Chapter 15

MiRNA and Proline Metabolism in Cancer

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1. Introduction

Tumor metabolism and bioenergetics are important areas for cancer research and present promising targets for anticancer therapy. Growing tumors alter their metabolic profiles to meet the bioenergetic and biosynthetic demands of increased cell growth and proliferation. These alterations include the well-known aerobic glycolysis, the Warburg effect, which has been considered as the central tenet of cancer cell metabolism for more than 80 years [1]. Interest in cancer cell metabolism has been refueled by recent advances in the study of signaling pathways involving known oncogene and tumor suppressor genes, which reveal their close interaction with metabolic pathways [2-4]. For example, recent studies document an important role of glutamine catabolism in tumor stimulated by the oncogenic transcriptional factor c-MYC (herein termed MYC) which has been previously shown to stimulate glycolysis [5, 6]. Although glucose and glutamine serve as the main metabolic substrate for tumor cells, proline as a microenvironmental stress substrate has attracted lots of attention due to its unique metabolic system, its availability in tumor microenvironments and its responses to various stresses.

1.1. Special features of proline metabolism

Proline is the only proteinogenic secondary amino acid, and it has special functions in biology [7-11]. Proline metabolism is distinct from that of primary amino acids. The inclusion of an alpha-nitrogen within its pyrrolidine ring precludes its being the substrate for the usual amino acid-metabolizing enzymes, such as, the decarboxylases, aminotransferases, and racemases. Instead, proline metabolism has its own family of enzymes with their tissue and subcellular localization and their own regulatory mechanisms. As shown in the schematic of proline metabolic pathway (Figure 1), these enzymes include proline dehydrogenase/oxidase (PRODH/POX) and pyrroline-5-carboxylate reductase (PYCR) catalyzing the interconversion of proline and Δ^1-pyrroline-5-carboxylate (P5C), P5C dehydrogenase (P5CDH) and P5C synthase (P5CS) mediating the

interconversion of P5C and glutamate, and ornithine aminotransferase (OAT) catalyzing the interconversion of P5C and ornithine. Glutamate can be converted to α-ketoglutarate (α-KG) entering the tricarboxylic acid (TCA) cycle, which is also the main pathway of glutamine catabolism. Ornithine can be converted to arginine entering the urea cycle. Thus proline metabolism is closely related with glutamine metabolism, TCA cycle, and urea cycle, the main metabolic pathways in human body.

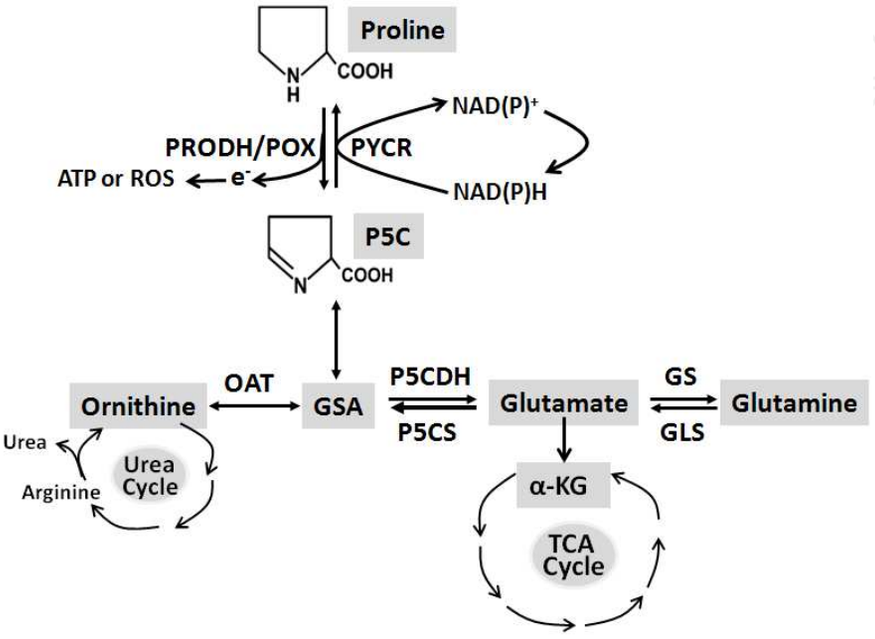

Figure 1. Proline metabolic pathway. Proline metabolism is closely related with glutamine metabolism, TCA cycle, urea cycle and pentose phosphate pathway (PPP). Abbreviations: P5C, $^{\Delta}$1 -pyrroline-5-carboxylate; GSA, glutamic-gamma-semialdehyde; PRODH/POX, proline dehydrogenase/oxidase; PYCR, P5C reductase; P5CDH, P5C dehydrogenase; GS, glutamine synthase; GLS, glutaminase; P5CS, P5C Synthase; OAT, ornithine aminotransferase. The interconversion between P5C and GSA is spontaneous.

Importantly, the interconversion between proline and P5C, catalyzed by PRODH/POX and PYCR, respectively, forms the "proline cycle" in the cytosol and mitochondria as shown in Figure 2, which acts as a redox shuttle transferring reducing and oxidizing potential. In the mitochondria, during the degradation of proline to P5C, PRODH/POX, the flavin adenine dinucleotide-containing enzyme tightly bound to mitochondrial inner membranes, donates electrons through its intervening flavine adenine dinucleotide into the electron transport chain (ETC) to generate ATP or ROS [7, 12, 13]. This characteristic of PRODH/POX serves as the basis of its function in human cancers, which will be discussed in detail in the following sections. P5C produced from the oxidation of proline, emerges from mitochondria and is converted back to proline in the cytosol using NADPH or NADH as cofactor, which interlock with the pentose phosphate pathway (Figure 1) or other metabolic pathways.

Proline metabolism has been shown to play an important role in various human physiologic and pathologic situations. For example, in the early 1970s, P5C, the immediate product of proline catabolism was found to be also the immediate biosynthetic precursor [7]. And in

the 1980s, the conversion of P5C to proline was recognized to regulate redox homeostasis as mentioned above [8, 14, 15]. A variety of evidence has shown the inborn errors of the proline metabolic pathway in several human genetic diseases and their potential roles [11, 16], such as familial hyperprolinemias [11, 17], mutations of PRODH/POX in neuropsychiatric diseases [18, 19], mutations of PYCR1 in cutis laxa [20], mutations of P5CS in hyperammonemia [21, 22], and so on. During the last decade, our understanding of the roles of proline metabolism as represented by the regulation and functions of PRODH/POX in tumorigenesis and tumor progression has made significant advances, which will be main focus in this chapter.

1.2. Proline availability in tumor microenvironment

Proline is one of the most abundant amino acids in the cellular microenvironment. Together with hydroxyproline, proline constitutes more than 25% of residues in collagen, the predominant protein (80%) in the extracellular matrix (ECM) of the human body. Although proline can be obtained from the dietary proteins, an important source of proline is from the degradation of collagen in the ECM by sequential enzymatic catalysis of matrix metalloproteinases (MMPs) and prolidase [9, 23]. The upregulation of MMPs in tumors has been considered a critical step for tumor progression and invasion [24-26]. A number of reports have shown that proline concentration is increased in various tumors, which may result from the upregulated MMPs degrading collagen. Previous work from our lab showed that glucose depletion activated MMP-2 and MMP-9 in cancer cells, which accompanied an increase in intracellular proline levels [27].

Autophagy-induced degradation of the intracellular protein, which has been shown to regulate cancer development and progression as a survival strategy of cancer cells [28, 29], may also provide an important source of free proline. Furthermore, proline can be biosynthesized from either glutamate or ornithine as shown in Figure 1 and Figure 2. Our latest finding showed that a large part of products from glutamine catabolism stimulated by MYC is proline [30], suggesting proline biosynthesis might serve as an additional source of proline availability in cancer. Taken together, the ample sources of proline in tumor microenvironment ensure its availability as an important stress substrate for metabolism in human cancers.

2. PRODH/POX as a mitochondrial tumor suppressor

2.1. PRODH/POX induces apoptosis through ROS generation

PRODH, the gene encoding PRODH/POX was discovered to be a p53-induced gene in a screening study in 1997 [31]. Importantly, the p53-initiated apoptosis was later found to depend on the induction of PRODH/POX [32]. To further study the function of PRODH/POX, we developed a DLD1-POX colorectal cancer cell line (designated as DLD1-POX tet-off cell line), which was stably transfected with the *PRODH* gene under the control of a tetracycline-controllable promoter [33]. When doxycycline (DOX) was removed from

the culture medium and the expression of PRODH/POX was induced, apoptotic cell death was initiated.

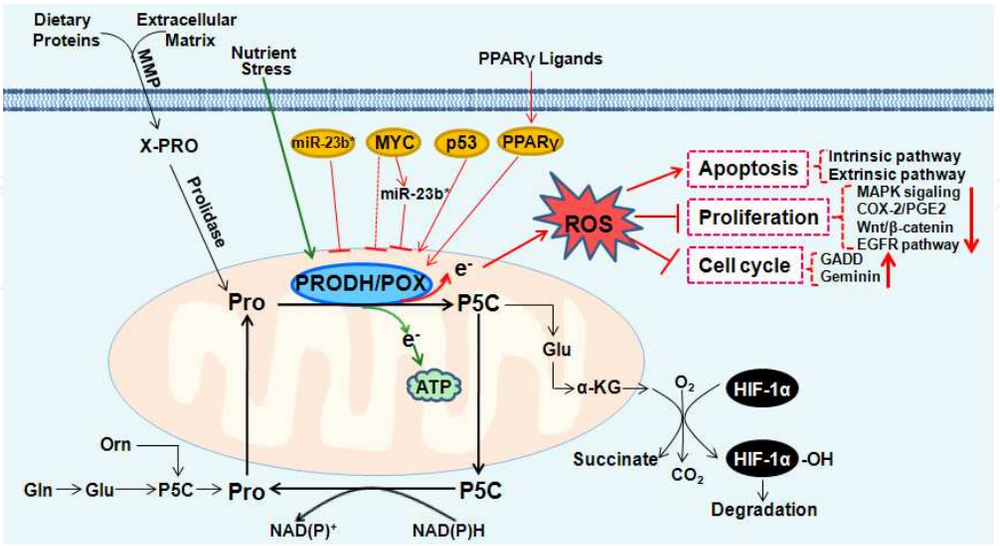

Figure 2. Proline metabolism in cancer. 1. Proline cycle: Interconversion of proline and P5C forms the proline cycle in the cytosol and mitochondria. Proline cycle acts as a redox shuttle transferring reducing potential generated by the pentose phosphate pathway or other metabolic pathway into mitochondria for the production of either ROS or ATP responding to different stresses. 2. Proline availability in human tumor microenvironment: dietary proteins, glutamate and ornithine catabolism, and degradation of extracellular matrix by matrix metalloproteinases (MMPs) are all important sources of proline, especially the last one. 3. The central enzyme of proline metabolism, PRODH/POX, localized in the mitochondrial inner membrane, function as a mitochondrial tumor suppressor. PRODH/POX is induced by p53, PPARγ and its ligands, and suppressed by miR-23b* and oncogenic protein MYC. PRODH/POX overexpression could initiate apoptosis, inhibit proliferation and induce G2 cell cycle arrest through ROS generation, and suppress HIF-1 signaling through increasing α-KG production. Abbreviations: X-PRO, x-prolyl dipeptide; Pro, proline; Orn, ornithine; Gln, glutamine; Glu, glutamate.

ROS, which include superoxide radical ($O_2^{-}\cdot$), hydroxyl radicals ($OH\cdot$) and the non-radical hydrogen peroxide (H_2O_2), play an important role in the induction of apoptosis [34]. PRODH/POX could donate electron to the ETC to generate ROS. In cells overexpressing PRODH/POX, the addition of proline increased ROS generation in a concentration-dependent manner, and the proline-dependent ROS increased with PRODH/POX expression [35]. N-acetyl cysteine (NAC), a widely used antioxidant agent, dramatically reduced PRODH/POX-induced apoptosis, indicating PRODH/POX induces apoptosis through ROS generation [13]. By introducing the recombinant adenoviruses containing different antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), Cu/Zn superoxide dismutase (CuZnSOD) or catalase (CAT) into the DLD1-POX tet-off cells, we found that only the expression of MnSOD, which localizes in the mitochondria, inhibited PRODH/POX-induced apoptosis, suggesting that it is superoxide as the form of ROS initially mediating PRODH/POX-induced apoptosis [13].

Further investigation on the molecular signaling involved in PRODH/POX-induced apoptosis showed that PRODH/POX activated both intrinsic and extrinsic apoptotic pathways [35, 36]. The DLD-1-POX cells overproducing PRODH/POX exhibited the

mitochondria (intrinsic pathway) and death receptor (extrinsic pathway)-mediated apoptotic responses in a proline-dependent manner [35]. Intrinsic pathway induced by PRODH/POX includes the release of cytochrome c, activation of caspase-9, chromatin condensation, DNA fragmentation, and cell shrinkage. Extrinsic pathway induced by PRODH/POX involves the stimulation of the expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL), and death receptor 5 (DR5) and then cleavage of caspase-8 [36]. Both pathways culminate in the activation of caspase-3 and cleavage of substrates. NFATc1, a member of the nuclear factor of activated T cells (NFAT) family of transcription factors is partially responsible for the TRAIL activity stimulated by PRODH/POX [36]. All of these effects mediated by PRODH/POX could be partially reversed by MnSOD, further confirming the role of ROS/superoxides in PRODH/POX-induced apoptosis [36].

Parallel studies showed that peroxisome proliferator activated receptor gamma (PPARγ) is another critical regulator of PRODH/POX, besides p53. PPARγ belongs to the nuclear hormone receptor superfamily and functions as a ligand-dependent transcription factor [37]. It is widely expressed in many malignant tissues, and its ligands can induce terminal differentiation, apoptosis, and cell growth inhibition in a variety of cancer cells [38-40]. Using a *PRODH*-promoter luciferase construct [41], we found that PPARγ was the most potent effector activating the *PRODH* promoter. PRODH/POX contributes greatly to apoptosis induced by the pharmacologic ligands of PPARγ through ROS signaling in human colorectal cancer cells and non-small cell lung carcinoma cells [41, 42].

More recently, we found that PRODH/POX was upregulated to contribute to ATP production under nutrient stress, such as glucose deprivation [27]. Under hypoxic conditions [43] or high levels of oxidized low-density lipoproteins (oxLDLs) [44], ROS produced by PRODH/POX contributes to autophagy as a survival signal. These effects seem paradoxical with PRODH/POX-induced apoptosis, but they can be well understood considering the temporal and spatial development of the evolving tumor, like the "two faces" of tumor suppressor p53 [45]. A detailed description of this point can be found in our recent review [9].

2.2. PRODH/POX inhibits tumor cell growth through ROS generation

In addition to initiating apoptosis, PRODH/POX also inhibits tumor cell growth and proliferation. In DLD1-POX tet-off cells, soft agar colony formation assays showed that the cells readily formed clones when PRODH/POX expression was inhibited by DOX, whereas the cloning ability of the cells was totally blocked when POX was overexpressed [46].

Several signaling pathways associated with tumor growth are downregulated by PRODH/POX. First, PRODH/POX suppresses the phosphorylation of three major subtypes of the mitogen-activated protein kinase (MAPK) pathways, including MEK/ERK, JNK, p38 [36]. In fact, MAPK pathways play an important role in a variety of cellular responses, including proliferation, differentiation, development, transformation, and apoptosis. The inhibition of MEK/ERK pathway is involved in PRODH/POX-induced apoptosis. Secondly,

PRODH/POX markedly reduces the expression of cyclooxygenase-2 (COX-2), and thus suppresses the production of prostaglandin E_2 (PGE_2) [47]. The addition of PGE_2 partially reverses the apoptosis and inhibits tumor growth induced by PRODH/POX. Cyclooxygenase is an enzyme that catalyzes the key step of the conversion of free arachidonic acid to prostaglandins. It has been widely accepted that elevated $COX2/PGE_2$ signaling plays a critical role in the initiation and development of various solid tumors, especially colorectal cancer [48-50]. Thirdly, PRODH/POX inhibits the phosphorylation of epidermal growth factor receptor (EGFR). Activating mutants and overexpression of EGFR signaling contributes to carcinogenesis of various tumors by inducing cell proliferation and counteracting apoptosis [51]. Fourthly, Wnt/β-catenin signaling is decreased by PRODH/POX [47]. Constitutive activation of this signaling pathway is found in many human cancers, which regulates proliferation, differentiation and cell fate [52]. Phosphorylation of β-catenin by GSK-3β leads to its ubiquitination and proteasomal degradation. PRODH/POX decreases phosphorylation of GSK-3β and thereby increases phosphorylation of β-catenin, resulting in the reduced activity of Wnt/ β-catenin signaling. All of aforementioned changes induced by PRODH/POX are partially reversed by MnSOD, further indicating the critical role of ROS/superoxides in PRODH/POX-mediated effects.

Furthermore, PRODH/POX induces G2 cell cycle arrest through affecting the regulators of cell cycle, such as geminin, cyclin-dependent kinase (CDC), and growth arrest and DNA damage inducible proteins (GADDs) [46]. Geminin is a nuclear protein that inhibits DNA replication, and has been used as a marker for G2 phase [53]. Its expression is up-regulated by PRODH/POX. CDC2 normally drives cells into mitosis and is the ultimate target of pathways that mediate rapid G2 arrest in response to DNA damage [54]. Although total CDC2 did not change with PRODH/POX expression, the phosphorylated CDC2 at tyrosine 15 increased, whereas phosphorylation at threonine 161 decreased when PRODH/POX was overexpressed, indicating that CDC2 is in an inactive status. CDC25C, the phosphatase that removes the inhibitory phosphates from CDC2 and activates cyclinB-CDC2, is downregulated by PRODH/POX. Additionally, the most important regulators of G2 cell cycle arrest, GADDs [55] also play a role in PRODH/POX-induced G2 cell cycle arrest, including GADD34, GADD45a, GADDh, GADDg [46].

2.3. PRODH/POX inhibits HIF signaling mainly through increasing α-KG production

The above described PRODH/POX-mediated induction of apoptosis together with the suppression of cell growth suggests that PRODH/POX could function as a tumor suppressor. PRODH/POX protein is located in the mitochondrial inner membrane, and has an anaplerotic role through glutamate and α-KG for the TCA cycle (Fig.1). The identification of several mitochondrial tumor suppressors has demonstrated that one of the critical ways they exert their antitumor effects is through hypoxia inducible factor-1 (HIF-1) signaling, which mediates the transcriptional response to hypoxia as a transcriptional factor and plays an important role in angiogenesis and tumor growth [56, 57]. Similarly, PRODH/POX also downregulates HIF-1 signaling including its downstream gene VEGF in both normoxic and

hypoxic conditions [46]. This is another mechanism, along with those described above, by which PRODH/POX exerts its tumor-suppressing role. However, unlike the effects of PRODH/POX on other signaling pathways, its effect on HIF-1 signaling could not be reversed by MnSOD, suggesting ROS is not the mediator for HIF inhibition.

The stability and transcriptional activity of HIF-1α are regulated through oxygen-sensitive modifications. Briefly, the posttranslational hydroxylation of specific prolyl and asparaginal residues in its α-subunits of HIF-1, catalyzed by prolyl hydroxylases (PHD), results in the degradation of HIF-1 through ubiquitinal and proteasomal degradation systems [58]. As an important substrate of PHD, the members of the 2-oxoglutarate (α-KG) dioxygenase family could increase the hydroxylation and degradation of HIF-1α [58]. HPLC analysis showed that α-KG was increased by overexpression of PRODH/POX [46]. When PRODH/POX expression is high, P5C, glutamate and α-KG are sequentially produced from proline, forming an important link between proline and the TCA cycle. The widely used cell-permeating α-KG analogue, dimethyloxalylglycine, was shown to block the inhibition of HIF-1 signaling by PRODH/POX, suggesting the pivotal role of α-KG in the down-regulation of HIF by PRODH/POX.

In addition, several TCA cycle intermediates and glycolytic metabolites, such as succinate and fumarate, have been revealed to inhibit PHD activity and stabilize HIF-1 signaling [58-61]. PRODH/POX expression could decrease succinate, fumarate and lactate as measured by gas chromatography-mass spectrometry (GC-MS) [46], which may also contribute to the impaired HIF-1 signaling.

2.4. PRODH/POX suppresses tumor formation *in vivo* and is downregulated in human tumors

The inhibitory effects of PRODH/POX on tumor cell growth are corroborated in a human colon cancer mouse xenograft model [46]. DLD-1 POX Tet-off cells were injected into immunodeficient mice. The expression of PRODH/POX was controlled by giving mice doxycycline in their drinking water. When PRODH/POX was suppressed by doxycycline, tumors readily formed in all the mice within a few days. By contrast, when PRODH/POX was overexpressed by removal of doxycycline in their drinking water, tumor development was greatly reduced and none of the mice developed tumors.

Further investigation on a variety of cancer tissues along with normal tissue counterparts including kidney, bladder, stomach, colon and rectum, liver, pancreas, breast, prostate, ovary, brain, lung, skin, etc., showed that 61% of all tumors had decreased expression of PRODH/POX compared to normal tissues, especially the tumor from kidney and digestive tract [46, 47, 62], suggesting tumor could eliminate the tumor suppressor roles of PRODH/POX. Suppression of PRODH/POX was more significant in kidney and digestive tract. More interestingly, PRODH/POX protein levels showed more striking decrease than mRNA levels in renal cancers, implicating that PRODH/POX might be regulated at the post-transcriptional level.

Sequencing the *PRODH* gene showed no somatic mutation or functionally significant single nucleotide polymorphisms (SNP) in tumor tissues. Hypermethylation analysis also didn't show any differences of PRODH genomic DNA between tumor and normal tissues. Therefore, PRODH does not satisfy the canonical requisite for tumor suppressor genes which often show genetic or epigenetic mutations in human cancers. With the discovery of microRNAs (miRNAs), a new mechanism to regulate protein expression has been revealed. Considering the inconsistency between PRODH/POX mRNA and protein expression and the importance of miRNAs in cancer, the regulation of miRNAs on PRODH/POX represented a very promising hypothesis.

3. MiRNA in cancer

3.1. Biogenesis and function of miRNAs

3.1.1. Discovery of miRNAs

MiRNAs are a class of post-transcriptional regulators. They are conserved, endogenously expressed, non-coding small RNAs of 18-25 nucleotides in length. MiRNAs were first discovered in 1993 by Lee RC *et al.* [63] and Wightman R *et al.* [64] in the nematode *Caenorhabditis elegans (C. elegans)* as a regulator of developmental timing regarding the gene lin-14. They found that the lin-14 could be regulated by the small RNA products from lin-4, a gene that does not code for any protein but instead produces a pair of small RNAs. These lin-4 RNAs had antisense complementarity to multiple sites in the 3' UTR of the lin-14 mRNA. However, it did not attract substantial attention until seven years later when let-7 was discovered to repress the expression of several mRNAs including lin-14 during transition in developmental stages in *C. elegans* [65]. Since then over 4000 miRNAs have been identified in eukaryotes including mammals, fungi and plants. More than 700 miRNAs have been found in humans.

3.1.2. Processing and biogenesis of miRNAs

In mammals, miRNA genes are usually transcribed as long primary transcripts (pri-miRNAs) by RNA polymerase II from DNA [66]. The pri-miRNAs then are cropped into the hairpin-shaped miRNA precursors (pre-miRNAs) by the RNase III enzyme Drosha [67, 68]. A single pri-miRNA may contain one to six pre-miRNAs which are composed of about 70 nucleotides. They are exported from the nucleus to the cytoplasm by exportin-5 (XPO5), a member of the Ran-dependent nuclear transport receptor family [69-71]. In cytoplasm, the pre-miRNA hairpin is subsequently cleaved by the endonuclease Dicer [72] into an imperfect miRNA:miRNA* duplex. Usually, only one strand of the duplex is incorporated into the RNA induced silencing complex (RISC) where the miRNA and its mRNA target interact. The thermodynamic stability, strength of base-pairing and the position of the stem-loop determine which strand becomes mature miRNA to incorporate into the RISC [73-75]. The other strand is normally degraded and is denoted with an asterisk (*) due to its lower levels in the steady state. However, recent evidence indicates that both strands of duplex are viable and become functional miRNA that target different mRNA populations [62, 76-78].

RISC is a multiprotein complex that incorporates mature miRNA to recognize complementary target mRNA. Once binding to target mRNA, miRNAs inhibit their target genes with the help of RISC. The key component of the RISC complex is the Argonaute (Ago) proteins, which are consistently found in RISC complexes from a variety of organisms [79]. Ago proteins directly interact with the miRNA [80, 81]. They are needed for miRNA-induced silencing and contain two conserved RNA binding domains: a PAZ domain, that can bind the single stranded 3′ end of the mature miRNA, and a PIWI domain, that structurally resembles ribonuclease-H (RNaseH) and functions in slicer activity through interacting with the 5′ end of the guide strand [82]. Most eukaryotes contain multiple Ago family members, with different Ago often specialized for distinct functions [83]. The human genome encodes four Ago proteins and Ago2 is the only Ago capable of endonuclease cleavage of target transcripts directly [84, 85].

Additional components of RISC involved in miRNA processing include the Vasa intronic gene (VIG) protein, the fragile X mental retardation protein (FMRP), human immunodeficiency virus transactivating response RNA binding protein (TARBP), protein activator of the interferon induced protein kinase (PACT), the SMN complex, Gemin3 and DICER1, and so on [86-92]. However their generality or precise function in miRNA silencing remains to be determined.

3.1.3. Stability of miRNAs

Turnover of mature miRNA is needed for rapid changes in miRNA expression profiles. Besides inducing the cleavage of the target mRNAs, Ago proteins have been recently reported to regulate the stability of miRNAs [93-98]. Mature miRNAs are stabilized after incorporation into Ago proteins, and release from this complex leaves miRNAs vulnerable to decay by exonucleases [94, 95]. Ectopic overexpression of Ago proteins prevents degradation of miRNAs, and loss of Ago2 significantly reduces miRNA stability and differentially regulates miRNAs production [93, 96].

In addition to taking refuge in protein complexes, mature miRNAs can undergo protective modifications [97]. For example, as indicated by work in the model organism *Arabidopsis thaliana*, mature plant miRNAs appear to be stabilized by the addition of methyl groups at the 3' end which prevents uridylation of miRNAs [99]. The addition of adenines to 3′ end of miRNAs detected in many different plant and animal miRNAs also has a stabilizing effect on miRNAs [100-104].

3.1.4. Function of miRNAs

MiRNAs inhibit the expression of their target genes through three different mechanisms [105, 106]. The first one is direct endonucleolytic cleavage of mRNAs supported by the slicer activity of specific Ago proteins present within RISC. As mentioned above, Ago2 is the only one of the four mammalian Ago proteins capable of directing cleavage [84, 85]. This mechanism is generally favored by a complete match of the so called seed-sequence of the miRNA (nucleotides 2-7 of 5′ end of miRNAs) and target mRNA [107], although some

mismatches can be tolerated and still allow cleavage to occur [108, 109]. The complementarity of the seed region defines the targets of the miRNA because the seed region binds to the mRNA as governed by binding of complementary nucleotides. The second mechanism is by inhibiting protein translation but without degradation of the mRNA [110-112]. It seems to be the most prevalent in mammals [113]. In this mechanism, the seed region of the miRNA does not need to be fully complementary; yet, efficient translation repression by miRNAs often requires multiple miRNA-binding sites, as suggested by the observations that the identified mRNA targets of miRNAs contained multiple sites for miRNA binding, either the same miRNA or a combination of several different miRNAs [114, 115]. However, many predicted mRNA targets of miRNAs contain only a single miRNA-binding site in their 3′UTR [107], indicating that such single sites may lead to fine "tuning" of mRNA function [116]. Distinct from the slicer activity of the specific Ago in the first manner, translation repression by miRNAs is common to all members of the Ago protein family. The third mechanism is called mRNA decay independent of slicer [117, 118]. In this manner, miRNAs either promote mRNAs decapping and 5′ to 3′ degradation, or target mRNAs by an unknown decay pathway. In the former way, the protecting poly-A-tail and ''cap'' of the mRNAs are removed, resulting in their rapid destruction by RNA splicing enzymes.

MiRNAs are now known to target thousands of genes. Bioinformatics analyses estimated that up to 30% of known human genes are under miRNAs′ control [107], whereas later reports increased this number to 74~92% [119]. A key issue in miRNAs function is the specificity of their interactions with their target mRNAs and how each interaction leads to discrete downstream consequences. Some miRNAs regulate specific individual targets, while others can function as master regulators of a process. Key miRNAs regulate the expression levels of hundreds of genes simultaneously, and many types of miRNAs regulate their targets cooperatively. Because of their potent and wide action on gene expression, miRNAs become critical regulators of cellular functions. They are involved in modulating a variety of biological processes, including cellular proliferation, differentiation, metabolic signaling, apoptosis and development. The aberrant expression or alteration of miRNAs has been linked to a range of human diseases, especially cancers.

3.2. Dysregulation of miRNA in cancer

In 2002, Calin *et al.* first demonstrated that miR-15 and miIR-16 are frequently deleted or down-regulated in chronic lymphocytic leukemia [120]. Subsequently, aberrant miRNA expression, and amplification or deletion of miRNAs are observed in various human tumors [121, 122]. MiRNAs are differentially expressed in cancer cells, in which they form distinct and unique miRNA expression patterns [123]. These properties make miRNAs become potential biomarkers for cancer diagnosis, in particular for the early detection of cancer [124]. The control of gene expression by miRNAs is seen in virtually all cancer cells. Their target genes are usually important proteins such as oncogenic factors (i.e., MYC, RAS), tumor suppressors (i.e., p53), or proteins regulating the cell cycle (i.e., the cyclin family). Even small changes in these crucial proteins can have profound effects on tumorigenesis or tumor development. Conversely, miRNAs are often critical downstream effectors of classic oncogene/tumor suppressor networks, such as MYC and p53 described below.

miRNAs can act as oncogenes or tumor suppressor genes in tumorigenesis depending on the targets they regulate. Oncogenic miRNAs repress known tumor suppressors, whereas tumor-suppressor miRNAs often negatively regulate protein-coding oncogenes (this has been reviewed in detail by others [125-127]). Oncogenic miRNAs are overexpressed in various human cancers. For example, the miR-17-92 cluster miRNAs which are transcribed as a polycistronic unit, are highly expressed in B-cell lymphoma and various solid cancer, such as breast, colon, lung, pancreas, prostate and stomach [128-130]. They function as oncogenes to promote proliferation, inhibit apoptosis, induce tumor angiogenesis, and augment the oncogenic effects of MYC [131-134]. Their effects on cell cycle and proliferation are at least in part through its regulation of E2F transcription factors [130, 135], and anti-apoptotic effects are through their inhibition of BIM, PTEN and p21 [135]. MiR-221 and miR-222 are frequently overexpressed in lung, liver and ERα- breast cancers. Their overexpression has been demonstrated to enhance tumorigenicity through suppressing the expression of different tumor suppressors, such as CDKN1B/C, BIM, PTEN, TIMP3 and FOXO3 [136, 137]. Overexpression of miR-504 promotes tumorgenicity of colon cancer *in vivo*, which directly targets tumor suppressor p53 and functions in apoptosis and cell cycle [138].

On the other hand, miRNAs that act as tumor suppressors are often found to be deleted or mutated in various human cancers. For example, Let-7 family miRNAs are frequently down-regulated in various cancers, including lung and colorectal cancers [139]. They can directly suppress the expression of oncogenes, including RAS and MYC, and therefore show tumor suppressive functions [139, 140]. MiR-15a and miR-16-1 are often deleted or down-regulated in B-cell chronic lymphocytic leukemia (B-CLL). They negatively regulate anti-apoptotic protein BCL2. Therefore, decreased expression of miR-15a and miR-16-1 up-regulates BCL2 levels and reduces apoptosis, contributing to malignant transformation [141].

Based on the critical role of miRNAs in tumorigenesis, recent research efforts are directed towards translating these basic discoveries into clinical applications in diagnosis, prognosis and therapy through identifying and targeting dysregulated miRNAs. Both silencing the oncogenic miRNAs and restoring the expression of silenced tumor-suppressor miRNAs have yielded positive results in mouse models of cancer and thus becomes promising therapeutic strategy for cancer [142, 143]. The silencing of oncogenic miRNAs can be achieved by using antisense oligonucleotides (antagomirs or anti-miRs), sponges or locked nucleic acid (LNA) constructs [144]. By contrast, the restoration of tumor-suppressor miRNA expression can be achieved by the use of synthetic miRNA mimics, adenovirus vectors, and pharmacological agents [144]. Although the drug delivery, proper drug composition and off-target effects are still the current challenges in the clinical application of miRNAs, the future is bright for miRNA-based therapy.

3.3. MiRNAs regulated by transcriptional factors, genetic and epigenetic changes

3.3.1. MiRNAs regulated by oncogenic transcriptional factor MYC

MiRNAs can be dysregulated by multiple transcription factors in cancer. Oncogenic transcriptional factor MYC regulates a variety of gene expression affecting a series of

cellular processes in cancer including cell growth and proliferation, metabolism, cell-cycle, differentiation, apoptosis, angiogenesis and metastasis [145-147]. Recently, it was found that MYC is also an important regulator of miRNAs. Consistent with their ability to potently influence cancer phenotypes, the regulation of miRNAs by MYC affects virtually all aspects of the MYC oncogenic program.

MYC directly activates the transcription of miR-17-92 polycistronic cluster though binding to an E-box within the first intron of the gene encoding the miR-17-92 primary transcript [148, 149]. Given its oncogenic role, the inhibition of key targets of miR-17-92 contributes to MYC-induced tumorigenesis. MiR-9 could also be activated directly by MYC, which regulates E-cadherin and cancer metastasis [150]. In contrast, MYC activity also results in repression of numerous miRNAs [151]. This repression involves the downregulation of miRNAs with antiproliferative, antitumorigenic and pro-apoptotic activity, such as let-7, miR-15a/16-1, miR-26a miR-29 or miR-34 family members [143, 151-153]. MiR-23a/b is an additional important example to be directly suppressed by MYC, which targets glutaminase to enhance glutamine catabolism [5]. MYC-driven reprogramming of miRNA expression patterns was shown to be a contributing factor in hepatoblastoma (HB), a rare embryonal neoplasm derived from liver progenitor cells [154]. Like an embryonic stem cell expression profile, undifferentiated aggressive HBs overexpress the miR-371-3 cluster with concomitant down-regulation of the miR-100/let-7a-2/miR-125b-1 cluster, which exerts antagonistic effects on cell proliferation and tumorigenicity. Chromatin immunoprecipitation (ChIP) and MYC inhibition assays in hepatoma cells demonstrated that both miR clusters are regulated by MYC in an opposite manner.

Although further investigation is necessary, the current studies have indicated that MYC uses both transcriptional and post-transcriptional mechanisms to modulate miRNA expression [151, 155]. Primary transcript mapping and ChIP revealed that MYC associates directly with evolutionarily conserved promoter regions upstream of several miRNAs [151], such as the direct activation of miR-17-92 cluster and direct suppression of miR-23a/b described above. MYC is also able to modulate the maturation of specific miRNAs without affecting transcription of the pri-miRNAs. For example, MYC activity results in repression of mature let-7 miRNAs while the expression of let-7 primary transcripts is unchanged [151, 156]. This phenomenon could be due to Lin28A and Lin28B being the direct target of MYC, which interacts with let-7 pre-miRNA stem-loops and may regulate let-7 at multiple levels including Drosha and Dicer processing [156, 157]. Additionally, interaction of Lin28A and Lin28B recruits the 3′ terminal uridylyl transferase 4 (TUT4) to pre-let-7, resulting in uridylation and subsequent decay of the pre-miRNA [158, 159].

3.3.2. MiRNAs regulated by tumor suppressor p53

The tumor suppressor p53 is another transcription factor that regulate the expression of a group of miRNAs mediating a variety of anti-proliferative processes [160]. The miR-34 family, which consists of miR-34a, miR-34b and miR-34c, was initially reported to be induced directly by p53 [161] and mediate some of the p53 effects. ChIP and luciferase assays showed that p53 binds to p53 response elements (REs) in miR-34 promoters and

activates their transcription [162]. MiR-34 family members directly repress the expression of several targets involved in the regulation of cell cycle and in the promotion of cell proliferation and survival. These targets include cyclin E2, cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), BCL2 and hepatocyte growth factor receptor c-Met [161]. Later on, p53 was reported to directly regulate the transcriptional expression of several additional miRNAs, including miR-145, miR-107, miR-192 and miR215, miR-149* [160, 163]. MiR-145 negatively regulates oncogene *MYC*, which accounts partially for the miR-145-mediated inhibition of tumor cell growth both *in vitro* and *in vivo* [164]. MiR-107 contributes to the role of p53 in the regulation of hypoxia signaling and anti-angiogenesis through repressing the expression of HIF-1β, which interacts with HIF-1α subunits to form a HIF-1 complex, a key player in tumor formation. MiR-192 and miR-215 induce cell cycle arrest and reduce tumor cell growth through targeting a number of regulators of DNA synthesis and cell cycle checkpoints, such as CDC7, MDA2L1 and CUL5 [165]. MiRNA-149* targets glycogen synthase kinase-3α, resulting in increased expression of Mcl-1 and resistance to apoptosis in melanoma cells [163].

Moreover, p53 also enhances the post-transcriptional maturation of miRNAs. In response to doxorubicin, P53 interacts with the Drosha processing complex through the association with DEAD box RNA helicases p68 (also known as DDX5) and p72 (also known as DDX17), and facilitates the Drosha-mediated processing of pri-miRNAs to pre-miRNAs. These miRNAs include miR-16-1, miR-143 and miR-145 with growth-suppressive functions. Transcriptionally inactive p53 mutants interfere with a functional assembly between Drosha complex and p68, leading to attenuation of miRNA processing activity [166].

3.3.3. MiRNAs regulated by other transcription factors

Estrogen receptor alpha (ERα), a member of the nuclear receptor superfamily of transcription factors, was found to negatively regulate expression of miR-221 and miR-222 by promoter binding and recruiting the corepressors NCoR and SMRT [137]. Overexpression of miR-221 and miR-222 conversely suppresses the expression of ERα, conferring estrogen-independent growth. They also suppress the expression of different tumor suppressors, such as CDKN1B, CDKN1C, BIM, PTEN, TIMP3, DNA damage-inducible transcript 4, and FOXO3, to promote high proliferation [137]. Transcription factor c-Jun could also activate miR-221 and miR-222 [136].

Microarray-based expression profiles reveal that a specific spectrum of miRNAs is induced in response to low oxygen, at least some via a HIF-dependent mechanism, such as miR-210, miR-26a-2, miR-24 and miR-181c [167]. Of these, miR-210 as a direct transcriptional target of HIF-1α has emerged as a critical element of the cellular hypoxia response in a broad variety of cell types ranging from cancer cell lines to human umbilical vein endothelial cells [168-170]. MiR-210 has diverse functions, including modulating angiogenesis [171], stem cell survival [172], and hypoxia-induced cell cycle arrest [173]. MiR-143 and miR-145 could be repressed by RAS-responsive element-binding protein 1 (RREB1), a zinc finger transcription factor which binds to RAS-responsive elements (RREs) of their promoters. Thus these two miRNAs are embedded in KRAS oncogenic network [174].

In general, miRNAs can be dysregulated by transcription factors and, therefore, genetic or epigenetic alterations that result in the dysregulation of transcription factors can cause miRNA dysregulation. Importantly, miRNAs can also be directly regulated by genetic or epigenetic alterations.

3.3.4. MiRNAs regulated by genetic and epigenetic changes

MiRNAs are frequently located in fragile regions of the chromosomes, such as common chromosomal-breakpoints that are associated with the development of cancer [175, 176]. These fragile regions are often missing, amplified or mutated in cancer cells, resulting in the genetic alterations of miRNAs. The genetic alterations can affect the production of the primary miRNA transcript, their processing to mature miRNAs and/or interactions with mRNA targets. The dysregulation of miR-15 and miR-16 in most B cell chronic lymphocytic leukemias, one of the first observations between miRNAs and cancer development, is the result from chromosome 13q14 deletion [120]. Interestingly, somatic translocations in miRNA target sites can also occur, representing a drastic means of altering miRNA function [177, 178].

In addition to the structural genetic alterations, dysregulation of miRNAs in cancer can occur through epigenetic changes, such as methylation of the CpG islands of their promoters, the modification of histone [179-181]. As the example, miR-127 is silenced by promoter methylation, which leads to the overexpression of BCL6, an oncogene involved in the development of diffuse large B cell lymphoma [179]. The expression of miR-127 could be restored by using hypomethylating agents such as azacytidine. MiRNA-200 family could serve as another example. The miR-200 family can be shifted to hypermethylated or unmethylated 5'-CpG island status corresponding to the epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) phenotypes, respectively, which contributes to the evolving and adapting phenotypes of human tumors [181].

4. miR-23b* targets PRODH/POX

Although numerous targets of miRNAs have been identified, miRNA regulators of critical cancer proteins and pathways remain largely unknown. As described above, PRODH/POX is frequently reduced in a variety of human cancers, including renal cancer, and PRODH/POX protein but not mRNA level is markedly down-regulated in renal cancers [46, 62]. The fact that miRNAs are critical post-transcriptional regulators, and miRNAs function as oncogenes to inhibit the expression of tumor suppressors raises attractive possibility that some specific miRNAs may regulate PRODH/POX and proline catabolism. Target-prediction algorithms have been used to identify the protein targets of miRNAs or miRNAs regulators of known protein, followed by experimental validation to eliminate false positives [141]. The bioinformatic analysis according to target-prediction algorithms predicted that 91 potential miRNAs could target PRODH/POX mRNA 3′UTR [62]. In miRNA microarrays, 10 miRNAs showed an increased expression in renal cancer cells relative to normal cells. However, only miR-23b* was shown to significantly inhibit

PRODH/POX protein expression, but not mRNA level. This is consistent with many previous reports, that is, in mammals, miRNAs more often inhibit protein translation of the target mRNA, other than inducing its degradation [113]. Subsequently, miR-23b* directly binding to PRODH/POX mRNA 3'UTR was experimentally confirmed through luciferase assays by co-transfecting the mimic miR-23b* and the luciferase reporter containing 3'UTR of PRODH/POX mRNA. Functional analysis showed that this miRNA impaired PRODH/POX functions, including PRODH/POX-mediated ROS generation, apoptosis, and PRODH/POX-inhibited HIF-1 signaling [62]. In contrast, the inhibitory antagomir of miR-23b* increased the expression of PRODH/POX protein in renal cancer cells. As a result, ROS production, the percentage of cells undergoing apoptosis increased, and HIF-1 signaling decreased.

The clinical relevance of these *in vitro* findings was substantiated by the data obtained in human renal carcinoma tissues *in vivo* [62]. There were statistical significant differences in both miR-23b* and PRODH/POX protein expression between carcinoma tissues and corresponding normal tissues, but not PRODH/POX mRNA levels. A negative correlation between miR-23b* and PRODH/POX protein was found.

In summary, PRODH/POX is subject to the negative regulation of miR-23b*, which is a novel mechanism for cells to regulate PRODH/POX protein level and functions. The increased miR-23b* might contribute to renal oncogenesis and progression by downregulating tumor suppressor PRODH/POX. This provides a possible strategic opening to inhibit tumor growth by decreasing the levels of miR-23b* or by blocking its function.

5. Regulation of miR-23b* in cancer

5.1. MiR-23b* regulation by oncogenic protein MYC

Recently, the oncogenic transcription factor MYC has been reported to transcriptionally suppress miR-23b to stimulate mitochondrial glutaminase expression and glutamine metabolism in lymphoma cells [5]. MiR-23b and miR-23b* are sibling miRNAs processed from the same transcript. Thus, this finding attracted our attention and compelled us to seek the potential effect of MYC on miR-23b* and related PRODH/POX expression and proline metabolism. As described above, MYC is a critical regulator of miRNAs expression at both transcriptional and post-transcriptional levels. Furthermore, proline and glutamine metabolism are closely related: not only their interconversions, but also both can be anaplerotic in the TCA cycle as an important energy source, as mentioned above. These facts strengthened our hypothesis that MYC may regulate the expression of miR-23b*, thereby PRODH/POX, and link proline and glutamine metabolism.

Using human Burkitt lymphoma model P493 cells that bear a tetracycline-repressible *MYC* construct, we found that MYC upregulated the expression of miR-23b* [30]. In PC3 prostate cancer cells which overexpress MYC, the same result was obtained, i.e., MYC knockdown by siRNA resulted in the decrease of miR-23b* expression. These results are distinct from the previous report which showed MYC directly bound to the transcriptional unit encompassing miR-23b, and regulated its expression at the transcriptional level [5]. Re-

examination of the expression of miR-23b*, miR-23b, and their primary transcript (pri-miR23b) showed that pri-miR23b increased about 50% with MYC suppression by tetracycline and then decreased on MYC re-induction in P493 cells [30]. Similarly, in PC3 prostate cancer cells, with MYC knockdown by siRNA, miR-23b* decreased 68%, while miR-23b and Pri-miR-23b increased 51% and 70%, respectively [30]. Thus, the level of miR-23b* is higher than miR-23b in cells without MYC knockdown. These results support previous work that MYC suppresses miR-23b expression at the transcriptional level. Considering the fact that MYC enhances the expression of miR-23b*, the sibling of miR-23b, we hypothesized that differential effects of MYC on the sibling miRNAs may be due to their differential stabilization and/or degradation mediated by MYC. As a consequence, even if MYC suppressed the expression of miR-23b primary transcript, its effects on miR-23b* stabilization and/or degradation could account for net higher levels of miR-23b* as observed in this report.

The mechanisms responsible for stabilized miRNA expression have been largely elusive. As mentioned above, Ago proteins, the key players in miRNA processing and function, recently have been shown to regulate miRNA stability [93-96]. Ago2 differentially regulates miRNAs expression [93, 96]. Not surprisingly, MYC significantly upregulated the expression of Ago2 [30]. Knockdown of Ago2 in P493 MYC-overexpressed cells, the expression of miR-23b* and miR-23b were differentially decreased (76% vs. 42%, respectively), but not Pri-23b. Although the differential effects on miR-23b* and miR-23b resulted from Ago2 regulation by MYC do not completely account for the observed differential effects of MYC, they do support our hypothesis that MYC may regulate miRNA levels by differential effects on the stabilization of miRNAs, which can serve as a model for the effects on sibling miRNAs.

Since a large number of RISC components are involved in the miRNA processing [86]. It is likely that MYC with its multitude of target genes may affect many proteins like Ago2 and differentially affect miR-23b* and miR-23b expression. In fact, several reports have described the regulation of MYC on other RISCs or accessory RISCs, such as the upregulation of XPO5 and DEAD box protein 5 (DDX5) [86, 182, 183], and the aforementioned Lin28A and Lin28B regulation by MYC which affects the expression of mature let-7 miRNAs at multiple levels including their processing and modification [151, 156-159], but further studies are needed to elucidate how they affect the final expression of mature miRNAs and their interaction.

5.2. miR-23b* regulation by other factors

As mentioned above, PRODH/POX is encoded by a p53-induced gene [31]. Maxwell SA *et al*. reported that reduced expression of PRODH/POX mRNA in renal cancer was due to a p53 mutation [184]. On the other hand, p53 is a critical regulator of miRNAs. Thus, the possibility exists that wild-type p53 may regulate the expression of PRODH/POX by both direct and indirect (miR-23b*-dependent) mechanism. Interestingly, the experiment showed that ectopic expression of p53 in p53-mutant renal cancer cell line TK10 increased the expression of miR-23b* [62]. This suggests that the upregulation of miR-23b* by p53 may counteract the direct induction of p53 on PRODH/POX gene expression in clear cell renal cell carcinoma. This interaction might also account for discrepancies between PRODH/POX mRNA and protein expression.

In addition, current evidence suggests that miR-23b* could be regulated by factors other than p53 and MYC. For example, as discussed above, several reports have shown the link between upregulation of miR-23b and hypoxia [167, 185, 186]. As miR-23b and miR-23b* share the same precursor, miR-23b* could also be regulated by HIF. In renal cell carcinoma, the constitutive expression of HIF due to VHL deficiency may link this regulation of miR-23b* with VHL. The fact that HIF-1 negatively regulates mitochondrial biogenesis by inhibiting MYC activity in VHL-deficient renal carcinoma cells [187] further increases the possibility that miR-23b* could be regulated by VHL, HIF, thereby affecting the expression of PRODH/POX. These regulatory interactions are of great interest and worth to be pursued.

6. Regulation of proline metabolism by MYC

6.1. MYC suppresses PRODH/POX primarily through miR-23b*

In view of the above findings, it is not surprising that MYC suppresses the expression of PRODH/POX through upregulating miR-23b*. First, PRODH/POX protein increased in a time-dependent fashion with diminished MYC expression and then decreased on MYC recovery in P493 cells. PRODH/POX mRNA expression also showed a significant increase with suppressed MYC expression, but the increase was far less than that of protein levels, raising the likelihood that miRNA mediates the effect of MYC on PRODH/POX at the post-transcriptional level. MYC knockdown in PC3 prostate cancer cells by siRNA resulted in the inhibition of PRODH/POX expression with a pattern similar to the P493 cells. Secondly, the inhibition of miR-23b* by its antagomirs in the P493 cells with MYC overexpression increased PRODH/POX protein level [30]. By contrast, the transfection of mimic miR-23b* into the P493 cells under MYC inhibition by tetracycline resulted in a marked decrease of PRODH/POX protein expression. However, the decrease of PRODH/POX still was not comparable with that without tetracycline treatment, indicating that MYC could suppress PRODH/POX expression through pathways other than miRNA, such as the regulation at the transcriptional level, which also is supported by the decrease of PRODH/POX mRNA by MYC. Thirdly, the luciferase assays in PC cells showed that knockdown of MYC increased the luciferase activity of the luciferase reporter containing POX 3′UTR with the binding site of miR-23b*, indicating the decrease of miR-23b* by siMYC. Without MYC knockdown, the luciferase activity of this reporter was much lower than that of the original reporter without POX 3′UTR, due to high levels of miR-23b* binding to PRODH/POX mRNA 3′UTR, thereby suppressing luciferase expression.

By transfecting the PRODH promoter/luciferase reporter construct containing PRODH promoter region in PC3 prostate cancer cells, knockdown of MYC resulted in the increase of PRODH promoter activity, which confirmed that MYC regulates PRODH/POX at the transcriptional level [41]. Analysis of PRODH promoter nucleotide sequence revealed one canonical MYC binding site 5′-CACGTG-3′ (E-box) and one noncanonical binding site (5′-ACGGTG-3′) at -2808 to -2813bp and -637 to -642bp of the PRODH promoter region, respectively. However, ChIP assay showed none of these PRODH promoter regions had significant PCR amplification, suggesting that MYC does not directly interact with the

PRODH gene, and the decreased PRODH/POX mRNA expression may be mediated through other transcription factors regulated by MYC [30].

6.2. Suppression of proline catabolism is essential for MYC-mediated cancer cell proliferation and survival

In addition to PRODH/POX, MYC also inhibits the expression of another enzyme in proline catabolism, P5CDH [30], but the mechanism remains unclear. However, the suppression of proline catabolism reflected by PRODH/POX inhibition by MYC has been shown to be essential for MYC-induced proliferation and cell survival. First, knockdown of PRODH/POX in P493 cells with MYC suppressed by tetracycline consistently reduced the production of ROS at different time points [30], although the suppression of MYC itself by tetracycline also decreased the accumulation of ROS at late stage which implicates the different effects of various MYC regulated genes on ROS production at various stages [188-190]. Correspondingly, the apoptosis assay by flow cytometry showed that PRODH/POX knockdown decreased the percentage of apoptotic and dead cells occurring with MYC suppression. In contrast, PRODH/POX siRNA significantly rescued 30~40% of the diminished growth rates resulting from MYC suppression by tetracycline [30]. These results indicated that PRODH/POX suppression is critical for MYC-mediated cancer cell proliferation and survival. The same assays performed in PC3 prostate cancer cells confirmed these results [30].

To summarize, oncogenic transcription factor MYC inhibits PRODH/POX expression and thereby inhibits its tumor suppressor function. When MYC is suppressed, the increase of PRODH/POX promotes proline catabolism to generate ROS, leading to the initiation of apoptosis and the decrease of cell proliferation and growth. MYC-induced suppression of PRODH/POX contributes to MYC-mediated changes of cell behavior including proliferation and metabolic reprogramming, which in turn may contribute to tumorigenesis and tumor progression. These findings further indicate the critical roles of proline catabolism catalyzed by PRODH/POX in human cancers.

6.3. MYC increases the biosynthesis of proline from glutamine

Since MYC plays an important role in glutamine metabolism which is closely related with proline metabolism due to the interconversion of proline and glutamate, we not only investigated the effect of MYC on proline catabolism catalyzed by PRODH/POX as shown above, but also examined proline biosynthesis, especially from glutamine. Western blots showed that MYC robustly increased the expression of GLS, P5CS and PYCR1 in the pathway from glutamine to proline biosynthesis [30]. PC3 prostate cancer cells displayed the same correlation between MYC and glutamine and proline metabolism. The measurement of the intracellular proline levels showed that MYC dramatically increased the intracellular levels of proline. Consistently, using [^{13}C,^{15}N]-Glutamine as a tracer, the direct production of proline from glutamine induced by MYC was confirmed by GC-MS and NMR analysis [30]. Thus, MYC not only suppresses proline catabolism and stimulates glutamine oxidation to glutamate, but also markedly enhances proline biosynthesis from glutamate.

Both normal and tumor cells depend on glucose and glutamine consumption as sources of metabolic energy, and as precursors for biosynthesis of macromolecules [6, 191]. *MYC* oncogene is considered a master regulator of tumor cell metabolism and proliferation. It not only promotes glucose uptake and induces aerobic glycolysis, but also enhances glutamine uptake and stimulates glutamine catabolism. Although glutamine catabolism is linked to biosynthesis of protein, nucleotides and lipids, redox homeostasis and energy metabolism, the report from Wise *et al*. suggests that little of the glutamine uptake stimulated by MYC is used for macromolecular synthesis [6]. MYC-induced glutamine catabolism is involved in reprogramming mitochondrial metabolism to sustain cellular viability and TCA cycle anapleurosis [6]. More recent findings reported by Le *et al*. [192] and Wang *et al*. [193] emphasized the metabolic reprogramming controlled by MYC in tumor cells and activated T cells. The latter showed that glutamine catabolism driven by MYC coupled with multiple biosynthetic pathways, especially ornithine and polyamine biosynthesis [193]. However, the importance of the biosynthesis of the ornithine and polyamine from glutamine is understood only in part. Similarly, the metabolic advantage afforded by the increased conversion of glutamine to proline and how biosynthetic pathway fits into the MYC-driven metabolic reprogramming also remain unclear. The connection between the conversion of P5C to proline, the last step of proline biosynthesis and pentose phosphate pathway through the oxidation-reduction reactions of NADPH and $NADP^+$ [8, 14, 15] provides us a clue to understand the importance of proline biosynthesis induced by MYC in cancer, since proline synthesis from P5C could also oxidize NADH to NAD^+ to maintain glucose metabolism, glycolysis. In fact, our unpublished data showed that the blockade of proline biosynthesis by knocking down P5CS or PYCR1 markedly decreased glycolysis, which supports our hypothesis.

It's noteworthy that glutamine may be not the only source of proline biosynthesis promoted by MYC, since the increase of PYCR1 is much greater than that of P5CS and GLS [30], and ornithine could also be converted to proline by ornithine aminotransferase and PYCR1 (see Figure 1). This possibility and its importance in MYC-induced metabolic reprogramming are also worth pursuing.

7. Conclusion

Proline, the unique proteinogenic secondary amino acid, is metabolized by its own family of enzymes. Early studies showed that proline metabolism is linked with TCA cycle, pentose phosphate pathway and urea cycle. During the conversion of proline to P5C, the central enzyme of proline metabolism, PRODH/POX, donates electron to ETC to generate ROS or ATP depending on context. As a tumor suppressor, PRODH/POX is induced by p53, PPARγ and its ligands, and contributes to the initiation of apoptosis and the inhibition of tumor growth through ROS generation (Figure 2). On the other hand, PRODH/POX is suppressed by miR-23b* and oncogene MYC. MYC not only suppresses proline catabolism, but increases proline biosynthesis from glutamine (Figure 3). Thus, these recent studies reveal a new link in human cancer between MYC, miRNA regulation, proline metabolism, glutamine metabolism, TCA cycle, and even glycolysis. These metabolic links emphasizes the

complexity of tumor metabolism. Further studies of proline metabolism in tumor microenvironment will provide a deeper understanding of tumor metabolism and novel therapeutic strategies in cancer.

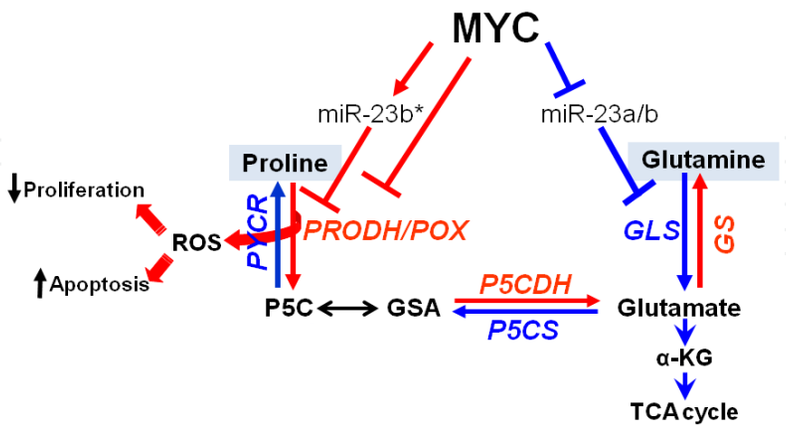

Figure 3. MYC regulation of proline and glutamine metabolism. MYC suppresses proline catabolism through its inhibition of the expression of PRODH/POX and P5CDH. MYC inhibits the expression of PRODH/POX at both transcriptional and post-transcriptional levels (upregulation of miR-23b*), which is essential for MYC-induced proliferation and cell survival. On the other hand, MYC stimulates glutamine catabolism through miR-23a/b-mediated glutaminase (GLS) upregulation. Furthermore, MYC not only suppresses proline catabolism, but also enhances proline biosynthesis from glutamine. Proline and glutamine metabolism are connected by MYC and miRNA regulation.

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