anti-SIRT2 (H-95, sc-20966) from Santa Cruz Biotechnology, Inc.

Measurement of PGAM activity

PGAM enzymatic activity was assessed as described previously (Mikawa *et al.* 2014). Briefly, total cell lysates were prepared, and 5 μg of the lysate was incubated with reaction buffer containing 0.2 mM NADH, 3 mM ADP, 10 μM 2, 3-diphosphoglycerate, 0.6 U/mL lactate dehydrogenase, 0.5 U/mL pyruvate kinase, 0.1 U/mL enolase. To start the reactions, 3-phosphoglyceric acid was added to 1 mM, and activity was measured as NADH degradation rate for 20 min by monitoring the absorbance at 340 nm in a Spectra max spectrophotometer (Molecular Devices). Measurements were carried out at 37 °C.

Acknowledgements

We thank the staff in the Diabetes, Endocrinology and Nutrition department of Kyoto University for their cooperation; Ms Eri Shibata for providing excellent technical assistance; Dr Eriko Michishita for human Sirtuins (SIRT1-7) expression vectors. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, from Japan Science and Technology Agency, and by JST, CREST (to H.K.).

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Received: 9 June 2014
Accepted: 31 July 2014

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1 Identification of the specific acetylated residues in the central region of PGAM.

Figure S2 Comparison of PCAF expression levels in HCT116 cells and HEK293 cells.

Tables S1 and S2 Plasmids (Table S1) and primers (Table S2) used in this study

Cell. Mol. Life Sci. (2015) 72:1881–1892
DOI 10.1007/s00018-015-1840-3

Cellular and Molecular Life Sciences

REVIEW

Dysregulated glycolysis as an oncogenic event

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Received: 30 September 2014 / Revised: 11 January 2015 / Accepted: 13 January 2015 / Published online: 22 January 2015

Abstract Enhanced glycolysis in cancer, called the Warburg effect, is a well-known feature of cancer metabolism. Recent advances revealed that the Warburg effect is coupled to many other cancer properties, including adaptation to hypoxia and low nutrients, immortalisation, resistance to oxidative stress and apoptotic stimuli, and elevated biomass synthesis. These linkages are mediated by various oncogenic molecules and signals, such as c-Myc, p53, and the insulin/Ras pathway. Furthermore, several regulators of glycolysis have been recently identified as oncogene candidates, including the hypoxia-inducible factor pathway, sirtuins, adenosine monophosphate-activated kinase, glycolytic pyruvate kinase M2, phosphoglycerate mutase, and oncometabolites. The interplay between glycolysis and oncogenic events will be the focus of this review.

Keywords Warburg effect · Cancer · HIF-1 · Sirtuin · Myc · PGAM

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Introduction

As glycolysis is essential for energy production in almost all mammalian cells, impaired glycolysis was assumed to have a pathological effect in various human diseases, including diabetes mellitus and muscle atrophy [1, 2]. Among the first descriptions of enhanced glycolysis in diseased states was the Warburg effect, which was proposed by Otto Warburg [3] after he observed that cancer cells preferably covert glucose into lactate even in the presence of oxygen. Indeed, enhanced glycolysis was subsequently found to be a metabolic characteristic of many cancers [3], and the upregulation of protein levels and enzymatic activities of many glycolytic enzymes was later confirmed [4, 5]. It was initially thought that enhanced glycolysis may provide an energy boost to meet the demands of the high proliferation rate of cancer cells. However, energy generation via glycolysis is relatively inefficient, as it generates only two ATP molecules per glucose, whereas the TCA cycle in mitochondria generates 36 ATPs per glucose [6]. Thus, the reason for cancer cells to favour enhanced glycolysis cannot be simply explained by the efficiency of energy production. Recent studies have revealed causal effects of enhanced glycolysis on cancerous growth, including an increase in biomass synthesis [7, 8] and radical scavenger activities [9]. These additional aspects of the Warburg effect might

partly explain the preference for enhanced glycolysis in cancer.

Inhibition of the Warburg effect has been proposed as a possible cancer therapy [6]; however, this strategy is problematic as the glycolytic pathway is also required in normal tissues. Thus, cancer therapies targeting the Warburg effect must induce cancer-specific and localised inhibition of glycolysis in order to minimise possible side effects. An important step will be to determine how glycolysis is dysregulated in cancer, while strictly regulated in healthy cells. In addition to their high proliferative capacity, cancer cells exhibit several cytological hallmarks. These include immortalisation, stress resistance mechanisms such as evasion from apoptotic stimuli, survival under nutrient-limited conditions, metastatic capacity, and anchorage-independent growth [10]. It is possible that the Warburg effect promotes these properties, which are known to be associated with genetic alterations and modulations in signalling pathways [11, 12]. Any links between the Warburg effect and oncogenic signalling pathways would be of great interest as potential targets for anticancer therapy [13, 14]. Here, we provide an overview of recent advances in our understanding of glycolysis regulation in cancer and the Warburg effect.

Cellular-context-dependent regulation of glycolysis

Glycolysis is a highly conserved metabolic process that involves sequential reactions mediated by several glycolytic enzymes. The sequences of the genes encoding these enzymes and the intermediate metabolites in glycolysis are highly conserved from bacteria to humans, implicating its fundamental importance for all living cells. It has been well established that phosphofructokinase (PFK) is the rate-limiting enzyme for the glycolytic pathway owing to its allosteric regulation, and this has been shown not only in bacteria and yeast, but also in cancerous cells and muscle cells in vitro [15, 16].

The regulation of glycolytic metabolism in mammalian cells depends on many factors, including differentiation status, growth conditions, and cellular environment (availability of oxygen, nutrients, etc.) [17, 18]. For example, normal cells might adapt to hypoxic conditions by enhancing anaerobic glycolysis and limiting energy demands. However, cancer cells continue growing even under hypoxic conditions in vivo, and this might require a maladaptive metabolic shift [19]. Thus, the fine tunings of glycolysis observed in normal cells are dysregulated in cancer cells to support their demand for excess glycolysis (Fig. 1). Indeed, recent studies have revealed that in addition to PFK, several glycolytic enzymes play key roles in establishing the Warburg effect in cancer.

Transport of glucose across the plasma membrane is the first rate-limiting step for glucose metabolism, which is mediated by GLUT proteins. Among them, GLUT1, GLUT3 and GLUT12 have been reported to be upregulated in some cancers [20]. Hexokinase (HK) mediates the critical first step of glycolysis; generation of glucose-6-phosphate (G-6-P) via phosphate transfer from ATP. Mammalian four isoforms of HK are designated as HK-1 to HK-4. Their intracellular localizations are variable; HK-1 and HK-2 mainly on the outer membrane of mitochondria, HK-3 in a perinuclear regions, and HK-4 in the cytosol. Their tissue distributions are also various. For example, HK-4, known as glucokinase, is mainly expressed in liver and pancreas. However, in cancer cells, HK-2 is predominantly overexpressed for following reasons. HK-1, -2, and -3 shows over 200-fold lower K_m for glucose compared to that of HK-4. Moreover, HK-2 has two functionally active kinase domains, while others not. HK-2 binds to voltage-dependent anion channels (VDACs), to smoothly access to mitochondria-generated ATP. VDAC-bound HK-2 is also insensitive to feedback inhibition of G-6-P as its product. Thus, HK-2 is more efficient to restore highly glycolytic flux than others. Moreover, the interaction between HK-2 and VDACs is critical to prevent apoptosis by proapoptotic factors, Bax and Bad, in tumours [21–23]. Interestingly, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) is also known to interact with VDACs [24].

The other key glycolytic enzyme is pyruvate kinase (PK), which converts phosphoenolpyruvate (PEP) into pyruvate in the final step of glycolysis. PKM1 and PKM2 are alternatively spliced isoforms of PK that differ in sequence by only 22 amino acids. PKM1 is expressed in normal adult tissues, while PKM2 is also detected in many tumours and embryonic tissues. Although there are some controversies regarding whether PKM2 is absolutely required for tumourigenesis in vivo [25, 26], PKM2 is designated as the oncogenic isoform of PK, not only because of its expression profile, but also because of its multifaceted functions in tumourigenesis [25]. The most striking evidence of PMK2 involvement in tumourigenesis is that the dimeric form of PKM2 also functions as a protein kinase that targets the tumourigenesis-associated factors STAT3 [27], β-catenin [28], histone H3 [29], BUB3 checkpoint protein [30], NF-κB p65 [31], OCT-4, CD44 (a cancer stem cell marker) [32], and HIF-1 [33]. These findings suggest that the regulation of PKM2 could be essential for cancer cell proliferation.

Interestingly, other glycolytic enzymes are involved in establishing the Warburg effect during the process of immortalisation and transformation. Normal primary cells cultured in vitro suffer irreversible cell cycle arrest, called senescence, which is induced by telomeric erosion or by stresses such as oxidative stress, DNA damage, and

oncogenic insult [34–36]. The latter is designated stress-induced senescence, which is often bypassed by cell immortalising events in vitro, such as the activation of some oncogenes and the ablation of tumour suppressor genes [27]. In vivo, cellular senescence forms a protective barrier against immortalisation [37]. During the senescence process, glycolysis declines in human and mouse primary cells, while cancerous cells maintain the Warburg effect even under standard tissue culture conditions (i.e. 20 % oxygen) [19]. Recent studies have uncovered roles for glycolytic enzymes in the bypass of senescence in cancer cells.

Phosphoglycerate mutase (PGAM) was reported to be an immortalising factor in mouse fibroblasts via its radical scavenging effects [9, 38]. This finding is supported by the notion that PGAM activation suppresses mitochondrial respiration in vivo and in vitro [38, 39], followed by decreased generation of reactive oxygen species (ROS). Moreover, 2-phosphoglycerate, the metabolic product of PGAM, also activates the pentose phosphate pathway, whose product, NADPH, is essential for maintaining reducing power [8]. Hexokinase 2 (HK2) was also identified as a senescence-bypassing gene [11]. HK2-expressing cells show activation of the hexosamine biosynthetic pathway (HBP), which branches from glycolysis. The HBP affects many cellular processes through protein modification, as it further branches into N-linked glycosylation and *O*-linked *N*-acetylglucosamine (O-GlcNAc) [40]. Moreover, the ectopic expression of the glucose transporter GLUT3 renders nonmalignant breast cells susceptible to experimental transformation under 3-D culture conditions, and this occurs via HBP activation coupled with the Warburg effect [12]. These phenotypic conversions are accompanied by the activation of some oncogenic signalling factors (EGFR, AKT, MEK, and β1 integrin) [12]. Thus, the activation of different glycolytic enzymes affects various metabolic and biological pathways, whose outcome similarly promotes the proliferation of cancer cells under Warburg effect conditions. These findings indicate that investigation of the complex relationship between glycolytic regulation and cancer metabolism is essential for understanding the Warburg effect.

Adaptation to hypoxia and transcriptional regulation of glycolytic enzymes

It is quite possible that the Warburg effect is the consequence of cellular adaptation to the hypoxic environment encountered by cancer cells, particularly inside the core of solid tumours outgrowing the oxygenating capacity of neovasculatures [41]. However, the molecular mechanism of the Warburg effect was unclear until breakthrough experiments on the transcriptional regulation of glycolysis, which led to the discovery of hypoxia-inducible transcription factor 1 (HIF-1). HIF-1 was identified by DNA affinity chromatography from large-scale cultures of HeLa cells based on its ability to bind to the hypoxia response element DNA sequence [42]. Subsequently, the functional homologue HIF-2 was identified, and was found to have targets that overlapped with those of HIF-1 in addition to its own distinct target genes [43–45]. HIF-1 is required to upregulate many glycolytic enzymes under hypoxic conditions [46]. In addition, pyruvate dehydrogenase kinase 1 is also upregulated directly by HIF-1, leading to the inhibition of pyruvate entry into the TCA cycle (Fig. 1) [47, 48]. HIF-1 also regulates MCT4 (monocarboxylate transporter), which is critical to prevent the intracellular lactic acidification in tumours [49]. While intracellular lactic accumulation provokes apoptosis in cells, exported lactate might protect tumours from attack by immune systems [22].

The accumulation of HIF-1 or HIF-2 has been observed in many cancer cells, and is associated with poor prognosis of patients [50]. However, several lines of evidence suggest that the Warburg effect cannot be simply explained as an adaptation to hypoxic conditions in vivo. First, cancer cells maintain a high level of glycolysis even in tissue culture conditions under normoxia (20 % oxygen) [51]. Second, the ectopic expression of HIF-1 causes cell cycle arrest in some cell lines [52]. Third, PGAM is not upregulated by HIF-1 during hypoxia [46]. Fourth, *HIF-1* knockdown hardly affects the mRNA profiles of glycolytic enzymes in some cells [12]. Fifth, recent work suggests that HIF-1 is also regulated by stimuli other than hypoxia [17]. Thus, the intriguing correlation between the Warburg effect and HIF-1 could be affected by the interplay between multiple factors in addition to hypoxia. In this context, it is noteworthy that the transcription factors STAT3 and NF-κB also regulate the transcription of glycolytic enzymes in cooperation with HIF-1 [53, 54], while ETS-1 cooperates with HIF-2 [55, 56].

Several other transcription factors are also involved in glycolytic regulation. Hepatocyte nuclear factor 1β (HNF-1β) is a homeodomain transcription factor that plays a critical role in pancreatic development, including the differentiation of pancreatic endocrine cells. *HNF-1β* mutations have been clinically reported in many cases of diabetes mellitus [57]. Recently, HNF-1β was reported to regulate the Warburg effect in ovarian cancer. Knockdown of *HNF-1β* in an ovarian clear cell carcinoma (OCCC) cell line downregulated the mRNA levels of many glycolytic enzymes, including HK, GPI, PFK, ALDO, TPI, PGK, PGAM, ENO, and LDH, leading to a reduction in glycolytic flux [58]. Interestingly, ablation of *HNF-1β* causes OCCC cells to proliferate more rapidly

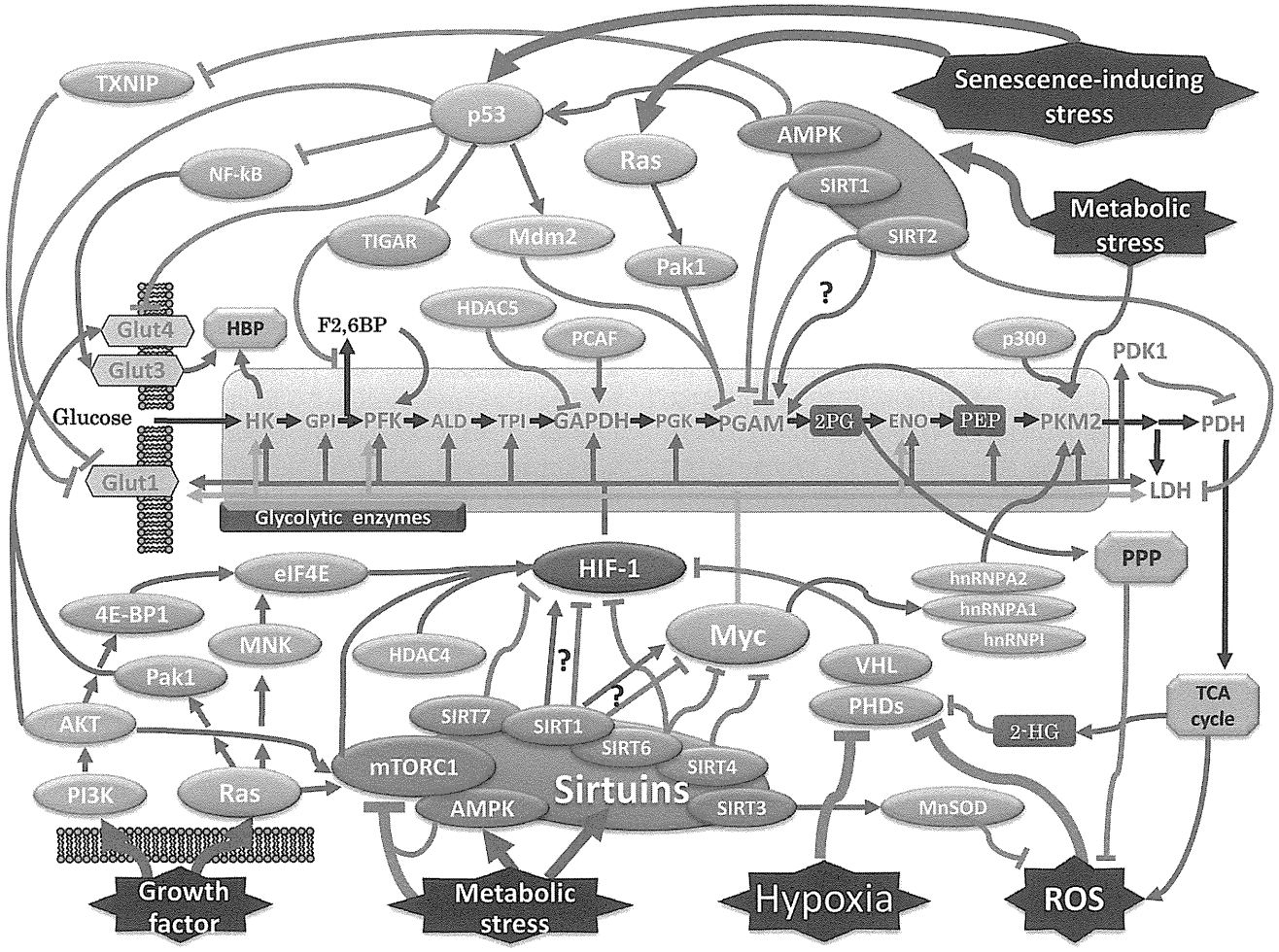

Fig. 1 Network of transcriptional and posttranscriptional regulation of glycolysis relevant to tumourigenesis. Classical oncogenic factors are indicated by *green circles*, while other signalling molecules are shown in *blue*. HIF-1 is indicated in *red*, and the metabolic sensor AMPK and sirtuins are shown in *orange*. The *arrow* indicates a positive effect, while the others are inhibitory effects. Pathways branching from glycolysis are described in the *grey box*, and some essential metabolites are in purple. See the text for additional mechanistic details and abbreviation definitions

with a reduced glycolytic rate. As OCCC is known to show slow progression but a poorer prognosis than other types of ovarian cancers [59], the Warburg effect in ovarian cancer might be associated with characteristics other than its proliferative potential. AD4BP/SF-1 (NR5A1), a steroidogenic tissue-specific nuclear receptor, was also recently reported as a transcriptional regulator of glycolysis [60]. Direct regulation of many glycolytic enzymes by AD4BP/SF-1 was clearly shown using a knockdown assay and CHIP analysis; these enzymes included HK, GPI, PFK, ALDO, TPI, GAPDH, PGK, PGAM, ENO, PKM2, and LDH. It would interesting to see whether AD4BP/SF-1 is also involved in tumourigenesis in relevant tissues [60]. Furthermore, the transcription factors specificity protein 1 (SP1) and SP3 induce PKM, enolase, and aldolase [61, 62], while peroxisome proliferator-activated receptor γ (PPAR γ) activates PKM and HK2 during hepatic tumourigenesis [63]. Additionally, microRNAs have been reported to be involved in glycolytic regulation; the details of this regulation have been described in other reviews [64, 65]. Although glycolytic regulation by HIF-1 and/or other transcription factors could also be required for normal cells under hypoxia or other conditions, these factors are known to be involved in oncogenic events, and their signalling in cancer cells maintains the Warburg effect, as discussed in "Classical oncogenic signals and glycolysis".

Classical oncogenic signals and glycolysis

It is known that several major oncogenic events constitute oncogenesis in vivo, including the activation of oncogenes (*Ras*, *Myc*, etc.) and inactivation of tumour suppressor

genes (p53, *Rb*, *Ink4*, etc.) [66]. These classical oncogenic signals are also required for the regulation of the Warburg effect (Fig. 1).

It has been suggested that several growth factors, including insulin, IGF-1, and IGF-2, stimulate glycolysis via the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT, also known as survival kinase)/mammalian target of rapamycin (mTOR) kinase pathway or the Ras/Raf/ERK pathway in cancer cells [67, 68]. The former pathway phosphorylates 4E-BP1, resulting in enhanced translation of *HIF-1* mRNA [69]. AKT kinase also promotes the translocation of the glucose transporter GLUT4 to the plasma membrane via phosphorylation of its target AS160 (AKT substrate of 160 kDa), a GTPase-activating protein of the small G protein Rab family [70]. Moreover, the ectopic expression of AKT kinase upregulates glycolysis in leukemic cells [71]. Oncogenic mutations in *Ras* and its downstream pathway are commonly observed in clinical and experimental tumourigenesis. Ras/Raf kinases activate the MAP kinases ERK1 and ERK2, and this is followed by the activation of MAP kinase-interacting kinases MNK1 and MNK2. Subsequently, MNK1 phosphorylates eIF-4E and promotes the translation of HIF-1. Furthermore, the Ras and insulin signalling pathways activate another small G protein, RAC1/CDC42, and its associated kinase, p21-activated protein kinase (PAK) [72, 73]. Although PAK is known to be involved in many tumourigenic processes, including cell motility, cytoskeleton reorganisation, apoptosis, and metastasis [74], its role in the Warburg effect is rather complicated. PAK directly phosphorylates and downregulates the glycolytic enzyme PGAM [75], while it facilitates insulin-stimulated GLUT4 translocation via actin remodelling [76]. These opposing roles of PAK in glycolysis are expected to be a topic of further investigation.

In early studies, Hunter et al. [77] pointed out the intriguing correlation between oncogenic kinases and glycolytic enzymes (Enolase, LDH, PGAM). More recently, PGAM was also reported to be regulated by oncogenic kinases [78], and the glycolytic enzyme PKM2 was found to be regulated by the oncogenic tyrosine kinases BCR-ABL, FGFR1, FLT3-ITD, and JAK2 [79]. Thus, phosphorylation is integral to the regulation of the Warburg effect. The counteracting activity of phosphatases might also be involved, as might other posttranscriptional modifications.

The function of HIF-1 is largely affected by two major cancer-related transcriptional regulators (c-MYC and p53) [80]. The Warburg effect is also induced by c-MYC activation or p53 inactivation, and this is associated with the senescence-bypassing ability of cancer cells [81, 82]. Several cancers frequently harbour oncogenic mutations or amplification of *c-Myc*, which directly affects the expression of several glycolytic enzymes including HK, PFK, TPI, GAPDH, ENO, and LDH [83, 84]. Moreover, c-MYC enhances the alternative splicing of *PKM2* rather than *PKM1* via upregulation of the RNA-binding proteins hnRNPA1, hnRNPA2, and hnRNPI [85, 86]. The tumour suppressor p53 also has several effects on glycolysis-related factors. For example, the inactivation of tumour suppressor p53 upregulates GLUT3 via NF-κB activation, and activates HK [82, 87]. Moreover, TP53-induced glycolysis and apoptosis regulator (TIGAR) is another glycolytic target of p53 [88]. The TIGAR protein shows a weak similarity to the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), but lacks the kinase domain. While fructose-2,6-bisphosphate, generated by PFK-2, is known as the most potent allosteric effector of PFK, the accumulation of fructose-6-phosphate generated by FBPase-2 or TIGAR inhibits PFK. Thus, the ectopic expression of TIGAR inhibits glycolysis but enhances the prosurvival ability of some cancer cells (U2OS and H1299), as TIGAR increases PPP activity, leading to increased reducing power and decreased ROS in cells. In this setting, p53 attenuates the Warburg effect to protect cancer cells from ROS-induced apoptosis.

While the knockdown of p53 mainly upregulates transcription of glycolytic enzymes or glycolytic flux in cancer cells [89], there is one exception; the glycolytic enzyme PGAM is positively regulated by p53 in muscle cells [90]. It is noteworthy that PGAM is also exempt from the regulation of glycolytic enzymes by other transcription factors, including HIF-1 and c-MYC. Thus, it is still not clear how the transcriptional regulation of PGAM is linked to the Warburg effect, although recent works have suggested that PGAM is subject to a high degree of posttranscriptional regulation. It was recently discovered that PGAM is posttranscriptionally regulated by the ubiquitin/proteasome pathway in primary cells under senescence-inducing stress, DNA damage, or oncogenic stress [91]. Proteolysis is an irreversible reaction that constitutes a regulatory mechanism for many cellular processes. Ubiquitination requires a substrate-specific E3 ubiquitin ligase and a substrate-nonspecific E1 ubiquitin-activating enzyme and E2 ubiquitin-conjugating enzyme [92]. Ubiquitinated proteins are degraded by proteasome pathway, unless ubiquitination is reversed by a deubiquitinase. Generally, ubiquitination requires an advance modification of the substrate (e.g. phosphorylation, acetylation). The RING finger protein MDM2, a transcriptional target of p53, is the ubiquitin ligase for PGAM, while PAK1 works as a priming kinase by facilitating the interaction between PGAM and MDM2 under stress [91]. *MDM2* has been perceived as an oncogene, because MDM2 also ubiquitinates the tumour suppressor p53 [93,

94]. Indeed, in certain cancers, gene amplification of *MDM2* is observed [95, 96]; however, in contrast, *MDM2* has also been reported to be a tumour suppressor [97, 98]. Thus, MDM2 may have opposing effects on the two different substrates, p53 and PGAM, in a cellular-context dependent manner. Under senescence-inducing stress, PGAM is degraded by the p53/MDM2 axis, whereas in the presence of some oncogenic signals, such as Ras-G12V and MDM2-M459I, PGAM is stabilised while p53 is impaired. In conclusion, p53 may regulate glycolysis directly by its transcriptional role or posttranscriptionally via its target *MDM2* [91].

New regulators for glycolysis and their oncogenic involvement

Besides hypoxia, low nutrient or low glucose conditions constitute critical metabolic stresses against rapidly growing solid tumours in vivo [19]. Recent advances in aging research have uncovered how adaption to low glucose modulates organismal longevity. Calorie restriction (CR) is a popular aging model proposed by McCay and Crowell in 1934 [99]. It has been well established that CR activates two crucial posttranscriptional regulators: adenosine monophosphate-activated kinase (AMPK) and sirtuins. AMPK is activated by an increase in the AMP/ATP ratio, while sirtuin is an NAD+-dependent deacetylase that is activated by the accumulation of nicotinamide adenine dinucleotide (NAD), a by-product of activated respiration during CR. Both molecules form an essential physiological energy sensor to regulate energy balance in vivo and in vitro [100]. Moreover, activation of mTOR signalling is also tightly linked to metabolic stress (starvation of amino acid or glucose) or hypoxia [101].

The core of mTOR signalling is mediated by mTORC (mTOR complex) kinase, which is activated by GTP-bound Rheb small G protein. TSC1/TSC2, the tuberous sclerosis complex (TSC) tumour suppressors, are GTPase-activating protein (GAP) for Rheb. TSC1/TSC2 is targeted by several kinases, AMPK, Akt kinase, ERK, and so on, as mTORC activation is essentially required for protein synthesis, autophagy, lipid synthesis and others. Interestingly, mTORC1 also upregulates glycolysis via enhanced translation of *HIF-1* mRNA. It is noteworthy that mTORC1 is aberrantly activated in 40–90 % of ten most frequently occurring cancers [102].

It is difficult to conclude whether *AMPK* behaves as an oncogene by supporting cancer survival under metabolic stress, or functions as a tumour suppressor by inhibiting anabolic metabolism. Several lines of evidence support the former model. *AMPK* is frequently amplified in human cancers [18], and is activated by oncogenic Ras-G12V [103]. AMPK directly activates the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, PFKFB3, leading to an increase in fructose-2,6-bisphosphate, which is an allosteric effector of PFK. Moreover, AMPK-dependent degradation of thioredoxin-interacting protein (TXNIP) enhances glucose uptake by activating its binding partner GLUT1 [104]. In support of the tumour suppressor model, AMPK belongs to the LKB1/mTOR tumour suppressor pathway; mutations to components of this pathway are known to cause predisposition to Peutz–Jeghers syndrome. Furthermore, AMPK directly activates p53 under low glucose conditions [105], and in a MYC-overexpressing state, *AMPK* ablation increases HIF-1-coupled glycolysis [106]. Thus, AMPK might augment or attenuate the Warburg effect in a cellular-context-dependent manner.

Sirtuins are the mammalian homologues of the *S. cerevisiae* silent information regulator 2 (*SIR2*) gene, which was initially identified as a pro-longevity gene under CR conditions. The sirtuin protein family has seven members, SIRT1-SIRT7, which share a central catalytic deacetylase domain and have distinct structures in the N- and C-termini. Initially, histones were proposed as the target for deacetylation by sirtuins [107]; however, recent studies revealed that sirtuins deacetylate not only histones, but also other metabolic regulators, including PGC-1, HIF-1, and MYC [99, 108]. As cancer cells adapt to different forms of metabolic stress, there has been keen interest as to whether sirtuins also function as metabolic modulators in cancer. Interestingly, many sirtuin knockout mice (SIRT2, SIRT3, SIRT4 and SIRT6) display a cancer-prone phenotype [109–112], while overexpression of the brain-specific SIRT1 and SIRT6 extended organismal lifespan in mice [113, 114].

Although elevated expression of SIRT1 has been observed in several cancers [115–117], opposing effects of SIRT1 on HIF-1 and MYC have been reported [118, 119], and it is not clear whether SIRT1 regulates the Warburg effect positively or negatively. The link between SIRT6 and glycolysis is more clear, as enhanced glycolysis in *SIRT6* knockout conditions was observed both in vivo and in vitro, consistent with its tumourigenic phenotype [112, 120]. Interestingly, several sirtuins (SIRT3, SIRT6, and SIRT7) inactivate HIF-1 and suppress the Warburg effect [120–122]. However, the inhibition of MYC by sirtuins (SIRT4, SIRT6, and SIRT7) has little effect on its glycolytic regulation [111, 112, 123], suggesting that unknown accessory regulation is operating for the MYC-induced Warburg effect. In addition, the deacetylase HDAC4 was also found to regulate and promote HIF-1 stability in a renal cancer cell line [124].

Glycolytic enzymes are also regulated by acetylation/deacetylation. The acetylation of LDH-A is downregulated in pancreatic cancer by SIRT2-mediated deacetylation, leading to increased LDH-A enzymatic

activity due to inhibition of protein degradation [125]. SIRT2 also regulates PGAM, although both negative and positive regulation has been reported [126, 127], and PGAM is also downregulated by SIRT1 [128]. Acetylation of GAPDH by PCAF increases its enzymatic activity and promotes cell proliferation after glucose stimuli, while GAPDH deacetylation by HDAC5 downregulates its enzymatic activity [129]. The acetylation of PKM2 is differently regulated by several different stimuli: glucose facilitates Lys305 acetylation of PKM2, leading to autophagic degradation [130], while oncogenic stimuli induce Lys433 acetylation by p300, which activates PKM2 kinase activity [131].

Regulation by ubiquitination and metabolites

Glycolysis is also controlled and greatly affected by the ubiquitin/proteasome system. While PGAM is degraded by MDM2 under stress, the HIF-1 protein is very unstable under normoxic conditions [42]. The E3 ubiquitin ligase for HIF-1 is the von Hippel–Lindau (VHL) protein, whose loss-of-function mutations are responsible for a renal cancer predisposition, termed VHL syndrome [132, 133], which involves the accumulation of HIF protein [134]. The competence of HIF-1 for ubiquitination is dependent upon hydroxylation of its proline-402 and -564 residues, which is induced under high oxygen conditions by the proyly-4-hydroxylase domain (PHD) proteins PHD1, PHD2, and PHD3 [135]. Hydroxylated HIF-1 binds more tightly to VHL and is therefore ubiquitinated more readily [134]. As PHD proteins are a subtype of dioxygenase, O_2 and α-ketoglutarate are utilised as substrates [17], and thus the dioxygenase activity of PHD proteins is impaired by ROS generated from dysfunctional mitochondria or from oncogenic signalling [136]. However, the activation of MnSOD by SIRT3-dependent deacetylation protects PHDs from ROS-dependent inactivation and facilitates HIF-1 activity [121, 137]. Together, these findings indicate that ubiquitin-mediated proteolysis is a key regulator of the Warburg effect.

Glycolytic regulation by metabolites has been well studied, but remains an intense focus of investigation. It has been well established that PFK1 is allosterically inhibited by the metabolites, citrate, and ATP, and allosterically activated by AMP and fructose 2,6-bisphosphate [16]. Thus, PFK is the rate-limiting step for glycolysis in cells. Surprisingly, recent developments in metabolomic analysis led to the identification of additional metabolites involved in glycolysis regulation. For example, lactate, fumarate, and succinate have been discovered to inhibit PHD activity under normoxic conditions, leading to an increase in HIF-1 stability [138–140]. It is noteworthy that α-ketoglutarate-dependent dioxygenases, which are PHD proteins, are competitively inhibited by another metabolite, 2-hydroxyglutarate (2-HG), which has been designated as an oncometabolite [141]. 2-HG is generated by oncogenic mutants of IDH1 and IDH2, which are observed frequently in gliomas and acute myeloid leukaemia, while their normal counterparts generate α-ketoglutarate (α-KG). Thus, in cancer cells bearing *IDH* mutations, the accumulation of 2-HG would disrupt the connection between environmental stress (oxygen or ROS condition) and the stabilisation of HIF-1, thereby causing constitutive activation of HIF-1.

PKM2 is also subject to metabolite-dependent regulation, including allosteric activation by fructose-1,6-bisphosphate, serine, and succinyl-5-aminoimidazole-4-carboxamide-1-ribose-50-phosphate (SAICAR), which is generated during de novo purine nucleotide biosynthesis. Curiously, oncogenic PKM2 shows much less pyruvate kinase activity than PKM1 [142], and PEP consequently accumulates in PKM2-expressing cancer cells. In this setting, phosphate from PEP is transferred to the catalytic histidine His11 on another glycolytic enzyme, PGAM, leading to a significant enhancement of PGAM activity [143]. Subsequently, pyruvate is generated from PEP by PGAM as an alternative glycolytic pathway in cancer cells [143]. This connection between PKM2 and PGAM via metabolites forms another positive feedback loop that maintains the Warburg effect. These findings suggest the possibility that as-yet-unknown metabolites could modulate the Warburg effect and potentially serve as anticancer therapies in the future. Indeed, the plant metabolite AICAR, which activates AMPK, has successfully been developed as a drug for the treatment of diabetes [144]. Human aetiology disclosed the positive statistical link between diabetes and several cancers (liver, pancreas, colon, etc.) [145], while recent data suggest that AICAR inhibits the proliferation of cancer in vitro [146]. Thus AICAR could potentially be a candidate for anticancer drug especially in diabetic cases.

In conclusion, the Warburg effect is not simply an energy boost mechanism in cancer cells. Rather, glycolysis in cancer is affected by several key factors, including hypoxia, ROS, metabolic stress, senescence-inducing stress, and growth factors. These factors are also coupled with other properties of cancer through the modulation of oncogenic signalling pathways. Furthermore, it is possible that oncogenic mutations or oncometabolites may disrupt the tight connection between glycolytic enzymes and their regulators, thereby maintaining a constitutively high flux of glycolysis. Thus, the Warburg effect connects many aspects of cancer to a metabolic shift that results from genetic reprogramming and oncogenic signalling.

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Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

REVIEW

MicroRNA-33a/b in Lipid Metabolism

– Novel "Thrifty" Models –

Koh Ono, MD, PhD; Takahiro Horie, MD, PhD; Tomohiro Nishino, MD; Osamu Baba, MD, PhD; Yasuhide Kuwabara, MD, PhD; Masayuki Yokode, MD, PhD; Toru Kita, MD, PhD; Takeshi Kimura, MD, PhD

MicroRNAs (miRNAs; miRs) are small non-protein-coding RNAs that negatively regulate gene expression. They bind to the 3' UTR of specific mRNAs and either inhibit translation or promote mRNA degradation. There is emerging evidence linking miR-33a/b to lipid homoeostasis, targeting *ABCA1*, *SREBF1*, etc and it would appear that they have acted as "thrifty genes" during evolution to maintain cholesterol levels both at the cellular and whole body level. As we are now living in a period of "satiation", miR-33a/b no longer seem to be useful and could be potential therapeutic targets for lipid disorders and/or atherosclerosis. In this review, we describe the current understanding of the function of miR-33a/b in lipid homeostasis, focusing on the "thrifty" aspect. (*Circ J* 2015; **79:** 278–284)

Key Words: High-density lipoprotein cholesterol; Lipid homeostasis; MicroRNA

MicroRNAs (miRNAs; miRs) are endogenous, small (approximately 20–22 nucleotides in length), non-protein-coding RNAs. miRNAs bind to the 3' untranslated region (UTR) of specific mRNAs according to the complementarity of their sequences and they either inhibit translation or promote mRNA degradation.[1,2] miRNAs were initially discovered in *Caenorhabditis elegans*[3,4] and were later found to be evolutionarily conserved.[5,6] More than 60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs, and so far, approximately 2,500 miRNAs have been identified in humans.[6,7]

miRNAs are usually transcribed as longer primary miRNAs (Pri-miRNAs) by RNA polymerase II (Pol II) and then processed by the Drosha (RNase III)/DGCR8 complex to premature miRNAs (Pre-miRNAs) in the nucleus. Pre-miRNAs are exported to the cytoplasm through exportin 5 and then processed by another ribonuclease enzyme, Dicer, to form mature miRNAs, which typically comprise 20–22 nucleotides. Moreover, it is known that miR-451 does not require Dicer. Instead, the pre-miRNA becomes loaded into Ago and is cleaved by the Ago catalytic center to generate an intermediate 3' end, which is further trimmed.[8] Mature miRNAs are assembled into an RNA-inducing silencing complex and post-transcriptionally inhibit mRNA expression by binding to the 3' UTR of their target mRNAs.[9]

In addition to their existence in tissues, recent studies have indicated that miRNAs also exist in serum, plasma, urine, and other body fluids in highly stable forms that are secure from endogenous RNase activity.[10] Altered levels of circulating miRNAs have been found in acute myocardial infarction,[11] acute coronary syndrome,[12] stable coronary artery disease (CAD),[13] heart failure,[14] essential hypertension,[15] and stroke.[16]

miRNAs have many functions in physiological and pathological states, and some miRNAs have been shown to have a significant effect on lipid homeostasis.[9,17,18] Dyslipidemia and related metabolic disorders continue to rise at an alarming rate worldwide and are associated with increased cardiovascular disease risk. A high plasma level of low-density lipoprotein cholesterol (LDL-C) is a major risk factor for CAD. Therapy with statins [inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (*HMGCR*)], which inhibit cholesterol biosynthesis, effectively reduces the levels of both LDL-C and modified forms of LDL in human plasma,[19] and significantly reduces the risk of CAD, as evidenced by primary and secondary clinical intervention studies.[20,21] However, patients who are treated with high doses of statins, regardless of their treated LDL-C level, are still at considerable risk for cardiovascular disease.[22,23] Thus, we still need other therapeutic options to treat the residual risk.[24–26] Elucidation of the function of miRNAs may provide avenues to developing novel treatments of dyslipidemia.

In particular, we have intensively investigated the functions of miR-33a/b, in vivo, using genetically modified mice.[27–30] In this review, we summarize the functions of miR-33a/b and therapeutic strategies to suppress these functions.

Received November 16, 2014; accepted December 11, 2014; released online January 8, 2015

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ISSN-1346-9843 doi:10.1253/circj.CJ-14-1252

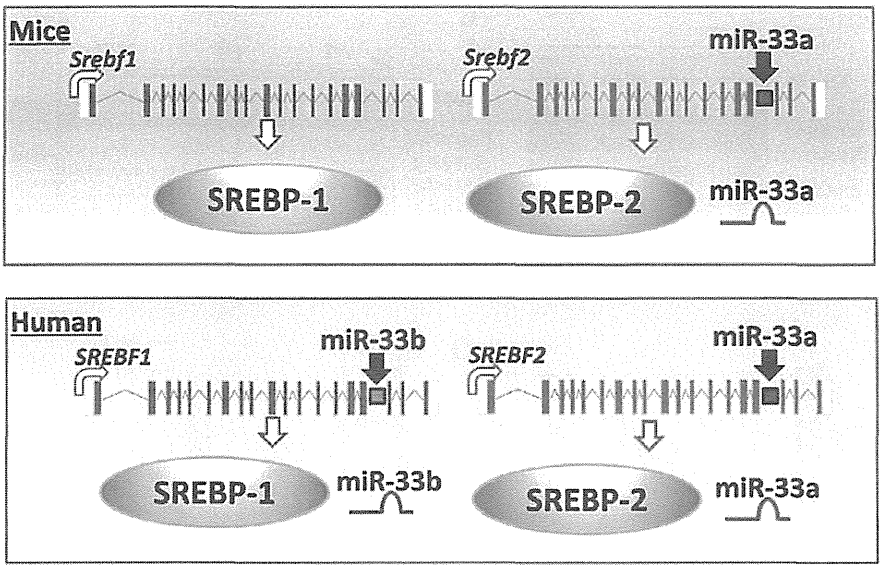

Figure 1. Mice have a single copy of microRNA (miR)-33, but humans have 2 copies (miR-33a and miR-33b). SREBP, sterol-regulatory element-binding protein.

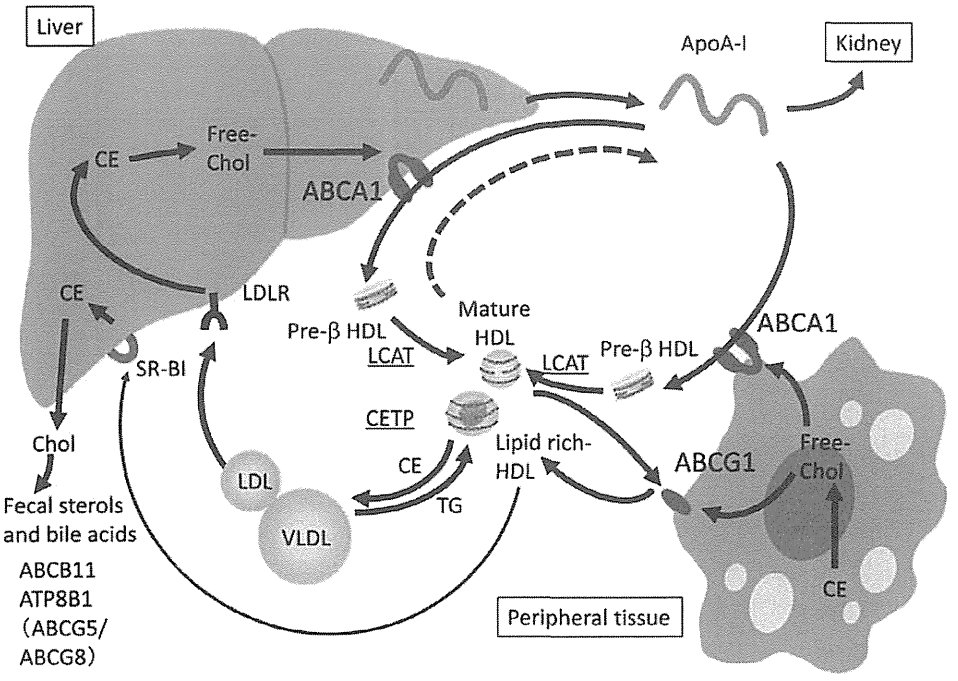

Figure 2. High-density lipoprotein cholesterol (HDL-C) and reverse cholesterol transport. The liver is the site of HDL biogenesis, where ABCA1-mediated lipidation of lipid-poor apolipoprotein A-I (apoA-I) generates nascent HDL. Free cholesterol on nascent HDL particles is esterified to cholesteryl esters (CE) by lecithin-cholesterol acyltransferase (LCAT). In turn, ABCG1 mediates cholesterol transfer to mature HDL. CETP mediates CE transfer from HDL to apolipoprotein B-containing lipoproteins (VLDL/LDL) in exchange for triglycerides (TG), promoting plasma cholesterol clearance by the uptake of VLDL/LDL lipoproteins through the LDLR pathway. Hepatic SR-BI mediates the removal of FC and CE from HDL, through the selective uptake pathway, and excess cholesterol is excreted from the liver into the bile. Both the ABCB11 and ATP8B1 transporters promote hepatic clearance, and have been shown to be the target genes of miR-33a.[40] LDLR, low-density lipoprotein receptor.

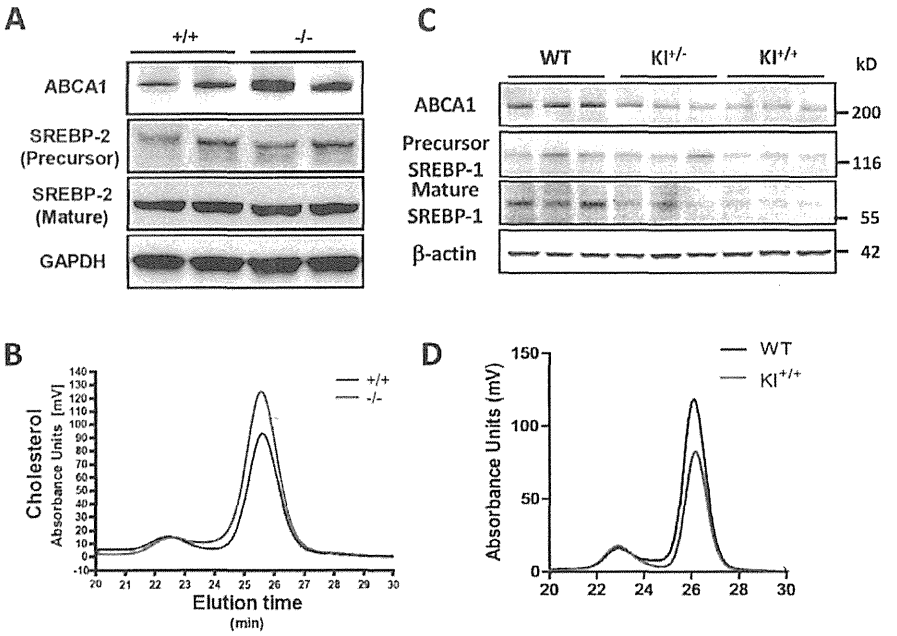

Figure 3. Western blot analysis and lipid profiles of wild-type, miR-33a-deficient mice, and miR-33b KI mice. (A) Western blot analysis of hepatic ABCA1 and SREBP-2 in 16-week-old male wild-type and miR-33a-deficient mice. GAPDH was used as a loading control. (B) Representative HPLC analysis of serum cholesterol from male wild-type and miR-33a-deficient mice. (C) Western blot analysis of hepatic ABCA1 and SREBP-1 in 8-week-old male wild-type and miR-33b $KI^{+/+}$ mice. β-actin was used as the loading control. (D) Representative HPLC analysis of serum cholesterol from male wild-type and miR-33b $KI^{+/+}$ mice. HPLC, high-performance liquid chromatography; KI, knock-in; miR, microRNA; SREBP, sterol-regulatory element-binding protein. (Cited and modified from references 27 and 30).

In Vivo Regulation of HDL-C by miR-33a/b

Recent studies have indicated that the miR-33s, located in the intron of sterol-regulatory element-binding proteins (SREBPs), control cholesterol homeostasis.[27,31–34] In humans, miR-33a and -33b are encoded in the introns of *SREBF2* and *SREBF1*, respectively,[32,35] whereas in rodents there is a deletion in part of the miR-33b encoding lesion and miR-33b cannot be expressed (Figure 1). Furthermore, miR-33a and -33b share the same seed sequence and differ in only 2 nucleotides. *SREBF1* encodes SREBP-1a and -1c, which mainly regulate lipogenic genes, such as fatty acid synthase (*FASN*), stearoyl-CoA desaturase (*SCD*), and acetyl-CoA carboxylase 1 (*ACC1*). *SREBF2* encodes SREBP-2, which mainly regulates cholesterol-regulating genes, such as *HMGCR*, and low-density lipoprotein receptor (*LDLR*).[36–38] miR-33a and -33b are considered to be cotranscribed and regulate lipid homeostasis with their host genes.

Several groups, including ours, have reported that miR-33a targets ATP-binding cassette transporter A1 (*Abca1*) in vivo, using either antisense technology or by generating miR-33a knockout mice.[27,31–33] ABCA1 mediates the efflux of cholesterol to lipid-poor apolipoprotein A-I (apoA-I) and forms nascent HDL. Therefore, ABCA1 is an essential molecule for HDL biogenesis and reverse cholesterol transport (RCT) (Figure 2). ABCA1 mRNA and protein half-lives are very short (1–2 h), suggesting that de novo transcription and translation are important for controlling its expression in response to environmental stimuli.[39] It was shown that mice treated with LNA antisense oligonucleotides or anti-miR-33a lentivirus exhibited increased ABCA1 expression in the liver and ABCA1 and ABCG1 expression in macrophages (*Abcg1* is another target of miR-33a in rodents). More importantly, in anti-miR-33a-treated mice, plasma HDL levels increased to 35–50%, without affecting other lipoproteins.[31–33] miR-33a knockout mice also showed a significant increase in the expression of ABCA1 in the liver and macrophages, and a 25–40% increase in serum HDL[27] (Figures 3A,B). Moreover, we analyzed the liver and measured the serum lipid profile of miR-33b knock-in (KI) mice, which have miR-33b in the same intron as humans.[30] In contrast to the results with miR-33a-deficient mice, HDL-C levels in these mice were reduced by almost 35%, even in miR-33b KI hetero mice compared with the control mice (Figures 3C,D).

Effect of miR-33 Inhibition on Atherosclerosis

In mammals, somatic cells do not catabolize cholesterol; thus, the removal of excess cellular cholesterol by HDL-C is central to the maintenance of sterol homeostasis. A major component of the atheroprotective function of HDL-C is the removal of cholesterol from lipid-loaded macrophages in the vessel wall, and its delivery to the liver for excretion, thereby playing a key role in RCT. Anti-miR-33 therapy contributes to enhancing of this pathway not only by increasing HDL-C through ABCA1 upregulation but also by increasing bile secretion through upregulation of ABCB11 and ATP8B1, which are the other targets of miR-33s.[40] It has already been proven that

antisense inhibition of miR-33a results in regression of atherosclerotic plaque in LDLR-deficient mice by promoting RCT.[41] Moreover, miR-33a-deficiency reduced the progression of atherosclerosis in apoE-deficient mice (Figure 4).[28] In that study, miR-33a-deficient mice not only had higher and functional HDL-C but also macrophages, which have a higher cholesterol efflux capacity, resulting in lower lipid accumulation in atherosclerotic areas, proven by bone marrow transplantation experiments. Other possible beneficial properties of anti-miR-33a therapy include an antiinflammatory response via upregulation of ABCA1. ABCA1 modulates cell-surface cholesterol levels, inhibits its partitioning into lipid rafts, and decreases the responsiveness of inflammatory signals from innate immune receptors. Furthermore, ABCA1 has been reported to directly act as an antiinflammatory receptor, independent of its lipid transport activities.[42]

Different results have been recently reported by 2 groups regarding the effect of antisense inhibition of miR-33a on atherosclerosis progression in LDLR-deficient mice. Marquart et al reported that anti-miR-33a therapy did not alter the progression of atherosclerosis in LDLR-deficient mice,[43] whereas Rotllan et al reported that silencing of miR-33a inhibited the progression of atherosclerosis in LDLR-deficient mice.[44] There are some discrepancies between these studies, which include differences in the antisense technology used and a considerable difference in the animals' diet (1.25% vs. 0.15% cholesterol).

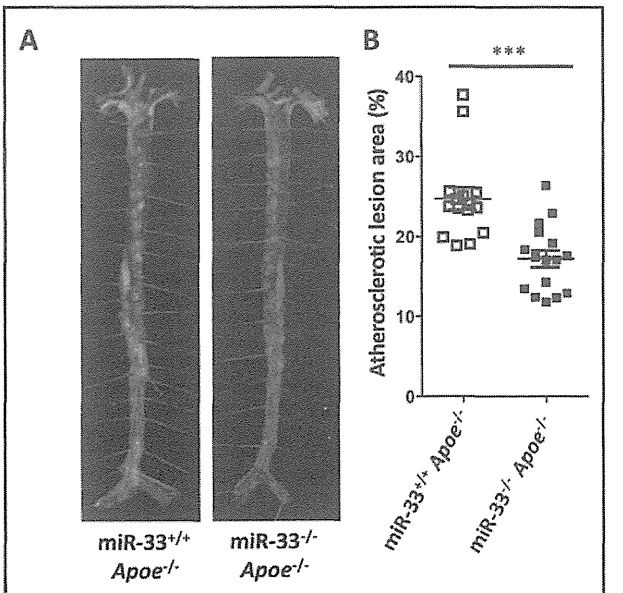

Figure 4. miR-33a-deficiency reduced atherosclerosis. (**A**) Representative images of the en face analysis of the total aorta in miR-33a$^{+/+}$*Apoe*$^{-/-}$ and miR-33a$^{-/-}$*Apoe*$^{-/-}$ male mice. (**B**) Quantification of the atherosclerotic lesion area in the *en face* analysis of the total aorta in male mice. Values are mean±SE (n=15–16 each). ***P<0.001. ApoE, apolipoprotein E; miR, microRNA; SE, standard error. (Cited and modified from reference 28.)

Obesity and Hepatic Steatosis in miR-33a-Deficient Mice

Although inhibition of the miR-33 s shows several beneficial effects, such as raising HDL-C and preventing atherosclerosis, we noticed that obesity and hepatic steatosis in the miR-33a-deficient mice at the age of 50 weeks or when fed a high-fat diet (HFD) for 12 weeks (Figure 5). In order to determine the cause of the phenotypic changes observed in miR-33a$^{-/-}$ mice fed a HFD or in older miR-33a$^{-/-}$ mice, we analyzed the gene expression profiles by microarray analysis using the livers of miR-33a$^{+/+}$ and miR-33a$^{-/-}$ mice fed NC at the age of 16 weeks when their weights were the same. Most strikingly, the genes classified in the fatty acid metabolism pathway showed the greatest change. We validated their expression levels in the liver by quantitative RT-PCR. Significant differences were observed in the expression levels of several lipogenic genes, including *Srebf1, Pparg,* and their downstream genes. We also measured de novo hepatic fatty acid synthesis rates, and they had significantly increased in the miR-33a$^{-/-}$ mice compared with those in the miR-33a$^{+/+}$ mice. *Srebf1* proved to be a good target of miR-33a, and miR-33a$^{-/-}$ mice had enhanced expression of SREBP-1 in the liver. To elucidate the role of SREBP-1 in the phenotypic changes in miR-33$^{-/-}$ mice fed a HFD, we generated miR-33a$^{-/-}$ mice that had SREBP-1 expression levels similar to wild-type mice. Protein levels of SREBP-1 were the same in miR-33a$^{-/-}$*Srebf1*$^{+/-}$ and miR-33a$^{+/+}$*Srebf1*$^{+/+}$ mice. Generation of miR-33a$^{-/-}$*Srebf1*$^{+/-}$ mice clearly showed that enhanced expression of SREBP-1 caused fatty liver and obesity in miR-33a$^{-/-}$ mice.

These results indicate a previously unrecognized association between SREBP-1 and SREBP-2 through miR-33a (Figure 6). Until now, it has only been shown that interactions between SREBP-1 and SREBP-2 were mediated by changes in sterol levels. It is known that in cholesterol-rich dietary conditions, SREBP-2 is downregulated at the cleavage level and SREBP-1c is transcriptionally activated through activation of liver X receptors (LXRs) by the binding of oxysterols.[45–48] However, in sterol-depleted conditions, SREBP-2 is cleaved in the Golgi and the active N-terminal region translocates to the nucleus. Reduction in oxysterol levels inactivates LXRs, resulting in a decrease in SREBP-1c mRNA levels. Therefore, suppression of SREBP-1 by miR-33a indicates the existence of a more direct and fine regulatory mechanism between SREBPs.

Novel "Thrifty" Models of miR-33a/b

From our findings, we speculate that miR-33a and -33b have evolved to keep cholesterol levels in 2 different modes. At the cellular level, they target ABCA1 and reduce cholesterol efflux to keep intracellular cholesterol levels under cholesterol-depleted conditions. In cholesterol-rich conditions, SREBP-2 and miR-33a levels decrease, resulting in the desuppression of ABCA1.

At the whole body level, miR-33a maintains cholesterol levels at the cost of fatty acid synthesis. Our data showed that miR-33a targeted the 3' UTR of *Srebf1* and upregulation of miR-33a by cholesterol depletion, considerably affecting the reduction of SREBP-1 expression.[29] Therefore, based on our finding that miR-33a regulates SREBP-1, miR-33a in the intron of *SREBF2* may amplify the reduction in SREBP-1 levels in sterol-depleted conditions (Figure 6). SREBP-1c is known to activate transcription genes involved in fatty acid and triglyceride synthesis, such as genes encoding *ACC1*, *FASN*, and *ELOVL6* and *SCD*. Therefore, it is possible that in sterol-depleted conditions, acetyl-CoA is the preferred substrate for cholesterol production, and not for fatty acid production, through upregulation of miR-33a. On the other hand, in cholesterol-rich conditions, the miR-33a level decreases[31] and

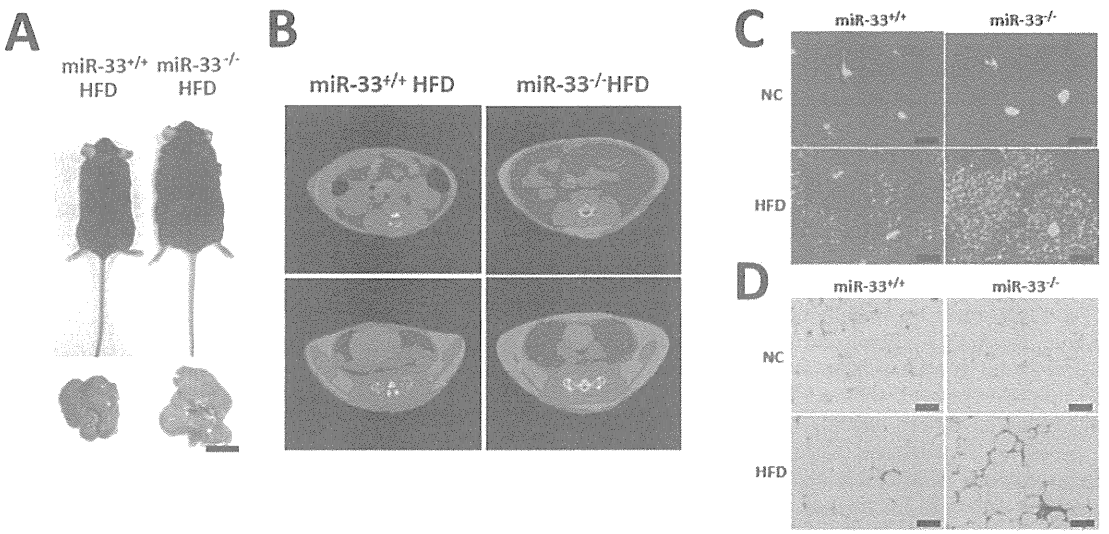

Figure 5. miR-33a$^{-/-}$ mice become obese and develop hepatic steatosis. (**A**) Representative image of miR-33a$^{+/+}$ and miR-33a$^{-/-}$ mice that were fed a high-fat diet (HFD). Lower images show the livers of the mice. Scale bar=1.0 cm. (**B**) Representative CT images of miR-33a$^{+/+}$ and miR-33a$^{-/-}$ mice fed a HFD. Pink color indicates abdominal fat in 2 different slices of each mice. (**C**) Representative microscopic images of the livers of miR-33a$^{+/+}$ and miR-33a$^{-/-}$ mice fed or not fed a HFD. Fatty change is shown by the increase in large fat vacuoles, which are optically "empty". Scale bars=200 μm. (**D**) Representative microscopic images of the adipose tissue (epididymal fat) of miR-33a$^{+/+}$ and miR-33a$^{-/-}$ mice fed or not fed a HFD. Scale bars=200 μm. miR, microRNA. (Cited and modified from reference 29.)

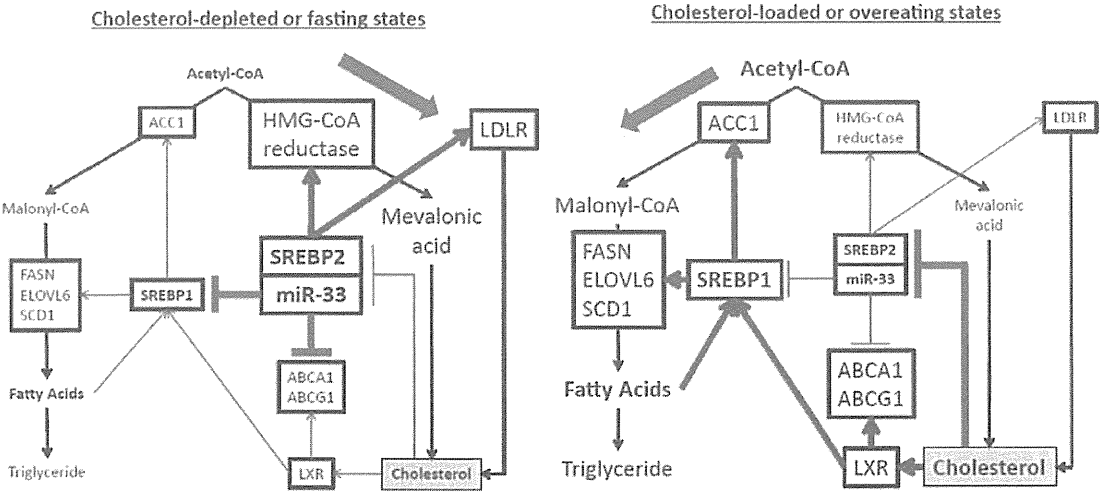

Figure 6. Schematic overview of the function of miR-33a in the regulation of SREBP-1 expression levels. Context-dependent change in the role of miR-33a in lipid metabolism. LDLR, low-density lipoprotein receptor; LXR, liver X receptor; miR, microRNA; SREBP, sterol-regulatory element-binding protein. (Cited from reference 29.)

its negative regulation of SREBP-1 may be reduced. Thus, in this situation, acetyl-CoA is the preferred substrate for fatty acid production (**Figure 6**).

We have found that miR-33b targets similar genes to miR-33a.[30] It is known that fasting-refeeding causes strong transcriptional activation of *SREBF1*. Therefore, we speculate that miR-33b is also involved in the maintenance of cholesterol levels at both the cellular and whole body level, even during famine (**Figure 7**). It is true that activation of miR-33a/b decreases HDL-C levels; however, low HDL-C is not a life-

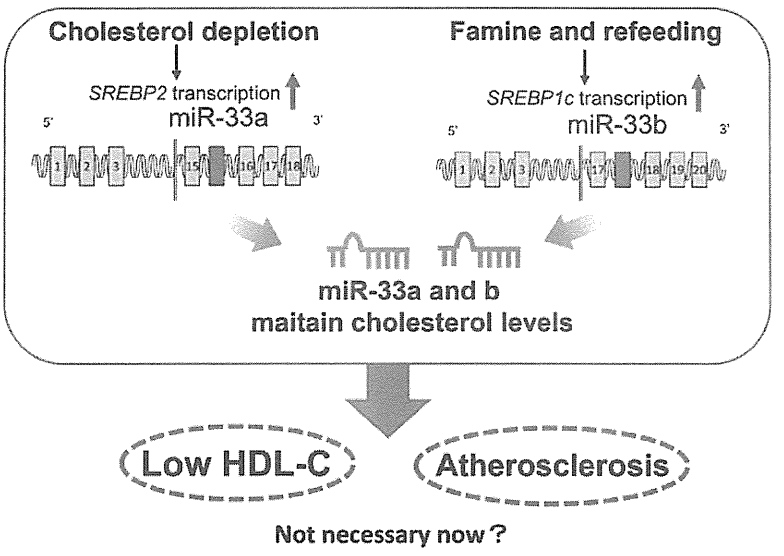

Figure 7. Schematic overview of the function of miR-33a and miR-33b in the regulation of cellular cholesterol levels. miR-33a and miR-33b may act as 'thrifty' genes to maintain intracellular cholesterol levels. HDL-C, high-density lipoprotein cholesterol; miR, microRNA; SREBP, sterol-regulatory element-binding protein.

threatening risk at that time most people/animals are dying of infectious diseases. At present, it is the time of "satiation"; thus, miR-33a/b no longer seems to be useful and could potentially be a therapeutic target for lipid disorders and/or atherosclerosis.

Future Direction of miR-Based Therapeutic Strategies

miRNAs are important regulators of physiological and pathological states. Recent studies provide considerable evidence about the effect of miR-33 s on lipid metabolism, particularly in the preclinical stage.[39] Because one miRNA can have many targets and affect the expression levels of many genes, further investigations are required to understand the complexity of miRNA biology. In the case of the miR-33s, complete inhibition leads to the development of obesity and liver steatosis;[29] therefore, spatiotemporal regulation may be required.

Conclusions

miR-33a/b are involved in lipid homoeostasis by targeting *ABCA1*, *SREBF1*, etc. and as such, appear to have acted as "thrifty genes" during evolution to maintain cholesterol levels at both the cellular and whole body level. Fine regulation of miR-33a/b could be a promising new approach to preventing or treating cardiovascular diseases in the future.

Acknowledgments

This work was supported in part by grants from the Japan Society for the Promotion of Science; by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to T. Kimura, T. Kita, K.H., T.H., and K.O.); by a Grant-in-Aid for Scientific Research on Innovative Areas "Crosstalk between transcriptional control and energy pathways, mediated by hub metabolites" (3307) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K.O.); by Otsuka Pharmaceutical Co, Ltd. Kyoto University SRP Program (to T. Kimura, T. Kita, M.Y., T.H., and K.O.); by grants from Banyu Life Science Foundation International, Takeda Memorial Foundation, Suzuken Memorial Foundation, Sakakibara Memorial Foundation, Japan Foundation of Applied Enzymology, SENSHIN Medical Research Foundation, Kowa Life Science Foundation, and ONO Medical Research Foundation (to T.H.); and by grants from ONO Medical Research Foundation, the Cell Science Research Foundation, Daiichi-Sankyo Foundation of Life Science, and Takeda Memorial Foundation (to K.O.).

Disclosures

Conflict of Interest: All authors report none.

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