We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

MiRNA and Proline Metabolism in Cancer

Wei Liu and James M. Phang

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/55139>

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, Δ^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 *PROD*H/*PO*X in neuropsychiatric diseases [18, 19], mutations of *PYCR*1 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 *PROD*H/*PO*X 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 prolydase [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. *PROD*H/*PO*X as a mitochondrial tumor suppressor

2.1. *PROD*H/*PO*X induces apoptosis through ROS generation

*PROD*H, the gene encoding *PROD*H/*PO*X 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 *PROD*H/*PO*X [32]. To further study the function of *PROD*H/*PO*X, we developed a DLD1-*PO*X colorectal cancer cell line (designated as DLD1-*PO*X tet-off cell line), which was stably transfected with the *PROD*H 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, PPAR γ 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 (PPAR γ) is another critical regulator of PRODH/POX, besides p53. PPAR γ 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 PPAR γ was the most potent effector activating the *PRODH* promoter. PRODH/POX contributes greatly to apoptosis induced by the pharmacologic ligands of PPAR γ 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 E₂ (PGE₂) [47]. The addition of PGE₂ 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/PGE₂ 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-3 β leads to its ubiquitination and proteasomal degradation. PRODH/POX decreases phosphorylation of GSK-3 β 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-1 α 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-1 α [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 Droscha [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 miR-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 ER α - 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 tumorigenicity 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 through 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 miR-215, 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-1 β , which interacts with HIF-1 α 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-3 α , 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 (ER α), 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 ER α , 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-1 α 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, PPAR γ 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.

Author details

Wei Liu and James M. Phang*

Metabolism and Cancer Susceptibility Section, Basic Research Laboratory, Frederick National Laboratory for Cancer Research, NIH. Frederick, MD

Acknowledgement

The work was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. This project also has been funded in part with Federal funds from the National Cancer Institute, NIH, under contract no. HHSN27612080001. The content of this review does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government. We thank Dr. Ziqiang Zhu for his reading of the manuscript.

8. References

- [1] Warburg O (1956) On the origin of cancer cells, *Science* 123, 309-314.

* Corresponding Author

- [2] Vander Heiden MG, Cantley LC, & Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation, *Science* 324, 1029-1033.
- [3] Fogal V, Richardson AD, Karmali PP, Scheffler IE, Smith JW, & Ruoslahti E (2010) Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation, *Mol Cell Biol* 30, 1303-1318.
- [4] Dang CV (2010) Rethinking the Warburg effect with Myc micromanaging glutamine metabolism, *Cancer Res* 70, 859-862.
- [5] Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, *et al.* (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism, *Nature* 458, 762-765.
- [6] Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, *et al.* (2008) Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction, *Proc Natl Acad Sci U S A* 105, 18782-18787.
- [7] Adams E (1970) Metabolism of proline and of hydroxyproline, *Int Rev Connect Tissue Res* 5, 1-91.
- [8] Phang JM (1985) The regulatory functions of proline and pyrroline-5-carboxylic acid, *Curr Top Cell Regul* 25, 91-132.
- [9] Phang JM, Liu W, & Zabirnyk O (2010) Proline metabolism and microenvironmental stress, *Annu Rev Nutr* 30, 441-463.
- [10] Phang JM & Liu W (2012) Proline metabolism and cancer, *Front Biosci* 17, 1835-1845.
- [11] Phang JM, Hu CA, & Valle D (2001) Disorders of proline and hydroxyproline metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. In *Metabolic and Molecular Bases of Inherited Disease*, New York: McGraw-Hill. pp. 1821-1838.
- [12] Adams E & Frank L (1980) Metabolism of proline and the hydroxyprolines, *Annu Rev Biochem* 49, 1005-1061.
- [13] Liu Y, Borchert GL, Donald SP, Surazynski A, Hu CA, Weydert CJ, *et al.* (2005) MnSOD inhibits proline oxidase-induced apoptosis in colorectal cancer cells, *Carcinogenesis* 26, 1335-1342.
- [14] Phang JM, Downing SJ, Yeh GC, Smith RJ, Williams JA, & Hagedorn CH (1982) Stimulation of the hexosemonophosphate-pentose pathway by pyrroline-5-carboxylate in cultured cells, *J Cell Physiol* 110, 255-261.
- [15] Yeh GC, Roth EF, Jr., Phang JM, Harris SC, Nagel RL, & Rinaldi A (1984) The effect of pyrroline-5-carboxylic acid on nucleotide metabolism in erythrocytes from normal and glucose-6-phosphate dehydrogenase-deficient subjects, *J Biol Chem* 259, 5454-5458.
- [16] Hu CA, Bart Williams D, Zhaorigetu S, Khalil S, Wan G, & Valle D (2008) Functional genomics and SNP analysis of human genes encoding proline metabolic enzymes, *Amino Acids* 35, 655-664.
- [17] Schafer IA, Scriver CR, & Efron ML (1962) Familial hyperprolinemia, cerebral dysfunction and renal anomalies occurring in a family with hereditary nephropathy and deafness, *N Engl J Med* 267, 51-60.
- [18] Willis A, Bender HU, Steel G, & Valle D (2008) PRODH variants and risk for schizophrenia, *Amino Acids* 35, 673-679.

- [19] Bender HU, Almashanu S, Steel G, Hu CA, Lin WW, Willis A, *et al.* (2005) Functional consequences of PRODH missense mutations, *Am J Hum Genet* 76, 409-420.
- [20] Reversade B, Escande-Beillard N, Dimopoulou A, Fischer B, Chng SC, Li Y, *et al.* (2009) Mutations in PYCR1 cause cutis laxa with progeroid features, *Nat Genet* 41, 1016-1021.
- [21] Baumgartner MR, Hu CA, Almashanu S, Steel G, Obie C, Aral B, *et al.* (2000) Hyperammonemia with reduced ornithine, citrulline, arginine and proline: a new inborn error caused by a mutation in the gene encoding delta(1)-pyrroline-5-carboxylate synthase, *Hum Mol Genet* 9, 2853-2858.
- [22] Baumgartner MR, Rabier D, Nassogne MC, Dufier JL, Padovani JP, Kamoun P, *et al.* (2005) Delta1-pyrroline-5-carboxylate synthase deficiency: neurodegeneration, cataracts and connective tissue manifestations combined with hyperammonaemia and reduced ornithine, citrulline, arginine and proline, *Eur J Pediatr* 164, 31-36.
- [23] Dixit SN, Seyer JM, & Kang AH (1977) Covalent structure of collagen: amino-acid sequence of chymotryptic peptides from the carboxyl-terminal region of alpha2-CB3 of chick-skin collagen, *Eur J Biochem* 81, 599-607.
- [24] Stallings-Mann M & Radisky D (2007) Matrix metalloproteinase-induced malignancy in mammary epithelial cells, *Cells Tissues Organs* 185, 104-110.
- [25] Deryugina EI & Quigley JP (2006) Matrix metalloproteinases and tumor metastasis, *Cancer Metastasis Rev* 25, 9-34.
- [26] Kakkad SM, Solaiyappan M, O'Rourke B, Stasinopoulos I, Ackerstaff E, Raman V, *et al.* (2010) Hypoxic tumor microenvironments reduce collagen I fiber density, *Neoplasia* 12, 608-617.
- [27] Pandhare J, Donald SP, Cooper SK, & Phang JM (2009) Regulation and function of proline oxidase under nutrient stress, *J Cell Biochem* 107, 759-768.
- [28] Klionsky DJ (2007) Autophagy: from phenomenology to molecular understanding in less than a decade, *Nat Rev Mol Cell Biol* 8, 931-937.
- [29] Mathew R, Karantza-Wadsworth V, & White E (2007) Role of autophagy in cancer, *Nat Rev Cancer* 7, 961-967.
- [30] Liu W, Le A, Hancock C, Lane AN, Dang CV, Fan TW, *et al.* (2012) Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC, *Proc Natl Acad Sci U S A* 109, 8983-8988.
- [31] Polyak K, Xia Y, Zweier JL, Kinzler KW, & Vogelstein B (1997) A model for p53-induced apoptosis, *Nature* 389, 300-305.
- [32] Rivera A & Maxwell SA (2005) The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway, *J Biol Chem* 280, 29346-29354.
- [33] Donald SP, Sun XY, Hu CA, Yu J, Mei JM, Valle D, *et al.* (2001) Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species, *Cancer Res* 61, 1810-1815.
- [34] Simon HU, Haj-Yehia A, & Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction, *Apoptosis* 5, 415-418.

- [35] Hu CA, Donald SP, Yu J, Lin WW, Liu Z, Steel G, *et al.* (2007) Overexpression of proline oxidase induces proline-dependent and mitochondria-mediated apoptosis, *Mol Cell Biochem* 295, 85-92.
- [36] Liu Y, Borchert GL, Surazynski A, Hu CA, & Phang JM (2006) Proline oxidase activates both intrinsic and extrinsic pathways for apoptosis: the role of ROS/superoxides, NFAT and MEK/ERK signaling, *Oncogene* 25, 5640-5647.
- [37] Willson TM, Brown PJ, Sternbach DD, & Henke BR (2000) The PPARs: from orphan receptors to drug discovery, *J Med Chem* 43, 527-550.
- [38] Robbins GT & Nie D (2012) PPAR gamma, bioactive lipids, and cancer progression, *Front Biosci* 17, 1816-1834.
- [39] Reka AK, Goswami MT, Krishnapuram R, Standiford TJ, & Keshamouni VG (2011) Molecular cross-regulation between PPAR-gamma and other signaling pathways: implications for lung cancer therapy, *Lung Cancer* 72, 154-159.
- [40] Phang JM, Pandhare J, Zabinryk O, & Liu Y (2008) PPARgamma and Proline Oxidase in Cancer, *PPAR Res* 2008, 542694.
- [41] Pandhare J, Cooper SK, & Phang JM (2006) Proline oxidase, a proapoptotic gene, is induced by troglitazone: evidence for both peroxisome proliferator-activated receptor gamma-dependent and -independent mechanisms, *J Biol Chem* 281, 2044-2052.
- [42] Kim KY, Ahn JH, & Cheon HG (2007) Apoptotic action of peroxisome proliferator-activated receptor-gamma activation in human non small-cell lung cancer is mediated via proline oxidase-induced reactive oxygen species formation, *Mol Pharmacol* 72, 674-685.
- [43] Liu W, Glunde K, Bhujwalla ZM, Raman V, Sharma A, & Phang JM (2012) Proline oxidase promotes tumor cell survival in hypoxic tumor microenvironments, *Cancer Res* 72, 3677-3686.
- [44] Zabinryk O, Liu W, Khalil S, Sharma A, & Phang JM (2010) Oxidized low-density lipoproteins upregulate proline oxidase to initiate ROS-dependent autophagy, *Carcinogenesis* 31, 446-454.
- [45] Smith ML & Kumar MA (2010) The "Two faces" of Tumor Suppressor p53-revisited, *Mol Cell Pharmacol* 2, 117-119.
- [46] Liu Y, Borchert GL, Donald SP, Diwan BA, Anver M, & Phang JM (2009) Proline Oxidase Functions as a Mitochondrial Tumor Suppressor in Human Cancers, *Cancer Res* 69, 6414-6422.
- [47] Liu Y, Borchert GL, Surazynski A, & Phang JM (2008) Proline oxidase, a p53-induced gene, targets COX-2/PGE2 signaling to induce apoptosis and inhibit tumor growth in colorectal cancers, *Oncogene* 27, 6729-6737.
- [48] Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, *et al.* (2009) The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment, *Carcinogenesis* 30, 377-386.
- [49] Brown JR & DuBois RN (2004) Cyclooxygenase as a target in lung cancer, *Clin Cancer Res* 10, 4266s-4269s.
- [50] Arun B & Goss P (2004) The role of COX-2 inhibition in breast cancer treatment and prevention, *Semin Oncol* 31, 22-29.

- [51] Henson ES & Gibson SB (2006) Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy, *Cell Signal* 18, 2089-2097.
- [52] Yao H, Ashihara E, & Maekawa T (2011) Targeting the Wnt/beta-catenin signaling pathway in human cancers, *Expert Opin Ther Targets* 15, 873-887.
- [53] Zhu W, Chen Y, & Dutta A (2004) Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint, *Mol Cell Biol* 24, 7140-7150.
- [54] Stark GR & Taylor WR (2006) Control of the G2/M transition, *Mol Biotechnol* 32, 227-248.
- [55] Liebermann DA & Hoffman B (2008) Gadd45 in stress signaling, *J Mol Signal* 3, 15.
- [56] Gottlieb E & Tomlinson IP (2005) Mitochondrial tumour suppressors: a genetic and biochemical update, *Nat Rev Cancer* 5, 857-866.
- [57] Yamakuchi M, Lotterman CD, Bao C, Hruban RH, Karim B, Mendell JT, *et al.* (2010) P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis, *Proc Natl Acad Sci U S A* 107, 6334-6339.
- [58] Verma A (2006) Oxygen-sensing in tumors, *Curr Opin Clin Nutr Metab Care* 9, 366-378.
- [59] Hewitson KS, Lienard BM, McDonough MA, Clifton IJ, Butler D, Soares AS, *et al.* (2007) Structural and mechanistic studies on the inhibition of the hypoxia-inducible transcription factor hydroxylases by tricarboxylic acid cycle intermediates, *J Biol Chem* 282, 3293-3301.
- [60] Koivunen P, Hirsila M, Remes AM, Hassinen IE, Kivirikko KI, & Myllyharju J (2007) Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF, *J Biol Chem* 282, 4524-4532.
- [61] Lu H, Dalgard CL, Mohyeldin A, McFate T, Tait AS, & Verma A (2005) Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1, *J Biol Chem* 280, 41928-41939.
- [62] Liu W, Zabirnyk O, Wang H, Shiao YH, Nickerson ML, Khalil S, *et al.* (2010) miR-23b targets proline oxidase, a novel tumor suppressor protein in renal cancer, *Oncogene* 29, 4914-4924.
- [63] Lee RC, Feinbaum RL, & Ambros V (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*, *Cell* 75, 843-854.
- [64] Wightman B, Ha I, & Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*, *Cell* 75, 855-862.
- [65] Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, *et al.* (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*, *Nature* 403, 901-906.
- [66] Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, *et al.* (2004) MicroRNA genes are transcribed by RNA polymerase II, *EMBO J* 23, 4051-4060.
- [67] Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, *et al.* (2003) The nuclear RNase III Drosha initiates microRNA processing, *Nature* 425, 415-419.

- [68] Denli AM, Tops BB, Plasterk RH, Ketting RF, & Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex, *Nature* 432, 231-235.
- [69] Yi R, Qin Y, Macara IG, & Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs, *Genes Dev* 17, 3011-3016.
- [70] Bohnsack MT, Czaplinski K, & Gorlich D (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs, *RNA* 10, 185-191.
- [71] Lund E, Guttinger S, Calado A, Dahlberg JE, & Kutay U (2004) Nuclear export of microRNA precursors, *Science* 303, 95-98.
- [72] Lund E & Dahlberg JE (2006) Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs, *Cold Spring Harb Symp Quant Biol* 71, 59-66.
- [73] Khvorova A, Reynolds A, & Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias, *Cell* 115, 209-216.
- [74] Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, & Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex, *Cell* 115, 199-208.
- [75] Lin SL, Chang D, & Ying SY (2005) Asymmetry of intronic pre-miRNA structures in functional RISC assembly, *Gene* 356, 32-38.
- [76] Kim S, Lee UJ, Kim MN, Lee EJ, Kim JY, Lee MY, *et al.* (2008) MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2), *J Biol Chem* 283, 18158-18166.
- [77] Nass D, Rosenwald S, Meiri E, Gilad S, Tabibian-Keissar H, Schlosberg A, *et al.* (2009) MiR-92b and miR-9/9* are specifically expressed in brain primary tumors and can be used to differentiate primary from metastatic brain tumors, *Brain Pathol* 19, 375-383.
- [78] Okamura K, Chung WJ, & Lai EC (2008) The long and short of inverted repeat genes in animals: microRNAs, mirtrons and hairpin RNAs, *Cell Cycle* 7, 2840-2845.
- [79] Carmell MA, Xuan Z, Zhang MQ, & Hannon GJ (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis, *Genes Dev* 16, 2733-2742.
- [80] Song JJ, Liu J, Tolia NH, Schneiderman J, Smith SK, Martienssen RA, *et al.* (2003) The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes, *Nat Struct Biol* 10, 1026-1032.
- [81] Ma JB, Ye K, & Patel DJ (2004) Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain, *Nature* 429, 318-322.
- [82] Lingel A & Sattler M (2005) Novel modes of protein-RNA recognition in the RNAi pathway, *Curr Opin Struct Biol* 15, 107-115.
- [83] Okamura K, Ishizuka A, Siomi H, & Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways, *Genes Dev* 18, 1655-1666.
- [84] Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, *et al.* (2004) Argonaute2 is the catalytic engine of mammalian RNAi, *Science* 305, 1437-1441.
- [85] Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, & Tuschl T (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs, *Mol Cell* 15, 185-197.
- [86] van Kouwenhove M, Kedde M, & Agami R (2011) MicroRNA regulation by RNA-binding proteins and its implications for cancer, *Nat Rev Cancer* 11, 644-656.

- [87] Melo SA, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA, *et al.* (2009) A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function, *Nat Genet* 41, 365-370.
- [88] Hill DA, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, Desruisseau D, *et al.* (2009) DICER1 mutations in familial pleuropulmonary blastoma, *Science* 325, 965.
- [89] MacRae IJ, Ma E, Zhou M, Robinson CV, & Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex, *Proc Natl Acad Sci U S A* 105, 512-517.
- [90] Murchison EP & Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery, *Curr Opin Cell Biol* 16, 223-229.
- [91] Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, *et al.* (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs, *Genes Dev* 16, 720-728.
- [92] Caudy AA, Myers M, Hannon GJ, & Hammond SM (2002) Fragile X-related protein and VIG associate with the RNA interference machinery, *Genes Dev* 16, 2491-2496.
- [93] Winter J & Diederichs S (2011) Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization, *RNA Biol* 8, 1149-1157.
- [94] Diederichs S & Haber DA (2007) Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression, *Cell* 131, 1097-1108.
- [95] O'Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, *et al.* (2007) A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway, *Genes Dev* 21, 1999-2004.
- [96] Zhang X, Graves PR, & Zeng Y (2009) Stable Argonaute2 overexpression differentially regulates microRNA production, *Biochim Biophys Acta* 1789, 153-159.
- [97] Kai ZS & Pasquinelli AE (2010) MicroRNA assassins: factors that regulate the disappearance of miRNAs, *Nat Struct Mol Biol* 17, 5-10.
- [98] Azuma-Mukai A, Oguri H, Mituyama T, Qian ZR, Asai K, Siomi H, *et al.* (2008) Characterization of endogenous human Argonautes and their miRNA partners in RNA silencing, *Proc Natl Acad Sci U S A* 105, 7964-7969.
- [99] Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, *et al.* (2005) Methylation as a crucial step in plant microRNA biogenesis, *Science* 307, 932-935.
- [100] Li J, Yang Z, Yu B, Liu J, & Chen X (2005) Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis, *Curr Biol* 15, 1501-1507.
- [101] Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, *et al.* (2007) A mammalian microRNA expression atlas based on small RNA library sequencing, *Cell* 129, 1401-1414.
- [102] Lu S, Sun YH, & Chiang VL (2009) Adenylation of plant miRNAs, *Nucleic Acids Res* 37, 1878-1885.
- [103] Reid JG, Nagaraja AK, Lynn FC, Drabek RB, Muzny DM, Shaw CA, *et al.* (2008) Mouse let-7 miRNA populations exhibit RNA editing that is constrained in the 5'-seed/cleavage/anchor regions and stabilize predicted mmu-let-7a:mRNA duplexes, *Genome Res* 18, 1571-1581.

- [104] Katoh T, Sakaguchi Y, Miyauchi K, Suzuki T, Kashiwabara S, & Baba T (2009) Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2, *Genes Dev* 23, 433-438.
- [105] Valencia-Sanchez MA, Liu J, Hannon GJ, & Parker R (2006) Control of translation and mRNA degradation by miRNAs and siRNAs, *Genes Dev* 20, 515-524.
- [106] Billeter AT, Druen D, Kanaan ZM, & Polk HC, Jr. (2012) MicroRNAs: new helpers for surgeons?, *Surgery* 151, 1-5.
- [107] Lewis BP, Burge CB, & Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, *Cell* 120, 15-20.
- [108] Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang G, Zamore PD, Barton MK, *et al.* (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region, *EMBO J* 23, 3356-3364.
- [109] Yekta S, Shih IH, & Bartel DP (2004) MicroRNA-directed cleavage of HOXB8 mRNA, *Science* 304, 594-596.
- [110] Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell* 116, 281-297.
- [111] Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, *et al.* (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells, *Science* 309, 1573-1576.
- [112] Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, *et al.* (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2, *Proc Natl Acad Sci U S A* 102, 13944-13949.
- [113] Williams AE (2008) Functional aspects of animal microRNAs, *Cell Mol Life Sci* 65, 545-562.
- [114] Zeng Y, Yi R, & Cullen BR (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms, *Proc Natl Acad Sci U S A* 100, 9779-9784.
- [115] Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, *et al.* (2004) A combined computational-experimental approach predicts human microRNA targets, *Genes Dev* 18, 1165-1178.
- [116] Bartel DP & Chen CZ (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs, *Nat Rev Genet* 5, 396-400.
- [117] Eulalio A, Huntzinger E, & Izaurralde E (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay, *Nat Struct Mol Biol* 15, 346-353.
- [118] Behm-Ansmant I, Rehwinkel J, & Izaurralde E (2006) MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay, *Cold Spring Harb Symp Quant Biol* 71, 523-530.
- [119] Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, *et al.* (2006) A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes, *Cell* 126, 1203-1217.

- [120] Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, *et al.* (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia, *Proc Natl Acad Sci U S A* 99, 15524-15529.
- [121] Caldas C & Brenton JD (2005) Sizing up miRNAs as cancer genes, *Nat Med* 11, 712-714.
- [122] Calin GA & Croce CM (2006) MicroRNAs and chromosomal abnormalities in cancer cells, *Oncogene* 25, 6202-6210.
- [123] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, *et al.* (2005) MicroRNA expression profiles classify human cancers, *Nature* 435, 834-838.
- [124] Sandhu S & Garzon R (2011) Potential applications of microRNAs in cancer diagnosis, prognosis, and treatment, *Semin Oncol* 38, 781-787.
- [125] Croce CM (2009) Causes and consequences of microRNA dysregulation in cancer, *Nat Rev Genet* 10, 704-714.
- [126] Nicoloso MS, Spizzo R, Shimizu M, Rossi S, & Calin GA (2009) MicroRNAs--the micro steering wheel of tumour metastases, *Nat Rev Cancer* 9, 293-302.
- [127] Lujambio A & Lowe SW (2012) The microcosmos of cancer, *Nature* 482, 347-355.
- [128] Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, *et al.* (2006) A microRNA expression signature of human solid tumors defines cancer gene targets, *Proc Natl Acad Sci U S A* 103, 2257-2261.
- [129] Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, *et al.* (2008) Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes, *Nat Immunol* 9, 405-414.
- [130] Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, *et al.* (2008) Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters, *Cell* 132, 875-886.
- [131] He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, *et al.* (2005) A microRNA polycistron as a potential human oncogene, *Nature* 435, 828-833.
- [132] Diosdado B, van de Wiel MA, Terhaar Sive Droste JS, Mongera S, Postma C, Meijerink WJ, *et al.* (2009) MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression, *Br J Cancer* 101, 707-714.
- [133] Mu P, Han YC, Betel D, Yao E, Squatrito M, Ogrodowski P, *et al.* (2009) Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas, *Genes Dev* 23, 2806-2811.
- [134] Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, *et al.* (2008) E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer, *Cancer Cell* 13, 272-286.
- [135] Mendell JT (2008) miRiad roles for the miR-17-92 cluster in development and disease, *Cell* 133, 217-222.
- [136] Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankeu A, *et al.* (2009) miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation, *Cancer Cell* 16, 498-509.

- [137] Di Leva G, Gasparini P, Piovan C, Ngankeu A, Garofalo M, Taccioli C, *et al.* (2010) MicroRNA cluster 221-222 and estrogen receptor alpha interactions in breast cancer, *J Natl Cancer Inst* 102, 706-721.
- [138] Hu W, Chan CS, Wu R, Zhang C, Sun Y, Song JS, *et al.* (2010) Negative regulation of tumor suppressor p53 by microRNA miR-504, *Mol Cell* 38, 689-699.
- [139] Roush S & Slack FJ (2008) The let-7 family of microRNAs, *Trends Cell Biol* 18, 505-516.
- [140] Bueno MJ, Gomez de Cedron M, Gomez-Lopez G, Perez de Castro I, Di Lisio L, Montes-Moreno S, *et al.* (2011) Combinatorial effects of microRNAs to suppress the Myc oncogenic pathway, *Blood* 117, 6255-6266.
- [141] Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, *et al.* (2008) MiR-15a and miR-16-1 cluster functions in human leukemia, *Proc Natl Acad Sci U S A* 105, 5166-5171.
- [142] Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, *et al.* (2008) The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities, *Nat Med* 14, 1271-1277.
- [143] Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, *et al.* (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model, *Cell* 137, 1005-1017.
- [144] Garzon R, Marcucci G, & Croce CM (2010) Targeting microRNAs in cancer: rationale, strategies and challenges, *Nat Rev Drug Discov* 9, 775-789.
- [145] Dang CV (1999) c-Myc target genes involved in cell growth, apoptosis, and metabolism, *Mol Cell Biol* 19, 1-11.
- [146] Eilers M & Eisenman RN (2008) Myc's broad reach, *Genes Dev* 22, 2755-2766.
- [147] Dang CV, Le A, & Gao P (2009) MYC-induced cancer cell energy metabolism and therapeutic opportunities, *Clin Cancer Res* 15, 6479-6483.
- [148] Dews M, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, *et al.* (2006) Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster, *Nat Genet* 38, 1060-1065.
- [149] O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, & Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression, *Nature* 435, 839-843.
- [150] Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, *et al.* (2010) miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis, *Nat Cell Biol* 12, 247-256.
- [151] Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, *et al.* (2008) Widespread microRNA repression by Myc contributes to tumorigenesis, *Nat Genet* 40, 43-50.
- [152] Bui TV & Mendell JT (2010) Myc: Maestro of MicroRNAs, *Genes Cancer* 1, 568-575.
- [153] Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, *et al.* (2010) The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia, *Cancer Cell* 17, 28-40.
- [154] Cairo S, Wang Y, de Reynies A, Duroire K, Dahan J, Redon MJ, *et al.* (2010) Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer, *Proc Natl Acad Sci U S A* 107, 20471-20476.

- [155] Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, *et al.* (2007) Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression, *Mol Cell Biol* 27, 2240-2252.
- [156] Chang TC, Zeitels LR, Hwang HW, Chivukula RR, Wentzel EA, Dews M, *et al.* (2009) Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation, *Proc Natl Acad Sci U S A* 106, 3384-3389.
- [157] Newman MA, Thomson JM, & Hammond SM (2008) Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing, *RNA* 14, 1539-1549.
- [158] Hagan JP, Piskounova E, & Gregory RI (2009) Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells, *Nat Struct Mol Biol* 16, 1021-1025.
- [159] Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, *et al.* (2009) TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation, *Cell* 138, 696-708.
- [160] Feng Z, Zhang C, Wu R, & Hu W (2011) Tumor suppressor p53 meets microRNAs, *J Mol Cell Biol* 3, 44-50.
- [161] He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, *et al.* (2007) A microRNA component of the p53 tumour suppressor network, *Nature* 447, 1130-1134.
- [162] Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, *et al.* (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis, *Mol Cell* 26, 731-743.
- [163] Jin L, Hu WL, Jiang CC, Wang JX, Han CC, Chu P, *et al.* (2011) MicroRNA-149*, a p53-responsive microRNA, functions as an oncogenic regulator in human melanoma, *Proc Natl Acad Sci U S A* 108, 15840-15845.
- [164] Sachdeva M, Zhu S, Wu F, Wu H, Walia V, Kumar S, *et al.* (2009) p53 represses c-Myc through induction of the tumor suppressor miR-145, *Proc Natl Acad Sci U S A* 106, 3207-3212.
- [165] Georges SA, Biery MC, Kim SY, Schelter JM, Guo J, Chang AN, *et al.* (2008) Coordinated regulation of cell cycle transcripts by p53-Inducible microRNAs, miR-192 and miR-215, *Cancer Res* 68, 10105-10112.
- [166] Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, & Miyazono K (2009) Modulation of microRNA processing by p53, *Nature* 460, 529-533.
- [167] Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, *et al.* (2007) A microRNA signature of hypoxia, *Mol Cell Biol* 27, 1859-1867.
- [168] Mutharasan RK, Nagpal V, Ichikawa Y, & Ardehali H (2011) microRNA-210 is upregulated in hypoxic cardiomyocytes through Akt- and p53-dependent pathways and exerts cytoprotective effects, *Am J Physiol Heart Circ Physiol* 301, H1519-1530.
- [169] Hu S, Huang M, Li Z, Jia F, Ghosh Z, Lijkwan MA, *et al.* (2010) MicroRNA-210 as a novel therapy for treatment of ischemic heart disease, *Circulation* 122, S124-131.
- [170] Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, *et al.* (2008) hsa-miR-210 Is induced by hypoxia and is an independent prognostic factor in breast cancer, *Clin Cancer Res* 14, 1340-1348.

- [171] Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, *et al.* (2008) MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3, *J Biol Chem* 283, 15878-15883.
- [172] Kim HW, Haider HK, Jiang S, & Ashraf M (2009) Ischemic preconditioning augments survival of stem cells via miR-210 expression by targeting caspase-8-associated protein 2, *J Biol Chem* 284, 33161-33168.
- [173] Zhang Z, Sun H, Dai H, Walsh RM, Imakura M, Schelter J, *et al.* (2009) MicroRNA miR-210 modulates cellular response to hypoxia through the MYC antagonist MNT, *Cell Cycle* 8, 2756-2768.
- [174] Kent OA, Chivukula RR, Mullendore M, Wentzel EA, Feldmann G, Lee KH, *et al.* (2010) Repression of the miR-143/145 cluster by oncogenic Ras initiates a tumor-promoting feed-forward pathway, *Genes Dev* 24, 2754-2759.
- [175] Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, *et al.* (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers, *Proc Natl Acad Sci U S A* 101, 2999-3004.
- [176] Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, *et al.* (2006) microRNAs exhibit high frequency genomic alterations in human cancer, *Proc Natl Acad Sci U S A* 103, 9136-9141.
- [177] Mayr C, Hemann MT, & Bartel DP (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation, *Science* 315, 1576-1579.
- [178] Veronese A, Visone R, Consiglio J, Acunzo M, Lupini L, Kim T, *et al.* (2011) Mutated beta-catenin evades a microRNA-dependent regulatory loop, *Proc Natl Acad Sci U S A* 108, 4840-4845.
- [179] Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, *et al.* (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells, *Cancer Cell* 9, 435-443.
- [180] Cao Q, Mani RS, Ateeq B, Dhanasekaran SM, Asangani IA, Prensner JR, *et al.* (2011) Coordinated regulation of polycomb group complexes through microRNAs in cancer, *Cancer Cell* 20, 187-199.
- [181] Davalos V, Moutinho C, Villanueva A, Boque R, Silva P, Carneiro F, *et al.* (2011) Dynamic epigenetic regulation of the microRNA-200 family mediates epithelial and mesenchymal transitions in human tumorigenesis, *Oncogene* doi: 10.1038/onc.2011.383.
- [182] Guo QM, Malek RL, Kim S, Chiao C, He M, Ruffey M, *et al.* (2000) Identification of c-myc responsive genes using rat cDNA microarray, *Cancer Res* 60, 5922-5928.
- [183] Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, & Ren B (2003) A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells, *Proc Natl Acad Sci U S A* 100, 8164-8169.
- [184] Maxwell SA & Rivera A (2003) Proline oxidase induces apoptosis in tumor cells, and its expression is frequently absent or reduced in renal carcinomas, *J Biol Chem* 278, 9784-9789.
- [185] Kulshreshtha R, Davuluri RV, Calin GA, & Ivan M (2008) A microRNA component of the hypoxic response, *Cell Death Differ* 15, 667-671.

- [186] Guimbellot JS, Erickson SW, Mehta T, Wen H, Page GP, Sorscher EJ, *et al.* (2009) Correlation of microRNA levels during hypoxia with predicted target mRNAs through genome-wide microarray analysis, *BMC Med Genomics* 2, 15.
- [187] Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, *et al.* (2007) HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity, *Cancer Cell* 11, 407-420.
- [188] Vafa O, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM, *et al.* (2002) c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability, *Mol Cell* 9, 1031-1044.
- [189] DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, *et al.* (2011) Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis, *Nature* 475, 106-109.
- [190] Wonsey DR, Zeller KI, & Dang CV (2002) The c-Myc target gene PRDX3 is required for mitochondrial homeostasis and neoplastic transformation, *Proc Natl Acad Sci U S A* 99, 6649-6654.
- [191] Fan TW, Tan JL, McKinney MM, & Lane AN (2011) Stable isotope resolved metabolomics analysis of ribonucleotide and RNA metabolism in human lung cancer cells *Metabolomics* doi:10.1007/s11306-011-0337-9.
- [192] Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, *et al.* (2012) Glucose-Independent Glutamine Metabolism via TCA Cycling for Proliferation and Survival in B Cells, *Cell Metab* 15, 110-121.
- [193] Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, *et al.* (2011) The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation, *Immunity* 35, 871-882.