleotides. As there is no ribonucleotide precursor for thymidylate, it is synthesized by thymidylate synthase (TS)-mediated methylation of deoxyuridine monophosphate [50].

Cells also have an endogenous mechanism to eliminate excess nucleotides and nucleosides that are generated by degradation of the nucleic acids. Pyrimidine catabolism uses the same set of enzymes for all pyrimidine molecules, as TMP and dTMP are sequentially dephosphorylated to their respective bases and then cleaved into open chain amino acids. Cytidine enters the catabolic pathway via deamination to uridine. The key enzymes of this pathway are thymidine phosphorylase (TYMP), 5’,3’-nucleotidase, cytosolic (NT5C), and dihydropyrimidine dehydrogenase (DPYD) [50].

2.2. Oncogenic activation of pyrimidine metabolism in cancer

A continuous supply of dNTPs is essential for survival of the cancer cells and, therefore, the perpetual activation of de novo PyM genes becomes incumbent for a growing tumor. Not surprisingly, the expression of de novo pathway genes (also nucleoside salvage pathway genes) is regulated by key oncogenes. Mutant p53 (p53[mut]) regulates the expression of an array of PyM genes and impacts the flux of the ribonucleotides phosphates to the dNTP pool by increasing the expression of the regulatory subunit of RNR [52]. p53[mut]-regulated expression of PyM has also been implicated in resistance against the drugs that target TS [53]. MYC (protein: c-myc), another key oncogene, binds to the regulatory region of several PyM genes and increases their expression [54,55] to maintain an elevated dNTP pool [56]. mTORC1 is a protein complex that is activated by loss of the tumor suppressor PTEN or activation of PISK- mTORC1 signaling. It integrates the oncogenic signals to mediate the major cancer-associated metabolic shifts. It phosphorylates S6K and the transcription factor E2F1 [57] to increase PyM. S6K post-translationally activates carbamoyl-phosphate synthase 2 (a component of CAD) [58,59] by phosphorylating Ser1059 and subsequent oligomerization [59], whereas E2F1 upregulates the expression of pyrimidine anabolic genes such as TYMS (gene coding TS) and TK and catabolic genes such as DPYD [60] at the transcriptional level. mTOR also controls the eflux of mitochondrial UTP to cytosol to support proliferation [61]. RAS [62] and EGFR [63] mediate the activation of MAPK phosphorylate CAD at Tyr456, resulting in desensitization to the UTP inhibition and increased sensitivity to the activation by PRPP. KRAS has been shown to drive tumor growth in the pancreatic cancer by activating PyM [64], and DHODH inhibition has been shown to be selectively responsive to the cells with KRAS mutation [65]. Thus, cancer cells can employ different mechanisms not only to activate the PyM genes but also to desensitize feedback regulatory pathways to circumvent allosteric inhibition, ensuring a continuous flow of the cellular nitrogen toward pathways that generate ribonucleotide phosphates and dNTPs. Since fueling of proliferation is the prime role of PyM in cancer cells, other functions have been under-appreciated. In the following sections, corroboration from literature will be discussed to emphasize the role of PyM in differentiation in leukemia and solid tumors.

3. PYRIMIDINE METABOLISM, LEUKEMIA, AND PROLIFERATION-DEPENDENT TERMINAL DIFFERENTIATION

The role of PyM in differentiation was first reported in late 1980 in leukemic cells, where the interplay between thymidine catabolic and anabolic pathways was shown to induce terminal differentiation [3,66]. These studies focused on acute myeloid lymphoma (AML) cells that originate from the hematopoietic progenitor cells (blast cells) that fail to differentiate and, therefore, rapidly proliferate [67]. A higher enzymatic activity of PyM anabolic enzymes (thymidine kinase and thymidylate synthase) was maintained in the leukemic cells compared with the normal leukocytes. In contrast, inducing differentiation in the leukemic cell line U-937 using 12-O-tetradecanoylphorbol-13-acetate (TPA) led to higher DPDY activity, indicating that the cells tend to eliminate thymidine via catabolic degradation [3]. In fact, treatment of AML cells with thymidine and inhibition of uridylic acid conversion to cytidine (thereby limiting the dNTP pool) promoted differentiation and maturation of leukemic cells [68]. Similarly, inhibition of DHODH [69–71] and UMPS [70] in AML released the blast cells from differentiation blockade (inability to differentiate) and resulted in their maturation. Considered together, these data present a proliferation-dependent model of PyM-mediated leukemic differentiation. In summary, as normal lymphoblasts undergo oncogenic transformation, they direct
thymidine toward thymidylate synthesis and maintain a balanced dNTP pool for proliferation. Whereas, following differentiation, thymidine is fluxed to catabolism, thus limiting the availability of thymidylate for cellular proliferation. However, as it will be discussed in the following section, the mode of PyM-mediated differentiation in solid tumors considerably varies from this mechanism.

4. PYRIMIDINE METABOLISM, SOLID TUMORS, AND PROLIFERATION-INDEPENDENT DIFFERENTIATION

4.1. PyM maintains the EMT phenotypes in epithelial tumors

The notion that EMT-driven aggressive epithelial tumors rely on PyM to maintain a functional phenotype is now well established in the following section, the mode of PyM-mediated differentiation in solid tumors considerably varies from this mechanism.

Figure 1: Pathway map of pyrimidine metabolism depicting de novo, salvage, and catalytic pathways. CAD: carbamoyl-phosphate synthetase 2, aspartate transcarbamylase 2, and dihydropyrimidinase 3, UMP5: uridine monophosphate synthetase (orotate phosphorybosyl transferase), and orotidylate decarboxylase, CMPK1/2: cytidine/uridine monophosphate kinase 1/2, RNR: ribonucleotide reductase, DTYMK: deoxythymidylate kinase, ENTPD8: ectonucleoside triphosphate diphosphohydrolase 8, NDK6: nucleoside diphosphate kinase 6, DUT: deoxyuridine triphosphatase, CTPS1/2: CTP synthase 1/2, ENTPD1: ectonucleoside triphosphate diphosphohydrolase 1, PRPS1/2: phosphoribosyl pyrophosphate synthetase 1/2, TS: thymidylate synthase, SMIT1: serine hydroxymethyltransferase 1, DHFR: dihydrofolate reductase, NTSC: 5'-3'-nucleotidase, cytosolic, TYMP: thymidine phosphorylase, DPYD: dihydropyrimidine dehydrogenase, DPKS: dihydropyrimidine, UPB1: beta-ureidopropionase 1, UCKL1: uridine-cytidine kinase 1 like 1, DCTD: dCMP deaminase, GLS: glutaminase, CPS1: carbamoyl-phosphate synthase.
literature. For instance, in aggressive lung cancers that are driven by simultaneous KRAS activation and loss of tumor suppressor STK11 (serine/threonine kinase 11; protein: liver kinase B1/LKB1), the mitochondrial carbamoyl phosphate from the urea cycle is diverted toward pyrimidine synthesis rather than the excretory pathway [72]. LKB1 has been previously shown to block EMT by inhibiting ZEB1 in lung [73] and hepatic cancer [74] and to co-localize with E-cadherin to promote the epithelial phenotype in breast cancer [75]. This might be the first indication that diversion of the mitochondrial carbamoyl phosphate to the production of pyrimidine could be a consequence of aggressive mesenchymal phenotype associated with the loss of LKB1. Indeed, several genes from the PyM correlate with the aggressive phenotypes in many tumors [76–78], but their impact on cancer malignancy has been exclusively attributed to their role in proliferation.

In an attempt to establish a fundamental link between the functional phenotypes of EMT and PyM in carcinoma, our laboratory discovered a strong correlation between TYMS mRNA (a gene that is central to the de novo pathway) and key markers of epithelial and mesenchymal-like phenotypes from a transcriptomic dataset from the NCI (NCI-60 panel, spanning nine different types of cancers). Among the EMT markers investigated, we could validate a functional association between TS and ZEB1 and observed that shRNA-mediated ZEB1 knockdown could not only suppress EMT, but also decrease TS protein level. Further, we identified a role for miR-375 in TYMS regulation, and the existence of a ZEB1/miR-375/Ts axis in regulating EMT in non-small-cell lung cancer (NSCLC) cells. However, more surprisingly, in the same work, a similar effect of EMT reversal was observed in cancer cells from different tumor types by direct shRNA-mediated targeting of TYMS mRNA, which was shown to suppress migration in a proliferation-independent manner [79].

When the model of poorly differentiated and mesenchymal-like triple-negative breast cancer (TNBC, breast cancer subtype with aggressive clinical behavior and predominantly basal-like phenotype) was tested, knockdown of TS led to an increase in the CD24+ population (markers of differentiated breast cancer cells) and reduced the migration across the wound in a monolayer culture [80]. The PyM-mediated EMT in breast epithelial cells further involves the enzymes for the catabolic degradation of thymidine, and a prominent role of DPYD, the only reversible enzyme in the catabolic pathway, has been recognized [80,81]. EMT induction in human breast epithelial cells by three independent overexpressions of EMT-TFs (TWIST, SNAI1, and GCS-gossecoid) led to an increased accumulation of the pyrimidine catabolic products [48]. Shaul et al. identified DPYD from a metabolic signature associated with EMT in a panel of 978 human cancer cell lines from different tumors, and demonstrated the importance of its catabolic products, dihydrothymine and dihydrooracil, in EMT in breast cancer [81]. The significance of DPYD in EMT has been further shown in the hepatocellular carcinomas (HCCs), where DPYD was shown to regulate the expression of EMT-TF SNAI1 through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling [82]. Hence, these data render a model with an anabolism/catabolism axis for EMT in aggressive cancers. Data from our laboratory further reinforce this notion, as we have shown that TS enzymatic activity is essential for the maintenance of the EMT phenotype of the TNBC cells and linked it to the catalytic degradation of thymidylate operated by DPYD [80]. We could further identify NFκB signaling as one of the most differentially regulated pathways in breast cancer cell lines with TS knockdown. Along with the involvement of the DPYD/NFκB axis in HCC [82], these observations suggest a TS/DPYD axis in the maintenance of an aggressive phenotype via regulation of EMT-TFs (Figure 2).

### 4.2. PyM affects aggressive behavior of non-epithelial tumors

Having professed a role for PyM in EMT in epithelial tumors, we wanted to understand its impact on non-epithelial tumors such as glioblastoma and melanoma, which have also been found to be driven by EMT-related processes [83–85]. In fact, in glioblastoma, the anabolic rate-limiting enzymes CAD and DHODH have been reported to be essential for self-renewal and maintenance of stem cells. A pyrimidine metabolic signature has been identified in glioblastoma, which stratified patients with higher grade from lower grades, and higher levels of pyrimidine metabolites were found in glioblastoma stem cells compared with the differentiated cells [86]. Further, ENPP1, a peripheral PyM enzyme, has been shown to regulate E2F1 to maintain stem cells in glioblastoma [87]. White et al. showed the importance of DHODH in self-renewal of neural-crest stem cells.

**Figure 2: Interplay between EMT-TFs and pyrimidine metabolism genes.** As the normal cells undergo oncogenic transformation, oncogenes activate and upregulate PyM genes. PyM in cancer is essential for proliferation, but the catabolic activity of PyM enzymes is further required to maintain aggressive cells in a mesenchymal-like state. This review aims to open a discussion that could lead to identification of possible feedback loops that connect the PyM back to EMT. The question marks in the figure indicate possibility of an existing feedback activation.

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cells in development of zebrafish embryos. Interestingly, melanoma cells, which originate from the neural crest [88] and are characterized by stem cell phenotypes [89], showed a marked decrease in growth in vivo and in vitro when treated with DHODH inhibitors [89].

Further, even in multiple myeloma stem cells, identified by low CD138 expression and high aldehyde dehydrogenase 1 family member A1 (ALDH1, a marker for CSCs) activity, inhibition of TS stimulated the proliferation and simultaneously broke the dormant phenotype associated with stemness [91]. Briefly, treatment of the CD27+CD138+ stem cells with a sub-lethal dose of 5-fluoro-2'-deoxyuridine (FdUrd, a competitive inhibitor of TS) resulted in a reduction in ALDH1 activity, marked the increase in the population of the CD138+ differentiated cells and increased the sensitivity to 125I-ITdU (an Auger electron emitting thymidine analogue [92]) [91]. Loss of the stem cells phenotype with TS inhibition, concurrent with an unexpected increase in proliferation, strongly indicated a proliferation-independent role of TS in stem cell phenotype.

Therefore, the above discussion supports that PyM can impact the aggressive nature of tumors that do not originate from the epithelial lineage. Although the exact mechanism has not yet been elucidated, a mechanistic overlap between carcinoma and non-epithelial aggressive tumors can be speculated in the context of PyM and validated experimentally.

### 4.3 PyM-mediated EMT and proliferation are independent modalities

Taking the discussed arguments into account, a strong case could be advocated for the role of PyM in cancer cell differentiation, which is a function of PyM anabolism as well as catabolism. It is of further interest to speculate about the connection between proliferation and EMT in the TS/DPYD model, and our observations strongly suggest that proliferation and differentiation are two independent modalities in epithelial tumors [70]. In our TNBC models, the effect on proliferation correlated with level of TS knockdown. Complete shRNA-induced ablation of TS resulted in growth arrest and cell death; strong TS reduction caused a proliferation defect; whereas, mild reduction in TS did not affect cell growth at all. Nevertheless, TS depletion always attenuated the mesenchymal-like differentiation, irrespective of loss in proliferation. When dNTPs were quantified in the TS knocked-down MDA-MB-231 cells with loss of CD24-negativity and suppression of EMT signature genes but without a detectable defect in proliferation, no imbalance in the dNTP pool was observed. These observations clearly decouple the TS-mediated EMT from proliferation, despite the necessity of TS enzymatic activity for maintenance of the mesenchymal-like state. These observations substantiate a model in which some highly aggressive cancer cells maintain thymidylate in excess of the threshold level required for proliferation. Excess thymidylate is then degraded by DPYD to promote and keep the EMT state intact [80,81].

The concept of the pyrimidine-mediated EMT adds another dimension to the existing studies that have shown that some metabolic pathways can act upstream of the EMT-TFs. For instance, fumarate acts as an epigenetic regulator of EMT [93], and excess glucose can enter the polyol pathway to regulate EMT by activating the autocrine TGF-β signaling [84]. As increase in glucose uptake is a salient feature of EMT [95,96], the existence of a polyol pathway could serve as a feedback channel to maintain it. Based on the discussed evidence, a reciprocal loop could be hypothesized between the EMT-induced PyM metabolism and sustained activation of EMT by activated PyM at transcriptional level. However, more studies are required to understand the downstream mechanism in detail, to uncover the whole network that transduces the PyM-mediated differentiation program, and to explore its possible connection with EMT-TFs such as ZEB1, TWIST, and SNAI1 (Figure 2). Moreover, the role of the peripheral PyM genes, such as ENPP1, a transmembrane protein that catalyzes the conversion of extracellular NTPs to NMPS, could be investigated in the context of its enzymatic activity, as it has been shown to promote the stem cell phenotype of breast cancer cells by promoting the cell surface localization of ABCG2 (ATP-binding cassette subfamily G member 2) transporter [97].

### 5. PYM CAN TUNE DIFFERENTIATION STATUS TO IMPACT METASTASIS

Differentiation is an important determinant of metastasis, thus, the effect of PyM on the metastatic outcome is a rational supposition. Indeed, in vivo mouse models have shown that suppression of PyM genes influences metastasis. In a colorectal cancer model, the reduction of DHODH did not affect the primary tumor growth from two different cell lines, but dramatically reduced liver metastasis (indicating that loss of DHODH affected the metastatic colonization although there was no proliferative defect in the primary tumors) [98]. In pancreatic cancer, TS knocked-down cells showed a marked reduction in metastatic colonization, whereas the supplementation of thymidylate rescued the effect of TS knockdown [99]. Similarly, DPYD knockdown in immortalized breast cells showed reduced metastatic behavior in vivo [81]. However, in our model from TNBC breast cancer cell lines, TS knockdown resulted in increased metastasis [80]. The variable outcome of PyM ablation on metastasis can be explained by the model of partial EMT and its association with differentiation status of the cells.

EMT is a non-binary process that includes multiple genetic and phenotypic alterations [100]. As epithelial cells lose their differentiation via EMT, they may acquire a complete mesenchymal phenotype, or they may acquire some of the several changes that define EMT [100]. The latter case is termed a partial/hybrid EMT state, and has now been reported in different cancer types [101–104]. Partial EMT does not only allow the transit to mesenchymal phenotype through selected functional changes, but the degree to which these changes are attained is also variable. This associates it with epithelial plasticity, and allows the tumor cells to exist in different states of differentiation [104]. The cells that undergo complete EMT are believed to lose their ability to interchange phenotypes and seed new tumors [101,103]. Thus, there exists a window of stemness, a zone marked by cells in partial EMT, that determines the metastatic potential of carcinomas [101] (Figure 3).

A correlation between partial EMT and enhanced metastasis is in fact now substantially documented in several in vivo models [105]. In addition, in certain cancers, such as breast cancer, cells with partial EMT have been shown as the main driver of metastasis [103,105,106]. Therefore, for a successful metastasis, reduction of PyM must direct the cells into the stemness window that is defined by the partial EMT state. Thus, in an experimental model with a knockdown approach, metastatic colonization depends on the initial EMT state of the cells and on the extent of change in EMT status after knockdown. For instance, in highly mesenchymal MDA-MB-231 cells, TS knockdown led to the loss of mesenchymal differentiation with a dramatic increase in the CD24+ cells and reduction of migration [80]. However, these cells intravasated and extravasated more readily compared with the control cells. Also, they colonized more in the lungs, indicating that the changes induced by the TS knockdown rendered the cells in partial EMT state with high metastatic potential compared with highly
mesenchymal parental controls. This model is supported by the fact that overexpression of miR-200c, a potent EMT inhibitor, promotes metastasis [15,107]; and, other studies have shown simultaneous loss of EMT in vitro and increased metastasis in vivo (reviewed in [108]). These results also have important clinical implications, because anti-PyM drugs are frequently administered to patients with aggressive cancers, and expression level of PyM genes, such as TS, could be elevated levels of dihydrothymine were reported in TWIST-induced tumors [109]. Extracellular purines generate signaling, classified as purinergic signaling, through a dedicated set of cell surface receptors [110] and can affect proliferation and metastasis [110–112]. A similar function could also be speculated and explored for pyrimidines. The endogenous accumulation of pyrimidine metabolites has been reported in the glycine decarboxylase-driven CD166+ CSCs from NSCLC [113]. In the hTERT-transformed mammary epithelial cells, elevated levels of dihydrothymine were reported in TWIST-induced EMT, and accumulation of dihydrothymine was shown to be essential for EMT [81]. However, the exact contribution of these metabolites has never been estimated.

Notably, in vitro the exogenous supplementation of pyrimidine metabolites has been used to rescue the EMT reversal induced by the knockdown of PyM genes [80,81], suggesting that the extracellular pyrimidine metabolites can be easily uptaken and work as oncometabolites. Along this line, recent reports in pancreatic cancer [114,115] have shown that tumor microenvironment can be enriched with pyrimidines secreted by non-tumor entities such as tumor-associated macrophages (TAMs) and stromal stellar cells. The macrophages secrete a broader spectrum of pyrimidines (thymidine, thymine, dCMP, cytosine, cytidine, dUMP, uridine, and uracil) with the highest secretion of thymidine [115], whereas stellar cells mainly secrete deoxycytidine (dC) with a detectable level of thymidine and deoxyuridine [114]. Although in both cases the assimilation of the deoxycytidine by the cancer cells was shown as a strategy to increase resistance against gemcitabine (a chemical analogue of dC), these observations open several possibilities in relation to metabolic control of EMT, such as cell intrinsic regulation, extracellular signaling, and assimilation of extracellular nucleotides. Although there is a lack of empirical data, based on the purinergic signals, a concept of pyrimidine-mediated cellular crosstalk can be hypothesized and validated experimentally. Therefore, it could be interesting to investigate the non-cancer components that might be involved in the enriching tumor microenvironment with pyrimidine metabolites and understand the mode of action of extracellular pyrimidines in differentiation.

6. PYRIMIDINES AS POTENTIAL ONCOMETABOLITES

The arguments presented in the preceding section establish a role of PyM genes in maintaining the EMT status of the cells. However, it is of great interest to understand if the pyrimidine metabolites play a direct and essential role in the process. Intracellular and extracellular accumulations of the pyrimidine metabolites are reported but not functionally described in detail. An oncogenic role of the pyrimidine metabolites could be anticipated, as purine molecules have been shown to act as receptor ligands in the microenvironment of many tumors [109]. Extracellular purines generate signaling, classified as purinergic signaling, through a dedicated set of cell surface receptors [110] and can affect proliferation and metastasis [110–112]. A similar function could also be speculated and explored for pyrimidines. The endogenous accumulation of pyrimidine metabolites has been reported in the glycine decarboxylase-driven CD166+ CSCs from NSCLC [113]. In the hTERT-transformed mammary epithelial cells, elevated levels of dihydrothymine were reported in TWIST-induced EMT, and accumulation of dihydrothymine was shown to be essential for EMT [81]. However, the exact contribution of these metabolites has never been estimated.

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7. THE LINK BETWEEN PYM, CHEMORESISTANCE, STEMNESS, AND EMT

As the cancer cells are addicted to the de novo pathway, competitive inhibitors of the de novo enzymes, such as 5-flourouracil (5-FU), capecitabine, and pemetrexed, have been used clinically for inducing DNA damage and cell death [116–120]. Although these drugs are widely used, their clinical efficacy is limited by chemoresistance attributed to the increased expression of the targeted PyM genes [121–123] and the reprogramming of the cells to the stem-like phenotype that is regulated by EMT [124]. Induction of the PyM genes is a common feature of the cells with the resistant phenotype. Several studies have reported an increase in the cellular levels of TS after cells are subjected to chemotherapeutic drugs [125–128]. Exposure of a TNBC cell line to doxorubicin has shown phosphorylation of CAD and induction of the de novo pyrimidine synthesis, leading to an increase in cellular triphosphate of cytidine and thymidine [129]. Colon cancer cells increased the expression of DPYD by suppressing miR-494 after exposure to 5-FU gradient at a sub-lethal dose [130].
However, chemoresistance has been shown to be promoted by EMT, which reprograms epithelial cells to mesenchymal-like cells with stem cell properties [131–133]. Many of the EMT TFs, TWIST [134–137], ZEB1/2 [138–141], SNAI1 [142–145], and SNAI2 [146–148], are upregulated in resistant cells and play a functional role in desensitizing cells to chemotherapy. Many of them do so by increasing the expression of the PyM genes. An EMT-promoting gene, astrocyte-elevated gene-1 (AEG-1) [149,150], confers resistance in HCC by inducing the expression of DPYD [151]. miRNAs that are associated with EMT have also been shown to regulate expression of the PyM genes and simultaneously modulate the drug response. For instance, miR-203, an important EMT inhibitor [152], binds directly to TS and sensitizes cells to 5-FU [153]. On the other hand, chemotherapeutic drugs induce the expression of EMT-TF; for example, gemcitabine has been shown to induce EMT through ZEB1 [154]. Since PyM plays a role in EMT, it would be interesting to evaluate the PyM-mediated EMT as a mechanism of acquired chemoresistance. In this direction, our laboratory has demonstrated that exposing NSCLC cell lines to increasing doses of 5-FU up-regulated the expression of its target, TS. The cells developed resistance against the drug and underwent EMT with an increase in ALDH1 activity. However, TS knockdown blocked these effects, indicating that TS is critical for the drug-induced cancer stem cell phenotype in these cells [79]. Cells with the stem-like phenotype have been found to be more resistant to antimetabolites that target the PyM pathway compared with the non-stem cells [155–158], and these antimetabolites, in turn, may enhance the stem-cell phenotype [159,160], similarly to other chemotherapeutic agents [161]. Therefore, there exists a clear network between EMT, stemness, PyM, and chemoresistance that could be investigated, with the possibility of feedback loops that regulate chemoresistance via PyM-mediated EMT.

8. THERAPEUTIC IMPACT

Oncogenic activation and elevation of the PyM genes are key features of the tumor cell, and PyM anabolic and catabolic pathways are associated with differentiation apart from maintaining a continuous proliferative state. The studies presented in this review strongly support a model in which some aggressive tumor types maintain their mesenchymal-like state by channeling the pyrimidine metabolites to the catabolic pathway, which is mediated via DPYD. Interestingly, diminution of the PyM can have different effects on metastasis in vivo, depending on the cellular context and level of suppression. Therefore, there is a need to extensively probe the role of PyM in differentiation and its subsequent effect on metastasis. This is essential, for instance, in the case of TS, which is an important clinical drug target, and TS-inhibiting drugs are often given to patients with highly aggressive cancers [120]. The effects of TS inhibition on the aggressive tumors also need to be re-evaluated in light of the new evidence. Simultaneously, chemotherapeutic inhibition of DHODH and other PyM enzymes could be tested for repressing EMT and metastatic behavior.

Based on the themes considered in this review, different strategies could be adopted to render solid tumors sensitive to chemotherapy (outlined in Figure 4). For instance, sources that contribute to the extracellular pyrimidines can be identified and eradicated to debilitating the protumorigenic microenvironment. In addition, a reduced dose of drugs (or novel improved agents) that could target PyM without stimulating expression of the target genes could be determined and used in the clinic to sensitize cells to combination chemotherapy. Finally, the network between PyM enzymes and EMT-TFs that maintains the cells in the stem-cell-like state could be disconnected to render cells more susceptible to existing chemotherapies and radiotherapy. Thus, overall, in-depth investigation of the non-proliferative functions of PyM can provide therapeutic options for difficult-to-treat cancers.

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CONFLICT OF INTEREST

None declared.

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