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The Intestinal Absorption of Folates

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Abstract

The properties of intestinal folate absorption were documented decades ago. However, it was only recently that the proton-coupled folate transporter (PCFT) was identified and its critical role in folate transport across the apical brush-border membrane of the proximal small intestine established by the loss-of-function mutations identified in the *PCFT* gene in subjects with hereditary folate malabsorption and, more recently, by the *Pcft*-null mouse. This article reviews the current understanding of the properties of PCFT-mediated transport and how they differ from those of the reduced folate carrier. Other processes that contribute to the transport of folates across the enterocyte, along with the contribution of the enterohepatic circulation, are considered. Important unresolved issues are addressed, including the mechanism of intestinal folate absorption in the absence of PCFT and regulation of *PCFT* gene expression. The impact of a variety of ions, organic molecules, and drugs on PCFT-mediated folate transport is described.

Keywords

folates; folic acid; folate absorption; transport; intestinal folate transport; pemetrexed; methotrexate; PCFT; HCP1; RFC; PCFT/HCP1

1. INTRODUCTION

Folates are a family of B₉ vitamins found in nature primarily as 5-methyltetrahydrofolate (5-methylTHF). Folates provide a methylene moiety for the de novo synthesis of thymidylate from deoxyuridylate and two formate moieties for the de novo synthesis of the purine ring. 5-MethylTHF is the source of the methyl group for the synthesis of methionine from homocysteine and is hence an important determinant of the formation of S-adenosylmethionine, which is required for a variety of methylation reactions (1–3). Thus, it is not surprising that folate deficiency is associated with profound disturbances that affect, in particular, tissues such as the bone marrow, the gastrointestinal tract, and (in infancy) the developing central nervous system (4, 5). After the biological importance of folates was recognized, research on their intestinal absorption began shortly thereafter, yielding the first studies to address the mechanism of membrane transport of folates (6). A more detailed understanding of folate transporters emerged with studies catalyzed by the introduction of antifolates as anticancer agents and by the recognition that the membrane transport of antifolates was an important determinant of tumor cell resistance to these drugs. Those

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studies led to the cloning of the major transporter of folates and antifolates into normal systemic tissues and cancer cells: the reduced folate carrier (RFC, or SLC19A1) (7). Because RFC is widely expressed in the apical brush-border membrane of the intestinal mucosa, this transporter was considered for many years to be the mechanism of transport of folates into intestinal cells and a key element in the absorptive process. This perception persisted despite the fact that the properties of intestinal folate absorption and RFC-mediated folate transport were different in several fundamental ways. Ultimately, the issue was resolved in studies on an antifolate that has a high affinity for a transport activity expressed in tumor cells with the same properties as intestinal folate transport. These studies resulted in the identification of the proton-coupled folate transporter (PCFT, or SLC46A1)—the primary mechanism by which folates are absorbed across the apical brush-border membrane of the small intestine under physiological conditions (8).

This article reviews the current understanding of the mechanisms by which folates are transported across the intestinal epithelium, the multiple transporters involved in the enterohepatic circulation of folates, and the factors that regulate and modulate these processes. The focus is on important recent developments; the reader is referred to reviews on folate and antifolate transport that also encompass earlier contributions to these fields and their pharmacological applications (9–15). In the terminology employed, folate(s) refers to the family of B₉ vitamins encompassing, among others, folic acid, 5-formyltetrahydrofolate (5-formylTHF), and 5-methylTHF.

2. FOLATE ABSORPTION IN THE PROXIMAL SMALL INTESTINE: THE APICAL MEMBRANE

Folates are absorbed primarily in the duodenum and jejunum within the acid microenvironment at the cell surface. A variety of model systems have been utilized to characterize this process, from the absorption of folates in humans and animals *in vivo* to studies in isolated intestinal segments, everted intestinal sacs, intestinal tissues and cells, and membrane vesicles (6). Other studies have been directed to mammalian cell systems, with a particular focus on Caco-2 cells, which grow to confluence, form tight junctions, differentiate, and polarize so that these cells, when grown on artificial membranes, mediate vectorial transepithelial transport. These cells have the appearance and function of enterocytes, although they originated from a human colon cancer. Studies have also utilized normal rodent small intestinal cells, such as IEC-6 cells, that have limited replicative capacity *in vitro* but that, in some cases, have been immortalized (16, 17).

Basically, these studies described a highly pH-dependent carrier-mediated process increasing with decreasing pH, with influx K_t s (where K_t denotes the substrate concentration at which influx is one-half the maximum rate) for folates in the low-micromolar range at low pH. Two important distinguishing features of folate transport in the various intestinal systems studied are the low pH optimum and the high affinity of the transporter for folic acid. These features contrast with **RFC's** pH optimum of 7.4 and very low affinity for folic acid. Methotrexate and folic acid are commonly used for studies on intestinal folate transport and absorption because of their availability specifically labeled with tritium, their stability, and their relatively low cost.

2.1. The Folate-Specific Solute Transport Carriers

Although a number of facilitative solute carriers have the capacity to transport folates, only two are specific for folates: RFC and PCFT. The properties of these folate-specific transporters are described in this section.

2.1.1. RFC—At approximately the same time that transport physiologists were studying the mechanism of intestinal folate absorption, cancer researchers became interested in membrane transport of antifolates when it was recognized, in the early 1960s, that impaired transport is an important mechanism of resistance to methotrexate, a member of the first class of antimetabolites to enter the clinics for the treatment of cancer (13, 18). RFC, characterized primarily with the L1210 murine leukemia model, has a high affinity (K_t of 2–7 μM) for the reduced folates, **5-methylTHF**, and **5-formylTHF** and for antifolates such as methotrexate, pralatrexate, and pemetrexed but a very low affinity ($K_i \sim 100\text{--}200 \mu\text{M}$) for folic acid. RFC functions optimally at a pH of 7.4. RFC was cloned in 1994 (7) and was subsequently designated as SLC19A1, a member of the superfamily of solute transport carriers (12). Other designations for this carrier have been used, but one, RFC1, is particularly inappropriate because it is now recognized that there is no RFC2. Indeed, the other two members of the SLC19 family, SLC19A2 and SLC19A3, are thiamine transporters that do not transport folates (12).

RFC is a typical facilitative carrier with 12 transmembrane domains; the N and C termini are located within the cytoplasm (11). The human *RFC* is located on chromosome 21q22; the protein consists of 591 amino acids. RFC is expressed in virtually all tissues and cell lines and is the major, if not sole, route of delivery of folates to cells within the systemic circulation under physiological conditions. RFC is also expressed in epithelia: the apical brush-border membrane of gastrointestinal cells, the basolateral membrane of the proximal renal tubule, and the apical membranes of the choroid plexus and retinal pigment epithelium. The location of RFC suggests that it plays a role in the vectorial transport of folates across these epithelia, although that role has not been defined in these tissues (19).

RFC is an organic phosphate antiporter capable of transporting its substrates into cells to achieve high electrochemical-potential differences across the cell membrane. Proof for this interaction came from studies that demonstrated clear heteroexchange between folates and thiamine phosphorylated derivatives. Thiamine is transported into cells via SLC19A2 and SLC19A3. Once inside the cell, thiamine rapidly forms the mono- and pyrophosphate derivatives that have good affinities for RFC and that can exit the cell by this mechanism (20, 21). The downhill flow of these organic phosphates out of the cell mediated by RFC provides the energy for the uphill flow of folates into the cells by the same carrier. A similar interaction among folates, RFC, and another intracellular organic phosphate [4-aminoimidazole-carboximide ribotide monophosphate (ZMP)] was recently reported (22). The properties of RFC were recently reviewed (10, 11).

2.1.2. PCFT—The identification of **PCFT** (SLC46A1) as the mechanism of folate transport across the apical brush-border membrane of the small intestine was accomplished via an unlikely route. The predominant interest in folate transporters in tumor cells emerged in the early 1960s and was focused, as described in the above section, on the antifolate methotrexate and on a process that was optimal at pH 7.4 and mediated by RFC. However, another prominent folate transport activity was also recognized for many years in human and murine cancer cell lines, a process with a low pH optimum. But little was done to characterize this activity in detail or to determine its molecular basis, and no connection was apparently made between this activity and the low-pH transport activity associated with intestinal folate absorption. Interest heightened when studies indicated that the low-pH activity in cancer cells exhibited a high affinity for a new-generation antifolate, pemetrexed, even at neutral pH. Further, when under methotrexate selective pressure, the RFC gene was deleted in a HeLa cell line (HeLa-R5) (23), there was no change in the low-pH transport activity, and there was sufficient retention of pemetrexed transport at neutral pH to sustain the antitumor activity of the drug in vitro (24–26). Hence, it became clear that transport at low pH in tumor cells must be mediated by a process genetically distinct from RFC.

Through further antifolate selective pressure, a derivative of HeLa-R5 cells, HeLa-R1-11, was obtained that had lost the low-pH transport activity and was now resistant to pemetrexed (26).

These two cell lines—HeLa-R5, which lacked the RFC activity but retained the low-pH activity, and HeLa-R1-11, which lacked both activities—provided the tools for the identification of the low-pH transporter. By using a data-mining approach, candidate genes were identified and screened for their message expression in the two cell lines. *PCFT* was identified as the one message expressed in HeLa-R5 cells, which manifested low-pH activity, but not expressed in the HeLa-R1-11 cells, which lacked low-pH activity (8).

PCFT resides on chromosome 17q11.2; the protein consists of 459 amino acids. The secondary structure of PCFT has been established by the substituted-cysteine accessibility method along with green fluorescent protein labeling of the N and C termini (27–29) (Figure 1). Like RFC, PCFT has 12 transmembrane domains, with the N and C termini directed into the cytoplasm. Human PCFT has two glycosylation sites (N58 and N68) located in the first extracellular loop between the first and second transmembrane helices; the integrity of these sites is not required for transport function (29). PCFT exists as a homo-oligomer; the C229 residue is responsible for the cross-link between PCFT molecules (30, 31).

PCFT properties have been characterized in *Xenopus* oocytes and in human and rodent cells (8, 28, 32, 33). The HeLa-R5 cells that express PCFT but lack RFC have been particularly useful for characterization of PCFT-mediated transport because there is essentially no background folate transport with substrate concentrations in the single-digit-micromolar range. These cells have allowed for characterization of PCFT-mediated transport across a spectrum of pH levels and a variety of other conditions. PCFT activity increases as the pH decreases. In most cells, maximum activity is achieved at a pH of ~5.5, although in some cases the activity continues to increase as the pH decreases further. However, below pH 5.5 there is protonation of the carboxyl groups of the glutamate moiety that results in increased passive diffusion across the cell membrane as the net charge of the folate molecule is decreased.

PCFT-mediated transport is electrogenic and proton coupled; studies in *Xenopus* oocytes demonstrate that acidification occurs concurrently with folate transport (8). Indeed, many aspects of this transport process could be characterized in oocytes on the basis of the current generated (8). Transport remains voltage dependent to some extent, even in the absence of a proton gradient at pH 7.4 (8, 32). A Hill analysis suggested one-for-one proton-folate coupling (32); however, a direct assessment of the relationship between folate and proton transport will be required to establish the stoichiometry of transport. PCFT also has channel-like activity in that protons are transported through PCFT even in the absence of an accompanying folate ion, a phenomenon that is increased with certain mutant forms of PCFT (34).

PCFT has a high affinity for its folate substrates, largely in the 1–3 μM range at pH 5.5. With an increase in pH, there is an increase in the influx K_t and a decrease in the influx V_{max} . These changes, however, differ among different folate substrates. For pemetrexed, the substrate with the highest affinity for PCFT, when the pH was increased from 5.5 to 7.4, the influx K_t increased 16-fold, from 0.8 to 12.7 μM , and the V_{max} decreased by a factor of only 1.6. However, for folic acid, when the pH was increased from 5.5 to 7.4, the influx K_t increased 39-fold from 1.3 to 131 μM , and the influx V_{max} decreased by a factor of 2.5 (8, 35).

It is generally assumed that the influx K_t is equivalent to the substrate-binding constant, which is essentially the case for the wild-type PCFT. However, there can be marked deviations from this scenario in mutated forms of the carrier, as occur in residues identified in subjects with hereditary folate malabsorption (HFM) (36, 37).

2.2. Establishing the Role of PCFT in Intestinal Folate Transport and Absorption

A confounding factor in establishing the role of **PCFT** in intestinal folate absorption was the omnipresence of **RFC** in the apical brush-border membrane of the entire gastrointestinal tract (19). Although the properties of intestinal folate transport (low pH optimum, high affinity for folic acid) were quite different from those of RFC (pH optimum of 7.4, very low affinity for folic acid) (see Figure 2), a literature spanning decades promulgated the concept that RFC is the intestinal folate transporter. This issue can now finally be put to rest, not only with the identification of PCFT and the correspondence of its folate transport properties to those of the intestinal absorptive process, but also with studies of the phenotypes of humans and mice in which the *PCFT* gene was inactivated, as described in the next section. Another distinguishing feature in the structural specificity of these transporters is the high affinity of RFC for PT523, an antifolate, and for thiamine pyrophosphate, in contrast to the extremely low affinity of PCFT for these molecules (20, 24, 25, 38, 39).

2.2.1. The *PCFT*-null human: hereditary folate malabsorption—Characterization of the *PCFT*-null human preceded the development of the *Pcft*-null mouse. First recognized in the 1960s, HFM is an autosomal recessive disorder in which there is impaired intestinal absorption of folates (5). The molecular basis for this disorder was established when subjects with HFM were shown to have loss-of-function mutations in the *PCFT* gene (8, 40). Since then, loss-of-function mutations in *PCFT* have been detected in 19 different families with HFM (4) (Figure 1). Although HFM is a rare disorder, a number of subjects of Puerto Rican heritage share a common mutated allele that appears to have originated in the central region of Puerto Rico (41). Newborns with HFM are normal; the disorder presents clinically several months after birth, apparently coincidental with the depletion of folates stored during gestation; and signs and symptoms progress thereafter. On the basis of current knowledge, the pathophysiology is due to two defects in epithelial transport: (a) a failure of intestinal folate absorption resulting in severe systemic folate deficiency and (b) a failure of folate transport into the central nervous system. The latter defect appears to be at the level of the choroid plexus, resulting in severe neural folate deficiency, a defect compounded by the lack of systemic folate available for transport into the central nervous system. The systemic folate deficiency in infants results in a clinical picture far more severe than that in folate-deficient adults. There is profound anemia and, not infrequently, pancytopenia. There can be hypogammaglobulinemia and T cell dysfunction, resulting in infections with unusual organisms, in particular, *Pneumocystis jiroveci*. There are often neurological developmental delays; other neurological deficits; and ultimately, if treatment is delayed, seizures. The systemic abnormalities in these patients do not improve with physiological, and modestly increased, oral doses of folates but rapidly correct with high doses of oral folate supplementation or with lower-dose parenteral folate. However, very high folate blood levels are necessary to restore physiological levels of cerebrospinal fluid folate. Recent reviews have described the complete clinical picture of this disorder, the spectrum of genotypes of subjects that have been studied (Figure 1), and their treatment (4, 5). As indicated below, *Pcft*-null mice are fertile, which also appears to be the case for humans. A woman with HFM who is known to the authors, and who has a mutation resulting in the complete loss of the PCFT protein, recently had two normal pregnancies and delivered normal infants.

2.2.2. The *Pcft*-null mouse—The *Pcft*-null mouse was reported in 2011, and the findings basically recapitulated the hematological abnormalities found in humans (42). These animals appear to be normal at birth but grow much more slowly than *Pcft*^{+/+} and *Pcft*^{+/-} mice. The growth rate and survival of the heterozygotes were normal. *Pcft*^{+/-} mice had a modest decrease in blood folate and diminished uptake of folate in the duodenum, jejunum, liver, and kidney with an oral folate load. The *Pcft*^{-/-} animals were pancytopenic with macrocytosis, had multilobed neutrophils, and showed evidence of ineffective erythropoiesis. There were elevated levels of homocysteine in plasma and tissues including the brain. The *Pcft*^{-/-} animals could be rescued with parenteral low-dose folates or high-dose oral folates. It was unclear as to whether and when the *Pcft*^{-/-} animals develop neurological findings, as occurs in humans. However, because animals did not survive on oral folic acid doses that corrected the hematological abnormality, death was likely due to the neurological consequences of the loss of both PCFT alleles, as indicated in a subsequent study (42a).

PCFT was initially reported to be a pH-independent, low-affinity, intestinal heme transporter (uptake K_t of ~125 μ M) that contributes to intestinal iron absorption and iron homeostasis (43). However, several other laboratories failed to confirm heme transport capacity for PCFT (8, 32, 33), although heme is a very weak inhibitor of PCFT-mediated folate transport (8, 33). Studies in humans with HFM have not revealed any parameter consistent with iron deficiency, and *Pcft*-null mice are not iron deficient. Indeed, such mice appear to have an excess of iron (42). Hence, at this point, it is clear that PCFT is a transporter highly specific for folates and that heme is a very weak inhibitor of this process (44). As an epiphenomenon, PCFT may have minimal heme transport capability in a low-affinity process that is not proton coupled, but there is no evidence that this transporter contributes to iron homeostasis.

2.3. The Mechanism of Intestinal Folate Absorption in the Absence of PCFT

The folate-deficient phenotype of *PCFT*-null humans with HFM and *Pcft*-null mice firmly establishes PCFT as the mechanism of transport of folates across the apical brush-border membrane of the proximal small intestine when there are physiological levels of folate intake. That **RFC** is expressed in the entire small intestine indicates that RFC function is inadequate under these conditions to compensate for the loss of PCFT function (Figure 3). This is not surprising for RFC expressed in the jejunum, considering the unfavorable pH (5.8–6.0) in the microenvironment in this region sustained by Na⁺/H⁺ exchangers; this pH is more than an order of magnitude less than optimal for RFC (45, 46). Furthermore, when absorption does not occur in the jejunum, folate is then delivered to more distal segments of the small intestine. Yet, RFC that is present in the brush-border membrane of this region, within a favorable pH environment, is not sufficiently functional to prevent a folate-deficient state.

The systemic folate deficiency that accompanies the complete loss of PCFT function can be corrected with supraphysiological oral folate supplementation. The doses required to achieve adequate intestinal folate absorption, and the advantages of one folate type versus another, have not been defined. Nor has the mechanism by which this absorption occurs been established. The higher concentrations of folate achieved with supplementation may be absorbed, albeit with low efficiency, by RFC in the proximal small intestine and/or with greater efficiency in the distal small intestine, and through this mechanism systemic folate levels could be restored to normal. If that were the case, then folic acid would be a very poor folate supplement because of the very low affinity of RFC for this folate form, although folic acid would be reduced to tetrahydrofolates, the preferred substrates for RFC, by bacteria in the small intestine.

A factor that may influence folate transport mediated by PCFT-independent routes is the impact of folate deficiency on transporter expression. For instance, there is marked upregulation of *Rfc* mRNA in mice on a folate-deficient diet; this upregulation, along with increased oral folate intake, may restore folate sufficiency in the absence of PCFT (47). Interestingly, folate receptor α (*Fra*), which is not expressed in the intestine under normal conditions, is overexpressed in the folate-deficient mouse intestine and may contribute to uptake through an endocytic process (47).

The organic anion-transporting polypeptide 2B1 (OATP2B1, or SLCO2B1) is expressed at the apical brush-border membrane of the proximal small intestine (48). Transport of folic acid, **5-methylTHF**, and **5-formylTHF** mediated by OATP2B1 is barely detectable. However, antifolates are better substrates (49). Pemetrexed transport mediated by OATP2B1 is pH dependent; there is a marked drop in influx with an increase in pH from 5.5 to 6.0. Although transport of other substrates is also optimal at low pH (48, 50, 51), transport of sulfobromophthalein (BSP), an excellent substrate for OATP2B1, is independent of pH. Hence, although this transporter is sensitive to pH in a substrate-specific manner, transport does not appear to be proton coupled (49). The impact of pH may be due to alterations in the protonation of the pyrrolopyrimidine ring, as suggested by studies on the pH sensitivity of pemetrexed transport mediated by the breast cancer resistance protein (BCRP) (see below) (52). There are polymorphisms of *OATP2B1* that impact its function. Among the nonsynonymous mutations, S486F is associated with impaired transport of estrone-3-sulfate (53) and, conversely, with enhanced transport of rosuvastatin (54). The functional impact of other *OATP2B1* polymorphisms is also substrate dependent (55). Although the R312Q-mutant OATP2B1 alters the pharmacokinetics of a drug unrelated to the folates (56, 57), there is no information on whether this or other polymorphisms impact transport of folates and/or antifolates. Nor is it known whether folate deficiency enhances OATP2B1 expression in the small intestine. Studies on the *Pcft*-null mouse would further clarify the PCFT-independent mechanism(s) of folate absorption across the apical brush-border membrane.

2.4. Hydrolysis of Folate Polyglutamates to Their Transportable Monoglutamate Forms

PCFT is highly specific for the monoglutamate form of its folate substrates, as is **RFC**. The polyglutamate forms of methotrexate from diglutamate to pentaglutamate have no effect on the influx of folic acid mediated by PCFT at a concentration ratio of 100:1 (28). Because dietary folates are present to a large extent in their polyglutamate forms, their absorption first requires hydrolysis to the monoglutamate. In humans, such hydrolysis is achieved by intestinal glutamate carboxypeptidase II (folate hydrolase), which is located within the brush-border membrane of the jejunum and has optimal activity at pH 6.5. This enzyme successively cleaves the terminal glutamate moiety of the folate molecule, ultimately yielding monoglutamate (58). γ -Glutamyl hydrolase, with similar enzymatic activity, is present in tissues that form and store high levels of folate polyglutamates, such as the liver and kidney. However, this enzyme is located within lysosomes and has optimal function at lower pH (4.5–6.0). Hydrolase activity within the organs that store folates as polyglutamates, such as the liver and kidney, is essential to ensure hydrolysis to the monoglutamate forms, which can be efficiently exported out of these cells to meet systemic folate needs (59, 60). This activity is of particular importance in the intestine, where folates cannot be transported as polyglutamates and must be in their monoglutamate form to allow for transit into the enterocyte and to achieve rapid and efficient transepithelial vectorial transport. It is of interest in this respect that the major dietary and blood folate, 5-methylTHF, is a very poor substrate for folypolyglutamate synthetase, the enzyme that successively adds glutamate moieties to the folate molecule within cells. This minimizes the formation of polyglutamate derivatives of this folate in enterocytes, facilitating its vectorial

transport across these cells into the blood. Likewise, folic acid, the folate form in most vitamins and in food supplements, is a poor substrate for this enzyme.

2.5. Role of the Multidrug-Resistance-Associated Proteins in Intestinal Folate Transport Across the Apical and Basolateral Membranes

MRP2 (multidrug-resistance-associated protein 2, or ABCC2) and BCRP (ABCG2) are expressed at the apical brush-border membrane of the small intestine (Figure 3). These ATP-binding-cassette transporters have the capacity to pump folates from enterocytes back into the intestinal lumen and thereby oppose the inward transport mediated by PCFT (61–63). The properties of these transporters are described in Section 3. However, the extent to which these exporters influence net folate absorption is not clear. Vectorial transport of folates across enterocytes requires export across the basolateral membrane for transit into the vascular system to occur. Several of the multidrug-resistance-associated proteins are expressed in the basolateral membrane: MRP3 (ABCC3) transports folic acid and 5-formylTHF with K_t s of ~2 and ~1.4 mM, respectively (64). Cells that overexpress MRP3 have 32–38% reductions in their intracellular folate levels (65). MRP3 appears to play a role in the absorption of oxidized folates, primarily in the duodenum. In *Mrp3*-null mice, the maximum folic acid and methotrexate blood levels and areas under the curve are markedly reduced following an oral load. However, this does not occur after oral administration of 5-formylTHF and 5-methylTHF, although net absorption of 5-formylTHF is reduced. Nor is the 5-methylTHF blood level decreased or the homocysteine blood level increased in *Mrp3*-null mice. Studies using everted sacs from *Mrp3*-null mice reported similar trends. In the *Mrp3*-null mice, folic acid transport across duodenal sacs decreased, but transport across jejunal sacs did not (66, 67). *Mrp3* expression is increased in the intestines of folate-deficient mice, as is the case for *Pcft*; both contribute to the vectorial transport of folates across the enterocyte (47).

MRP5 (ABCC5) is expressed at the basolateral membrane of enterocytes (68) but has a very low affinity for folates with uptake K_t s of 1.3 and 1.0 mM for methotrexate and folic acid, respectively. However, folate/antifolate accumulation and sensitivity to methotrexate are reduced in MRP5-overexpressing cells (69). MRP1 (ABCC1) is minimally expressed at the basolateral membrane of the enterocyte and transports folic acid and 5-formylTHF at rates far lower than for methotrexate (64). However, cells that overexpress MRP1 have lower intracellular folate levels (65), and *Mrp1* expression is increased in the intestines of folate-deficient mice (47). The impact of the MRP family of transporters on folate homeostasis was recently reviewed (14).

3. THE ENTEROHEPATIC CIRCULATION

The net intestinal absorption of folates not only is determined at the intestinal level but also is influenced by the flow of folates from the liver via the bile back into the intestine. This process is mediated largely by MRP2 and BCRP, which are expressed in the apical bile canalicular membrane (62, 63), and by OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) (70–72), which are expressed in the basolateral membrane of the hepatocyte, in apposition to the hepatic sinusoid, which receives blood from the hepatic portal vein and the hepatic artery (Figure 3).

The influx K_t for MRP2-mediated uptake of 5-methylTHF into bile canalicular membrane vesicles is ~125 μ M (61). In *Mrp2*-deficient rats, the biliary excretion of endogenous tetrahydrofolate and tetrahydrofolate cofactors is markedly decreased (61), and plasma levels of methotrexate are increased after oral administration of the drug compared with levels in wild-type rats (73–75). A single-nucleotide polymorphism, –24C > T in the 5' untranslated region of the *MRP2* gene, is essential for liver-specific expression (76) and is

associated with increased methotrexate blood levels in females (77, 78) due to a reduction in hepatobiliary excretion (79). Subjects heterozygous for the R412G MRP2 mutation (associated with diminished folate transport activity) have a threefold reduction in methotrexate clearance (80).

In *Bcrp*-null mice, methotrexate blood levels are increased after an oral load. Likewise, methotrexate levels in the liver are increased in these animals; however, there is no change in methotrexate levels in the small intestine compared with levels in wild-type animals (74, 81). Cells deprived of folic acid downregulate BCRP, consistent with a regulatory mechanism that recognizes folate deficiency (82). Pemetrexed and methotrexate transport mediated by BCRP increases as the pH decreases (52, 83). For pemetrexed, the influx K_t at pH 5.2 is $\sim 390 \mu\text{M}$. Homology modeling attributed the increased function at low pH to protonation of N1 within the pemetrexed pyrrolopyrimidine ring, resulting in an electrostatic interaction with the R482 residue of the protein (52). pH-dependent transport was substantially diminished when this residue was mutated to a noncharged moiety (52). The S441N, F431L, and F489L BCRP variants lack transport activity for methotrexate in vitro (84).

OATP1B1 and OATP1B3 are liver-specific organic anion transporters (70–72). *OATP1B3* expression is approximately one-tenth that of *OATP1B1*. Whereas *OATP1B1* is expressed at the basolateral membrane of all hepatocytes, *OATP1B3* expression is largely in the basolateral membrane of hepatocytes in the area of the central vein. These organic anion transporters mediate the uptake of a variety of substrates from sinusoidal blood into the hepatocyte (85–87), from where the folates are then available for export across the bile canalicular membrane. Both transporters have a methotrexate influx K_t in the range of 25 to $40 \mu\text{M}$; OATP1B3 does not transport folic acid (71, 72). There is no information on whether these transporters mediate 5-methylTHF transport. When transgenic mice expressing OATP1B1 specifically in the liver are given intravenous methotrexate, the area under the plasma concentration–time curve for intravenous methotrexate is decreased, and the liver content doubles compared with content in wild-type mice (88). A role for OATP1B1 in folate homeostasis is supported by the observation that polymorphisms in this transporter are associated with alterations in methotrexate clearance in children with leukemia treated with this agent. Most of these variants are noncoding single-nucleotide polymorphisms, suggesting that the association may be due to changes in the regulation and expression of this gene (89). These results were recently reproduced in a large cohort of patients treated with high-dose methotrexate (90). Assessment of the impact of these polymorphisms on 5-methylTHF transport and folate homeostasis would be of considerable interest.

MRP4 (ABCC4) is also expressed at the basolateral membrane of hepatocytes (91). The K_t s for methotrexate, folic acid, and **5-formylTHF** are 220, 170, and $640 \mu\text{M}$, respectively, and the relative V_{max} values are 1:2.8:8 (92). Hence, OATP1B1 and OATP1B3, MRP2, and BCRP work in concert to enhance uptake of folates from hepatic venous blood into hepatocytes (processes opposed by MRP4) and export them into the bile. From there, folates are delivered to the intestine, where they are excreted in the feces or reabsorbed.

The role of **PCFT** in the delivery of folates to the liver is not clear. This transporter is highly expressed in the liver (8, 28) and appears to be localized to the basolateral membrane (Y. Wang & I.D. Goldman, unpublished data). Earlier studies clearly demonstrated a low-pH folate transport activity in hepatocyte basolateral membrane vesicles (93). The extent to which PCFT functions in this environment would depend upon the pH at the transport interface; sodium-proton exchangers are expressed in the basolateral membranes of hepatocytes (94). RFC is also expressed in hepatocytes (19), and although its location has

not been established, its transport function into cells would make the basolateral membrane a logical site.

It is uncertain as to whether the diminished folate levels in the livers of *Pcft*-null mice are due to impaired transport into hepatocytes across the basolateral membrane, beyond what might be expected because of decreased intestinal absorption and low folate blood levels. This is another issue that further studies in *Pcft*-null mice could resolve (42). The liver is an important site of storage of folate polyglutamates that are mobilized when needed to meet systemic folate requirements. After folate polyglutamates are hydrolyzed to their monoglutamate forms, they may be (a) secreted in the bile, followed by intestinal absorption and delivery to the hepatic vein via the portal vein, and (b) transported across the basolateral membrane into the hepatic sinusoid if the net flow of folates across this membrane is reversed under conditions of folate deficiency.

4. THE ROLE OF THE LARGE INTESTINE IN FOLATE ABSORPTION

The proximal small intestine is the major site of folate absorption, consistent with the high level of *PCFT* expression in the duodena and jejunum in humans and rodents (8, 28). *PCFT* is expressed at much lower levels in the colon. However, there is a high level of folate transport activity at pH 5.5 in proximal and distal human colonic apical brush-border membranes, with uptake K_s for folic acid of 8 and 5 μ M, respectively (95). Similar observations were made with a transformed NCM 460 normal human colon cell line (96). Caco-2 cells, derived from a human colon cancer, have been an important model for the study of intestinal transport processes. These cells manifest the highest level of low-pH folate transport activity measured in any tissue. When *PCFT* expression was decreased by ~75–80% in Caco-2 cells by using a combined sh/si-RNA strategy, folate transport activity at pH 5.5 was suppressed by a similar amount, indicating that at least this percentage of transport activity at this pH is *PCFT* mediated (8).

The colon is a rich breeding ground for bacteria that synthesize folates and hence are a potential endogenous source of folates (97). Tritiated *p*-amino-benzoic acid introduced into the rat cecum is rapidly converted to folates by intestinal bacteria, is absorbed in part, and is distributed to the liver and other tissues (97, 98). A recent study quantified the extent of folate absorption from the large intestine by the infusion of [13 C] 6*S*-5-formylTHF into the cecum during screening colonoscopies (99). Whereas the rate of folate absorption from the colon was estimated to be approximately one-fiftieth the rate of absorption from the small intestine, the difference in net absorption was estimated to be much smaller (approximately one-tenth) due to the much longer transit time in the colon compared with that in the small intestine. Hence, the colon may make a small but significant contribution to folate homeostasis. Although *PCFT* is present in the colonic mucosa, the extent of absorption of folates from the colon in vivo by this mechanism is not clear. The pH is not especially favorable: The bulk pH in the right-, mid-, and left colon was 6.37, 6.71, and 7.04, respectively (100), and the mucosal pH in those regions was 7.05, 7.42, and 7.46, respectively (101). In any event, whatever other transporters might be present in the colon, they cannot compensate for the loss of folate absorption in the proximal small intestine, or for whatever is normally *PCFT* mediated in the colon, in the absence of *PCFT*. Bacteria that populate the proximal small intestine also produce folates that complement dietary sources of folate (102). The presence of these organisms in the intestine is the basis for the requirement for antibiotic administration in experiments designed to produce folate deficiency in rodents.

5. FURTHER CHARACTERIZATION OF THE PROPERTIES OF PCFT

5.1. The Impact of Inorganic Ions, Organic Molecules, and Drugs on PCFT Function

PCFT has a high level of specificity among folates and antifolates, particularly at neutral pH. At pH 7.4, the influx K_t for pemetrexed is 12–15 μM , whereas the K_i values for 5-formylTHF, 5-methylTHF, and folic acid are 45, 80, and 92 μM , respectively, and $>100 \mu\text{M}$ for PT523 (25). Despite the fact that PCFT's substrates are bivalent anions, organic phosphates and divalent inorganic anions have little effect on PCFT-mediated transport (103). This is unlike the case for **RFC**, which is an organic anion antiporter with a preference for organic phosphates, such as the phosphorylated derivatives of thiamine (12).

Of particular interest is the observation that bicarbonate is an inhibitor of PCFT-mediated transport. Although the inhibition is weak, bicarbonate markedly suppresses PCFT function at the high physiological bicarbonate concentrations that are present in blood. The inhibition is of a mixed nature, and the mechanism by which bicarbonate alters PCFT function is not clear; this inhibitory effect is less relevant to studies that are performed under conditions of low pH and very low bicarbonate concentrations. However, when PCFT function is assessed at neutral pH, a HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer that lacks bicarbonate is often used in which transport is much more rapid than when bicarbonate is present under physiological conditions (103).

Another important consideration is the stability of intracellular pH when studies are performed in cell lines that do not derive from tissues chronically exposed to a low-pH environment and that do not, therefore, have the homeostatic mechanisms required to sustain a normal intracellular pH. Because the transmembrane pH gradient determines PCFT function, a fall in the gradient due to an increase in the intracellular proton concentration rapidly decreases transport. Hence, accurate initial rate (influx) measurements require very brief uptake intervals in these cells. Reliable steady-state measurements of net transport require confirmation that transmembrane pH gradients are sustained. An important factor in the design and interpretation of studies is the impact of potential transport inhibitors on the transmembrane pH gradient. For instance, nitrite and bisulfite are inhibitors of PCFT-mediated folate transport at low pH. However, the acid form of these molecules rapidly diffuses into HeLa cells, and their inhibitory effect on PCFT function is largely indirect due to a collapse of the pH gradient rather than inhibition of PCFT through a direct interaction with the carrier. Likewise, PCFT-independent transport of the monocarboxylic acids lactate and acetate into cells results in a collapse of the pH gradient, which, in turn, impairs PCFT-mediated folate transport (103). Hence, alterations in intracellular pH should be considered in the interpretation of the effects of agents that appear to inhibit PCFT transport function.

Recent studies assessed the inhibitory effects of a variety of drugs on PCFT-mediated folate transport. In one study using HEK293 cells stably transfected with *PCFT*, 200 nM sulfasalazine and indomethacin produced ~49 and 19% inhibition of 10 nM folic acid uptake, respectively. The IC_{50} (concentration at which transport is inhibited by 50%) for sulfasalazine was 60 nM (33). In another study (104), the K_i for sulfasalazine was 42 μM on the basis of inhibition of folic acid transport in PCFT-transfected MDCKII cells. These concentrations of sulfasalazine are achievable in clinical regimens. However, although sulfasalazine has been reported to inhibit intestinal folate absorption (105), there is no firm evidence that this inhibition is the basis for the macrocytic anemia associated with the use of this drug for the treatment of inflammatory disorders. Nor is it clear whether sulfasalazine impairs the intestinal absorption of methotrexate, an agent with which it is frequently coadministered, diminishing the pharmacological effect of the antifolate. Whereas sulfasalazine inhibited PCFT-mediated transport of folic acid, PCFT-mediated transport of 0.5 μM tritiated sulfasalazine was not detected (104).

Other organic compounds inhibit PCFT-mediated transport at high concentration ratios (20,000:1). These include BSP, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), and *p*-amino-benzylglutamic acid (106). The underlying studies were performed in cells exposed for variable intervals to buffer at pH 5.5 so that the pH gradient may have been decreased under these conditions.

Another study, with a small number of patients, assessed the impact of proton-pump inhibitors on *PCFT* expression in biopsies obtained during diagnostic esophago-gastro-duodenoscopic and colonoscopic examinations (104). The level of *PCFT* expression in the duodenum exceeded the level of expression in the terminal ileum by a factor of nearly seven, and the latter level exceeded that in the colon by a factor of approximately five. In nine subjects treated with proton-pump inhibitors, there was a significant reduction (of ~40%) in *PCFT* expression. However, there was no difference in red-blood-cell folate levels in treated versus control subjects (104). Furthermore, despite the reduction in gastric acid that enters the proximal small intestine from the stomach in subjects treated with these agents, there is no evidence that these drugs are associated with an increased incidence of folate deficiency. Earlier studies suggested that antacids and H₂ receptor antagonists inhibit intestinal folate absorption (107), whereas, conversely, absorption is increased in subjects with chronic pancreatitis who are deficient in alkaline intestinal secretions (108).

5.2. Regulation of PCFT

Because of the role that folates play in biosynthetic and regulatory reactions, and the requirement for intestinal folate absorption in the maintenance of folate sufficiency, the mechanism of regulation of this process is of considerable interest. This section reviews the current status of understanding of the factors that influence folate absorption and modulate the expression of the folate-specific transporters.

5.2.1. Factors that alter PCFT expression—Of all the transporters potentially involved in the absorptive process for folates, only two, **PCFT** and **RFC**, are folate specific, although other transporters are required for the vectorial flow of folates across the enterocyte, and only PCFT has a role in absorption across the apical brush-border membrane under physiological conditions. Of particular interest is the impact of (*a*) folate deficiency and (*b*) alcohol on expression of these transporters and on intestinal folate transport in vitro and in vivo.

5.2.1.1. Folate deficiency: From the organismal perspective, folate restriction with the addition of succinyl sulfathiazole markedly (10-fold) increased *Pcft* mRNA expression in mice (28). This increase was accompanied by a comparable increase in *Rfc* mRNA and by a very high level of expression of *Fra* mRNA; the latter is not expressed in the normal gut. Interestingly, *Mrp1* and *Mrp3* mRNA levels were also markedly (100-fold) increased; there was no change in *Mrp4* expression (47). In an earlier study (109) that used a similar method to achieve folate deficiency in mice, there was a 3.3–4.1-fold increase in *Rfc* mRNA in various segments of the small and large intestines; this increase was not accompanied by an increase in *Fra* message. There was a 2-fold increase in transport of folic acid from mucosa to serosa across jejunal sacs and a 10-fold increase in the folic acid uptake $V_{\max}$ into jejunal brush-border membrane vesicles. Because these studies were conducted at pH 5.8 and 5.5, respectively, they reflected PCFT-mediated transport (109). However, in Wistar rats on a folate-deficient diet that also included succinylsulfathiazole, there were only very modest (~20 and ~50%) increases in *Pcft* and *Rfc* mRNA expression and only an ~30% increase in the transport $V_{\max}$ at pH 5.5 in brush-border membrane vesicles (110).

Methodology that reliably alters PCFT expression in vitro in response to folate restriction would allow for a careful analysis of transcriptional events that reflect the regulatory changes that occur in the response to folate restriction observed in vivo. In one study, there were decreases in *RFC* and *PCFT* message (~30 and ~65%, respectively) by semiquantitative PCR in Caco-2 cells when the folic acid concentration in the growth medium was increased from 0.25 to 100 μ M folic acid. These decreases were accompanied by a small decrease (~50%) in folic acid uptake into these cells at pH 5.5, but there was no change at pH 7.4. Hence, changes in expression were accompanied by changes in function only for PCFT (111). This laboratory has not achieved a consistent and substantial increase in *PCFT* expression with severe folate starvation in either Caco-2 or HeLa-R5 cells in vitro that would inform studies on transcriptional regulation (N. Diop-Bove & I.D. Goldman, unpublished results).

5.2.1.2. Alcohol: Alcoholics are notoriously folate deficient. Although this deficiency has, in part, a nutritional basis, there is substantial evidence from studies in humans, monkeys, and pigs that alcohol results in impaired intestinal folate absorption (112). Many of these studies were done prior to the identification of *PCFT*, and hence alterations in folate absorption and transport into intestinal tissues associated with the ingestion of alcohol were attributed to the changes observed in *RFC* expression. Numerous recent studies demonstrated decreased *Pcft* expression in animals subjected to chronic ingestion of alcohol. Rats subjected to alcohol ingestion for 3 months had a decrease in serum and red-blood-cell folate levels (113, 114). Folate deficiency in those animals was associated with small decreases in intestinal *Pcft* and *Rfc* mRNA expression and with decreased uptake into apical brush-border membranes obtained from intestinal epithelial cells at both pH 5.0 and 7.4, reflecting modest decreases in the activities of both transporters (115). PCFT and RFC were localized to lipid raft microdomains of the membrane vesicles, suggesting that the lipid composition of the membranes altered by chronic alcoholism may affect the distribution of these two folate transporters and, as a result, affect their function (115).

5.2.2. Transcription of *PCFT*—Basic information on the regulatory region of the *PCFT* gene is emerging. The human *PCFT* basal promoter is located within the region 138 to 157 bp upstream of the translation start site in HeLa cells (116, 117). Vitamin D₃, nuclear respiratory factor 1 (NRF1), and Kruppel-like factor 4 (KLF4) have been implicated in the regulation of PCFT expression in Caco-2, HeLa, and HEK293 cells, respectively.

5.2.2.1. Vitamin D₃: Expression of *PCFT* message and low-pH folate transport activity were increased in Caco-2 cells treated with vitamin D₃. There was also an increase in rat duodenal *PCFT* mRNA levels in vivo in response to vitamin D₃ (118). Vitamin D₃ activated the *PCFT* promoter in Caco-2 cells, with an additional increase in the presence of the vitamin D receptor (VDR) partner RXR α (retinoic X receptor α) and its ligand, retinoic acid. At least one vitamin D₃-binding element was identified between -2231 and -1674 bp relative to the transcriptional start site, further refined to between -1694 and -1680, by using PCFT promoter luciferase reporter constructs. Mutation or deletion of that putative binding site (1694 to -1680) resulted in decreased binding of a VDR:RXR α heterodimer and a 50% decrease in PCFT promoter activity. This finding suggested the presence of additional binding elements between -843 and +96 or vitamin D interactions with other, unrelated DNA-binding factors (118). Vitamin D₃ and the VDR have been associated with modulation of the expression of various transporters, such as the sodium-dependent bile acid transporter (119), oligopeptide transporter 1, and the multidrug-resistance-associated proteins (120, 121), are found in the intestine.

Studies in mouse and rat intestines revealed higher mRNA and PCFT and RFC protein levels in the villus tip compared with expression in the crypt base (122, 123). Similarly, as

Caco-2 cells became confluent and differentiated, expression of both transporters increased (122). Although vitamin D₃ promotes differentiation (124, 125), it is uncertain whether the increase in *PCFT* expression that occurs with the differentiation of Caco-2 cells, or in the villus tip versus crypt cells, is related specifically to vitamin D₃ regulation.

5.2.2.2. Nuclear respiratory factor 1: The *PCFT* basal promoter contains three sites for NRF1, a master regulator of mitochondrial biogenesis and respiration (127). Transient expression of a constitutively active NRF1-VP16 fusion protein resulted in *PCFT* promoter activation in HeLa cells, and NRF1 suppression by siRNA resulted in a parallel decrease in *NRF1* and *PCFT* mRNA levels (127). Electrophoretic mobility shift assays using HeLa nuclear extracts and P³²-labeled probes for each NRF1 site contained in the *PCFT* basal promoter indicated specific DNA-protein bands; competition was demonstrated with cold probes, and NRF1 antibodies resulted in a supershift (127). These observations support the concept that NRF1 regulates *PCFT* expression. Mitochondria and mitochondrial damage play an important role in NRF1 expression, and this organelle also plays an important role in one-carbon metabolism (128). Furthermore, folate deficiency in rats has been associated with mitochondrial damage (129). However, it is unclear as to whether, beyond these nonspecific *NRF1* responses to mitochondrial damage, conditions associated with an alteration in *PCFT* expression, as occurs with folate deficiency in the absence of structural damage, are NRF1 mediated or modulated.

The small salutary effects of hypoxia and cadmium chloride on *PCFT* expression and heme and folate transport may be mediated by mitochondrial damage (43, 130, 131). However, in studies from this laboratory, exposure of HeLa or Caco-2 cells to hypoxia sufficient to detect the hypoxia-inducible factor 1 α (HIF1 α) protein did not alter *NRF1* or *PCFT* mRNA levels or folate transport. Moreover, treatment of cells with 10 μ M cadmium chloride, resulting in an ~20-fold increase in heme oxygenase levels, did not alter *PCFT* mRNA expression or transport activity. Likewise, when Caco-2 cells were exposed to cobalt chloride sufficient to detect HIF1 α , there was no change in *PCFT* or *NRF1* mRNA expression (N. Diop-Bove & I.D. Goldman, unpublished data).

5.2.2.3. Kruppel-like factor 4: KLF4 is highly expressed in the differentiated cells at the intestinal villus tip (132) and stimulates *PCFT* promoter activity (133). This stimulation was further enhanced by the coexpression of KLF4 and hepatic nuclear factor 4 α (HNF4 α) in a synergistic manner in HEK293 cells. However, expression of only HNF4 α —another important regulator of many intestinal and hepatic genes, including transporter genes (134–136)—had little effect on *PCFT* promoter activation. The mechanism by which HNF4 α cooperates with KLF4 to enhance *PCFT* promoter activation is unclear. Chromatin immunoprecipitation analysis was consistent with a physical association between KLF4 and the region of –209 to –18 bp relative to the transcriptional start site within the *PCFT* promoter; however, a binding site was not identified (133).

5.2.2.4. DNA methylation: DNA methylation is an important epigenetic factor in *PCFT* expression in cell lines. Hypermethylation of the promoter was responsible, in part, for *PCFT* gene silencing in an antifolate-resistant HeLa-derived cell line, HeLa-R1-11 (116), and in T cell leukemia cell lines (137). In both cases, 5-aza-2'-deoxycytidine, a demethylating agent, partially restored *PCFT* expression and function.

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KEY ABBREVIATIONS

5-FormylTHF	5-formyltetrahydrofolate
5-MethylTHF	5-methyltetrahydrofolate
BCRP	breast cancer resistance protein
MRP	multidrug-resistance-associated protein
OATP	organic anion-transporting polypeptide
PCFT	proton-coupled folate transporter
RFC	reduced folate carrier

LITERATURE CITED

1. Tibbetts AS, Appling DR. Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annu Rev Nutr.* 2010; 30:57–81. [PubMed: 20645850]
2. Salbaum JM, Kappen C. Genetic and epigenomic footprints of folate. *Prog Mol Biol Transl Sci.* 2012; 108:129–58. [PubMed: 22656376]
3. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. *Vitam Horm.* 2008; 79:1–44. [PubMed: 18804690]
4. Diop-Bove, N.; Kronn, D.; Goldman, ID. Hereditary folate malabsorption. In: Pagon, RA.; Bird, TD.; Dolan, CR.; Stephens, K., editors. *GeneReviews*. Seattle: Univ. Wash; 2011.
5. Geller J, Kronn D, Jayabose S, Sandoval C. Hereditary folate malabsorption: family report and review of the literature. *Medicine.* 2002; 81:51–68. [PubMed: 11807405]
6. Mason, JB.; Rosenberg, IH. Intestinal absorption of folate. In: Johnson, LR., editor. *Physiology of the Gastrointestinal Tract*. New York: Raven; 1994. p. 1979-95.
7. Dixon KH, Lanpher BC, Chiu J, Kelley K, Cowan KH. A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. *J Biol Chem.* 1994; 269:17–20. [PubMed: 8276792]
8. Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, et al. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell.* 2006; 127:917–28. [PubMed: 17129779]
9. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. *Expert Rev Mol Med.* 2009; 11:e4. [PubMed: 19173758]
10. Zhao R, Diop-Bove N, Visentin M, Goldman ID. Mechanisms of membrane transport of folates into cells and across epithelia. *Annu Rev Nutr.* 2011; 31:177–201. [PubMed: 21568705]
11. Matherly LH, Hou Z. Structure and function of the reduced folate carrier: a paradigm of a major facilitator superfamily mammalian nutrient transporter. *Vitam Horm.* 2008; 79C:145–84. [PubMed: 18804694]
12. Zhao R, Goldman ID. Folate and thiamine transporters mediated by facilitative carriers (SLC19A1-3 and SLC46A1) and folate receptors. *Mol Aspects Med.* 2013; 34:373–85. [PubMed: 23506878]
13. Goldman ID, Chattopadhyay S, Zhao R, Moran RG. The antifolates: evolution, new agents in the clinic, and how targeting delivery via specific membrane transporters is driving the development of a next generation of folate analogs. *Curr Opin Investig Drugs.* 2010; 11:1409–23.
14. Assaraf YG. The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. *Drug Resist Updat.* 2006; 9:227–46. [PubMed: 17092765]
15. Desmoulin SK, Hou Z, Gangjee A, Matherly LH. The human proton-coupled folate transporter: biology and therapeutic applications to cancer. *Cancer Biol Ther.* 2012; 13:1355–73. [PubMed: 22954694]

16. Rajgopal A, Sierra EE, Zhao R, Goldman ID. Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities. *Am J Physiol Cell Physiol.* 2001; 281:C1579–86. [PubMed: 11600421]
17. Wang Y, Rajgopal A, Goldman ID, Zhao R. Preservation of folate transport activity with a low-pH optimum in rat IEC-6 intestinal epithelial cell lines that lack reduced folate carrier function. *Am J Physiol Cell Physiol.* 2005; 288:C65–71. [PubMed: 15385270]
18. Zhao R, Goldman ID. Resistance to antifolates. *Oncogene.* 2003; 22:7431–57. [PubMed: 14576850]
19. Wang Y, Zhao R, Russell RG, Goldman ID. Localization of the murine reduced folate carrier as assessed by immunohistochemical analysis. *Biochim Biophys Acta.* 2001; 1513:49–54. [PubMed: 11427193]
20. Zhao R, Gao F, Wang Y, Diaz GA, Gelb BD, Goldman ID. Impact of the reduced folate carrier on the accumulation of active thiamin metabolites in murine leukemia cells. *J Biol Chem.* 2000; 276:1114–18. [PubMed: 11038362]
21. Zhao R, Gao F, Goldman ID. Reduced folate carrier transports thiamine monophosphate: an alternative route for thiamine delivery into mammalian cells. *Am J Physiol Cell Physiol.* 2002; 282:C1512–17. [PubMed: 11997266]
22. Visentin M, Zhao R, Goldman ID. Augmentation of reduced folate carrier-mediated transport of folates/antifolates through an antiport mechanism with 5-aminoimidazole-4-carboxamide riboside monophosphate. *Mol Pharmacol.* 2012; 82:209–16. [PubMed: 22554803]
23. Zhao R, Gao F, Hanscom M, Goldman ID. A prominent low-pH methotrexate transport activity in human solid tumor cells: contribution to the preservation of methotrexate pharmacological activity in HeLa cells lacking the reduced folate carrier. *Clin Cancer Res.* 2004; 10:718–27. [PubMed: 14760095]
24. Wang Y, Zhao R, Goldman ID. Characterization of a folate transporter in HeLa cells with a low pH optimum and high affinity for pemetrexed distinct from the reduced folate carrier. *Clin Cancer Res.* 2004; 10:6256–64. [PubMed: 15448015]
25. Zhao R, Hanscom M, Chattopadhyay S, Goldman ID. Selective preservation of pemetrexed pharmacological activity in HeLa cells lacking the reduced folate carrier; association with the presence of a secondary transport pathway. *Cancer Res.* 2004; 64:3313–19. [PubMed: 15126375]
26. Zhao R, Chattopadhyay S, Hanscom M, Goldman ID. Antifolate resistance in a HeLa cell line associated with impaired transport independent of the reduced folate carrier. *Clin Cancer Res.* 2004; 10:8735–42. [PubMed: 15623659]
27. Zhao R, Unal ES, Shin DS, Goldman ID. Membrane topological analysis of the proton-coupled folate transporter (PCFT-SLC46A1) by the substituted cysteine accessibility method. *Biochemistry.* 2010; 49:2925–31. [PubMed: 20225891]
28. Qiu A, Min SH, Jansen M, Malhotra U, Tsai E, et al. Rodent intestinal folate transporters (SLC46A1): secondary structure, functional properties, and response to dietary folate restriction. *Am J Physiol Cell Physiol.* 2007; 293:C1669–78. [PubMed: 17898134]
29. Unal ES, Zhao R, Qiu A, Goldman ID. N-linked glycosylation and its impact on the electrophoretic mobility and function of the human proton-coupled folate transporter (HsPCFT). *Biochim Biophys Acta.* 2008; 1178:1407–14. [PubMed: 18405659]
30. Hou Z, Desmoulin SK, Etnyre E, Olive M, Hsiung B, et al. Identification and functional impact of homo-oligomers of the human proton-coupled folate transporter. *J Biol Chem.* 2012; 287:4982–95. [PubMed: 22179615]
31. Zhao R, Shin DS, Fiser A, Goldman ID. Identification of a functionally critical GXXG motif and its relationship to the folate binding site of the proton-coupled folate transporter (PCFT-SLC46A1). *Am J Physiol Cell Physiol.* 2012; 303:C673–81. [PubMed: 22785121]
32. Umapathy NS, Gnana-Prakasam JP, Martin PM, Mysona B, Dun Y, et al. Cloning and functional characterization of the proton-coupled electrogenic folate transporter and analysis of its expression in retinal cell types. *Investig Ophthalmol Vis Sci.* 2007; 48:5299–305. [PubMed: 17962486]
33. Nakai Y, Inoue K, Abe N, Hatakeyama M, Ohta KY, et al. Functional characterization of human PCFT/HCP1 heterologously expressed in mammalian cells as a folate transporter. *J Pharmacol Exp Ther.* 2007; 322:469–76. [PubMed: 17475902]

34. Unal ES, Zhao R, Chang MH, Fiser A, Romero MF, Goldman ID. The functional roles of the His247 and His281 residues in folate and proton translocation mediated by the human proton-coupled folate transporter SLC46A1. *J Biol Chem*. 2009; 284:17846–57. [PubMed: 19389703]
35. Zhao R, Qiu A, Tsai E, Jansen M, Akabas MH, Goldman ID. The proton-coupled folate transporter (PCFT): impact on pemetrexed transport and on antifolate activities as compared to the reduced folate carrier. *Mol Pharmacol*. 2008; 74:854–62. [PubMed: 18524888]
36. Shin DS, Zhao R, Yap EH, Fiser A, Goldman ID. A P425R mutation of the proton-coupled folate transporter causing hereditary folate malabsorption produces a highly selective alteration in folate binding. *Am J Physiol Cell Physiol*. 2012; 302:C1405–12. [PubMed: 22345511]
37. Shin DS, Zhao R, Fiser A, Goldman DI. Functional roles of the A335 and G338 residues of the proton-coupled folate transporter (PCFT-SLC46A1) mutated in hereditary folate malabsorption. *Am J Physiol Cell Physiol*. 2012; 303:C834–42. [PubMed: 22843796]
38. Wright JE, Vaidya CM, Chen Y, Rosowsky A. Efficient utilization of the reduced folate carrier in CCRF-CEM human leukemic lymphoblasts by the potent antifolate N^Q -(4-amino-4-deoxypteroyl)- N^5 -hemiphthaloyl-L-ornithine (PT523) and its B-ring analogues. *Biochem Pharmacol*. 2000; 60:41–46. [PubMed: 10807943]
39. Inoue K, Nakai Y, Ueda S, Kamigaso S, Ohta KY, et al. Functional characterization of PCFT/HCP1 as the molecular entity of the carrier-mediated intestinal folate transport system in the rat model. *Am J Physiol Gastrointest Liver Physiol*. 2008; 294:G660–68. [PubMed: 18174275]
40. Zhao R, Min SH, Qiu A, Sakaris A, Goldberg GL, et al. The spectrum of mutations in the PCFT gene, coding for an intestinal folate transporter, that are the basis for hereditary folate malabsorption. *Blood*. 2007; 110:1147–52. [PubMed: 17446347]
41. Mahadeo K, Diop-Bove N, Ramirez SI, Cadilla CL, Rivera E, et al. Prevalence of a loss-of-function mutation in the proton-coupled folate transporter gene (PCFT-SLC46A1) causing hereditary folate malabsorption in Puerto Rico. *J Pediatr*. 2011; 159:623–27. [PubMed: 21489556]
42. Salojin KV, Cabrera RM, Sun W, Chang WC, Lin C, et al. A mouse model of hereditary folate malabsorption: Deletion of the PCFT gene leads to systemic folate deficiency. *Blood*. 2011; 117:4895–904. [PubMed: 21346251]
- 42a. Wang X, Cabrera RM, Li Y, Miller DS, Finnell RH. Functional regulation of P-glycoprotein at the blood-brain barrier in proton-coupled folate transporter (PCFT) mutant mice. *FASEB J*. 2013; 27:1167–75. [PubMed: 23212123]
43. Shayeghi M, Latunde-Dada GO, Oakhill JS, Laftah AH, Takeuchi K, et al. Identification of an intestinal heme transporter. *Cell*. 2005; 122:789–801. [PubMed: 16143108]
44. Andrews NC. When is a heme transporter not a heme transporter? When it's a folate transporter. *Cell Metab*. 2007; 5:5–6. [PubMed: 17189201]
45. McEwan GT, Lucas ML, Denvir M, Raj M, McColl KE, et al. A combined TDDA-PVC pH and reference electrode for use in the upper small intestine. *J Med Eng Technol*. 1990; 14:16–20. [PubMed: 2342080]
46. Said HM, Smith R, Redha R. Studies on the intestinal surface acid microclimate: developmental aspects. *Pediatr Res*. 1987; 22:497–99. [PubMed: 3684377]
47. Liu M, Ge Y, Cabelof DC, Aboukameel A, Heydari AR, et al. Structure and regulation of the murine reduced folate carrier gene: identification of four noncoding exons and promoters and regulation by dietary folates. *J Biol Chem*. 2005; 280:5588–97. [PubMed: 15579899]
48. Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther*. 2003; 306:703–8. [PubMed: 12724351]
49. Visentin M, Chang MH, Romero MF, Zhao R, Goldman ID. Substrate- and pH-specific antifolate transport mediated by organic anion-transporting polypeptide 2B1 (OATP2B1-SLCO2B1). *Mol Pharmacol*. 2012; 81:134–42. [PubMed: 22021325]
50. Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, Stieger B. Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. *Am J Physiol Cell Physiol*. 2009; 296:C570–82. [PubMed: 19129463]

51. Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther.* 2004; 308:438–45. [PubMed: 14610227]
52. Li L, Sham YY, Bikadi Z, Elmquist WF. pH-dependent transport of pemetrexed by breast cancer resistance protein. *Drug Metab Dispos.* 2011; 39:1478–85. [PubMed: 21628496]
53. Nozawa T, Nakajima M, Tamai I, Noda K, Nezu J, et al. Genetic polymorphisms of human organic anion transporters OATP-C (SLC21A6) and OATP-B (SLC21A9): allele frequencies in the Japanese population and functional analysis. *J Pharmacol Exp Ther.* 2002; 302:804–13. [PubMed: 12130747]
54. Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, et al. Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. *Gastroenterology.* 2006; 130:1793–806. [PubMed: 16697742]
55. Nakanishi T, Tamai I. Genetic polymorphisms of OATP transporters and their impact on intestinal absorption and hepatic disposition of drugs. *Drug Metab Pharmacokinet.* 2012; 27:106–21. [PubMed: 22185815]
56. Laitinen A, Niemi M. Frequencies of single-nucleotide polymorphisms of *SLCO1A2*, *SLCO1B3* and *SLCO2B1* genes in a Finnish population. *Basic Clin Pharmacol Toxicol.* 2011; 108:9–13. [PubMed: 20560925]
57. Mougey EB, Feng H, Castro M, Irvin CG, Lima JJ. Absorption of montelukast is transporter mediated: A common variant of OATP2B1 is associated with reduced plasma concentrations and poor response. *Pharmacogenet Genomics.* 2009; 19:129–38. [PubMed: 19151602]
58. Darcy-Vrillon B, Selhub J, Rosenberg IH. Analysis of sequential events in intestinal absorption of folylpolyglutamate. *Am J Physiol Gastrointest Liver Physiol.* 1988; 255:G361–66.
59. Halsted CH. The intestinal absorption of dietary folates in health and disease. *J Am Coll Nutr.* 1989; 8:650–58. [PubMed: 2695555]
60. Shafizadeh TB, Halsted CH. γ -Glutamyl hydrolase, not glutamate carboxypeptidase II, hydrolyzes dietary folate in rat small intestine. *J Nutr.* 2007; 137:1149–53. [PubMed: 17449573]
61. Kusuhara H, Han YH, Shimoda M, Kokue E, Suzuki H, Sugiyama Y. Reduced folate derivatives are endogenous substrates for cMOAT in rats. *Am J Physiol Gastrointest Liver Physiol.* 1998; 275:G789–96.
62. Fetsch PA, Abati A, Litman T, Morisaki K, Honjo Y, et al. Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues. *Cancer Lett.* 2006; 235:84–92. [PubMed: 15990223]
63. Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* 2001; 61:3458–64. [PubMed: 11309308]
64. Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res.* 2001; 61:7225–32. [PubMed: 11585759]
65. Hooijberg JH, Peters GJ, Assaraf YG, Kathmann I, Priest DG, et al. The role of multidrug resistance proteins MRP1, MRP2 and MRP3 in cellular folate homeostasis. *Biochem Pharmacol.* 2003; 65:765–71. [PubMed: 12628490]
66. Kitamura Y, Hirouchi M, Kusuhara H, Schuetz JD, Sugiyama Y. Increasing systemic exposure of methotrexate by active efflux mediated by multidrug resistance-associated protein 3 (Mrp3/*Abcc3*). *J Pharmacol Exp Ther.* 2008; 327:465–73. [PubMed: 18719291]
67. Kitamura Y, Kusuhara H, Sugiyama Y. Basolateral efflux mediated by multidrug resistance-associated protein 3 (Mrp3/*Abcc3*) facilitates intestinal absorption of folates in mouse. *Pharm Res.* 2010; 27:665–72. [PubMed: 20148291]
68. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. *Annu Rev Biochem.* 2002; 71:537–92. [PubMed: 12045106]
69. Wielinga P, Hooijberg JH, Gunnarsdottir S, Kathmann I, Reid G, et al. The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. *Cancer Res.* 2005; 65:4425–30. [PubMed: 15899835]

70. König J, Cui Y, Nies AT, Keppler D. A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol Gastrointest Liver Physiol*. 2000; 278:G156–64. [PubMed: 10644574]
71. Abe T, Kakyo M, Tokui T, Nakagomi R, Nishio T, et al. Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. *J Biol Chem*. 1999; 274:17159–63. [PubMed: 10358072]
72. Abe T, Unno M, Onogawa T, Tokui T, Kondo TN, et al. LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. *Gastroenterology*. 2001; 120:1689–99. [PubMed: 11375950]
73. Naba H, Kuwayama C, Kakinuma C, Ohnishi S, Ogihara T. Eisai hyperbilirubinemic rat (EHBR) as an animal model affording high drug-exposure in toxicity studies on organic anions. *Drug Metab Pharmacokinet*. 2004; 19:339–51. [PubMed: 15548845]
74. Vlaming ML, van Esch A, van de Steeg E, Pala Z, Wagenaar E, et al. Impact of Abcc2 [multidrug resistance-associated protein (Mrp) 2], Abcc3 (Mrp3), and Abcg2 (breast cancer resistance protein) on the oral pharmacokinetics of methotrexate and its main metabolite 7-hydroxymethotrexate. *Drug Metab Dispos*. 2011; 39:1338–44. [PubMed: 21566011]
75. Chen C, Scott D, Hanson E, Franco J, Berryman E, et al. Impact of Mrp2 on the biliary excretion and intestinal absorption of furosemide, probenecid, and methotrexate using Eisai hyperbilirubinemic rats. *Pharm Res*. 2003; 20:31–37. [PubMed: 12608533]
76. Tanaka T, Uchiumi T, Hinoshita E, Inokuchi A, Toh S, et al. The human multidrug resistance protein 2 gene: functional characterization of the 5'-flanking region and expression in hepatic cells. *Hepatology*. 1999; 30:1507–12. [PubMed: 10573531]
77. Rau T, Erney B, Gores R, Eschenhagen T, Beck J, Langer T. High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. *Clin Pharmacol Ther*. 2006; 80:468–76. [PubMed: 17112803]
78. Simon N, Marsot A, Villard E, Choquet S, Khe HX, et al. Impact of ABCC2 polymorphisms on high-dose methotrexate pharmacokinetics in patients with lymphoid malignancy. *Pharmacogenomics J*. 2013; 13:507–13. [PubMed: 23069858]
79. Masuda M, Iizuka Y, Yamazaki M, Nishigaki R, Kato Y, et al. Methotrexate is excreted into the bile by canalicular multispecific organic anion transporter in rats. *Cancer Res*. 1997; 57:3506–10. [PubMed: 9270020]
80. Hulot JS, Villard E, Maguy A, Morel V, Mir L, et al. A mutation in the drug transporter gene *ABCC2* associated with impaired methotrexate elimination. *Pharmacogenet Genomics*. 2005; 15:277–85. [PubMed: 15864128]
81. Vlaming ML, Pala Z, van EA, Wagenaar E, de Waart DR, et al. Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate in vivo. *Clin Cancer Res*. 2009; 15:3084–93. [PubMed: 19383815]
82. Ifergan I, Shafran A, Jansen G, Hooijberg JH, Scheffer GL, Assaraf YG. Folate deprivation results in the loss of breast cancer resistance protein (BCRP/ABCG2) expression: a role for BCRP in cellular folate homeostasis. *J Biol Chem*. 2004; 279:25527–34. [PubMed: 15047700]
83. Breedveld P, Pluim D, Cipriani G, Dahlhaus F, van Eijndhoven MA, et al. The effect of low pH on breast cancer resistance protein (ABCG2)-mediated transport of methotrexate, 7-hydroxymethotrexate, methotrexate diglutamate, folic acid, mitoxantrone, topotecan, and resveratrol in in vitro drug transport models. *Mol Pharmacol*. 2007; 71:240–49. [PubMed: 17032904]
84. Tamura A, Watanabe M, Saito H, Nakagawa H, Kamachi T, et al. Functional validation of the genetic polymorphisms of human ATP-binding cassette (ABC) transporter ABCG2: identification of alleles that are defective in porphyrin transport. *Mol Pharmacol*. 2006; 70:287–96. [PubMed: 16608919]
85. Hagenbuch B, Stieger B. The SLCO (former SLC21) superfamily of transporters. *Mol Aspects Med*. 2013; 34:396–412. [PubMed: 23506880]
86. Hagenbuch B. Drug uptake systems in liver and kidney: a historic perspective. *Clin Pharmacol Ther*. 2010; 87:39–47. [PubMed: 19924123]

87. Hagenbuch B, Gui C. Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica*. 2008; 38:778–801. [PubMed: 18668430]
88. van de SE, van der Kruijssen CM, Wagenaar E, Burggraaff JE, Mesman E, et al. Methotrexate pharmacokinetics in transgenic mice with liver-specific expression of human organic anion-transporting polypeptide 1B1 (*SLCO1B1*). *Drug Metab Dispos*. 2009; 37:277–81. [PubMed: 19022939]
89. Trevino LR, Shimasaki N, Yang W, Panetta JC, Cheng C, et al. Germline genetic variation in an organic anion transporter polypeptide associated with methotrexate pharmacokinetics and clinical effects. *J Clin Oncol*. 2009; 27:5972–78. [PubMed: 19901119]
90. Ramsey LB, Panetta JC, Smith C, Yang W, Fan Y, et al. Genome-wide study of methotrexate clearance replicates *SLCO1B1*. *Blood*. 2013; 121:898–904. [PubMed: 23233662]
91. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. *Hepatology*. 2003; 38:374–84. [PubMed: 12883481]
92. Chen ZS, Lee K, Walther S, Raftogianis RB, Kuwano M, et al. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. *Cancer Res*. 2002; 62:3144–50. [PubMed: 12036927]
93. Horne DW, Reed KA, Hoefs J, Said HM. 5-Methyltetrahydrofolate transport in basolateral membrane vesicles from human liver. *Am J Clin Nutr*. 1993; 58:80–84. [PubMed: 8317394]
94. Arias IM, Forgac M. The sinusoidal domain of the plasma membrane of rat hepatocytes contains an amiloride-sensitive Na^+/H^+ antiport. *J Biol Chem*. 1984; 259:5406–8. [PubMed: 6325437]
95. Dudeja PK, Torania SA, Said HM. Evidence for the existence of a carrier-mediated folate uptake mechanism in human colonic luminal membranes. *Am J Physiol Gastrointest Liver Physiol*. 1997; 272:G1408–15.
96. Kumar CK, Moyer MP, Dudeja PK, Said HM. A protein-tyrosine kinase-regulated, pH-dependent, carrier-mediated uptake system for folate in human normal colonic epithelial cell line NCM460. *J Biol Chem*. 1997; 272:6226–31. [PubMed: 9045638]
97. Rong N, Selhub J, Goldin BR, Rosenberg IH. Bacterially synthesized folate in rat large intestine is incorporated into host tissue folyl polyglutamates. *J Nutr*. 1991; 121:1955–59. [PubMed: 1941259]
98. Asrar FM, O'Connor DL. Bacterially synthesized folate and supplemental folic acid are absorbed across the large intestine of piglets. *J Nutr Biochem*. 2005; 16:587–93. [PubMed: 16081276]
99. Aufreiter S, Gregory JF III, Pfeiffer CM, Fazili Z, Kim YI, et al. Folate is absorbed across the colon of adults: evidence from cecal infusion of ^{13}C -labeled [6S]-5-formyltetrahydrofolic acid. *Am J Clin Nutr*. 2009; 90:116–23. [PubMed: 19439459]
100. Evans DF, Pye G, Bramley R, Clark AG, Dyson TJ, Hardcastle JD. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. *Gut*. 1988; 29:1035–41. [PubMed: 3410329]
101. McDougall CJ, Wong R, Scudera P, Lesser M, DeCosse JJ. Colonic mucosal pH in humans. *Dig Dis Sci*. 1993; 38:542–45. [PubMed: 8444087]
102. Camilo E, Zimmerman J, Mason JB, Golner B, Russell R, et al. Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. *Gastroenterology*. 1996; 110:991–98. [PubMed: 8613033]
103. Zhao R, Visentin M, Suadicani SO, Goldman ID. Inhibition of the proton-coupled folate transporter (PCFT-SLC46A1) by bicarbonate and other anions. *Mol Pharmacol*. 2013; 84:95–103. [PubMed: 23609145]
104. Urquhart BL, Gregor JC, Chande N, Knauer MJ, Tirona RG, Kim RB. The human proton-coupled folate transporter (hPCFT): modulation of intestinal expression and function by drugs. *Am J Physiol Gastrointest Liver Physiol*. 2010; 298:G248–54. [PubMed: 19762432]
105. Halsted CH, Gandhi G, Tamura T. Sulfasalazine inhibits the absorption of folates in ulcerative colitis. *N Engl J Med*. 1981; 305:1513–17. [PubMed: 6117796]
106. Inoue K, Nakai Y, Ueda S, Kamigaso S, Ohta KY, et al. Functional characterization of PCFT/HCP1 as the molecular entity of the carrier-mediated intestinal folate transport system in the rat model. *Am J Physiol Gastrointest Liver Physiol*. 2008; 294:G660–68. [PubMed: 18174275]

107. Russell RM, Golner BB, Krasinski SD, Sadowski JA, Suter PM, Braun CL. Effect of antacid and H₂ receptor antagonists on the intestinal absorption of folic acid. *J Lab Clin Med.* 1988; 112:458–63. [PubMed: 2902178]
108. Russell RM, Dhar GJ, Dutta SK, Rosenberg IH. Influence of intraluminal pH on folate absorption: studies in control subjects and in patients with pancreatic insufficiency. *J Lab Clin Med.* 1979; 93:428–36. [PubMed: 34665]
109. Said HM, Chatterjee N, Haq RU, Subramanian VS, Ortiz A, et al. Adaptive regulation of intestinal folate uptake: effect of dietary folate deficiency. *Am J Physiol Cell Physiol.* 2000; 279:C1889–95. [PubMed: 11078704]
110. Wani NA, Thakur S, Kaur J. Mechanism of intestinal folate transport during folate deficiency in rodent model. *Indian J Med Res.* 2012; 136:758–65. [PubMed: 23287122]
111. Ashokkumar B, Mohammed ZM, Vaziri ND, Said HM. Effect of folate oversupplementation on folate uptake by human intestinal and renal epithelial cells. *Am J Clin Nutr.* 2007; 86:159–66. [PubMed: 17616776]
112. Halsted CH, Villanueva JA, Devlin AM, Chandler CJ. Metabolic interactions of alcohol and folate. *J Nutr.* 2002; 132:S2367–72.
113. Hamid A, Kaur J, Mahmood A. Evaluation of the kinetic properties of the folate transport system in intestinal absorptive epithelium during experimental ethanol ingestion. *Mol Cell Biochem.* 2007; 304:265–71. [PubMed: 17530184]
114. Hamid A, Wani NA, Rana S, Vaiphei K, Mahmood A, Kaur J. Down-regulation of reduced folate carrier may result in folate malabsorption across intestinal brush border membrane during experimental alcoholism. *FEBS J.* 2007; 274:6317–28. [PubMed: 18005257]
115. Wani NA, Kaur J. Reduced levels of folate transporters (PCFT and RFC) in membrane lipid rafts result in colonic folate malabsorption in chronic alcoholism. *J Cell Physiol.* 2010; 226:579–87. [PubMed: 21069807]
116. Diop-Bove NK, Wu J, Zhao R, Locker J, Goldman ID. Hypermethylation of the human proton-coupled folate transporter (SLC46A1) minimal transcriptional regulatory region in an antifolate-resistant HeLa cell line. *Mol Cancer Ther.* 2009; 8:2424–31. [PubMed: 19671745]
117. Stark M, Gonen N, Assaraf YG. Functional elements in the minimal promoter of the human proton-coupled folate transporter. *Biochem Biophys Res Commun.* 2009; 388:79–85. [PubMed: 19643086]
118. Eloranta JJ, Zair ZM, Hiller C, Hausler S, Stieger B, Kullak-Ublick GA. Vitamin D₃ and its nuclear receptor increase the expression and activity of the human proton-coupled folate transporter. *Mol Pharmacol.* 2009; 76:1062–71. [PubMed: 19666701]
119. Chen X, Chen F, Liu S, Glaeser H, Dawson PA, et al. Transactivation of rat apical sodium-dependent bile acid transporter and increased bile acid transport by 1 α ,25-dihydroxyvitamin D₃ via the vitamin D receptor. *Mol Pharmacol.* 2006; 69:1913–23. [PubMed: 16481392]
120. Maeng HJ, Durk MR, Chow EC, Ghoneim R, Pang KS. 1 α ,25-Dihydroxyvitamin D₃ on intestinal transporter function: studies with the rat everted intestinal sac. *Biopharm Drug Dispos.* 2011; 32:112–25. [PubMed: 21341280]
121. McCarthy TC, Li X, Sinal CJ. Vitamin D receptor-dependent regulation of colon multidrug resistance-associated protein 3 gene expression by bile acids. *J Biol Chem.* 2005; 280:23232–42. [PubMed: 15824121]
122. Subramanian VS, Reidling JC, Said HM. Differentiation-dependent regulation of the intestinal folate uptake process: studies with Caco-2 cells and native mouse intestine. *Am J Physiol Cell Physiol.* 2008; 295:C828–35. [PubMed: 18650265]
123. Dev S, Ahmad WN, Kaur J. Regulatory mechanisms of intestinal folate uptake in a rat model of folate oversupplementation. *Br J Nutr.* 2011; 105:827–35. [PubMed: 21092376]
124. Suda T, Shinki T, Takahashi N. The role of vitamin D in bone and intestinal cell differentiation. *Annu Rev Nutr.* 1990; 10:195–211. [PubMed: 2200463]
125. Halline AG, Davidson NO, Skarosi SF, Sitrin MD, Tietze C, et al. Effects of 1,25-dihydroxyvitamin D₃ on proliferation and differentiation of Caco-2 cells. *Endocrinology.* 1994; 134:1710–17. [PubMed: 8137734]
126. Deleted in proof

127. Gonen N, Assaraf YG. The obligatory intestinal folate transporter PCFT (SLC46A1) is regulated by nuclear respiratory factor 1. *J Biol Chem*. 2010; 285:33602–13. [PubMed: 20724482]
128. Pike ST, Rajendra R, Artzt K, Appling DR. Mitochondrial C₁-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. *J Biol Chem*. 2010; 285:4612–20. [PubMed: 19948730]
129. Kim JM, Lee H, Chang N. Hyperhomocysteinemia due to short-term folate deprivation is related to electron microscopic changes in the rat brain. *J Nutr*. 2002; 132:3418–21. [PubMed: 12421861]
130. Laftah AH, Latunde-Dada GO, Fakhri S, Hider RC, Simpson RJ, McKie AT. Haem and folate transport by proton-coupled folate transporter/haem carrier protein 1 (SLC46A1). *Br J Nutr*. 2009; 101:1150–56. [PubMed: 18782461]
131. Latunde-Dada GO, Takeuchi K, Simpson RJ, McKie AT. Haem carrier protein 1 (HCP1): expression and functional studies in cultured cells. *FEBS Lett*. 2006; 580:6865–70. [PubMed: 17156779]
132. Flandez M, Guilmeau S, Blache P, Augenlicht LH. KLF4 regulation in intestinal epithelial cell maturation. *Exp Cell Res*. 2008; 314:3712–23. [PubMed: 18977346]
133. Furumiya M, Inoue K, Ohta K, Hayashi Y, Yuasa H. Transcriptional regulation of PCFT by KLF4, HNF4 α , CDX2 and C/EBP α : implication in its site-specific expression in the small intestine. *Biochem Biophys Res Commun*. 2013; 431:158–63. [PubMed: 23313509]
134. Trauner M, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev*. 2003; 83:633–71. [PubMed: 12663868]
135. Klein K, Kullak-Ublick GA, Wagner M, Trauner M, Eloranta JJ. Hepatocyte nuclear factor-4 α and bile acids regulate human concentrative nucleoside transporter-1 gene expression. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:G936–47. [PubMed: 19228884]
136. Knauer MJ, Girdwood AJ, Kim RB, Tirona RG. Transport function and transcriptional regulation of a liver-enriched human organic anion transporting polypeptide 2B1 transcriptional start site variant. *Mol Pharmacol*. 2013; 83:1218–28. [PubMed: 23531488]
137. Gonen N, Bram EE, Assaraf YG. PCFT promoter methylation and restoration of gene expression in human leukemia cells. *Biochem Biophys Res Commun*. 2008; 376:787–92. [PubMed: 18817749]
138. Shin DS, Mahadeo K, Min SH, Diop-Bove N, Clayton P, et al. Identification of novel mutations in the proton-coupled folate transporter (PCFT-SLC46A1) associated with hereditary folate malabsorption. *Mol Genet Metab*. 2011; 103:33–37. [PubMed: 21333572]

FUTURE ISSUES

A number of important questions can be addressed now that there is a mouse model of hereditary folate malabsorption in which *Pcft* has been inactivated. Other unresolved issues will require different experimental approaches.

1. What is the mechanism(s) of folate absorption across the apical brush-border membrane of the intestine in the absence of PCFT? What role do **RFC** and potentially other transporters play? Where are folates absorbed within the large and small intestines in the absence of PCFT?
2. Which multidrug resistance-associated protein(s) is required for transport of folates across the serosal membrane of the human intestine?
3. What is the role of PCFT and RFC in folate transport in hepatocytes?
4. To what extent is the expression of organic anion transporters and the multidrug-resistance-associated proteins with folate transport capabilities changed in folate-deficient, *Pcft*-null mice?
5. What is the impact of vitamin D on the regulation of PCFT expression in the small intestines of folate-deficient mice?
6. Further characterization of the transcriptional regulation of *PCFT* expression awaits the development of a reliable in vitro model of regulation.
7. What is the role of NRF1 in the regulation of *PCFT* expression in vivo? Which signals control the expression of this factor?

Figure 1.

The confirmed secondary structure of the proton-coupled folate transporter. The sites of mutations identified in subjects with hereditary folate malabsorption are shown. Modified from Reference 138.

Figure 2.

Distinguishing between reduced folate carrier (RFC)- and proton-coupled folate transporter (PCFT)-mediated transport into rat small intestinal epithelial cells. (a) Influx of [^3H]methotrexate (MTX) as a function of pH. MTX has a high affinity for both PCFT, at low pH, and RFC, at neutral pH. The graph clearly distinguishes between the pH optima for these two processes, as indicated by the vertical lines. (b) Influx of [^3H]folic acid as a function of pH. Folic acid has a high affinity for PCFT at low pH but a very low affinity for RFC at neutral pH. In this case, only the low pH activity is seen; transport at neutral pH is too low to be detected. Panels a and b illustrate the minimal overlap between these processes. (c) Influx of 0.5 μM MTX in the presence and absence of 20 μM folic acid at pH 5.5. At low pH, at which transport is mediated solely by PCFT, for which folic acid has high affinity, folic acid abolishes influx of MTX. (d) Influx of 0.5 μM MTX in the presence and absence of 20 μM folic acid at pH 7.4. Because MTX influx at pH 7.4 is mediated solely by RFC, and folic acid has a very low affinity for this transporter, folic acid has no impact on MTX influx. The data are from Reference 16.

Figure 3.

The intestinal absorption and enterohepatic circulation of folates. Shown are the transporters that are expressed in the apical brush-border and basolateral membranes of the enterocyte and that impact intestinal folate absorption. The primary role for the proton-coupled folate transporter (PCFT) in transport across the apical brush-border membrane of the enterocyte has been validated on the basis of the consequences of the loss-of-function mutations in this transporter in humans and mice that ingest physiological amounts of folates. Also shown is the enterohepatic circulation as folates from the intestine are delivered via the hepatic portal vein, and as systemic folates are delivered via the hepatic artery, to the hepatic sinusoid. From there, folates reach the systemic circulation via the hepatic veins or are transported into hepatocytes at the basolateral membrane. Folates are then stored as polyglutamate derivatives or are transported into the biliary system at the canalicular membrane. PCFT is expressed in the basolateral membrane, but its function there is not clear. RFC, an organic phosphate (OP^-) antiporter, is also expressed in hepatocytes, but its location and function have not been established. The acidic pH at the mucosal surface is generated by Na^+/H^+ exchangers.