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Research article

Jmjd6 regulates ES cell homeostasis and enhances reprogramming efficiency☆

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ABSTRACT

Jmjd6 is a conserved nuclear protein which possesses histone arginine demethylation and lysyl hydroxylase activity. Previous studies have revealed that Jmjd6 is essential for cell differentiation and embryo development. However, the role of Jmjd6 in mammalian ES cell identity and reprogramming has been unclear. Here we report that depletion of Jmjd6 not only results in downregulation of pluripotency genes but also is implicated in apoptosis, glycolysis, cell cycle and protein hydroxylation. We also revealed the reduction of BrdU incorporation in *Jmjd6* depleted cells. Reprogramming efficiency of MEFs can be enhanced with Jmjd6 overexpression while the efficiency was reduced upon *Jmjd6* depletion. Together, these results suggest that Jmjd6 can regulate ES cell homeostasis and enhance somatic cell reprogramming.

1. Introduction

Jumonji domain containing 6 (Jmjd6) is a conserved nuclear protein which possesses bifunctional histone arginine demethylation and lysyl hydroxylase activities [1]. Jmjd6 has also been reported to be a marker of poor prognosis in several types of cancers such as breast, lung and colon cancer [2, 3, 4, 5]. Chang *et al.* firstly reported that Jmjd6 showed histone arginine demethylase activity [6], while this has been challenged by other research groups that, unlike other family member of Jmjd proteins, such activity of Jmjd6 could not be detected [7, 8, 9]. Although there have been conflicting reports with regards to the histone arginine demethylase activity of Jmjd6, it has been found that Jmjd6 exhibits lysyl-hydroxylase activity, implying a role in epigenetic regulation of gene transcription [9, 10]. Jmjd6 can also affect alternative splicing by catalysing posttranslational lysyl-5-hydroxylation of the RNA splicing factor U2 small nuclear ribonucleoprotein auxiliary factor 65-kilodalton subunit (U2AF65) and promote colon carcinogenesis through negative regulation of p53 by hydroxylation [5, 7].

Jmjd6 is essential for embryonic development. *Jmjd6* knockout led to prenatal death and caused severe developmental defects in many tissues, such as heart, kidney, eyes and brain [11, 12, 13, 14]. Liu et al discovered that bromodomain-containing protein 4 (BRD4) can recruit Jmjd6 to distal enhancers known as anti-pause enhancers (A-PEs), and release RNA polymerase II promoter-proximal pause in a large population of transcription units, which possibly contribute to the regulation of gene expression and thus affect processes involved in differentiation and development [15]. Notably this BRD4-dependent transcriptional pause release can guard productive transcriptional elongation of pluripotency genes, thus maintaining the pluripotent state and ensuring efficient somatic cell reprograming [16, 17, 18]. Furthermore, Jmjd6 likely is a direct binding target of master pluripotency factors Oct4/Sox2 [19]. Taken together, we hypothesized that Jmjd6 could be an essential pluripotency factor.

Here we report that Jmjd6 expression is associated with the pluripotent state of ES cells. Depletion of Jmjd6 in ES cells leads to reduction of pluripotency genes, up-regulation of lineage genes. In addition, upon Jmjd6 depletion, genes involved in apoptosis were up-regulated while glycolysis related genes and some hydroxylase-encoding genes were down-regulated. Jmjd6 also promotes the generation of iPSCs in OKSM-mediated somatic reprogramming. Our results suggest that Jmjd6 plays multiple roles in ES cell self-renewal, cell proliferation and metabolism.

2. Materials and methods

2.1. Cell culture and alkaline phosphatase (AP) staining

Murine E14 ES cells were cultured on gelatin-coated plates in filtered cell medium consisting of Glasgow Minimum Essential Medium (GMEM;

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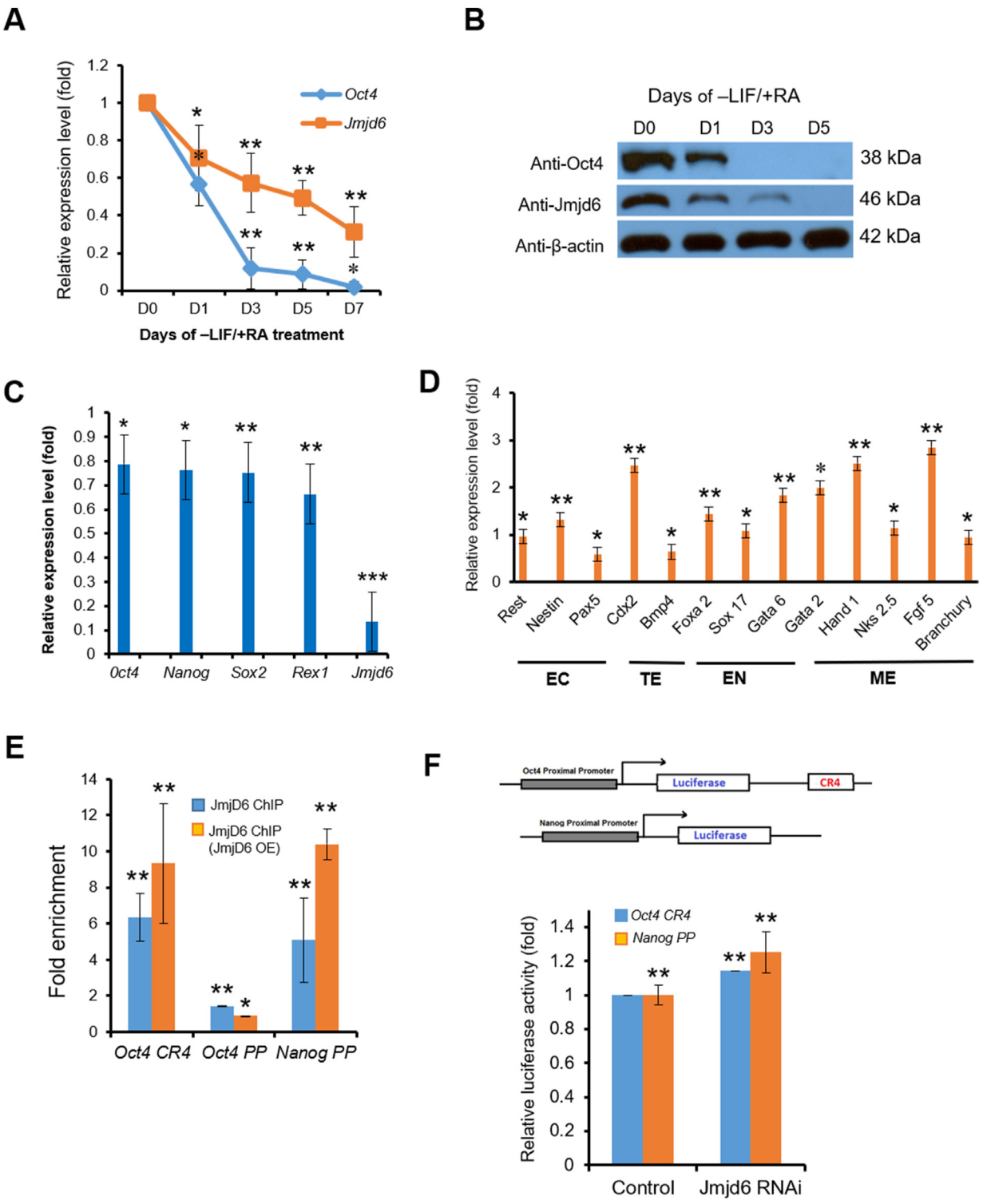


Figure 1. Jmjd6 is associated with ES cell pluripotency. (A) Jmjd6 expression decreases upon ES cell differentiation. E14 ES cells were treated with 0.1 μM RA without the presence of LIF for seven consecutive days. RNA and protein were extracted at alternative days of treatment. The relative level of Jmjd6 and Pou5f1 were compared with undifferentiated ES cells and normalised against beta-actin. Duplicate experiments were performed to obtain the mean transcript levels. (B) Protein levels of Oct4 and Jmjd6 upon –LIF/+RA treatment. Beta-actin serves as a loading control. (C) Pluripotency markers were down-regulated in *Jmjd6* depleted cells. Data were normalized against *β-actin* expression. (D) *Jmjd6* depletion up-regulates some lineage markers. (E) Jmjd6 binds to *Oct4* CR4 region and *Nanog* proximal promoters. E14 chromatin were precipitated with anti-JMJD6 antibody were analyzed by real-time PCR with primers flanking *Oct4* CR4 region, *Oct4* proximal promoter (Oct4 PP) and *Nanog* proximal promoter (Nanog PP) regions. Enrichment folds were calculated by comparison with input chromatin DNA and normalized against an intergenic region. (F) Jmjd6 depletion does not alter activities of Oct4 CR4 region and Nanog proximal promoter. Jmjd6 knockdown plasmids were co-transfected with luciferase reporter vectors *Oct4* proximal promoter CR4-luciferase and *Nanog* proximal promoter-luciferase. Luciferase activities were measured 48 h after transfection. The numeric value was presented as mean ± S.E. Student's t-tests were completed and statistical significance is indicated. *p < 0.05; **p < 0.01; ***p < 0.001.

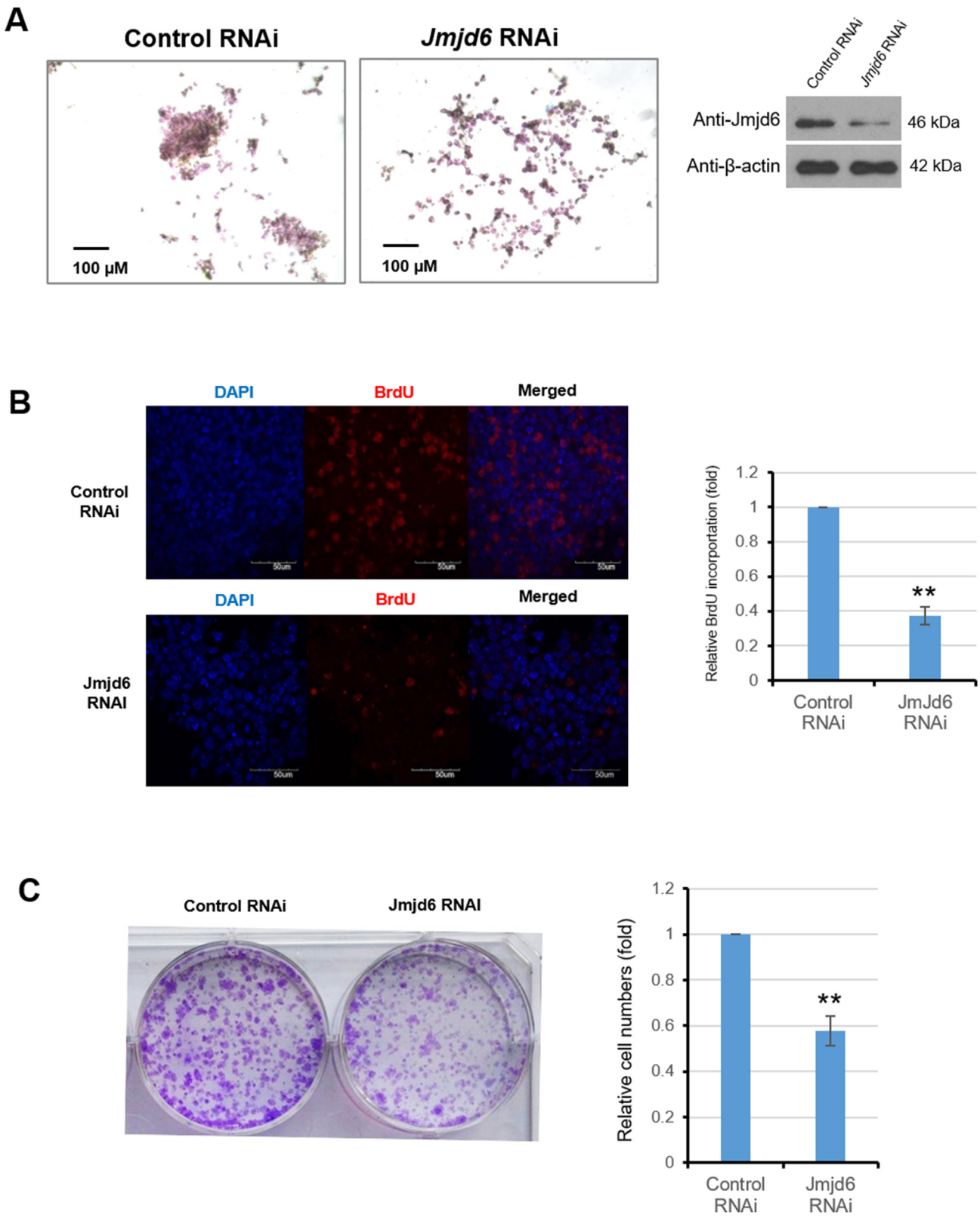
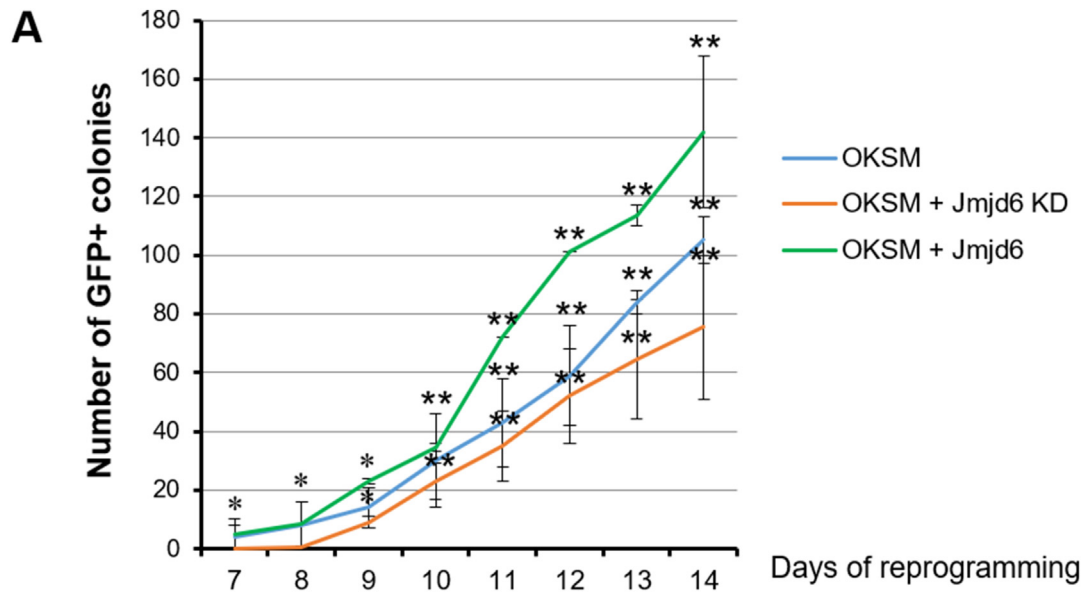
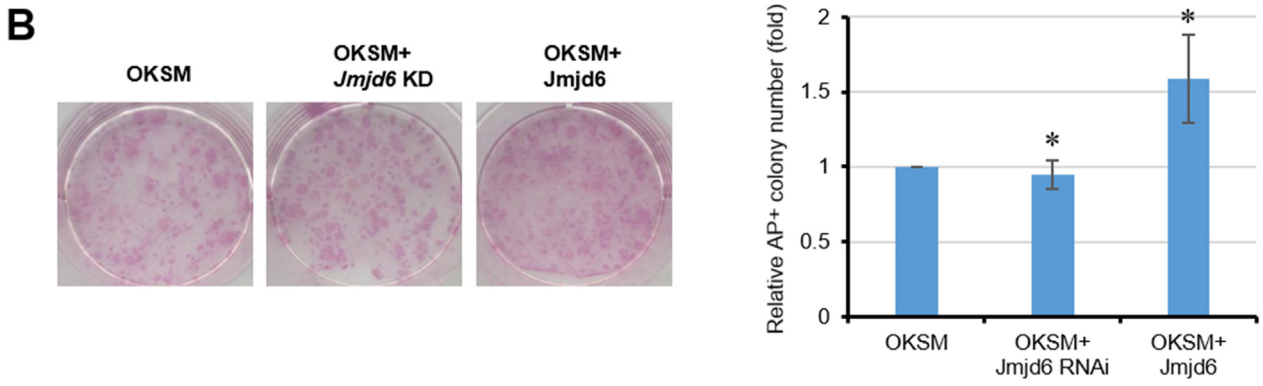
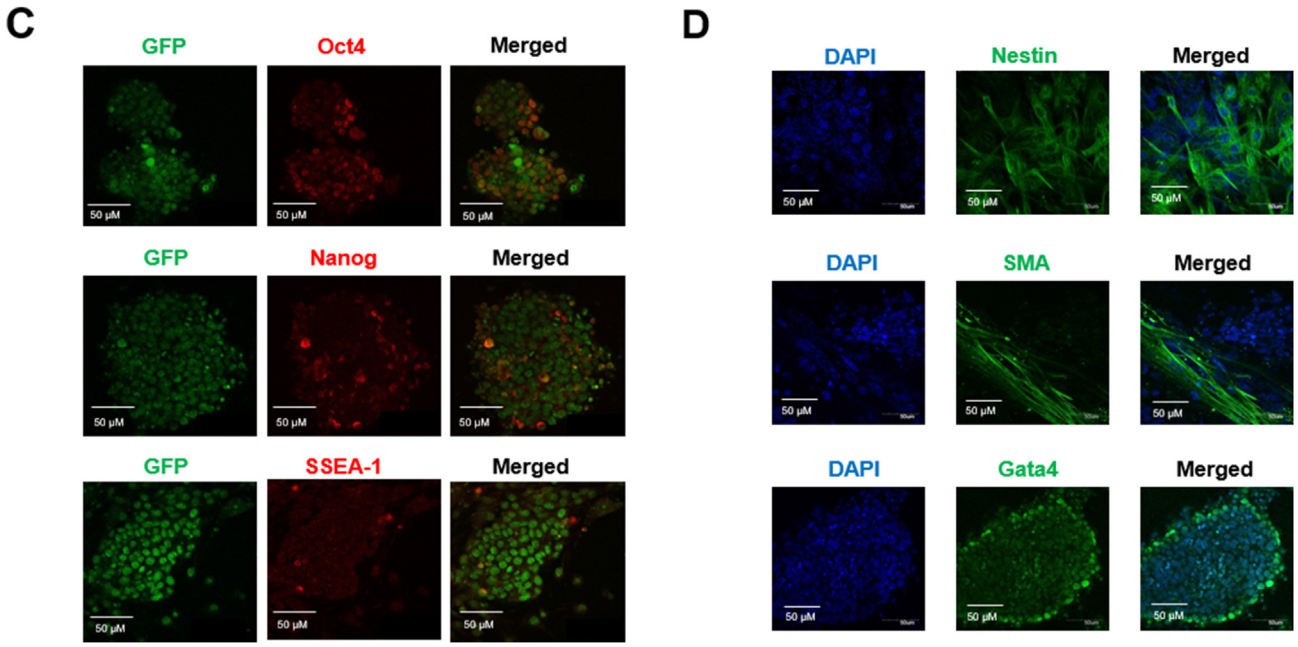


Figure 2. Depletion of *Jmjd6* impairs ES cell proliferation. (A) ES cells could not form colonies upon *Jmjd6* RNAi. Alkaline phosphatase staining was performed 4 days after E14 cells were transfected with control vector or *Jmjd6* knockdown construct. Western blot showed that Jmjd6 protein was significantly depleted upon *Jmjd6* RNAi. (B) BrdU incorporation was decreased in *Jmjd6* knock-down E14 cells. BrdU staining was performed 4 days after E14 cells were transfected with control vector or Jmjd6 knock-down construct. BrdU was added into the cell medium and incubated for 2 h prior to immunostaining. BrdU can be incorporated into the newly synthesized DNA of replicating cells which can then be detected by anti-BrdU antibodies. (C) ES cell proliferation rate was reduced upon Jmjd6 RNAi. Crystal violet assay kit was used to measure the difference between jmjd6 depleted cells and control cells. Data was presented as mean ± SEM; n = 3; *P ≤ 0.05, **P ≤ 0.01.







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Invitrogen), 15% ES cell qualified fetal bovine serum (FBS; Invitrogen), 0.055 mM ß-mercaptoethanol (Sigma-Aldrich), 100 mM sodium pyruvate (Invitrogen), 0.1 mM MEM nonessential amino acid (NEAA, Invitrogen) and 1,000 units/ml of Leukemia Inhibitory Factor (LIF, Millipore). Platinum-E (Plat-E) cells were cultured in filtered Plat-E medium containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) and 10% FBS. SNL medium for maintenance of SNL feeder cells consists of GMEM and 10% FBS. AP staining was carried out using Alkaline Phosphatase Detection Kit (Millipore) according to the instructions provided in the kit. Stained colonies were viewed under an Axio Observer A1 inverted light microscope (Zeiss).

Pou5f1-GFP mouse embryonic fibroblasts (MEFs) infected with retroviruses for iPS cell formation were cultured in mES cell medium without LIF for 5 days post infection followed by maintenance in Knockout Serum Replacement (KSR) medium consisting of DMEM, 15% KSR (Invitrogen), 2 mM L- Glutamine (PAA), 1 mM sodium pyruvate, 1000 units/ml of LIF, 0.055 mM ß-mercaptoethanol and 0.1 mM MEM NEAA. All the cells were kept in a 37°C with 5% CO_2 incubator.

2.2. Plasmid construction

For the generation of RNAi vectors, pSUPER.puro plastmid (Oligoengine) and pSUPER.retro.puro plasmid (Oligoengine) were digested with HindIII and BglII restriction enzymes (New England Biolabs), and ligated with annealed shRNA primers. The primers used to target *Jmjd6* were as followed: F 5′–gatccccAATGAAACCCTTTACCTAttcaagagaTAGGTAAAGGGTTTCATTGttttta–3’; R 5′–agcttaaaaaCAATGAAACCCTTTACCTAtctcttgaaTAGGTAAAGGGTTTCATTggg–3’.

Primers for overexpression of Jmjd6 were designed using the coding sequence with the addition of BamHI and NotI restriction sites. Amplified Jmjd6 cDNA were then ligated pMX plasmid (Addgene). The primers used were as followed: F 5’ – atatggatccATGAACCACAAGAGCAAGAAGC – 3’; R 5’ – taatagcggccgcTCACCTGGAGGAGCTGCGCTCT – 3’.

2.3. Transfection, reverse transcription and qPCR

Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the product manual. After selected by puromycin for 4 days, RNA was extracted by TRIzol (Invitrogen). Real time PCR was performed as previously described [20]. Quantitative real time PCR was performed using Fast SYBR® Green Master Mix (Bio-Rad Laboratories). The relative quantification of mRNA levels between *Jmjd6* depleted cells and control cells was computed using the 2-ΔΔCT method.

2.4. Chromatin immunoprecipitation and dual luciferase assay

Chromatin immunoprecipitation assays using Anti-JMJD6 antibody (ab64575) was performed as previously described [21]. Primers used for qPCR are as follows:

Oct4 CR4 region: F 5′-GTGGTGGAGAGTGCTGTCTAGGCCTTAG-3′,
R 5′-AGCAGATTAAGGAAGGGCTAGGACGAGAG-3’;
Oct4 proximal promoter (Oct4 PP): F 5′-GGATTGGGGAGGGAGAGGTGAAACCGT-3’;
R 5′-TGGAAGCTTAGCCAGGTTCGAGGATCCAC-3’;
Nanog proximal promoter (Nanog PP): F 5′-CTCTTTCTGTGGGAAGGCTGCGGCTCACTT-3’;
R 5′-CATGTCAGTGTGATGGCGAGGGAAGGGA-3’.

Oct4 Proximal P_CR4-luciferase and Nanog proximal P-luciferase reporter vectors were constructed as in previous studies [33]. Lluciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Measurement of luminescence was performed using the Ultra 384 Microplate Reader (Tecan).

2.5. Immunofluorescence staining

Cells were cultured on coverslips were fixed and then probed with primary antibody, washed with PBST, probed with secondary antibody in the dark and washed with PBST again. The cells were stained with Vectashield mounting medium containing 4′, 6- diamidino-2-phenylindole (DAPI; Vector Laboratories) before observed under a confocal microscope (Olympus FV1000). Primary antibodies used for probing iPS cells were anti-Oct4 (sc-8628, Santa Cruz), anti-SSEA-1 (mab34301, Millipore) and anti-Nanog (sc-33760, Santa Cruz). As for probing EBs, primary antibodies used were anti-alpha smooth muscle Actin (ab5694, Abcam), anti-Gata4 (sc-25310, Santa Cruz) and anti-Nestin (mab2736, R&D). Secondary antibodies used to probe iPS cells were Alexa Fluor 594 lgG antibody (Invitrogen) while Alexa Fluor 488 lgG antibody (Invitrogen) was used to probe EBs.

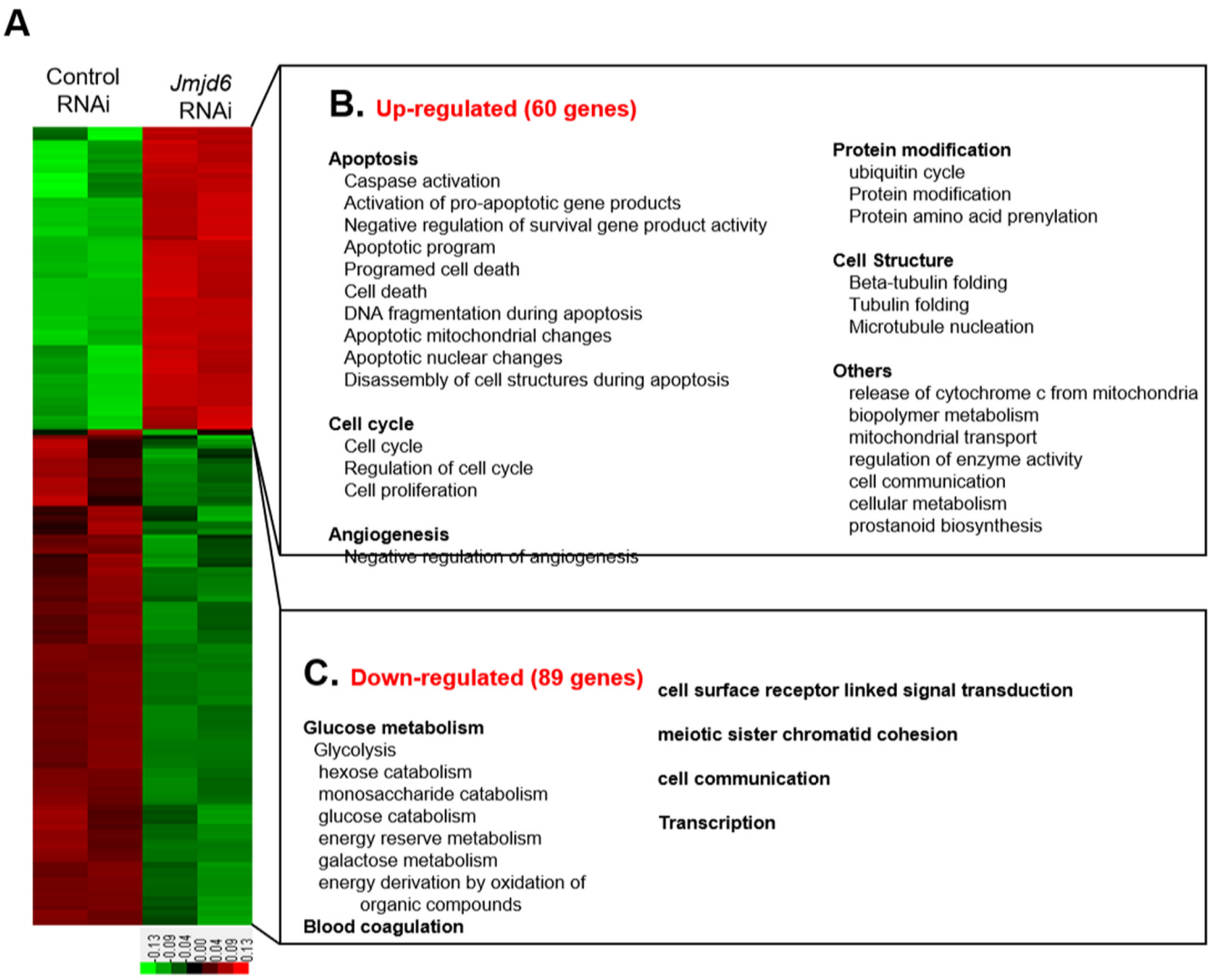
2.6. Bromodeoxyuridine (BrdU) incorporation assay

E14 cells were cultured on coverslips and BrdU at a concentration of 3 μg/ml was added into the medium. The cells were fixed with 4% paraformaldehyde (PFA), washed with PBS. The primary antibody used to probe the cells was anti-BrdU (sc-32323, Santa Cruz) while the secondary antibody used was Alexa Fluor 594 lgG antibody (Invitrogen).

2.7. Gene expression microarray analysis

RNA extracted from Jmjd6 depleted cells and control was analysed with Affymetrix Mouse Genome MG430 Plus 2.0 Array according to the manufacturer's instructions. Differential expression between knockdown and wild type was identified using a fold change of 1.5. log2 transformation was performed on the data followed by subtraction from the mean of the means of knockdown and wild type sample groups before hierarchical clustering was carried out. GO TermFinder was used to identify enriched “Gene Ontology” (GO) terms of the differentially expressed genes. Hierarchical clustering was performed with Cluster 3.0 on differentially expressed genes. Raw fold change data was adjusted to both genes and array by its mean prior to hierarchical clustering by the Euclidean distance similarity metric and average linkage. The heat-map was visualized with Java Treeview. Gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8. The p-value cut-off was set at 0.05 for the significant enriched GO terms.

Figure 3. Overexpression of *Jmjd6* enhances reprogramming efficiency. Oct4-GFP MEFs were infected with retrovirus carrying Yamanaka's factors OKSM together with overexpression or knock-down of *Jmjd6* to form induced pluripotent stem cells (iPSCs). (A) The appearance of GFP^+ colonies were observed. MEFs infected with only OKSM serves as a control experiment. Overexpression of *Jmjd6* enhances reprogramming efficiency by generating more GFP^+ colonies while knock-down of *Jmjd6* reduces the efficiency of reprogramming. (B) The iPSCs generated were stained for alkaline phosphatase activity on the 14^{th} day of reprogramming. Lesser colonies generated from OKSM + *Jmjd6* knock-down were observed to be stained as compared to OKSM while more colonies generated from OKSM + *Jmjd6* overexpression were observed to be stained as compared to OKSM. (C) Jmjd6 overexpression iPSCs were ESC like. GFP^+ iPSCs generated from *Jmjd6* overexpression were subjected to immunostaining using anti-Oct4, anti-Nanog and anti-SSEA-1 antibodies and the expression of these genes observed indicating that the iPSCs were ES-cell like. (D) Jmjd6 overexpression iPSCs were able to differentiate into all three germ layers. EBs generated from *Jmjd6* overexpression iPSCs were stained with anti-Nestin, anti-SMA and anti-Gata4 antibodies and it was observed that the iPSCs could differentiate into ectoderm, endoderm and mesoderm lineages respectively. The numeric value was presented as mean ± S.E. Student's t-tests were completed and statistical significance is indicated. *$p < 0.05$; **$p < 0.01$.



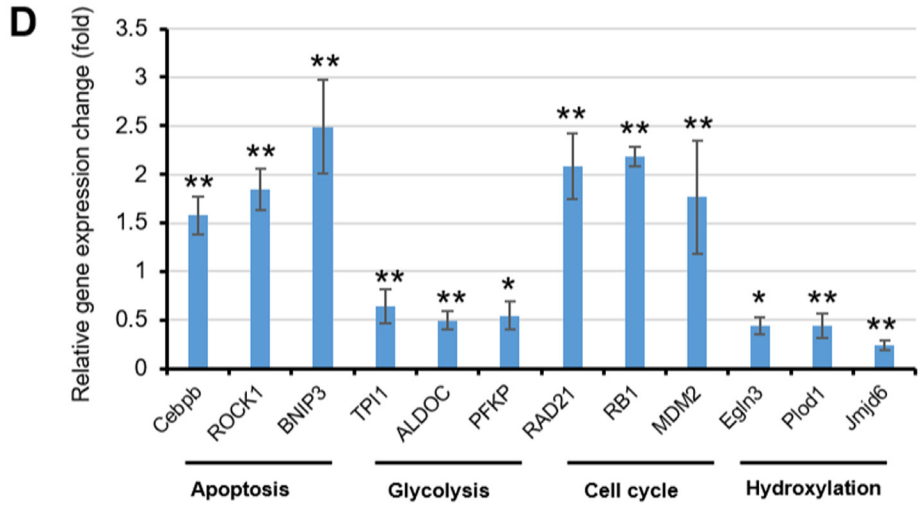


Figure 4. Gene expression microarray profiling of changes in ES cells after *Jmjd6* RNAi. (A) Microarray heat map with enriched Gene Ontology biological process terms for the up-regulated genes (366 genes) and down-regulated genes (685 genes). Relative highly expressed genes were shown in red and low expressed genes in green. (B) Gene ontology analysis of up-regulated genes in Jmjd6 depleted cells. (C) Gene ontology analysis of down-regulated genes. Enriched terms ($p < 0.05$) were classified and grouped according to the biological processes. (D) Validation of gene expression microarray results. qRT-PCR was performed with cDNA from *Jmjd6* RNAi and control cells on selected genes related to apoptosis, glycolysis, cell cycle and protein hydroxylation. Gene expression was normalized to the expression of *β-actin*. Data was presented as mean ± SEM; n = 2. *P ≤ 0.05, **P ≤ 0.01.

2.8. iPSC formation assay

Retrovirus packing and infection in Plat-E cells were carried out as previously described [20]. The virus containing medium was concentrated through centrifugation using Amicon Centrifugal Filter Units-100 kDa (Millipore) and were then stored at -80°C. At 6 h prior to infection, *Pou5f1*-GFP MEFs at 50–70% confluency were seeded onto gelatin-coated 24 well plates using mES cell medium without LIF. During infection, 8 mg/ml of polybrene (Sigma) was supplemented to the medium together with the addition of 10 μl of each concentrated retrovirus. At 2 days post infection, the MEFs were passaged onto mitomycin-C (Sigma) treated SNL feeder layer and cultured in mES cell medium without LIF for 5 days followed by maintenance in KSR medium. KSR medium was changed every day and the appearance was GFP + colonies were monitored.

2.9. Embryoid body (EB) formation assay

iPS cells were treated with 0.1% Trypsin and cultured in Ultra Low Adhesion Culture Dish (Coring). The EB obtained were transferred to coverslips coated with gelatin and cultured for 14 days. Expression of specific lineage markers was detected through immunostaining with anti-Nestin, anti-Gata4 and anti-smooth muscle actin antibodies. Fluorescence images were captured at 60X magnification using a confocal microscope (Olympus FluoView 1000).

2.10. Statistical analysis

Numerical data were presented as mean ± SEM. Differences between groups were determined by Student's t-test and significant differences were determined by $p \leq 0.05$.

3. Results

3.1. Jmjd6 is a pluripotency-associated factor

First, we examined the expression of Jmjd6 upon ES cell differentiation. ES cells were induced by the presence of 0.1 μM retinoic acid (RA) and withdrawal of LIF. After the cells were treated for 3 days, we observed that Jmjd6 expression was significantly down-regulated at both RNA level and protein level (Figure 1A, B). As shown in Figure 1B, JMJD6 was barely detected at Day 5 upon ES cell differentiation (the master pluripotency factor Oct4 served as a positive control). This suggests that Jmjd6 is a pluripotency-associated factor.

In order to examine whether the high expression of Jmjd6 in ES cells is specifically required for pluripotency regulation, we next performed *Jmjd6* RNAi and overexpression assay in ES cells. As shown in Figure 1C, *Jmjd6* was efficiently reduced to 10% upon *Jmjd6* depletion. We then found that pluripotency marker genes *Oct4*, *Nanog*, *Sox2* and *Rex1* showed a down-regulated trend. Further examination of the lineage specific genes revealed that trophectoderm marker *Cdx2* and mesoderm markers were up-regulated, implying that these depleted ES cells underwent differentiation (Figure 1D).

In addition, ES cells overexpressing Jmjd6 displayed normal ES cell morphology, positive alkaline phosphatase staining with elevated expression of Nanog (Supplementary Figure 1), suggesting that high level of Jmjd6 promotes pluripotency maintenance.

3.2. Jmjd6 binds to promoter regions of Oct4 and Nanog

Since *Jmjd6* RNAi resulted in reduced expression of *Oct4* and *Nanog*, we next examined whether Jmjd6 can bind to their promoter regions to regulate their transcription. We performed ChIP assay using anti-JMJD6 antibody. Our real time-PCR results showed that Jmjd6 is highly enriched at *Oct4* conserved region 4 (CR4) and *Nanog* transcription starting site (TSS region) (Figure 1E). Next, we performed reporter assays to examine whether Jmjd6 can regulate the transcription of *Oct4* and *Nanog*. *Jmjd6* shRNA plasmids or control plasmids were co-transfected with the reporter vector in which CR4 region of *Oct4* gene was inserted downstream of luciferase gene. As shown in Figure 1F, *Jmjd6* RNAi did not change the luciferase activity through CR4 region. Similarly, activity of luciferase downstream of *Nanog* proximal promoter was not changed significantly in *Jmjd6* depleted cells as compared to control cells. It seems that Jmjd6 can bind to promoters of *Oct4* and *Nanog*, which however did not alter the downstream luciferase activities. This suggests that the binding of Jmjd6 to the promoter regions is redundant and could not significantly change the transcription activity.

3.3. Jmjd6 depletion inhibits ES cell proliferation

Given that Jmjd6 was known as a driver of cell proliferation, we indeed observed that Jmjd6 depleted cells could not proliferate properly. Alkaline phosphatase staining showed that, although Jmjd6 knockdown cells were still AP positive, these cells could not form typical ES colonies (Figure 2A). Bromodeoxyuridine (BrdU) incorporation assay further revealed that *Jmjd6* depletion clearly reduced the percentage of BrdU-stained cells to 37.5% (Figure 2B). Furthermore, our cell viability experiment using crystal violet staining demonstrated that Es cell proliferation rate was decreased to 57.8% (Figure 2C). These strongly suggest that decrease in Jmjd6 expression reduces cell proliferation, suggesting a role of Jmjd6 in promoting ES cell proliferation.

3.4. Jmjd6 can enhance OKSM reprogramming of MEFs into iPS cells

We nest asked whether Jmjd6 would promote iPS cell generation during the process of OKSM reprogramming. We infected Oct4-GFP MEFs with retrovirus carrying Yamanaka's factors OKSM together with overexpression or knockdown constructs of *Jmjd6*. We found that *Jmjd6* overexpression can enhance OKSM reprogramming as more GFP^+ colonies were generated throughout the process of reprogramming as compared to the control where only OKSM were used. As of 14 days of reprogramming, the numbers of GFP + positive colonies generated from OKSM plus Jmjd6 was 1.35-fold that of the control (Figure 3A). When OKSM is co-infected with *Jmjd6* RNAi virus, a lower number of GFP^+ colonies were generated as compared to control throughout the reprogramming process and as of 14 days of reprogramming, the number of GFP^+ colonies generated from OKSM plus *Jmjd6* knockdown was 0.7-fold that of the control (Figure 3A).

These iPS cells were also stained for alkaline phosphatase activity which is an marker of pluripotent cells [22]. More AP^+ colonies (about 1.6 folds) were produced from OKSM + *Jmjd6* overexpression as compared to OKSM (Figure 3B). This further confirmed that Jmjd6 can enhance reprogramming by generating more colonies of iPS cells. However, the effect of *Jmjd6* RNAi on generating AP stained iPS cells was not very prominent (Figure 3B). Since AP activity is an early pluripotency marker in reprogramming, it possibly implied that Jmjd6 is redundant in the early stage of pluripotency reactivation.

We also examined whether Jmjd6 overexpressing iPS cells were pluripotent. We performed immunostaining iPS cells generated from Jmjd6 overexpression. We found that Jmjd6 iPS cells were able to express pluripotency marker genes such as Oct4 and Nanog, as well as the mES cell marker SSEA-1 (Figure 3C). Furthermore, these cells were able to express ectoderm, endoderm and mesoderm markers when they were induced to differentiate into embryoid bodies (EBs) (Figure 3D), suggesting that these iPS cells produced from Jmjd6 overexpression are pluripotent.

3.5. Gene expression microarray profiling of changes in ES cells after Jmjd6 RNAi

To gain more insight into the functions of Jmjd6 in pluripotency regulation, gene expression microarray assay was carried out to

investigate the change in global gene expression levels when Jmjd6 was knocked-down. Figure 4 showed the microarray heat map generated from relative expression levels of genes. Relatively high expressed genes were represented in red while relatively low expressed genes in green. Expression of as many as 60 genes was shown to be increased by more than 1.9-fold while 89 genes showed a more than 1.5-fold reduction in expression after *Jmjd6* knockdown. We validated expression changes of some selected genes correlated with apoptosis, glycolysis, cell cycle and protein hydroxylation (Figure 4D). We listed 20 most upregulated and downregulated genes in Supplementary Tables 1 and 2.

Notably, our gene ontology (GO) analysis showed that genes related to angiogenesis were enriched, corresponding with the observation of severe cardiopulmonary malformations in *Jmjd6* knockout mice, [12]. As presented in Figure 4, many up-regulated genes were related to apoptosis, negative regulation of the cell cycle and cell death, which ties in with our observation that *Jmjd6* depletion reduced cell proliferation. Among those down-regulated genes, we found many terms related to cellular metabolism, particularly glycolysis. Considering that ES cells and iPSC cells rely on glycolysis pathway for ATP production [23, 24], it is highly possible that *Jmjd6* depletion causes cellular metabolic reduction and cannot meet the energy demand of highly proliferating cells, thus repressing cell proliferation. Interestingly, some protein hydroxylase encoding genes were also found in the down-regulated genes, such as *Plod1*, *Egln3* and *Jmjd6* (Figure 4D). Hence, it seems that Jmjd6 plays vital roles in regulating hydroxylation.

4. Discussion

Though Oct4/Sox2/Nanog transcriptional network governs ES cell pluripotency, still it is important to identify novel pluripotency factors as the depletion of a single factor may result in changes in ES cell characteristics [25]. Here we showed that Jmjd6 is associated with pluripotency and Jmjd6 overexpression enhances the generation of iPSCs. Knockdown of *Jmjd6* reduces ES cell proliferation, suggesting an impaired self-renewal. However, lineage marker genes did not increase significantly upon *Jmjd6* depletion and *Jmjd6* depleted ES cells can still maintain AP activity. Hence, Jmjd6 could be a redundant factor in pluripotency maintenance, but it is essential for ES cell self-renewal. Our results showed that Jmjd6 seems not directly regulate the transcription of master pluripotency genes *Oct4* and *Nanog*. The main functions of Jmjd6 in mouse ES cells might be the regulator of cell homeostasis including cell proliferation, metabolism and cell survival. Also, Jmjd6 may be involved in protein modification and stability. To further understand how Jmjd6 regulates ES cell identity, conditional Jmjd6 knockout in ES cells and identification of Jmjd6-interacting proteins could shed more lights on functions of Jmjd6.

Jmjd6 has been reported to have multiple substrates and biological functions. Jmjd6 is known to act as: 1) demethylase of histone arginine and non-histone substrate estrogen receptor alpha [6,26]; 2) lysine hydroxylase to target U2AF65, a protein associated with mRNA splicing, p53, histone H3/H4 and H2A/H2B [5,7,8]; 3) associates with BRD4 to promote Pol II promoter-proximal pause release via long-range interactions between promoters and anti-pause enhancers (A-PEs) [15], which collectively suggest that Jmjd6 is an important epigenetic regulator of gene transcription and elongation. Since histone arginine methylations are generally associated with transcriptional activation [27, 28, 29, 30]. Indeed, JMJD6 promotes self-renewal of hematopoietic stem cells and adipose-derived mesenchymal stem cells [31, 32]. Interestingly, Jmjd6 may regulates gene expression by integrating with chromatin [33]. Furthermore, as Jmjd6 can affect alternative splicing by catalysing posttranslational lysyl-5-hydroxylation RNA splicing factor U2AF65 [7], it will be interesting to look at how Jmjd6 regulates alternative RNA splicing.

There are several possible ways by which Jmjd6 can enhance reprogramming. Firstly, the histone modification activity of Jmjd6 may play a role in the epigenetic remodelling during the reprogramming process. Jmjd6 was reported to hydroxylate histone lysyl residues of histones H3/H4 and H2A/H2B to generate 5-hydroxylysine [10]. 5-hyxdroxylysine generated from 2OG-dependent dioxygenase activity of Jmjd6 can alter the epigenetic status of histones and even regulate other epigenetic modifiers to influence the regulation of the transcriptional network during the reprogramming process. Indeed, hydroxylation of the methyl group in lysine residues has been found to catalyse the demethylation of these lysine residues in histones, which is then linked to potency and cell fate determination [34].

Secondly, Jmjd6 may regulate reprogramming through interaction with BRD4 since transcriptional pause release mediated by BRD4 is a rate-limiting step for somatic cell reprogramming [17]. Jmjd6 was reported to interact with BRD4 and act as a functional unit which binds to distal enhancers and result in the regulation of RNA polymerase II promoter-proximal pausing in a large population of genes [15]. Binding of Jmjd6 to the distal enhancers is a Brd4 dependent process, and this leads to the removal of inhibitory factors and activation of p-TEFb complex and results in the increase in efficiency of transcriptional elongation. Since the expression of many pluripotency genes are regulated by RNA polymerase II promoter-proximal pausing, the interaction of Jmjd6 and Brd4 could lead to the efficient transcriptional reactivation of pluripotency genes and thus contribute to the enhancement of reprogramming [16, 17].

In conclusion, our results demonstrate that Jmjd6 is a pluripotency-related factor which can enhance OKSM-mediated reprogramming process. Jmjd6 can also regulate ES cell homeostasis. Given that Jmjd6 can interact with many proteins and plays pleiotropic functions in diverse context [35, 36], More studies are required to understand multifactorial roles of Jmjd6. Furthermore, since Jmjd6 is a conserved protein present in human and mouse [37], we propose that Jmjd6 may have similar functions in human ES cells and reprogramming process.

Declarations

Author contribution statement

Guanxu Ji, Xiaoxiao Xiao: Performed the experiments; Analyzed and interpreted the data.

Min Huang: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Qiang Wu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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