miR-290/371-Mbd2-Myc circuit regulates glycolytic metabolism to promote pluripotency

Yang Cao1‡, Wen-Ting Guo2‡, Shengya Tian1, Xiaoping He3, Xi-Wen Wang2, Xiaomeng Liu3, Kai-Li Gu2, Xiaoju Ma1, De Huang1, Lan Hu1, Yongping Cai4, Huafeng Zhang1, Yangming Wang2* & Ping Gao1**

Abstract

Enhanced glycolysis is a main feature of pluripotent stem cells (PSCs) and is proposed to be important for the maintenance and induction of pluripotency. The molecular mechanism underlying enhanced glycolysis in PSCs is not clear. Using Dgcr8−/− mouse embryonic stem cells (ESC)s that lack mature miRNAs, we found that miR-290 cluster of miRNAs stimulates glycolysis by upregulating glycolytic enzymes Pkm2 and Ldh, which are also essential for the induction of pluripotency during reprogramming. Mechanistically, we identified Mbd2, a reader for methylated CpGs, as the target of miR-290 cluster that represses glycolysis and reprogramming. Furthermore, we discovered Myc as a key target of Mbd2 that controls metabolic switch in ESCs. Importantly, we demonstrated that miR-371 cluster, a human homolog of miR-290 cluster, stimulates glycolysis to promote the reprogramming of human fibroblasts. Hence, we identified a previously unappreciated mechanism by which miR-290/371 miRNAs orchestrate epigenetic, transcriptional and metabolic networks to promote pluripotency in PSCs and during reprogramming.

Keywords glycolysis; microRNA; Mbd2; metabolism; pluripotency

Subject Categories Metabolism; RNA Biology; Stem Cells

DOI 10.15252/embj.2014409441 | Received 30 October 2014 | Revised 19 December 2014 | Accepted 23 December 2014 | Published online 20 January 2015


Introduction

PSCs, including ESCs and induced pluripotent stem cells (iPSCs), are unique cells with specific properties such as the ability to self-renew indefinitely and the potential to differentiate into almost any somatic cells. Though genetic and epigenetic mechanisms are extensively studied in PSCs (Jaenisch & Young, 2008), the role of metabolism in controlling pluripotency is poorly understood. PSCs prefer glycolysis rather than more efficient aerobic respiration for energy supply (Kondoh et al, 2007; Prigione et al, 2010; Varum et al, 2011), which is reminiscent of the Warburg effect, or aerobic glycolysis in cancer cells, described almost 90 years ago by Otto Warburg that, compared to normal cells, cancer cells utilize a high rate of glycolysis followed by lactic acid fermentation in cytosol rather than a relatively low rate of glycolysis followed by oxidative phosphorylation (OXPHOS) in mitochondrial as energy sources even with ambient oxygen supply (Heiden et al, 2009; Hanahan & Weinberg, 2011). More interestingly, recent studies indicated that reprogramming from fibroblast cells to iPSCs is accompanied by metabolic switch from OXPHOS to glycolysis (Zhu et al, 2010; Folmes et al, 2011, 2013a; Panopoulos et al, 2012; Zhang et al, 2012) and that stimulating glycolysis by forced expression of Lin28, c-Myc (hereafter referred to as Myc) and Hif1α, or adding a small molecule, PS48, increased reprogramming efficiency (Hanna et al, 2009; Zhu et al, 2010; Folmes et al, 2013b; Prigione et al, 2014). Moreover, modulating the activity of AMP-activated protein kinase (AMPK), the master inhibitor of glycolysis, by small molecule inhibitors or miRNAs was also reported to improve the reprogramming of both mouse and human fibroblasts into iPSCs (Vazquez-Martín et al, 2012; Faubert et al, 2013; Judson et al, 2013). Nevertheless, compared to significant progress in understanding the Warburg effect in cancer cells, the enhanced glycolysis in stem cells has only attracted attention recently. It remains largely unclear whether this type of metabolic switch is critical for key stem cell properties such as pluripotency and self-renewal. Even less is known about how the metabolic switch in pluripotent stem cells is regulated.

MicroRNAs (miRNAs) are small non-coding RNAs with an average length of ~22 nucleotides that act by binding a partial complementary sequence within a target mRNA, usually in the 3′ untranslated region (UTR) (Bartel, 2004; Nilsen, 2007; Winter et al, 2009). Using mouse cells lacking miRNA processing proteins such as DICER1 or Dgcr8, we and others have shown that mature miRNAs are essential for ESC proliferation and differentiation (Bernstein et al, 2003; Kanellopoulou et al, 2005; Murchison et al,
2005; Wang et al., 2007). In addition, through a miRNA mimic screening in Dgcr8<sup>−/−</sup> ESCs, we identified ESC-specific cell cycle regulating (ESCC) miRNAs that enable rapid proliferation by promoting the G1/S transition in ESCs (Wang et al., 2008). The ESCC miRNAs include multiple members with a seed sequence ‘AAGUGCU’ from the miR-290–295 and miR-302–367 clusters that are more specifically and highly expressed in pluripotent stem cells. More interestingly, these miRNAs are demonstrated to be critical in modulating ESC self-renewal and promoting the induction of pluripotency during somatic cell reprogramming (Judson et al., 2009; Melton et al., 2010; Subramanyam et al., 2011; Wang et al., 2013). Opposite to ESCC miRNAs, early reports have also identified multiple miRNAs that can silence the self-renewal and induce the differentiation of ESCs (Melton et al., 2010; Wang et al., 2013). These studies clearly demonstrated that miRNAs are extensively involved in maintaining stem cell properties and cell fate decisions. However, how these miRNAs achieve these important functions is still not clear. Previously, we and others have shown that miRNAs facilitate aerobic glycolysis or Warburg effect to promote the proliferation and metastasis of cancer cells (Gao et al., 2009; Eichner et al., 2010; Singh et al., 2011; Jiang et al., 2012). Although cancer cells share a variety of properties with pluripotent stem cells, such as self-renewal, metabolism and cell cycle control, it is still not clear whether the unique metabolic properties of ESCs are also regulated by miRNAs and whether this regulation plays critical roles in controlling the self-renewal and pluripotency in ESCs and/or during the somatic cell reprogramming.

In the current study, we investigated the potential role of miRNAs in the regulation of metabolic switch in ESCs using Dgcr8<sup>−/−</sup> ESC model. We uncovered that miRNAs are indispensable for sustaining the glycolytic characteristics of ESCs. Furthermore, we identified an unexpected regulatory circuit consisting of a cluster of miRNAs (miR-290 cluster), a transcriptional repressor (Mbd2) and a transcriptional activator (Myc) that controls the metabolic switch to promote pluripotency in ESCs and enhance the induction of pluripotency during somatic cell reprogramming.

Results

miRNAs are critical for glycolytic metabolic signatures of ESCs

Previous reports demonstrated that PSCs depend on enhanced glycolysis for energy supply (Holmes et al., 2011; Panopoulos et al., 2012; Zhang et al., 2012). Hence, we first confirmed these results by comparing the metabolic signatures of mouse embryonic fibroblasts (MEFs) and ESCs and found that ESCs indeed showed a higher rate of cellular glucose utilization and lactate production but reduced oxygen consumption rate (OCR), an index for OXPHOS activity (Supplementary Fig S1A). Accordingly, proteins of several typical glycolytic enzymes including HK2, TPI1, PKM2, and LDHA (Varum et al., 2011; Chen & Russo, 2012; Shyh-Chang et al., 2013c) were highly expressed in ESCs compared to MEFs (Supplementary Fig S1B). Similar results were observed in iPSCs that were derived from reprogramming of MEFs with four Yamanaka transcription factors (4F) (Supplementary Fig S1C and D). These results confirmed previous reports that PSCs display glycolytic metabolic phenotype that resembles the Warburg effect in cancer cells and indicated that the higher expression of certain glycolytic enzymes may account for the phenotype observed in PSCs.

To investigate the role of miRNAs in metabolic regulation in stem cells, we used Dgcr8<sup>−/−</sup> mouse ESCs that lack all mature miRNAs (Wang et al., 2007). Interestingly, compared to wild-type (WT) ESCs, Dgcr8<sup>−/−</sup> cells showed a significant decrease in cellular glucose utilization and lactate production (Fig 1A). Measurement of OCR uncovered higher rates of aerobic respiration in Dgcr8<sup>−/−</sup> versus WT ESCs (Fig 1A), demonstrating that glycolysis was decreased while OXPHOS activity was increased in Dgcr8<sup>−/−</sup> ESCs. These results indicate that miRNAs are essential to sustain the enhanced glycolytic metabolism in ESCs.

To study whether the metabolic changes in Dgcr8<sup>−/−</sup> ESCs are associated with corresponding changes in the expression of metabolic enzymes, we analyzed the expression of metabolic enzymes in WT and Dgcr8<sup>−/−</sup> ESCs using quantitative real-time PCR (qRT–PCR) and Western blotting. Consistent with diminished glycolysis, qRT–PCR data showed that several enzymes involved in glycolysis, includingHK2, Pfk1, Aldoa, Tpi1, Pgk1, Pgam1, Eno1, Pkm2, and LdhA, were downregulated in Dgcr8<sup>−/−</sup> ESCs, while enzymes involved in TCA cycle, such as Pdha1 and Sdha (Varum et al., 2011; Chen & Russo, 2012; Shyh-Chang et al., 2013c), were slightly upregulated (Fig 1B). Consistently, Western blotting analysis showed decreased protein levels of HK2, PKF1, ALDOA, TPI1, PGK1, PGM1, PKM2, and LDHA, and mildly increased protein levels of PDHAI and SDHA (Fig 1C). These results indicated that canonical miRNAs play essential roles for enhanced glycolysis in ESCs by inducing the expression of multiple glycolytic enzymes.

miR-290 cluster enhances glycolysis by upregulating Pkm2 and LdhA in ESCs

To screen for miRNAs that are key to enhanced glycolysis in ESCs, we reintroduced the most highly enriched miRNA clusters in ESCs, including miR-290 cluster, miR-302 cluster, miR-1066 cluster, and miR-17 cluster, into Dgcr8<sup>−/−</sup> ESCs (Calabresi et al., 2007; Babiarz et al., 2008; Vidigal & Ventura, 2012). Interestingly, miR-290 cluster showed the most potent effect on stimulating glycolysis, markedly increasing cellular glucose utilization (Fig 2A) and lactate production (Fig 2B). Accordingly, OCR was decreased most significantly upon the transfection of miR-290 cluster (Fig 2C). It should be noted that, albeit much weaker than miR-290, miR-302 cluster also showed promoting effect on glycolysis (Fig 2A–C). Furthermore, by transfecting individual miRNA members of miR-290 cluster into Dgcr8<sup>−/−</sup> ESCs, we found that among the miR-290 family miRNAs tested, miR-291a, miR-291b, miR-294, and miR-295 showed notable effects on glucose uptake and lactate production, with miR-294 being the most effective one (Supplementary Fig S3A). Interestingly, all these miRNAs are members of ESCC miRNAs that share the same seed sequence ‘AAGUGCU’. qRT–PCR and Western blotting results showed that overexpression of miR-290 cluster in Dgcr8<sup>−/−</sup> ESCs significantly increased the mRNA and protein expression of glycolytic enzymes, especially Pkm2 and LdhA, but not Pdha1 and Sdha, two enzymes involved in TCA cycle (Fig 2D and E). In addition, ESCs lacking miR-290 cluster, generated by Crispr/Cas9 (Supplementary Fig S2A and B), also showed reduced glucose utilization and lactate production but increased OCR when compared to WT ESCs (Fig 2F), demonstrating the endogenous function of miR-290...
cluster in modulating glycolysis in ESCs. These phenotypic changes were associated with corresponding changes in the expression of Pkm2 and Ldha (Fig 2G). These results suggested that miR-290 cluster enhances glycolysis by upregulating the expression of Pkm2 and Ldha in ESCs. Consistent with our observation that miR-294 enhanced glycolysis most effectively, transfection of miR-294 markedly increased the expression of Pkm2 and Ldha both at mRNA and protein levels in Dgcr8−/− ESCs (Supplementary Fig S3B). Of note, we also observed that miR-290 cluster or miR-294 enhanced the expression of other enzymes such as Hk2, but not as highly as of Pkm2 and Ldha; hence, we focused on these two glycolytic enzymes for further study.

Figure 1. miRNAs are critical for glycolytic metabolic signatures of ESCs.
A Cellular glucose uptake, lactate production, and oxygen consumption rates were measured in WT and Dgcr8−/− ESCs.
B Relative mRNA levels of metabolic enzymes were determined by qRT–PCR in WT and Dgcr8−/− ESCs.
C Western blotting analysis of metabolic enzymes in WT and Dgcr8−/− ESCs. Actin serves as a loading control.
Data information: Data are presented as mean (± SD). *P < 0.05 as compared to WT group. In (A, B) n = 3; in (C) n = 6.
Source data are available online for this figure.
Figure 2.

The EMBO Journal
Vol 34 | No 5 | 2015

© 2015 The Authors
Since Pkm2 and Ldha are critical enzymes regulating glycolytic metabolism, we next sought to determine whether Pkm2 and Ldha are involved in elevated glycolysis regulated by miR-290 cluster. We found that when Pkm2 was knocked down by lentiviral short hairpin RNAs (shRNAs) in Dgcr8−/− ESCs, miR-290-induced increase in glucose uptake and lactate production was clearly attenuated (Fig 2H). Similar results were observed in Dgcr8−/− ESCs infected with lentiviral shRNAs specifically targeting Ldha (Fig 2I), indicating that both Pkm2 and Ldha are involved in miR-290-mediated upregulation of glycolytic metabolism in ESCs. Moreover, forced expression of Pkm2 or Ldha markedly promoted the glycolytic metabolism in Dgcr8+/− ESCs, increasing cellular glucose uptake and lactate production, while decreasing cellular OCR to levels similar to Dgcr8−/− ESCs transfected with miR-290 cluster (Fig 2A–C, Supplementary Fig S3C and D). Taken together, these data demonstrated that miR-290 cluster promotes glycolysis through enhancing Pkm2 and Ldha expression in ESCs.

**Pkm2 and Ldha are essential for miR-290-mediated reprogramming**

While miR-290 cluster is known to promote mouse somatic cell reprogramming with Oct4, Sox2, and Klf4 (Judson et al, 2009), the underlying mechanism remains as areas of active investigation. To test whether miR-290 cluster could promote reprogramming through upregulating the expression of Pkm2 or Ldha, we introduced miR-290 cluster along with retroviruses expressing Oct4, Sox2, and Klf4 (3F) into MEFs containing an Oct4-GFP reporter which is activated upon successful induction of pluripotency. qRT–PCR analysis of Pkm2 and Ldha expression on days 0, 2, and 4 post-infection revealed that the expression of Pkm2 and Ldha was significantly upregulated by miR-290 cluster (Fig 3A), suggesting that miR-290 cluster stimulates glycolysis during reprogramming. To test whether the overexpression of Pkm2 or Ldha can substitute miR-290 cluster to promote reprogramming, we introduced lentiviruses expressing Pkm2 or Ldha along with 3F into MEFs. Interestingly, similar to miR-290 cluster, overexpression of Pkm2, Ldha, or both (P+L) markedly increased the number of Oct4-GFP-positive or alkaline phosphatase (AP)-positive colonies (Fig 3B). Moreover, iPSC colonies reprogrammed from 3F plus Pkm2- or Ldha-transfected MEFs exhibited similar morphology to ESCs in terms of the round shape and undifferentiated state (Fig 3C). In addition, an iPSC line induced by 3F plus Pkm2 formed teratomas containing cell types of all three germ layers, indicating the true pluripotency of these iPSCs (Fig 3D). These data suggested that the upregulation of Pkm2 and Ldha by miR-290 cluster is sufficient to promote the induction of pluripotency during reprogramming.

To further determine whether Pkm2 and Ldha are required for enhanced reprogramming by miR-290 cluster, we infected MEFs with lentiviruses expressing shRNAs against Pkm2 or Ldha and then introduced 3F plus control oligo (CTR) or miR-290 cluster into these cells for reprogramming induction. Counting of Oct4-GFP-positive colonies revealed that when Pkm2 or Ldha was knocked down, the efficiency of reprogramming was drastically decreased, and addition of miR-290 cluster no longer promoted reprogramming in this context (Fig 3E), demonstrating that both Pkm2 and Ldha are required for miR-290 to promote reprogramming. Knocking down Pkm2 or Ldha also reduced the iPSC colonies in 4F-induced reprogramming (Supplementary Fig S3E). Taken together, these data suggested that miR-290-mediated upregulation of glycolytic enzymes Pkm2 and Ldha is essential for somatic reprogramming.
results validated the higher expression level of both Mbd2 mRNA and protein in MEFs compared to ESCs (Supplementary Fig S4A) and loss of miRNAs in ESCs led to the upregulation of Mbd2 (Fig 4B). We further demonstrated that miR-290 cluster greatly inhibited the expression of Mbd2, at both mRNA and protein levels (Fig 4C and D, and Supplementary Fig S4B). To determine whether miR-290 cluster directly regulates Mbd2, we generated a luciferase reporter by inserting WT or mutated (Mut) 3′ UTR region of Mbd2 gene containing the potential miR-294 binding site into psiCheck2 vector (Fig 4E). Dual luciferase reporter analysis revealed that the luciferase activity of the reporter containing WT but not Mut 3′ UTR was markedly higher in Dgcr8−/− versus WT ESCs and reduced upon the transfection of miR-294 in Dgcr8−/− ESCs (Fig 4E). Consistent with previous reports in human cells (Subramanyam et al, 2011; Lee et al, 2013), these data proved that Mbd2 is a direct target of miR-290 cluster in mouse ESCs.

To examine the functional impact of Mbd2 repression by miR-294 on gene expression in ESCs, we performed RNA-seq analysis on CTR-, miR-294-, and siMbd2-transfected Dgcr8−/− ESCs (Supplementary Table S2). We found that 819 genes were upregulated and 1,441 genes were downregulated greater than twofold upon miR-294 transfection, among which 166 genes (20%, fold of enrichment = 9.1, P < 0.0001) were upregulated and 180 genes (12%, fold of enrichment = 4.3, P < 0.0001) were downregulated greater than
Figure 4. MicroRNAs regulate glycolysis in PSCs.
Figure 4. Mbd2 is a direct target of miR-290 cluster that represses glycolytic metabolism and reprogramming.

A ECAR screening of Dgcr8−/− ESCs introduced with siRNAs against selected targets.
B qRT–PCR and Western blotting analysis of Mbd2 expression in WT and Dgcr8−/− ESCs.
C qRT–PCR and Western blotting analysis of Mbd2 expression in Dgcr8−/− ESCs transfected with miR-294 mimic.
D qRT–PCR and Western blotting analysis of Mbd2 expression in WT and miR-290 cluster−/− ESCs.
E Luciferase reporter assay for Mbd2 WT and Mut 3′ UTRs. Dgcr8 WT or miR-294 reduced luciferase activity compared to Dgcr8−/− or CTR group, respectively.
F RNA-seq analysis for CTR- and miR-294-transfected Dgcr8−/− ESCs. Each point represents FPKM value for a given transcript. Only genes that were upregulated or downregulated greater than twofold in miR-294-transfected Dgcr8−/− ESCs were plotted. Genes that were also upregulated or downregulated greater than twofold in siMbd2-transfected Dgcr8−/− ESCs are highlighted in red or blue, respectively.
G Cellular glucose uptake, lactate production, and oxygen consumption rates were measured in Dgcr8−/− ESCs stably expressing Mbd2 shRNAs.
H qRT–PCR and Western blotting showing that Pkm2 and Ldha were increased in Dgcr8−/− ESCs stably expressing Mbd2 shRNAs.
I Cellular glucose uptake and lactate production were measured in Mbd2-overexpressing Dgcr8−/− ESCs transfected with miR-290 cluster.
J Western blotting showing protein levels of PKM2 and LDHA in Mbd2-overexpressing Dgcr8−/− ESCs transfected with miR-290 cluster. A representative result is shown.
K Glucose uptake and lactate production were measured in WT and miR-290−/− ESCs transfected with Mbd2 siRNAs.
L Western blotting showing protein levels of Mbd2, PKM2, and LDHA in WT and miR-290−/− ESCs transfected with Mbd2 siRNAs. A representative result is shown.
M, N Oct4-GFP MEFs were introduced with the indicated factors, and Oct4-GFP-positive colonies were counted on day 16 after virus infection. AP staining of iPSC colonies was shown below.

Data information: Data are presented as mean ± SD. *p < 0.05 as compared to CTR group in (A, G, H, M), WT group in (B, D), CTR group in (C), indicated group in (E), EV+CTR group in (I, N) and WT+CTR group in (K), respectively. In (A, C–E, G–L) n = 3, in (B, M, N) n = 4. Actin serves as a loading control in Western blotting analyses. Source data are available online for this figure.

twofold in the same direction in siMbd2-transfected ESCs (Fig 4F). These data suggested that Mbd2 is responsible for the change of expression of a significant portion of genes that are regulated by miR-290 cluster.

We then tested whether Mbd2 is a functional target of miR-290 cluster in promoting glycolysis and reprogramming. Metabolism analysis revealed that suppression of Mbd2 in Dgcr8−/− ESCs with shRNAs led to increased cellular glucose uptake and lactate production and decreased oxygen consumption (Fig 4G). Consistently, expression of Pkm2 and Ldha was enhanced in Dgcr8−/− ESCs expressing shMbd2 both at mRNA and protein levels (Fig 4H). On the other hand, ectopic expression of Mbd2 without its native 3′ UTR in Dgcr8−/− ESCs diminished the promoting effect of miR-290 cluster on glycolysis (Fig 4I), as well as on Pkm2 and Ldha expression (Fig 4J). More importantly, suppression of Mbd2 with siRNAs rescued the decrease in glucose uptake and lactate production in miR-290−/− ESCs, accompanied with the upregulation of Pkm2 and Ldha expression (Fig 4K and L). These data indicated that Mbd2 is a downstream target of miR-290 cluster that inhibits glycolytic metabolism in ESCs.

We then performed reprogramming experiments using MEFs containing the Oct4-GFP reporter upon knocking down or ectopic expression of Mbd2. Knocking down Mbd2 by shRNAs significantly enhanced the reprogramming efficiency (Fig 4M), while forced expression of exogenous Mbd2 without its native 3′ UTR in MEFs abolished the increased reprogramming efficiency induced by miR-290 cluster (Fig 4N). Collectively, these data demonstrated that Mbd2 is a critical target, whose suppression is essential for miR-290-facilitated glycolysis and reprogramming.

Mbd2 suppresses glycolysis by repressing the transcription of Myc

Next, we sought to determine how Mbd2 suppresses glycolysis in ESCs and during somatic cell reprogramming. Mbd2 is known to repress gene expression via recruiting nucleosome remodeling and deacetylation complex (NuRD) to methylated promoters (Berger & Bird, 2005; Lai & Wade, 2011). A plausible explanation for how Mbd2 suppresses glycolysis is that Mbd2 directly represses the expression of glycolytic genes by binding to their promoters. To test this hypothesis, we re-analyzed previously published Mbd2 ChIP-seq data in ESCs (Baubec et al., 2013). Disappointingly, we did not find any evidence that MBD2 directly binds to the promoters of glycolytic genes such as Ldha or Pkm2; so, we refined our hypothesis that MBD2 represses another gene which activates the expression of glycolytic genes. To identify this gene, we focused on transcription factors that are directly bound by MBD2. In addition, we required that the expression of this candidate gene is upregulated (greater than twofold) in miR-294- and siMbd2- versus CTR-transfected Dgcr8−/− ESCs. Four transcription factors Myc, Hopx, Tafl7, and Pou42 were identified in this analysis (Fig 5A and Supplementary Table S3). Among them, Myc is an oncogenic transcription factor that is well known to promote glycolysis in numerous cancer cells (Shim et al., 1997; Collier et al., 2003; David et al., 2010). Furthermore, we found that Myc is enriched at the promoters of Pkm2 and Ldha in ESCs (Supplementary Fig S5A). In addition, we analyzed the change of expression of Myc target genes after knocking down Mbd2 by gene set enrichment analysis (GSEA) (Fig 5B). We found that the loss of Mbd2 caused a significant upregulation of Myc target genes indicating that upregulation of Myc upon knockdown of Mbd2 has a functional consequence on the transcription of Myc targets (Fig 5B). For these reasons, we hypothesized that Myc might provide the missing link in the miR-290-Mbd2-Pkm2/Ldha regulatory pathway, acting as a downstream target of Mbd2.

To further address whether Mbd2 directly repressed the transcription of Myc, we first performed ChIP–quantitative PCR (ChIP–qPCR) and found that Mbd2 was significantly enriched on the promoter region of Myc (Fig 5C and D). Consistently, promoter activity assay showed that knocking down Mbd2 with siRNAs notably increased the activity of a luciferase reporter bearing Myc promoter in Dgcr8−/− ESCs (Fig 5E). We then further confirmed that Mbd2 is a suppressor for Myc expression in Dgcr8−/− ESCs stably expressing shRNAs against Mbd2 (Fig 5F). In addition, we found that Myc protein level is significantly lower in MEFs, which express higher level of Mbd2, as compared to ESCs (Supplementary Figs S5B and S4A). Furthermore, forced expression of exogenous Mbd2 without its
Figure 5.

The EMBO Journal  Vol 34 | No 5 | 2015  617

© 2015 The Authors
endogenous 3′ UTR blocked the increase in Myc induced by miR-290 cluster (Fig 5G), suggesting that miR-290 cluster upregulated Myc expression through targeting Mbd2. Collectively, these data demonstrated that Mbd2 directly binds to Myc promoter to repress Myc expression and that its repression by miR-290 cluster is required for sustaining the high expression level of Myc in ESCs.

We then investigated the role of Myc in metabolic regulation in ESCs by Myc gain of function and loss-of-function experiments in Dgcr8⁻/⁻ and WT ESCs, respectively. These experiments revealed that Myc overexpression, similar to miR-290 overexpression, resulted in increased glucose utilization and lactate production, and decreased OCR in Dgcr8⁻/⁻ ESCs (Fig 5H). These phenotypic changes were associated with increased expression of Pkm2 and Ldha (Fig S1), consistent with the enrichment of Myc on the promoters of Pkm2 and Ldha (Supplementary Fig S5A). Moreover, Myc⁻/⁻ ESCs (Dgcr8 intact) showed significantly diminished glycolytic signatures (Supplementary Fig S5C). These data suggested that Myc enhances glycolysis by promoting the expression of glycolytic enzymes in ESCs.

To further determine the role of Myc played in miR-290-Mbd2 pathway, we knocked down Myc and then introduced miR-290 cluster or Mbd2 siRNAs into Dgcr8⁻/⁻ ESCs. Consistent with the epistatic interaction between Myc and miR-290-Mbd2, the promoting effects on glycolysis and glycolytic enzyme expression by miR-290 cluster or siMbd2 were completely eliminated upon knocking down Myc (Fig S3–M), suggesting that Myc is critical for miR-290-/Mbd2-regulated glycolysis in ESCs. Moreover, we introduced Pkm2 or Ldha into Dgcr8⁻/⁻ ESCs expressing shMyc to investigate the rescue function. As downstream factors regulated by Myc, Pkm2 and Ldha rescued significantly the negative effect shMyc has on glycolysis (Supplementary Fig S5D). Therefore, we identified here a regulatory circuit by which miR-290-mediated suppression of Mbd2 promotes glucose metabolism via Myc activation in ESCs. Of additional note, with concern over the potential effects of cell healthy status when analyzing the metabolic results, we measured caspase-3 protein levels of the cells with miR-290, Mbd2, Pkm2, and Myc overexpressed or knocked down. As indicated by the levels of pro-caspase-3 and cleaved caspase-3, manipulation of these genes did not have marked effects on cell viability or death (Supplementary Fig S5E).

### miR-371-MBD2-MYC circuit promotes glycolysis and reprogramming of human fibroblasts

Next, we tested whether the regulation of glycolysis and somatic cell reprogramming by miR-290-Mbd2-Myc circuit is conserved in human cells (Supplementary Fig S5F). Consistent with a previous study (Varum et al., 2011) and our results in mouse cells, metabolic analysis showed that a human ESC line H9 had a higher level of glycolysis than a human fibroblast cell line IMR90 (Fig 6A–C). Moreover, Western blotting analysis revealed that IMR90 cells expressed higher level of MBD2 and lower level of MYC, Pkm2, and LDHA than H9 ESCs (Fig 6D). These data supported the existence of a similar pathway in human cells that involves MBD2 and MYC to regulate glycolysis as observed in mouse cells. We then tested whether human homolog of miR-290 family, and miR-371 family, increases human fibroblast reprogramming (Subramanyam et al., 2011) by enhancing glycolytic metabolism through the Mbd2-Myc pathway. Introduction of miR-371–373 mimics into IMR90 cells caused a decrease in MBD2 expression and an increase in the expression of MYC, Pkm2, and LDHA (Fig 6E). Addition of Pkm2, LDHA, or both (P+L) into 3F during reprogramming increased the reprogramming efficiency as determined by TRA-1-81 staining (Fig 6G), supporting the function of Pkm2 and LDHA in promoting reprogramming of human somatic cells. Furthermore, it is interesting to observe that knocking down Pkm2 or LDHA with shRNAs (Fig 6F) almost completely blocked human somatic cell reprogramming even in the presence of miR-371 cluster (Fig 6G), suggesting that these enzymes are potent reprogramming stimuli, whose removal would represent a significant barrier for human somatic reprogramming. Together with our results in mouse cells, these data suggested the conservation of miR-290/371-Mbd2-Myc circuit that regulates glycolysis and reprogramming in both mouse and human cells.

**Figure 5.** miR-290-mediated suppression of Mbd2 induces Myc expression and glycolysis.

A Expression of the candidates of MBD2 target genes in miR-290- or siMbd2-transfected Dgcr8⁻/⁻ ESCs, compared to control group. Only 33 MBD2 targets upregulated greater than twofold in both miR-290- and siMbd2-transfected Dgcr8⁻/⁻ ESCs were shown. Transcription factors were highlighted in red.

B GSEA of a gene set comprising Myc targets [from ChIP-seq of Myc (Subramanian et al, 2005)] in siMbd2- versus CTR-transfected Dgcr8⁻/⁻ ESCs.

C Mbd2 binding sites on Myc promoter in ESCs. Arrow denotes the position of ChIP-qPCR primers.

D ChIP-qPCR analysis of Myc promoter.

E Promoter activity assay for Myc promoter upon Mbd2 depletion by siRNAs in Dgcr8⁻/⁻ ESCs. Empty pGL3-enhancer plasmid was used as a control.

F qRT-PCR and Western blotting showing Myc expression in Dgcr8⁻/⁻ ESCs infected with Mbd2 shRNAs.

G Western blotting showing Myc protein expression in Dgcr8⁻/⁻ ESCs stably expressing Mbd2 and further infected with miR-290 cluster. A representative result is shown.

H Glucose uptake, lactate production, and oxygen consumption rates were measured in Dgcr8⁻/⁻ ESCs expressing exogenous Myc.

I qRT-PCR and Western blotting showing Pkm2 and Ldha levels in Dgcr8⁻/⁻ ESCs expressing exogenous Myc.

J Glucose uptake and lactate production were measured in Dgcr8⁻/⁻ ESCs stably expressing Myc shRNAs and further infected with miR-290 cluster.

K Western blotting showing protein levels of MYC, Pkm2, and LDHA in Dgcr8⁻/⁻ ESCs stably expressing Myc shRNAs and further infected with miR-290 cluster. A representative result is shown.

L Glucose uptake and lactate production were measured in Dgcr8⁻/⁻ ESCs stably expressing Myc shRNAs and further infected with Mbd2 siRNAs.

M Western blotting showing protein levels of MYC, Pkm2, and LDHA in Dgcr8⁻/⁻ ESCs stably expressing Myc shRNAs and further infected with Mbd2 siRNAs. A representative result is shown.

Data information: Data are presented as mean (± SD). *P < 0.05 as compared to CTR group in (D), indicated group in (E), NTC group in (F), EV group in (H, I), NTC+CTR group in (J) and NTC+NTC group in (L), respectively. In (D–H, J, M) n = 3; in (I, L) n = 4; in (K) n = 5. Actin serves as a loading control in Western blotting analyses. Source data are available online for this figure.
Figure 6. miR-371 cluster, human homolog of miR-290 cluster, stimulates the metabolic switch and reprogramming of human fibroblasts.

A–C Cellular glucose uptake (A), lactate production (B), and oxygen consumption rates (C) were measured in IMR90 and H9 cell lines.

D Western blotting for MBD2, MYC, PKM2, and LDHA in IMR90 and H9 cells. A representative result is shown.

E Western blotting for MBD2, MYC, PKM2, and LDHA in IMR90 cells transfected with mimics of miR-371 cluster. A representative result is shown.

F Western blotting showing knockdown of PKM2 and LDHA in IMR90 cells. A representative result is shown.

G Tra-1-81-positive colonies were counted on day 35 after infection of IMR90 cells using the indicated factors.

H A working model of the miR-290-Mbd2-Myc axis in regulating metabolism and reprogramming. miR-290/371 cluster posttranscriptionally represses Mbd2, leading to the downregulation of MBD2 protein and reactivation of Mbd2 target gene Myc. Subsequently, Myc activates glycolysis through directly stimulating the transcription of glycolytic enzymes Pkm2 and Ldha. This regulatory circuit orchestrated by miRNAs facilitates metabolic switch in reprogramming and enhances glycolysis in ESCs.

Data information: Data are presented as mean (± SD). *P < 0.05 as compared to IMR90 group in (A–C), EV or NTC+CTR group in (G), respectively. In (A–E, G) n = 3; in (F) n = 2. Actin serves as a loading control in Western blotting analyses.

Source data are available online for this figure.
MicroRNAs regulate glycolysis in PSCs

Yang Cao et al

Discussion

PSCs such as cancer cells display unusually high level of glycolysis and rely heavily on it for energy supply (Varum et al., 2011). The molecular mechanisms that regulate metabolism in cancer cells have been well studied (Cairns et al., 2011; Schulze & Harris, 2013). In contrast, little is known about how the metabolism in PSCs is regulated to cope with the need of self-renewal and pluripotency. In this study, we uncovered an unexpected hierarchical pathway formed by three classes of well-known gene regulators: miRNAs, epigenetic reader, and transcriptional activator. Genetic epistasis analysis demonstrated that miR-290-Mbd2-Myc circuit is responsible for glycolytic signatures that are essential for stem cell pluripotency and somatic cell reprogramming. Moreover, we showed two downstream targets of this circuit, Pkm2 and Ldha, both as glycolytic enzymes, are strong promoters of somatic cell reprogramming. In addition, we demonstrated the existence and function of this regulatory circuit is well conserved in human cells. Together, these findings identified a previously unappreciated mechanism by which miR-290/371 family of miRNAs orchestrate epigenetic, transcriptional, and metabolic networks to regulate important characteristics of PSCs and reprogramming (Fig 6H).

Myc is an important transcription factor that is involved in the self-renewal and pluripotency network of ESCs (Chappell & Dalton, 2013). Interestingly, the Myc interaction network is largely independent from the well-established Oct4/Sox2/Nanog core regulatory loop (Chen et al., 2008; Kim et al., 2008; Ng & Surani, 2011), raising important questions such as how this gene enhances the self-renewal and pluripotency. Recent studies showed that Myc regulates the transcriptional pause release of thousands of genes (Rahl et al., 2010). More interestingly, Myc is shown to differentially control the transcription initiation and elongation of miRNAs and long non-coding RNAs (Zheng et al., 2014). Our study provides another possibility that Myc contributes to the self-renewal and pluripotency by enhancing favorable metabolism pattern in ESCs. Previous studies identified the canonical Dgcr8-Dicer-miRNA pathway that plays a role in sustaining the expression of Myc (Melton et al., 2010). However, the direct target that links miRNAs to Myc has never been shown. This study fills this gap by revealing that repression of a direct target of miR-290 cluster, Mbd2, is both required and sufficient to promote Myc expression. More interestingly, miR-290 cluster was shown to be able to replace Myc during somatic cell reprogramming, and it is suggested that Myc may function partially through increasing the transcription of miR-290 cluster (Judson et al., 2009). Here, we showed that miR-290/371 cluster can also promote the expression of Myc by repressing one of its repressors. Therefore, miR-290/371 and Myc form a positive feed-forward loop that robustly controls the metabolism, pluripotency, and somatic cell reprogramming. On the other hand, the amplification effects by this positive feed-forward loop may allow efficient differentiation upon specific differentiation cues.

Mbd2 has been shown to repress gene expression by recruiting NuRD complex to methylated DNA regions (Berger & Bird, 2005; Lai & Wade, 2011). A recent study showed that Mbd2 can bind to promoters of Oct4 and Nanog, therefore represses pluripotency when overexpressed in H1 ESCs (Lu et al., 2014). Mbd2 was also shown to affect miR-302-mediated somatic cell reprogramming via repressing the expression of NANOG (Lee et al., 2013). Our study identified a master transcription factor Myc as another important direct target of Mbd2, suggesting a very complicated regulatory network. Elucidating this regulatory network requires genome-wide identification of Mbd2 targets in pluripotent and differentiated cells. More interestingly, as metabolism is often linked with epigenetics by providing substrates such as methyl and acetyl groups for epigenetic modifications (Wellen et al., 2009; Katada et al., 2012; Shyh-Chang et al., 2013b), future studies are required to understand how metabolism may affect the function of Mbd2 in reverse. It should be noted from our RNA-seq dataset that miR-294 OE has a stronger effect on Myc and Pkm2 expression compared to Mbd2 knockdown, suggesting additional miR-294 targets involved potentially to contribute to this phenotype.

Metabolism is essential for key properties of stem cells such as self-renewal, pluripotency, and quiescence (Zhang et al., 2012; Shyh-Chang et al., 2013a; Ito & Suda, 2014). How metabolism pathways are integrated into the network regulating the key stem cell properties is not well understood. Our study shows how glucose metabolism is regulated by two well-known self-renewal regulating factors miR-290 cluster and Myc through an epigenetic reader Mbd2. However, metabolism includes a variety of chemical reactions that are beyond glucose metabolism (Ito & Suda, 2014). How other metabolism pathways such as those involving amino acids and lipids are regulated and integrated into the stem cell regulating network warrants future studies. These studies may further open the possibility to explore the metabolic phenotype of pluripotent stem cells for the induction of reprogramming or differentiation and hence their potential application for cell therapy. As stem cell research holds great promises for regenerative medicine, there is no doubt that the complicated metabolic network in stem cells warrants more investigations to match the tremendous efforts in elucidating Warburg effect in cancer cells that are opening new windows for cancer therapy (Xu et al., 2005; Pelicano et al., 2006; Heiden et al., 2009; Vander Heiden, 2011).

Materials and Methods

miR-290 cluster knockout strategy

A pair of guide RNA sequences, targeted against the miR-290 cluster genomic locus (containing miR-290, miR-291a, miR-292, miR-291b, miR-293 miR-294, and miR-295), was designed by http://crispr.mit.edu/. Knockout of the miR-290 cluster was verified by genomic PCR and miRNA RT–PCR.

Metabolism assay

ESCs were maintained without feeder layers for metabolism measurements. The intracellular glucose uptake and extracellular lactate production were measured in the cell lysates or culture medium using the glucose or lactate assay kit (BioVision), respectively. ESCs were first seeded in a well of 6-well plate without feeders. Forty-eight hours later, culture medium was collected for lactate measurement and cells were harvested and lysed for glucose detection. Relative glucose and lactate levels were normalized to protein.
concentration. For oxygen consumption rate (OCR) detection, 2.5 million trypsinized cells were suspended in 0.5 ml medium and measured by an Oxytherm unit (Strathkelvin Instrument Ltd.). Extracellular acidification rate screening (ECAR) was measured by Seahorse system. A total of 80,000 cells were transfected with siRNAs of specified genes and plated in Seahorse XF24 plates pretreated with 0.1% gelatin. One hour before measurement, culture medium was replaced with DMEM supplemented with 2 mM NEAA. Glucose (10 mM) was injected into medium as substrate during detection. Data were analyzed by Seahorse XF24. siRNA sequences used in ECAR assay are listed in Supplementary Table S4.

ChIP–qPCR

Dgcr8−/− ESCs overexpressing FLAG-tagged Mbd2 were cross-linked with 1% formaldehyde for 10 min at room temperature followed by the addition of 125 mM glycine to inactivate formaldehyde. Chromatin extracts containing DNA fragments with an average size of 200–500 bp were immunoprecipitated using IgG or anti-FLAG (Sigma-Aldrich) overnight at 4°C. Immunoprecipitated complexes were successively washed with low-salt buffer, high-salt buffer, LiCl buffer and TE buffer, and eluted with TE added with proteinase K. The reverse cross-link was performed by incubation of the samples overnight at 65°C. After reverse cross-linking, DNA was purified using minimal PCR Purification Kit (Kangweishiji) according to the manufacturer’s instructions. Primer sequences used in ChIP assay are listed in Supplementary Table S5.

Supplementary information for this article is available online: http://emboj.empress.org

Acknowledgements

We thank Dr. Tao Wei for providing the plasmid encoding Mbd2. Our work is supported in part by Chinese Academy of Sciences (XDA01010404), National Basic Key Research Program of China (2011CB011000, 2014CB910600, and 2012CB966700), and National Nature Science Foundation of China (31222102, 31171358, 31371429, 81372148, and 31071257).

Author contributions

YC, W-TG, H-FZ, Y-MW, and PG designed the project; YC, W-TG, S-YT, X-PH, X-WW, Y-PC, X-YM, LH, and DH performed experiments; X-ML and K-LG performed bioinformatic analysis; YC, W-TG, Y-MW, and PG coordinated the project and wrote the manuscript; and YC, W-TG, H-FZ, Y-MW, and PG performed bioinformatic analysis; YC, W-TG, Y-MW, and PG coordinated the project and wrote the manuscript; and YC, W-TG, H-FZ, Y-MW, and PG performed bioinformatic analysis; YC, W-TG, S-YT, X-PH, Ikeda Y, Perez-Terzic C, Terzic A (2011) Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metab 12: 352–361.


References


Published online: January 20, 2015

The EMBO Journal

MicroRNAs regulate glycolysis in PSCs  Yang Cao et al


Shenoy A, Blelloch R (2009) Genomic analysis suggests that miRNA destabilization by the Microprocessor is specialized for the auto-regulation of Dgr5. PLoS ONE 4: e6971


622 The EMBO Journal Vol 34 | No 5 | 2015 © 2015 The Authors