Bridging epigenetics and metabolism
Role of non-essential amino acids

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Recent research suggests that chromatin-modifying enzymes are metabolic sensors regulating gene expression. Epigenetics is linked to metabolomics in response to the cellular microenvironment. Specific metabolites involved in this sensing mechanism include S-adenosylmethionine, acetyl-CoA, alphaketoglutarate and NAD+. Although the core metabolic pathways involving glucose have been emphasized as the source of these metabolites, the reprogramming of pathways involving non-essential amino acids may also play an important role, especially in cancer. Examples include metabolic pathways for glutamine, serine and glycine. The coupling of these pathways to the intermediates affecting epigenetic regulation occurs by “parametabolic” mechanisms. The metabolism of proline may play a special role in this parametabolic linkage between metabolism and epigenetics. Both proline degradation and biosynthesis are robustly affected by oncogenes or suppressor genes, and they can modulate intermediates involved in epigenetic regulation. A number of mechanisms in a variety of animal species have been described by our laboratory and by others. The challenge we now face is to identify the specific chromatin-modifying enzymes involved in coupling of proline metabolism to altered reprogramming of gene expression.

Introduction

Over the past 30 years, the genetic basis of cancer has been firmly established, from Knudson’s two-hit hypothesis to the identification of numerous specific mutations characterized as oncogenes and suppressor genes.2,3 The clinical application of these seminal discoveries, however, has been disappointing. Attempts to repair or replace mutated genes, by “gene therapy” have been hindered not only by technical difficulties in delivery, but, more importantly, by the redundancy and plasticity of the cancer genome.3 In this regard, the discoveries in cancer epigenetics offer new opportunities and strategies.4-6 For this Point-of-View, epigenetic mechanisms refer to those structural and enzymatic interactions that regulate transcription independently of the DNA primary sequence. We will focus on the links between epigenetics and the metabolic mechanisms mediating adjustments to the tumor microenvironment.

The expression of genes is modulated by the interaction between DNA and chromatin, including acetylation of histones and methylation of both DNA and histones. A number of excellent reviews describe the families of histone and DNA methylases,7 and the histone acetylases and deacetylases.8 Thus, cancer genetics not only depends on mutations in the primary sequence of oncogenes and suppressor genes, but also on subsequent gene regulation. Importantly, the expression of genes downstream from oncogenes and suppressor genes are ultimately dependent on these regulated modifications of DNA and histones.

Although mutations have been found in the genes encoding DNA/histone modifying enzymes,6 targeting them for repair or replacement faces the same obstacles as therapy directed...
against primary oncogenes or suppressor genes. Importantly, as pointed out in recent reviews, the modulation of the epigenome by these enzymes depends on the level of metabolic intermediates and cofactors. They could be considered a minute-by-minute sensing mechanism of the cellular metabolic state. Examples of these linkages include the metabolism-dependent fluctuations in acetyl CoA, the substrate for histone acetyltransferases. On the other hand, sirtuins remove acetyl groups from lysine in histones requiring NAD+, a sensitive and dynamic indicator of energy metabolism. Methylation of both DNA and histones by their respective methyltransferases requires 1-carbon transfers from S-adenosylmethionine, the substrate for histone acetyltransferases.

The emphasis on the core metabolic pathways for glucose and glutamine into pathways generating substrates (amino acids, ribonucleotides and lipids) for building cell mass is a sensitive and dynamic indicator of energy metabolism. Methylation of both DNA and histones by their respective methyltransferases requires 1-carbon transfers from S-adenosylmethionine, whereas TET2- and JMDH2-mediated demethylation depends, in large part, on α-ketoglutarate (α-KG) as substrate for α-KG dioxygenases for oxidizing methionine to hydroxymethyl-cysteine. Thus, epigenetic enzymes not only depend on metabolites but they regulate genes which reprogram metabolism.

About 15 years ago, the resurgence of research interest led to the identification of reprogramming in metabolism in cancer. These metabolic changes are not due to so-called “passenger genes” but, instead, are the integrated responses to “driver genes” such as c-MYC and PI3K/PTEN. The constellation of metabolic changes in cancer cells includes aerobic glycolysis first described by Warburg, changes in TCA cycle function, activation of the pentose phosphate pathway and the “addiction” to glutamine. An attractive explanation is the rerouting of glucose and glutamine into pathways generating substrates (amino acids, ribonucleotides and lipids) for building cell mass. Pyruvate is diverted to lactate to recycle NADH to NAD+ for glycolysis. Pharmacologic intervention of reprogrammed metabolic pathways starves tumor cells and halts tumor progression. However, the success of this strategy was limited presumably because these core pathways have numerous compensatory mechanisms, including the network of endocrine factors providing homeostatic compensation.

**Non-Essential Amino Acids**

The emphasis on the core metabolic pathways for glucose and glutamine metabolism in tumor cells is understandable since “addiction” to glucose and glutamine has been demonstrated. However, recent discoveries that a number of non-essential amino acids play a critical role in cancer metabolism deserve consideration. Non-essential amino acids (NEAA) may not be important nutritionally, but may make critical contributions to metabolism.

Prokaryotes can synthesize every proteinogenic amino acid but, as organisms evolved to consume other organisms, this biosynthetic capability is no longer necessary. Certain biosynthetic pathways are evolutionarily conserved because they preserve other necessary metabolic functions. For example, the biosynthesis of arginine is involved in the incorporation of ammonia into urea and the biosynthesis of cysteine is a product in the degradation of homocysteine, an important component of the pathway for generating S-adenosylmethionine. Alanine, aspartic acid and glutamic acid are amino acid analogs of metabolically important α-keto-acids.

Recently, the syntheses of glycine and serine have been identified as essential for tumor growth. Glutamine is critical for de novo purine and pyrimidine synthesis as well as a source of TCA cycle intermediates. In the physiologic setting, glutamine is the currency for transferring carbons and nitrogens from proteolysis to central tissues for further carbon and nitrogen processing. The tiny genome of Cryptosporidium, a protozoan parasite found in human intestine, lacks genes for the TCA cycle and biosynthetic pathways for sugars and nucleotides, yet it has the synthetic pathways for asparagine, glutamine, glycine and proline. In fact, proline biosynthesis is retained from S. cerevisiae, D. discoideum to H. sapiens.

The functions of these aforementioned NEAA are recognized, but “the utility of the pathway to proline is not immediately obvious.” However, work from our laboratory has shown important functions for proline metabolism to explain the basis for its evolutionary conservation.

To understand the regulatory functions of NEAA, we must introduce a “parametabolic” paradigm (Fig. 1). For most biosynthetic pathways, the enzyme is regulated to produce a specific product from a precursor. However, for those in the pathways for NEAA, the specific AA is the byproduct of coupled reactions. An example of this is in the serine metabolic pathway, where the conversion of serine and glycine transfers a carbon group to tetrahydrofolate and 5-methyltetrahydrofolate regenerates methionine from homocysteine. In an ATP-dependent reaction, methionine is converted to S-adenosylmethionine, which is the principal methyl donor for biosynthesis of a variety of intermediates as well as the methylation of DNA.
reducing potential across mitochondrial membranes; for example, the malate-aspartate shuttle.29

Acetylation of histones is dependent on the availability of acetyl-CoA, and levels of acetyl-CoA can fluctuate as much as 10-fold;11,30 its metabolism primarily reflects the abundance of glucose and the flux through glucose metabolic pathways. On the other hand, the production of serine from 3-phosphoglycerate is promoted by the shift of pyruvate kinase isozymes from PK to PKM2.19,31 The latter has much lower catalytic activity, allowing the buildup of pyrrolidine ring, proline is not a substrate for generic amino acid metabolizing enzymes. Thus, the augmented production of serine would accommodate the substrates for production of SAM and methylation. These observations suggest that for some of the NEAA, their metabolic pathway serves as a parametabolic link between metabolism and epigenetics.

The Regulatory Role of Proline Metabolism

An intriguing question is whether the metabolism of the non-essential amino acid proline also plays such a parametabolic role and whether the proline regulatory axis plays a role not only in the metabolism of core substrates, such as glucose or glutamine, but also in regulating epigenetics. With its α-nitrogen sequestered within a pyrrolidine ring, proline is not a substrate for generic amino acid metabolizing enzymes. Instead, it has its own metabolic system, which has been recently reviewed.32,33 Of special interest, proline dehydrogenase (oxidase) is bound to mitochondrial inner membranes. Although the catalytic product of the reaction pyrroline-5-carboxylate (P5C) can be sequentially converted to glutamate and α-KG, an epigenetic metabolite, electrons from proline are donated to the mitochondrial electron transport chain accompanying the production of P5C.34 In addition, this enzyme is under diverse regulation making it an ideal candidate as an epigenetic regulator. It is robustly and rapidly induced by p5335-37 and by PPARγ38 leading to ROS generation,39,40 cell cycle arrest,41 apoptosis39,42 and, depending on the cellular context, prosurvival autophagy.43 The enzyme is upregulated by AMPK3,44 but downregulated by a specific microRNA, miR-23b*,45 which in turn is modulated by c-MYC.46

Table 1. Proline axis: regulatory effects on metabolic reprogramming and epigenetics

| Pathway       | Enzyme       | Regulation                                      | Metabolic effect                                      | Clinical, cellular findings epigenetic mechanism                  |
|---------------|--------------|-------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------------------|
| Degradative   | PRODH (POX)  | Increased by AMPK4,44 under glucose deprivation | ROS3,42; Increased α-KG41 Decreased glycolysis Decreased oxidative phosphorylation (Hancock, unpublished results) | Apoptosis39,41 Blockade cell cycle41 Stem cell pluripotency49,52 Ins/IGF1 prosurvival signaling dependent on proline metabolism50,71 |
| Synthetic     | P5CS         | Induced by c-MYC46                              | Necessary for c-MYC stimulated proliferation48 Essential for T-cell activation53 | Permissive effect on cell proliferation46 Neuropsychiatric disorders |
| Inborn error  | PYCR1        | Induced by c-MYC46                              | As for P5CS (Liu et al., unpublished results) Knockdown in xenograft tumors inhibits tumor growth25 | Neurodegeneration, mental deficiency and neuropathy Connective tissue disorder29 |
|               |              | More sensitive to peroxide36                     | Multiple genetic defects Cutis laxa, progeroid58 |                                                                   |

Proline Biosynthesis

We have shown that the enzymes in the pathway from glutamine to proline catalyzed sequentially by glutaminase (GLS), pyrroline-5-carboxylate synthase (P5CS)46,53 and pyrroline-5-carboxylate reductase 1 (PYCR1)46 are markedly increased by the oncogene c-MYC. This response occurred in the face of results) enabled redox signaling proposed by others through complex III.48 As first pointed out by Adams, P5C is not only the committed precursor for proline but also its immediate degradative product.49 Thus, the interconversions of proline and P5C constitute a redox cycle.50 The cycling was thought to function in a metabolic interlock with the oxidative arm of the pentose phosphate pathway,50 but recent findings by our lab and others have added several additional functions to the original hypothesis (Liu et al., unpublished results). Mutations in PRODH in humans may be associated with hyperprolinemia and neuropsychiatric disorders, but the clinical reports are controversial.51 Metabolic findings may be masked by the overlap in redox function between PRODH/POX and hydroxyproline oxidase PRODH2/HYPOX.52
physiologic concentrations of medium proline; others have shown the net synthesis of proline by cultured cells resulting in an increase in medium proline concentration.\(^{34}\) Therefore, the upregulation of the proline synthetic pathway by c-MYC is not to meet demands for protein synthesis. These findings are consistent with the observations of Windmueller et al., which demonstrate intestines containing proliferating cells primarily use glutamine and a major product is proline.\(^{35}\) Our findings showed that the enzymes of this pathway are markedly upregulated by c-MYC with correspondingly increased flux demonstrated by stable isotope-resolved metabolomics.\(^{46}\)

### Effects of Proline Metabolism

Humans with PYCR1 mutation have altered mitochondrial function and progeroid changes in connective tissues, including cutis laxa.\(^{36}\) These findings cannot be explained on the basis of inadequate proline for protein synthesis. Interestingly, the crystal structure for human PYCR1 has been solved: the native enzyme is a decamer with a conformation suggesting transporter or chaperone functions.\(^{37}\) Furthermore, others have characterized 3 isozymes of PYCR in cultured melanoma cells.\(^{38}\) They showed that PYCR1/2 prefer NADH as a cofactor whereas PYCLR mainly uses NADPH. PYCR1 appears associated with mitochondrial outer membranes whereas PYCR2 is in the matrix and PYCRL is in the cytosol. Although the localization and properties of these isozymes were shown in melanoma cells and need generalization to other tumors as well as normal tissue, they provide versatility for collagen, the most abundant protein by weight in the body with 25% of its amino acid residues either as proline or hydroxyproline, can serve as reservoir or metabolic dump for proline.\(^{39}\) Cells under metabolic stress upregulate metalloproteinases and PRODH/POX. Cells starved for substrates increase proline degradation through AMPK\(^{43,44}\) to provide an alternative mitochondrial substrate and generate ROS signals to activate prosurvival autophagy.\(^{45}\) Under conditions of rapid proliferation induced by growth factors and MYC activation, proline degradation is downregulated\(^{46}\) whereas robust induction of P5CS and PYCR1, PYCR mediates increased turnover of NAD(P)H to NAD(P)\(^+\) (ref. 46 and Liu et al., unpublished results). The increased availability of NADP\(^+\) and NAD\(^+\) provides oxidizing potential for critical steps in the oxidative arm of the pentose phosphate pathway and perhaps in glycolysis, respectively. The latter not only spares pyruvate for conversion to other vital intermediates, but NAD\(^+\) also supports the activity of sirtuins in deacetylating histones. The resultant proline can be incorporated into collagen thereby eliminating it from the bloodstream, much as excreted lactate results in the recycling of cellular NADH to NAD\(^+\) for glycolysis. Breast cancer provides an example of such coupling between proliferation and collagen synthesis. Although mechanoregulation between collagen fibrils and tumor cells has been proposed,\(^{60}\) the robust increase in collagen production also provides metabolic advantages for tumor growth. These metabolic features of proline and collagen metabolism may not be provided by a single cell type. In fact, it is likely that asymmetry in metabolism between tumor and stromal cells are a necessary feature. Metabolic/signaling collaboration between tissues and/or different cell types have been proposed\(^{61,62}\) and an intercellular complementation in proline metabolism between erythrocytes and hepatocytes based on the asymmetry of cellular enzymes has been described.\(^{63,64}\)

The redox turnover of pyridine nucleotides has been suggested, albeit direct demonstration of the effect requires further studies. Since the steady-state ratio of NAD(P)H to NAD(P)\(^+\) is difficult to ascertain, the demonstration of turnover can be inferred by flux through pathways in metabolic interlocks. For example with induction of PRODH/POX, the flux through the oxidative arm of the pentose phosphate pathway is markedly increased.\(^{44,65}\) Similarly, with MYC induction of P5CS and PYCR1, glycolysis is markedly increased by c-MYC expression.\(^{46}\) However, in the face of this Warburg effect, the knockdown of PYCR1 or P5CS markedly inhibited the response (Liu et al., unpublished results). These findings suggest that the proline regulatory axis is providing the redox turnover to optimally activate the c-MYC activation of glycolysis. Thus, from both proline degradation and proline biosynthesis, we have robust, albeit indirect, evidence of the recycling of pyridine nucleotides and the parametabolic regulation of glucose metabolism.

### Evidence for Cellular Response to Epigenetic Modulation by Proline

Is there experimental evidence that the parametabolic effect of the proline regulatory axis influences epigenetic mechanisms? Certainly, epigenetic changes induced by ROS have been demonstrated and reviewed.\(^{66}\) Others have shown that treatment of cultured hepatocellular carcinoma cells with hydrogen peroxide caused methylation of the E-cadherin promoter.\(^{67}\) We have shown that generation of ROS due to upregulation of PRODH/POX can activate a parametabolic response or program ranging from downregulation of COX-2 to the activation of prosurvival autophagy depending on the metabolic context.\(^{23}\) There are a number of published findings from other investigators suggesting that the proline regulatory axis is involved in epigenetics. First, Washington et al., using cultured mouse embryonic stem (ES) cells, showed that of all media constituents, proline uniquely regulated pluripotency.\(^{68}\) This finding was corroborated by Casalino et al.,\(^{69}\) who showed that the effect of proline in maintaining the metastability of ES cells required proline metabolism. Another recent example of proline-dependent epigenetic regulation was described by Zarse et al.\(^{70,71}\) Their studies in *C. elegans* and
in MEFs showed the life span extension with defective insulin/IGF1 (daf-2 in C. elegans) signaling was dependent on the catabolism of proline. Their interpretation is that the ROS generated by proline degradation induces the expression of a genetic program including antioxidant enzymes protecting against ROS so that lifespan is extended.

In summary, we propose that the metabolism of NEAA not only regulates core metabolism, but also produces substrates or cofactors participating in epigenetic mechanisms. Thus, the metabolism of NEAA acts as a coupling mechanism between metabolomics and epigenetics in cancer. Several of the NEAA mechanisms involve the cycling of substrates, cofactors or transfer mechanisms necessary for epigenetic regulation. We also introduce parametabolic regulation to understand the metabolic interlocks between NEAA and critical core pathways, i.e., glucose metabolism and ribonucleotides synthesis. We propose that the regulation of the enzymes of the proline regulatory axis provides plasticity in the production of various intermediates as well as in the recycling of pyridine nucleotides (Table 1). We hope that this regulatory axis will offer novel therapeutic targets for the treatment of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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