LncRNA-MIF, a c-Myc-activated long non-coding RNA, suppresses glycolysis by promoting Fbxw7-mediated c-Myc degradation

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Abstract

The c-Myc proto-oncogene is activated in more than half of all human cancers. However, the precise regulation of c-Myc protein stability is unknown. Here, we show that the lncRNA-MIF (c-Myc inhibitory factor), a c-Myc-induced long non-coding RNA, is a competing endogenous RNA for miR-586 and attenuates the inhibitory effect of miR-586 on Fbxw7, an E3 ligase for c-Myc, leading to increased Fbxw7 expression and subsequent c-Myc degradation. Our data reveal the existence of a feedback loop between c-Myc and lncRNA-MIF, through which c-Myc protein stability is finely controlled. Additionally, we show that the lncRNA-MIF inhibits aerobic glycolysis and tumorigenesis by suppressing c-Myc and miR-586.

Keywords c-Myc; Fbxw7; glycolysis; lncRNA; microRNA
Subject Categories Cancer; RNA Biology
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Introduction

The c-Myc gene was originally discovered as the cellular homolog of the retroviral c-Myc oncogene [1,2]. The c-Myc proto-oncogene was later revealed to be activated in over half of human cancers [3]. Many mechanisms are involved in c-Myc activation during tumorigenesis, including chromosomal rearrangement [4,5], gene amplification [6], and point mutations in the coding sequence [7,8]. The potent transforming activity of c-Myc to promote tumorigenesis has been well documented by extensive studies using both in vitro cell culture and in vivo mouse models [9].

As a master transcriptional factor, c-Myc has been previously reported to bind to approximately 10–15% of genes in the genome. Recent studies also suggest that c-Myc may function as a global amplifier of already active promoters [10–12]. By modulating expression of a variety of protein-coding genes, c-Myc has been shown to regulate various cellular processes impacting on cell growth, differentiation, and metabolism [13,14]. However, protein-coding genes only account for <2% of the human genome, and the majority of transcripts are non-coding RNAs [15,16]. Among them are long non-coding RNAs (lncRNAs), which are defined as transcripts longer than 200 nucleotides lacking significant protein-coding capacity. Thus far, more than 10,000 lncRNAs have been identified in the human genome [15]. The lncRNAs are emerging as an important regulator of biological process and have diverse functions including their involvement in the regulation of gene expression at different levels, such as chromatin remodeling, transcription, and post-transcriptional processing [17,18]. Of note, lncRNA has recently been shown to function as microRNA (miRNA) sponge or competing endogenous RNA (ceRNA) to regulate gene expression [19,20]. Dysregulation of lncRNAs has also been implicated in a variety of human diseases including cancer [21,22]. Despite these advances, most lncRNAs remain functionally uncharacterized. Particularly, it remains largely unknown how lncRNAs are involved in the regulation of c-Myc function.

Considering c-Myc has strong growth-promoting ability, so a small change in c-Myc levels may have a global impact on the cell. It is therefore not surprising that levels of c-Myc are under extraordinarily tight regulation in normal cells. c-Myc is an immediate-early gene, and its transcription is controlled at the level of initiation in response to a range of growth stimuli [23,24]. In addition, c-Myc mRNA is highly unstable, with a half-life of ~30 min. The export and translation of c-Myc mRNA are also highly controlled [25,26]. Furthermore, c-Myc is a labile protein, and its protein stability is regulated by multiple E3 ubiquitin ligases [27], among which SCF (Skp–Cullin–F-box)-Fbxw7 (F-box and WD repeat domain-containing 7) is the best-characterized E3 ubiquitin ligase for c-Myc. The SCF-Fbxw7-mediated degradation of c-Myc involves the recognition of phosphorylated c-Myc on threonine 58 (T58) and serine 62 (S62) by Fbxw7 [28,29]. It has been widely accepted that the c-Myc oncogene becomes dysregulated when those control mechanisms are compromised.

The tightly controlled expression of c-Myc is essential for many cellular processes. Deficiency in c-Myc is embryonic lethal in...
animal models, whereas the increased expression of c-Myc is oncogenic. It is interesting to note that c-Myc haploinsufficient (Myc<sup>+/−</sup>) mice are metabolically healthier and surviving longer than wild-type mice [30]. It is unclear whether this also holds true for human beings. In normal human cells, c-Myc is kept at a relatively low level, whereas c-Myc exhibits high-level expression in cancer cells. How this is achieved in their respective cells has not yet been fully addressed.

In this study, we demonstrate that lncRNA-MIF (Myc inhibitory factor), which is transcribed by c-Myc, is able to reduce c-Myc expression. Mechanistically, lncRNA-MIF competes with coding mRNA Fbxw7 for miR-586 and relieves the inhibitory effect of miR-586 on Fbxw7, thereby leading to increased Fbxw7 expression and decreased c-Myc level. Our data indicate the existence of a feedback loop between c-Myc and lncRNA-MIF, through which c-Myc protein stability is delicately controlled. Our study also suggests that lncRNA-MIF exerts its tumor-suppressive function by regulating c-Myc-mediated glycolysis and tumorigenesis.

Results

LncRNA-MIF reduces c-Myc protein stability

To identify novel long non-coding RNAs involved in the regulation of c-Myc function, we first analyzed lncRNA expression profile of P493-6 cells carrying a c-Myc tet-off system. By performing long non-coding RNA microarray analysis (Dataset EV1), we found that levels of three lncRNAs in P493-6 cells were decreased when c-Myc expression was suppressed by doxycycline addition (Fig EV1A). Among these c-Myc responsive lncRNAs, RP11-320M2.1 (ENST00000547349) particularly attracted our attention because knockdown of this lncRNA showed the most pronounced elevation of c-Myc in HeLa, H1299 and A549 cells (Figs 1A and EV1B). We named this lncRNA as c-Myc-inhibitory factor (lncRNA-MIF). To avoid off-target effect, three different lncRNA-MIF shRNA-1, -2, and -3 were used to knock down lncRNA-MIF, and each shRNA was shown to result in marked decrease in lncRNA-MIF and elevated c-Myc protein level (Fig 5D, lanes 2, 3, and 4, second panel from the top). Furthermore, knockdown effect on upregulation of c-Myc by lncRNA-MIF shRNA-1 and -2 can be rescued by overexpressed shRNA-1 and -2 resistant lncRNA-MIF (Fig EV1C and D). These results demonstrated that lncRNA-MIF shRNA-1 and -2 will not be off-targeting. LncRNA-MIF shRNA-1 showed the strongest suppression of lncRNA-MIF and therefore was used throughout this study. LncRNA-MIF was expressed from a locus between ODC1 and NOL10, and was predicted to have three exons by UCSC (University of California, Santa Cruz) Genome Browser (Fig 1B). LncRNA-MIF is readily detectable by Northern blotting in HeLa and HCT116 cells, and was approximately 800 bp in length that was the same as predicted by UCSC (Fig 1C). When lncRNA-MIF was introduced into HeLa cells, c-Myc levels were greatly reduced (Fig 1D). However, overexpression of lncRNA-MIF-AS (antisense) in HeLa cells showed no effect on c-Myc (Fig 5A, lanes 2 and 3, second panel from the top). These data indicate that lncRNA-MIF is fully functional for its inhibitory effect on c-Myc expression.

We next investigated how lncRNA-MIF decreased c-Myc protein levels. c-Myc mRNA levels were not affected by either overexpression or knockdown of lncRNA-MIF (Figs 1E and 5C and E), as determined by real-time RT–PCR analysis. However, the proteasome inhibitor MG132 was able to reverse the inhibitory effect of lncRNA-MIF on c-Myc protein level (Fig 1F and G). c-Myc half-life was decreased by lncRNA-MIF induction and increased by lncRNA-MIF knockdown (Fig 1H–K). These data suggest that lncRNA-MIF reduces c-Myc protein stability by promoting its degradation through the proteasome pathway.

LncRNA-MIF is a direct transcriptional target of c-Myc

Since lncRNA-MIF was identified as a c-Myc upregulated gene by lncRNA microarray as mentioned above, we sought to further validate the effect of c-Myc on lncRNA-MIF expression. The real-time RT–PCR analysis showed that levels of lncRNA-MIF were greatly decreased when c-Myc expression was suppressed in P493-6 cells.
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Figure 1.

A. Western blot analysis of c-Myc and GAPDH expression in HeLa, H1299, and A549 cells treated with sh-control and sh-lncRNA-MIF. The blots show the expression levels of c-Myc and GAPDH at 66 kDa and 35 kDa, respectively.

B. Chromosome 2 localization of lncRNA-MIF. The figure shows the location of lncRNA-MIF on Chromosome 2.

C. RT-qPCR analysis of lncRNA-MIF expression in Mi (DP), HeLa, and HCT 116 cells treated with actin. The expression levels are shown in various bands.

D. Western blot analysis of c-Myc and GAPDH expression in cells treated with MG132 and sh-control or sh-lncRNA-MIF. The blots show the expression levels of c-Myc and GAPDH at 66 kDa and 35 kDa, respectively.

E. Relative RNA level of lncRNA-MIF and c-Myc in HeLa cells treated with different conditions. The graph shows the relative RNA level of lncRNA-MIF and c-Myc.

F. Western blot analysis of c-Myc and GAPDH expression in cells treated with MG132 and sh-lncRNA-MIF-AS. The blots show the expression levels of c-Myc and GAPDH at 66 kDa and 35 kDa, respectively.

G. Western blot analysis of c-Myc and GAPDH expression in cells treated with MG132 and sh-lncRNA-MIF-1, sh-lncRNA-MIF-2, and sh-lncRNA-MIF-3. The blots show the expression levels of c-Myc and GAPDH at 66 kDa and 35 kDa, respectively.

H. Western blot analysis of c-Myc and GAPDH expression in cells treated with CHX and different conditions. The blots show the expression levels of c-Myc and GAPDH at 66 kDa and 35 kDa, respectively.

I. % c-Myc protein remaining after CHX treatment with different conditions. The graph shows the % c-Myc protein remaining over time.

J. Western blot analysis of c-Myc and GAPDH expression in cells treated with CHX and different conditions. The blots show the expression levels of c-Myc and GAPDH at 66 kDa and 35 kDa, respectively.

K. % c-Myc protein remaining after CHX treatment with different conditions. The graphs show the % c-Myc protein remaining over time.

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Figure 2.

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LncRNA-MIF is a direct transcriptional target of c-Myc.

A P493-6 cells (human B-cell lymphoma cell line) carrying a c-Myc tet-off system were treated with doxycycline (1 μg/ml) for the indicated periods of time. Total RNA was subjected to real-time RT-PCR analysis. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test). Cell lysates were analyzed by Western blotting with the indicated antibodies.

B HeLa, HCT116, and MCF10A cells were infected with lentviruses expressing control shRNA or c-Myc shRNA. Forty-eight hours after infection, total RNA and cell lysates were analyzed by real-time RT–PCR and Western blotting, respectively. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test). Cell lysates were also analyzed by Western blotting.

C HeLa, HCT116, and MCF10A cells were transfected with control vector or Flag-c-Myc. Twenty-four hours after transfection, total RNA was extracted from these cells and subjected to real-time RT–PCR analysis. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test). Cell lysates were also analyzed by Western blotting.

D Schematic illustration of consensus c-Myc binding sites in LncRNA-MIF gene promoter. The indicated pGL3-based luciferase reporter constructs were generated to examine transcriptional activities of three putative c-Myc binding sites MIF1, MIF2, and MIF3 in response to c-Myc induction. MIF1-M, MIF2-M, and MIF3-M indicate their corresponding mutant binding sites which are shown in the open boxes.

E HeLa cells were co-transfected with either Flag-c-Myc or control vector plus the indicated reporter constructs and Renilla luciferase plasmid. Twenty-four hours after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. Data shown are mean ± SD (n = 3; **P < 0.01, two-tailed t-test).

F HeLa cells expressing control shRNA or c-Myc shRNA were co-transfected with the indicated reporter constructs and Renilla luciferase plasmid. Twenty-four hours after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. Data shown are mean ± SD (n = 3; *P < 0.05, two-tailed t-test).

G P493-6 cells were co-transfected with the indicated reporter constructs and Renilla luciferase plasmid. Twenty-four hours after transfection, cells were treated with doxycycline for 0 and 12 h, respectively. Then reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test).

H Lysates from HeLa cells were subjected to ChIP assay. ChIP products were amplified by qPCR with the indicated pairs of primers. Data shown are mean ± SD (n = 3; *P < 0.05, two-tailed t-test).

LncRNA-MIF increases Fbxw7 expression by acting as a molecular sponge for miR-586

It has been recognized that stability of c-Myc is tightly controlled by Fbxw7-dependent ubiquitination and subsequent proteasome-dependent degradation. The above finding that LncRNA-MIF reduced c-Myc half-life (Fig 1H–K) led us to test the possibility that whether LncRNA-MIF could interact with c-Myc and/or Fbxw7 and thus to accelerate Fbxw7-mediated c-Myc degradation. We performed two types of RNA-pull-down experiments. Firstly, we incubated in vitro-synthesized full-length biotinylated LncRNA-MIF (antisense LncRNA-MIF transcripts were used as negative control) with HeLa cell lysates and isolated coprecipitated proteins by using streptavidin beads. Secondly, we incubated biotinylated antisense DNA probes targeted to LncRNA-MIF with HeLa cell lysates to pull down endogenous LncRNA-MIF and its interacting protein (sense DNA probes were used as negative control). The results showed no obvious interaction of LncRNA-MIF with either Fbxw7 or c-Myc (Fig 3A; PTBP1 was used as positive control, since PTBP1 was also shown to associate with LncRNA-MIF in our mass spectrometry analysis). However, we found that knockdown of LncRNA-MIF decreased, whereas induction of LncRNA-MIF increased both Fbxw7 mRNA and protein levels (Fig 3B and C). These data indicate that LncRNA-MIF may decrease c-Myc half-life via elevating Fbxw7 expression. We next explored how LncRNA-MIF regulated Fbxw7 expression. Since there is no direct association between LncRNA-MIF and Fbxw7, we asked whether LncRNA-MIF could act as a microRNA sponge to regulate Fbxw7 expression. To test this possibility, we first performed the bioinformatics analysis using TargetScan Human web server and miR-586 was predicted to target both LncRNA-MIF and Fbxw7 (Fig 3D). The RNA target specificities of miRNAs in animals are primarily encoded within a 7-nt “seed region” mapping to positions 2–8 at the molecule’s 5’ end [32]. LncRNA-MIF has five putative 7-mer complementary sequences for miR-586, and two of five have 7-nt perfect match, whereas the remaining three have 1 base variation. Concurrently, the 3’
The untranslated region (3' UTR) of the Fbxw7 gene contains one putative 8-mer site (nucleotides 636–643) that matches to the miR-586 seed region. We next determined whether lncRNA-MIF interacted with miR-586 and whether miR-586 targeted Fbxw7 to inhibit its translation. We showed that both biotinylated antisense DNA probe-enriched endogenous lncRNA-MIF and in vitro-synthesized c-Myc PTBP1 Fbxw7.
biotinylated lncRNA-MIF were able to successfully pull down miR-586 (Fig 3E and F). As a negative control, lncRNA-MIF failed to pull down miR-34a (Fig 3F), since it does not contain any putative miR-34a complementary sequence. To further validate the association of lncRNA-MIF with miR-586, we constructed a luciferase reporter plasmid containing wild-type lncRNA-MIF and a mutant reporter construct in which two putative miR-586 binding sites within lncRNA-MIF were mutated (Fig 3G). These reporter constructs were transfected into HeLa cells together with the indicated psicheck2-based luciferase reporter construct. Twenty-four hours after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test).

Taken together, these data suggest that lncRNA-MIF functions as miR-586 sponge and reinforces this conclusion, we performed an endogenous

To determine whether Fbxw7 is a bona fide target of miR-586, we introduced miR-586 mimics into HeLa cells together with luciferase reporter plasmids containing wild-type or mutant 3′ UTR of Fbxw7 (Fig 4A). The reporter activity was noticeably suppressed by the presence of miR-586 mimics, however, this activity remained largely unaffected when this 3′ UTR was mutated (Fig 4B). In addition, the Fbxw7 3′ UTR-luciferase activity was increased by treatment with miR-586 inhibitors (Fig 4C). These data indicate that 3′ UTR of Fbxw7 is inhibited by miR-586. To reinforce this conclusion, we performed an endogenous experiment. Treatment by miR-586 mimics led to a decrease in both protein and mRNA levels of endogenous Fbxw7, whereas miR-586 inhibitors showed the opposite effect (Fig 4D and E).Taken together, these data suggest that Fbxw7 is post-transcriptionally inhibited by miR-586.

Given that miR-586 was able to target both lncRNA-MIF and Fbxw7, we asked whether lncRNA-MIF competed with Fbxw7 mRNA for miR-586 binding. If there indeed exists a competition between lncRNA-MIF and Fbxw7 mRNA for shared miR-586, the copy number ratio of lncRNA-MIF to miR-586 is then expected to be within a rational range, too high or too low will make the competition become unworkable. We used quantitative real-time PCR to quantify the copy numbers of lncRNA-MIF and miR-586 per cell in HeLa, MCF7, and HCT116 cells. The ratio of miR-586 copy number to lncRNA-MIF copy number ranged from 5 to 20 in different types of cells (Fig 4F). Considering lncRNA-MIF has 5 putative miR-586 complementary sites, it is reasonable to speculate that lncRNA-MIF is able to efficiently inhibit miR-586 function. We also found that overexpression of lncRNA-MIF reduced, whereas knockdown of lncRNA-MIF increased levels of miR-586 (Fig 4G). To further determine whether lncRNA-MIF regulates Fbxw7 and c-Myc expression via miR-586, miR-586 mimics and inhibitor were utilized. We showed that induction of miR-586 by its mimics greatly reversed lncRNA-MIF-mediated up-regulation of Fbxw7 and down-regulation of c-Myc (Fig 4H). In addition, lncRNA-MIF knockdown caused Fbxw7 down-regulation and c-Myc up-regulation were strongly minimized by miR-586 inhibitor (Fig 4I). Furthermore, ectopic expression of lncRNA-MIF or lncRNA-MIF harboring nonsense mutations was shown to up-regulate Fbxw7 and down-regulate c-Myc, whereas overexpression of miR-586 binding-defective lncRNA-MIF which had lost the ability to bind miR-586 showed no effect on either Fbxw7 or c-Myc (Fig 4J and K). All together, our data suggest that lncRNA-MIF functions as miR-586 sponge and

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Figure 3. LncRNA-MIF is a molecular sponge for miR-586.

A Left panel: Lysates from HeLa cells were incubated with in vitro-synthesized biotin-labeled sense or antisense DNA probes against lncRNA-MIF for biotin pull-down assay, followed by Western blot analysis with the indicated antibodies. Right panel: Lysates from HeLa cells were incubated with in vitro-synthesized biotin-labeled lncRNA-MIF or biotin-labeled antisense RNA for biotin pull-down assay, followed by Western blot analysis with the indicated antibodies.

B HeLa cells were infected with lentiviruses expressing control RNA, lncRNA-MIF, control shRNA, or lncRNA-MIF shRNA as indicated. Forty-eight hours after infection, cell lysates were analyzed by Western blotting with the indicated antibodies.

C HeLa cells were infected with lentiviruses expressing control RNA, lncRNA-MIF, control shRNA, or lncRNA-MIF shRNA as indicated. Forty-eight hours after infection, total RNA was subjected to real-time RT–PCR analysis. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test).

D Illustration of the base pairing between miR-586 and lncRNA-MIF. The base pairing between miR-586 and Fbxw7 3′ UTR is also shown.

E Lysates from HeLa cells were incubated with in vitro-synthesized biotin-labeled sense or antisense DNA probes against lncRNA-MIF for biotin pull-down assay, followed by real-time RT–PCR analysis to examine miR-586 levels. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test).

F Lysates from HeLa cells were incubated with in vitro-synthesized biotin-labeled lncRNA-MIF and antisense RNA for biotin pull-down assay, followed by real-time RT–PCR analysis to examine miR-586 and miR-34a levels. Data shown are mean ± SD (n = 3; *P < 0.01, two-tailed t-test).

G Schematic representation of psicheck2-based luciferase reporter plasmid containing wild-type lncRNA-MIF (psicheck2-lncRNA-MIF) and a mutant reporter construct in which two putative miR-586 binding sites were mutated (psicheck2–lncRNA-MIF-M), and mutated bases are indicated in red.

H miR-586 or control mimics were transfected into HeLa cells together with the indicated psicheck2-based luciferase reporter construct. Twenty-four hours after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. Data shown are mean ± SD (n = 3; *P < 0.05, two-tailed t-test).

I miR-586 or control inhibitors were transfected into HeLa cells together with the indicated psicheck2-based luciferase reporter construct. Twenty-four hours after transfection, reporter activity was measured and plotted after normalizing with respect to Renilla luciferase activity. Data shown are mean ± SD (n = 3).

J HeLa cells were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. Total RNA extracted from each fraction was analyzed for the indicated RNA expression by real-time RT–PCR. Data shown are mean ± SD (n = 3). Actin and PARP were used as markers for cytoplasmic and nuclear fractions, respectively.

K HeLa cells were subjected to cytoplasm or nucleus fractionation before each fraction was incubated with in vitro-synthesized biotin-labeled sense or antisense DNA probes of lncRNA-MIF for biotin pull-down assay, followed by real-time RT–PCR analysis to examine miR-586 levels. Data shown are mean ± SD (n = 3; *P < 0.05, **P < 0.01, two-tailed t-test).
attenuates the inhibitory effect of miR-586 on Fbxw7, thereby leading to elevated Fbxw7 and reduced c-Myc.

Actually, there are two major isoforms of lncRNA RP11-320M2.1. LncRNA-MIF is isoform 001, which is largely overlapped with a longer isoform 002 named as lncRNA-MIF-L (long). We found that c-Myc did not affect lncRNA-MIF-L (Fig EV3A and B). Moreover, the copy number of lncRNA-MIF-L was only 2% of that in MCF7, HeLa, or H1299 cells (Fig EV3C). These results indicate that lncRNA-MIF-L will have little, if any, sponge effect on miR-586. We also knocked down lncRNA-MIF-L in HeLa cells and showed it did not affect the level of c-Myc.
miR-586, FBXW7, or c-Myc (Fig EV3D and E). Based on these results, lncRNA-MIF-L is unlikely to serve as a ceRNA for miR-586.

**LncRNA-MIF decreases c-Myc and c-Jun levels by increased FBXW7 expression**

The substrates of FBXW7 include several widely studied proteins such as cyclin E1, c-Myc, and c-Jun [33]. In addition to c-Myc, we demonstrated that lncRNA-MIF also affected c-Jun, which was required for progression through the G1 phase of the cell cycle. When lncRNA-MIF was introduced into HeLa cells, c-Myc and c-Jun protein but not mRNA levels were greatly reduced (Fig 5A and C). However, lncRNA-MIF-AS had no effect on either c-Myc or c-Jun (Fig 5A and C). Consistently, depletion of lncRNA-MIF by lncRNA-MIF shRNA-1, -2, and -3 led to an increase in c-Myc and c-Jun protein but not mRNA levels (Fig SD and E). Interestingly, lncRNA-MIF showed no effect on cyclin E1 (Fig 5A, C, D and E).

**LncRNA-MIF inhibits the glycolysis via miR-586**

c-Myc has been well known for its ability to promote the glycolysis under normoxia through transcriptionally regulating its target genes involved in the glycolysis pathway. Given that lncRNA-MIF was able to decrease c-Myc protein level, we sought to determine whether lncRNA-MIF could inhibit the glycolysis. Knockdown of lncRNA-MIF led to the acidification of the culture medium, whereas overexpression of lncRNA-MIF showed the opposite phenotype (Fig 6A). Consistent with these, knockdown of lncRNA-MIF reduced, whereas overexpression of lncRNA-MIF increased c-Myc target genes involved in the glycolysis pathway such as GLUT1, LDHA, PKM2, and HK2 (Fig 6B). To further confirm the effect of lncRNA-MIF on the glycolysis, we examined the glucose uptake and lactate production in HeLa cells with overexpression or knockdown of lncRNA-MIF. We found that lncRNA-MIF induction led to a strong decrease in glucose uptake and lactate production (Fig 6C and D). In contrast, lncRNA-MIF knockdown increased glucose uptake and lactate production (Fig 6E and F). These data strongly indicate that lncRNA-MIF inhibits the glycolysis. We next investigated whether miR-586 mediated the inhibitory effect of lncRNA-MIF on the glycolysis. Induction of miR-586 by its mimics markedly recovered lncRNA-MIF-decreased glucose uptake and lactate production (Fig 6C and D). Also, knockdown of miR-586 by its inhibitors greatly reversed lncRNA-MIF knockdown-increased glucose uptake and lactate production (Fig 6E and F). These results indicate that lncRNA-MIF inhibits the glycolysis via miR-586.

**LncRNA-MIF functions as a tumor suppressor to inhibit cell proliferation and cell cycle progression**

We measured the growth rate of HeLa cells expressing control RNA, lncRNA-MIF, and lncRNA-MIF-AS, respectively. A marked inhibition of cell proliferation was observed in HeLa cells expressing lncRNA-MIF, but not expressing lncRNA-MIF-AS or control RNA (Fig 7A). Growth rates of HeLa cells expressing control shRNA, lncRNA-MIF shRNA-1, -2, and -3 were also measured. HeLa cells expressing lncRNA-MIF shRNA-1, -2, or -3 exhibited higher growth rate than HeLa cells expressing control shRNA (Fig 7B), indicating that lncRNA-MIF inhibits cell proliferation. Moreover, knockdown of c-Myc nullified the effect of MIF depletion on cell growth rate (Fig EV4), indicating that the effect of lncRNA-MIF on cell proliferation is dependent on c-Myc.

We further performed a flow cytometric analysis for cell cycle and higher percentage of HeLa cells expressing lncRNA-MIF were in the G1 phase compared with the cells expressing control RNA or lncRNA-AS (Fig 7C and E). Consistently, a higher percentage of HeLa cells expressing lncRNA-MIF shRNA-1, -2, or -3 were in the S and G2/M phases compared with the cells expressing control shRNA (Fig 7D and F). These data suggest the inhibitory effect of lncRNA-MIF on cell cycle regulation.

Considering the inhibitory effect of the lncRNA-MIF–miR-586–FBXW7–cMyc axis on the glycolysis, we speculated that lncRNA-MIF...
may also inhibit cell proliferation via miR-586. To test this possibility, we performed the colony formation assay. As was expected, ectopic expression of lncRNA-MIF decreased the number of colonies from control HeLa cells, but not that from miR-586-overexpressing HeLa cells (Fig 8A). In addition, knockdown of lncRNA-MIF increased the number of colonies from control HeLa cells, but not from miR-586 knockdown HeLa cells (Fig 8B). Together, these results suggest that lncRNA-MIF inhibits cell proliferation via miR-586.

To further determine whether lncRNA-MIF regulates tumorigenesis, we used a xenograft mouse model. HeLa cells stably expressing exogenous lncRNA-MIF or lncRNA-MIF shRNA were injected subcutaneously into the dorsal flanks of the nude mice (n = 7 for each group). Three weeks after injection, mice were sacrificed and tumors were excised. Knockdown of lncRNA-MIF indeed increased tumorigenicity of HeLa cells (Fig 8C and D). In contrast, induction of lncRNA-MIF suppressed HeLa cell tumorigenicity (Fig 8C and D).

We further examined the expression levels of lncRNA-MIF and miR-586 in patients’ colorectal carcinoma and their para-carcinoma tissues. Both lncRNA-MIF and miR-586 in carcinoma tissues exhibited higher expression compared with that in para-carcinoma tissues (Fig EV5A). Based on the TCGA dataset, we found that lncRNA-MIF
expression level was higher in head and neck carcinoma than normal tissues (Fig EV5B).

**Discussion**

Cellular levels of c-Myc are nevertheless prone to fluctuation with the ever-changing environment, which may yield global effects on cells. How c-Myc level is regulated under physiological and/or cancerous conditions remains an unaddressed question. LncRNAs have recently attracted a lot of attention due to their abundance in the genome and biological significance in gene regulation. In this study, we show that as a target gene of c-Myc, lncRNA-MIF acts as a non-coding ceRNA (competing endogenous RNA) to compete with Fbxw7 mRNA for miR-586 and relieves the inhibitory effect of miR-586 on Fbxw7. This in turn increases Fbxw7 and accelerates Fbxw7-dependent degradation of c-Myc (Fig 8D). These data suggest that lncRNA-MIF suppresses c-Myc expression via absorbing more miR-586 to up-regulate Fbxw7, an E3 ligase for c-Myc. As a transcriptional factor, c-Myc is able to modulate expression of a large number of target genes and regulate multiple cellular processes including metabolism [34]. Recent studies have shown that many newly identified lncRNAs and microRNAs are c-Myc target genes [35–38]. The c-Myc-responsive lncRNAs are able to regulate cancer
Figure 7.

**A**

- ctrl
- IncRNA-MIF
- IncRNA-MIF-AS

**B**

- sh-ctrl
- sh-IncRNA-MIF-1
- sh-IncRNA-MIF-2
- sh-IncRNA-MIF-3

**C**

Cell number

- ctrl
  - G1: 61%
  - S: 15%
  - G2/M: 24%

- IncRNA-MIF
  - G1: 67%
  - S: 11%
  - G2/M: 22%

- IncRNA-MIF-AS
  - G1: 59%
  - S: 15%
  - G2/M: 26%

**D**

Cell number

- sh-ctrl
  - G1: 63%
  - S: 16%
  - G2/M: 21%

- sh-IncRNA-MIF-1
  - G1: 58%
  - S: 19%
  - G2/M: 23%

- sh-IncRNA-MIF-2
  - G1: 56%
  - S: 19%
  - G2/M: 25%

- sh-IncRNA-MIF-3
  - G1: 59%
  - S: 19%
  - G2/M: 22%

**E**

The proportion of the HeLa cells in each phase

- ctrl
- IncRNA-MIF
- IncRNA-MIF-AS

**F**

The proportion of the HeLa cells in each phase

- sh-ctrl
- sh-IncRNA-MIF-1
- sh-IncRNA-MIF-2
- sh-IncRNA-MIF-3
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Expression, lncRNA-MIF plays an important role in inhibiting cell proliferation and invasion [39–43]. We here also show that as a novel transcript by c-Myc, lncRNA-MIF strongly inhibits the glycolysis as well as tumor formation in nude mice. Taken together, these findings imply lncRNAs can serve as a novel class of tumor suppressive non-coding RNAs controlling c-Myc-driven tumorigenesis.

Despite that lncRNAs expression can be regulated by c-Myc, several lncRNAs have recently been shown to regulate c-Myc expression at multiple levels. For example, lncRNA-CCAT1-L (colon cancer-associated transcript 1, the long isoform) is able to promote Myc transcription via establishing an intra-chromosome looping between Myc and its upstream enhancer element [44]. LncRNA-GHET1 (gastric carcinoma high expressed transcript 1) promotes gastric carcinoma cell proliferation by increasing c-Myc mRNA stability [45]. LncRNA-GASS (growth arrest-specific transcript 5) binds to c-Myc mRNA and suppresses c-Myc translation via cooperatively with the eukaryotic translation initiation factor 4E (eIF4E) at position 96. LncRNA-PCAT1 (prostate cancer-associated transcript 1) post-transcriptionally regulates c-Myc expression by abrogating the down-regulation of c-Myc by miR-34a [47]. In addition, lncRNA-PVT1 (plasmacytoma variant translocation 1) increases protein expression of c-Myc via reducing its phosphorylation at threonine 58 (Thr58) and protecting it from proteasome-dependent degradation [48]. In our study, we show that lncRNA-MIF is able to negatively regulate c-Myc expression by acting as miR-586 sponge to increase Fbxw7 expression. Our findings therefore uncover a novel mechanism that depends on both long and small non-coding RNAs to control c-Myc stability, and add another layer of complexity to the c-Myc regulation.

C-Myc is also known to be dysregulated in a variety of human cancers, and many of them are addicted to c-Myc oncogenic signaling [49]. c-Myc is believed to be a promising target for treatment of cancer [50]. A casual link between lncRNAs and human cancer has been clearly established as many lncRNAs are dysregulated in various human cancers [21,51]. By suppressing c-Myc expression, lncRNA-MIF plays an important role in inhibiting aerobic glycolysis and tumorigenesis. As shown above, lncRNA-MIF overexpression decreases, whereas lncRNA-MIF knockdown increases tumorigenicity of HeLa cells. In addition, lncRNA-MIF down-regulates c-Jun, which has important function in progression through the G1 phase of the cell cycle, demonstrating that lncRNA-MIF is able to inhibit cancer cell proliferation via cell cycle arrest. These data suggest that lncRNA-MIF may represent a potential candidate for cancer therapy.

In summary, our findings in this study have uncovered a long non-coding RNA lncRNA-MIF, which links c-Myc transcription and its degradation. When c-Myc is overexpressed, it drives transcription of lncRNA-MIF, which functions as molecular sponge to absorb more cytosolic miR-586, thus leading to reduction in miR-586-mediated translational repression of Fbxw7. Conversely, in the case where c-Myc is reduced, lncRNA-MIF transcription is attenuated, which leads to increased free miR-586 in the cytosol that in turn triggers down-regulation of Fbxw7. Whether the c-Myc–miR-586–Fbxw7 may represent a novel mechanism to regulate c-Myc protein homeostasis still awaits further investigation. We realize that there are many other uncharacterized factors which are involved in the regulation of c-Myc stability besides lncRNA-MIF, Fbxw7, or miR-586.

Materials and Methods

Antibodies and reagents

The following antibodies were used for Western blot assays in this study: anti-Fbxw7 (R&D Systems); anti-c-Myc, anti-GAPDH, anti-GLUT1, anti-LDHA, and anti-PDK1 (Cell Signaling Technology); anti-PKMY and anti-HK2 (ImmunoWay); anti-PTBP1 (ProteinTech). Anti-c-Myc for ChIP assay was from Santa Cruz. Cycloheximide, doxycycline, cholaer toxin, and hydrocortisone were from Sigma–Aldrich.

Figure 7. LncRNA-MIF inhibits cell proliferation and cell cycle progression.

A HeLa cells were infected with lentiviruses expressing control RNA, lncRNA-MIF or lncRNA-MIF-AS as indicated. Forty-eight hours after infection, growth curves were measured for the indicated periods of time. Data shown are mean ± SD (n = 3).
B HeLa cells were infected with lentiviruses expressing control shRNA, lncRNA-MIF shRNA-1, -2, or -3. Forty-eight hours after infection, growth curves were measured for the indicated periods of time. Data shown are mean ± SD (n = 3).
C HeLa cells were infected with lentiviruses expressing control RNA, lncRNA-MIF, or lncRNA-MIF-AS as indicated. Forty-eight hours after infection, cells were stained with PI and analyzed by flow cytometry to identify phases of cell cycle.
D HeLa cells were infected with lentiviruses expressing control shRNA, lncRNA-MIF shRNA-1, -2, or -3. Forty-eight hours after infection, cells were stained with PI and analyzed by flow cytometry to identify phases of cell cycle.
E The percentage numbers of cells from (C) in G1, S or G2/M phase were analyzed by FlowJo 7.6 software. Data shown are mean ± SD (n = 3).
F The percentage numbers of cells from (D) in G1, S or G2/M phase were analyzed by FlowJo 7.6 software. Data shown are mean ± SD (n = 3).

Figure 8. LncRNA-MIF functions as a tumor suppressor to inhibit colony formation and cell proliferation.

A HeLa cells expressing either lncRNA-MIF or control RNA were transfected with miR-586 mimics or NC mimics as indicated. Colonies were stained with crystal violet and counted after 14 days incubation.
B HeLa cells expressing either MIF shRNA or control shRNA were transfected with miR-586 inhibitors or NC inhibitors as indicated. Colonies were stained with crystal violet and counted after 14 days incubation.
C 2 × 10^5 HeLa cells expressing either lncRNA-MIF, MIF control RNA, MIF shRNA, or MIF control shRNA were individually injected subcutaneously into flanks of nude mice (n = 7 for each group). Representative photographs of xenograft tumors in situ were taken 3 weeks after injection.
D Three weeks post-graft, mice were euthanized, tumors were excised and weighed, and the mean tumor weight was plotted on the graph. Data shown are mean ± SD (n = 7, *P < 0.05, two-tailed t-test).
E A schematic illustration of the proposed model depicting a role of c-Myc-induced lncRNA-MIF in regulating glycolysis via suppression of c-Myc.
Figure 8.

A

| IncRNA-MIF | + | - | + | + |
| miR-586 mimics | - | - | + | + |

B

| sh-IncRNA-MIF | + | - | + | + |
| miR-596 inhibitor | - | - | + | + |

C

Left(L): ctrl   Right(R): IncRNA-MIF

D

Tumor Weight (g)

E

Tumorigenesis
Glycolysis
Cell proliferation

Transcriptional activation

miR-586

Fbxw7 mRNA

Post-transcriptional inhibition

Fbxw7

Transcriptional activation

IncRNA-MIF

Proteasomal degradation

miR-586
Cell culture

HeLa, A549, H1299, HCT116, and MCF7 cell lines were cultured in DMEM (Dulbecco’s modified Eagle’s medium) medium containing 10% fetal bovine serum. P493-6 cell lines were cultured in RPMI medium 1640 containing 10% fetal bovine serum. MCF10A cell line was cultured in DMEM/F12 medium containing 5% horse serum, 20 μg/ml EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and 10 μg/ml insulin. All cells were tested for mycoplasma contamination and had no mycoplasma contamination.

ChiP assay

HeLa cells were cross-linked with 1% formaldehyde for 10 min. The ChiP assay was performed by using anti-c-Myc antibody and the Pierce Agarose ChiP kit (ThermoScientific, USA) according to the manufacturer’s instructions. Anti-rabbit immunoglobulin G was also used as a negative control. The bound DNA fragments were subjected to real-time PCR using the specific primers (Table EV1).

Luciferase reporter assay

To determine the effect of c-Myc on IncRNA-MIF promoter, either p3xFlag-Myc-CMV-24 or p3xFlag-Myc-CMV-24-c-Myc was co-transfected into HeLa cells together with individual PGL3-MIF1/2/3/1-M/2-M/3-M construct plus Renilla luciferase reporter plasmid. Twenty-four hours after transfection, firefly and Renilla luciferase activity were measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The data are represented as mean ± SD of three independent experiments. To evaluate the effect of miR-586 on Fbxw7 3’ UTR, HeLa cells were co-transfected with the psicheck2-based constructs containing Fbxw7 3’ UTR or Fbxw7 3’ UTR-M plus miR-586 mimics or inhibitors. Twenty-four hours after transfection, firefly and Renilla luciferase activity were measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The data are represented as mean ± SD of three independent experiments.

Colony formation assay

HeLa cells expressing control RNA, IncRNA-MIF, control shRNA, or IncRNA-MIF shRNA were transfected with miR-586 mimics or inhibitors as indicated. Twenty-four hours after transfection, HeLa cells (5 × 10⁴) in each condition were cultured in a six-well plate. Fourteen days later, cells were fixed, stained with crystal violet, and photographed.

Northern blot analysis

Northern blot analysis was performed as described previously [18] with minor modifications. Briefly, 20 μg of RNA was resolved by 1% denaturing agarose gel electrophoresis and transferred to Hybond-N membrane (GE Healthcare) by capillary transfer, followed by UV cross-linking. For Northern blots using digoxin-labeled oligonucleotide probes, the prehybridization/hybridization buffer (Ambion) was used according to the manufacturer’s instructions. After hybridization, blots were incubated with HRP-conjugated anti-digoxin antibody. Immunolabelling was developed with ECL Western Blotting Detection Reagent (GE Amersham). Visualized images were obtained using Image Quant LAS-4000 mini (GE Fujifilm).

Real-time RT–PCR

Total RNA was isolated by TRIzol reagent (Invitrogen). One μg of RNA was used to synthesize cDNA using the First-strand cDNA Synthesis System (Marligen Biosciences). Real-time PCR was performed using SYBR Green real-time PCR analysis (Takara) with the specific primers (Table EV1). PCR results, recorded as cycle threshold (Ct), were normalized against an internal control (β-actin).

Quantitation of IncRNA-MIF, IncRNA-MIF-L, and miR-586 expression levels

The exact copy numbers of IncRNA-MIF, IncRNA-MIF-L, and miR-586 transcripts per HeLa cell were quantified by using quantitative real-time RT–PCR assay. In this assay, serially diluted RT–PCR products of IncRNA-MIF, IncRNA-MIF-L, and miR-586 were used as templates to formulate standard curves, and then, the exact copies of IncRNA-MIF, IncRNA-MIF-L, and miR-586 per cell were calculated accordingly.

Cytosolic/nuclear fractionation

HeLa cells (1 × 10⁷) were incubated with hypotonic buffer (25 mM Tris–HCl, PH 7.4, 1 mM MgCl2, 5 mM KCl) on ice for 5 min. An equal volume of hypotonic buffer containing 1% NP-40 was then added, and each sample was left on ice for another 5 min. After centrifugation at 5,000 g for 5 min, the supernatant was collected as the cytosolic fraction. The pellets were re-suspended in nucleus resuspension buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated at 4°C for 30 min. Nuclear fraction was collected after removing insoluble membrane debris by centrifugation at 12,000 g for 10 min.

Biotin pull-down assay

All processes were performed in the RNase-free conditions. For antisense oligomer affinity pull-down assay, sense or antisense biotin-labeled DNA oligomers corresponding to human IncRNA-MIF (1 μg) were incubated with lysates from HeLa cells (2 × 10⁷) or the cytosolic/nuclear extracts. One hour after incubation, streptavidin-coupled agarose beads (Invitrogen) were added to isolate the RNA–protein complex or RNA–RNA complex. For in vitro RNA pull-down assay, 3 μg in vitro-synthesized biotin-labeled IncRNA-MIF was incubated with lysates from HeLa cells (2 × 10⁷) for 3 h. Streptavidin-coupled agarose beads (Invitrogen) were then added to the reaction mix to isolate the RNA–protein complex or RNA–RNA complex.

Glucose uptake assay

Glucose uptake assay was performed as previously described [52].
Lactate production assay

Lactate production assay was performed as previously described [52].

Cell cycle analysis

HeLa cells were infected with lentiviruses and screened by puromycin. HeLa cells (1 × 10⁶) were plated into 6-mm plates. During the proliferative exponential phase (50% confluency), cells were fixed in 70% ethanol overnight. Cells were then stained with propidium iodide and analyzed by flow cytometry.

Xenograft mouse model

HeLa cells expressing control RNA, lncRNA-MIF, control shRNA, or lncRNA-MIF shRNA (2 × 10⁶) were subcutaneously injected into the dorsal flank of 4-week-old male athymic nude mice (Shanghai SLAC Laboratory Animal Co. Ltd.) (n = 7 mice per group). After 3 weeks, mice were sacrificed, and tumors were excised and weighed. Mice were used in the experiment at random. During testing the tumors’ weight, the experimentalists were blinded to the information and shape of tumor tissue masses. Studies on animals were conducted with approval from the Animal Research Ethics Committee of the University of Science and Technology of China.

Expanded View for this article is available online.

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Author contributions

PFZ, LMC, and MW designed research; PFZ and LMC performed experiments and analyzed data; PSF provided material support; PFZ, YDM, and MW wrote and revised the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


