JHDM3A Module as an Effector Molecule in Guide-directed Modification of Target Chromatin

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With the objective of returning cells to their undifferentiated state through alteration of epigenetic states, small molecules have been used that specifically inhibit proteins involved in sustaining the epigenetic system. However, this chemical-based approach can cause chaotic epigenetic states due to random actions of the inhibitors. We investigated whether JHDM3A/JMJD2A, a trimethylated histone H3-lysine 9 (H3K9me3)-specific demethylase, could function as an effector molecule to selectively demethylate target chromatin, with the aid of a guide protein to serve as a delivery vehicle. JHDM3A, a molecule to selectively demethylate target chromatin, with the aid of a guide protein to serve as a delivery vehicle. JHDM3A/JMJD2A, a trimethylated histone H3-lysine 9 (H3K9me3)-specific demethylase, could function as an effector molecule to selectively demethylate target chromatin, with the aid of a guide protein to serve as a delivery vehicle. JHDM3A/JMJD2A, a trimethylated histone H3-lysine 9 (H3K9me3)-specific demethylase, could function as an effector molecule, together with various guide proteins that served as delivery vehicles to targeted chromatin regions.

A widely accepted hypothesis about the origins of cancer proposes that it is caused, at least in part, by the silencing of genes implicated in cell death and differentiation (1). Similarly, induced pluripotent stem cells (iPSCs) (2) and somatic nuclear transfer clones are preceded by derepression of groups of genes responsible for stem cell properties and dedifferentiation, which are otherwise tightly suppressed in somatic cells (3). Fundamental changes in the nature of cells generally stem from perturbations in the transcriptional regulatory machinery; for example, changes in DNA methylation states at the regulatory DNA regions, mutations in chromatin remodeling complexes such as SWI/SNF, and/or changes in post-translational modifications of histone tails (4). Attempts to modulate DNA methylation by DNA methyltransferase inhibitors (5, 6), histone deacetylation by HDAC inhibitors (7–9), and histone methylation by histone demethylase inhibitors (10, 11) have received a great deal of attention. However, treating cells with small molecules such as these can lead to total inactivation of the target proteins, which could cause a chaotic alteration of epigenetic states by influencing the chromatin in its entirety and driving it to a structurally and functionally disorganized state. To avoid this, the epigenetic state of chromatin must be controlled at a local level. One means of achieving this is by targeting proteins that affect chromatin structure to specific genomic loci.

Histone lysine demethylases (12) have roles in transcriptional regulation, cancer cell proliferation (13), and reprogramming of differentiated cells (14); this suggests that they could be used to alter the histone methylation states of specific chromatin regions. In an attempt to selectively modulate the epigenetic states of target loci, we tested the ability of histone H3-lysine 9 (H3K9)-specific demethylase JHDM3A/JMJD2A (15, 16) to function as an effector molecule, together with various guide proteins that served as delivery vehicles to targeted chromatin regions.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—NIH3T3 and HEK293T cells were cultured in DMEM (Invitrogen) with 10% heat-inactivated fetal bovine serum (Invitrogen) and 50 μg/ml of streptomycin/penicillin (Invitrogen). Culture was kept in a humidified atmosphere with 5% CO₂ at 37 °C. Plasmids were transfected into NIH3T3 cells using Lipofectamine™ (Invitrogen) according to the manufacturer’s instructions. 24 or 40 h after transfection, the cells were fixed with 4% (w/v) formaldehyde solution (Sigma Aldrich).

Mouse Embryo Culture and Microinjection—For embryo collection, female BDF1 mice at 5 weeks of age were injected with 5 international units of pregnant mare serum gonadotrophin (Sigma Aldrich) followed by 5 international units of human chorionic gonadotropins (Sigma Aldrich) 48 h (h) apart and mated with male mice. Successful mating was determined...
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at the following morning by detection of a vaginal plug. 18 to 20 h after human chorionic gonadotropin injection, mouse zygotes were collected from mouse oviduct into M2 medium (Sigma Aldrich) and then were treated with 0.1% (w/v) hyaluronidase to remove cumulus cells. Embryos were cultured in M16 medium (Sigma Aldrich) at 37 °C and 5% CO₂ in air. For microinjection, mouse zygotes showing pronuclei were microinjected with JHDM3AEGFP 21 h after human chorionic gonadotropin injection. Microinjected eggs were allowed to develop in vitro in M16 media. Two- to eight-cell stage embryos were fixed and immunostained with anti-H3K9me3 antibody (Upstate).

Vector Constructions—For construction of the JHDM3Agfg expression vector, cDNA obtained from human embryonic stem cells (17, 18) were amplified by PCR. Primers we used are listed in supplemental Table 1. The PCR products were digested with NotI (Roche Applied Science) and BamHI (Roche Applied Science) and cloned into pCMV-Tag1 vector (Stratagene). For JHDM3AEGFP expression vector, PCR was performed using JHDM3Agfg plasmid as template. The PCR products were digested with HindIII (Roche Applied Science) and BamHI (Roche Applied Science) and cloned into pEGFP-C2 vector (Clontech). For constructions of JHDM3AgfgHP1α, JHDM3AgfgHP1β, and JHDM3AgfgHP1γ expression vectors, mouse cDNA was amplified by PCR. The PCR products were digested BglII (NEB) and HindIII (New England Biolabs) and cloned into pCMV-Tag1 vector. For JHDM3AgfgHP1α vector, the PCR products were digested BamHI (Roche Applied Science) and XbaI (Roche Applied Science) and cloned into JHDM3AEGFP. For truncated JHDM3A constructs, PCR amplification was done using JHDM3Agfg plasmid as template. The PCR products were digested with HindIII (NEB) and Sall (Roche Applied Science) and cloned into pEGFP-C2 vector. For amplifications of cDNA-encoding tagging proteins such as CCCTC-binding factor (CTCF), HP1α, and OCT4, cDNAs were amplified by PCR. The PCR products were digested BglII (NEB) and HindIII (New England Biolabs) and cloned into pCMV-Tag1 vector. For JHDM3AEGFP expression vector, we performed two separate PCR using two sets of primers. The resulting PCR products were mixed and used as template for secondary PCR. The PCR products were digested with HindIII (NEB) and BamHI (Roche Applied Science) and cloned into pEGFP-C2 vector. All vectors constructed were confirmed by enzyme digestion and sequencing analysis.

Immunocytochemistry—NIH3T3 cells or mouse embryos were fixed in 4% (w/v) formaldehyde for 15 min at room temperature and washed in PBS with 0.05% Tween 20 (PBST, Fisher Scientific). After permeabilization for 30 min in PBS containing 1% Triton X-100 (MP Biomedicals), samples were blocked in 2% BSA-PBS (Sigma Aldrich) for 1 h at room temperature and incubated for 1.5 h at 37 °C with primary antibodies. The secondary antibodies that we used were chick anti-rabbit conjugated Alexa Flour 488 or 594 or chick anti-mouse conjugated Alexa Flour 488 or 594 (1:300, Molecular Probes). Stained samples were mounted on slide glass with mounting media containing DAPI (Vectashield). Antibodies recognizing H3K9me1, H3K9me2, and H3K9me3 were purchased from Upstate Biotechnology, and FLAG was purchased from Sigma Aldrich. Samples were observed with Carl Zeiss Axiovert 200 M fluorescence microscope equipped with an Apotome apparatus. Images were captured digitally and merged using Axiovision (version 4.7) or Adobe Photoshop software (version 7.0). Fluorescence intensity profile was measured using the profiling tool in Axiovision software.

Dot Blot Analysis—For dot blot analysis, N-terminal peptides of histone H3 that were mono-, di-, and tri-methylated at H3K9 residue (H3K9me1, H3K9me2, and H3K9me3, respectively) were purchased from Abcam. Five micrograms of each peptide were dotted onto nitrocellulose membrane and dried. The membranes were blocked with 5% (w/v) skim milk in PBS for 1 h at room incubated with anti-H3K9me1 (1:1000; Abcam), anti-H3K9me2 (1:1000; Abcam), and anti-H3K9me3 (1:1000; Upstate) antibodies overnight at 4 °C. HRP-conjugated goat anti-rabbit IgG (1:2000, Santa Cruz Biotechnology) or goat anti-mouse IgG (1:2000, Santa Cruz Biotechnology) was added to react with primary antibodies for 1 h at room temperature. Immunoreactivity was determined using ECL method (Roche Applied Science) and was detected by LAS3000 imaging system.

RT-PCR—Total RNAs were isolated from FACS-sorted NIH3T3 cells using an RNasy mini kit (Qiagen). Complementary DNAs (cDNA) were synthesized with SuperScript™ II first-strand synthesis system (Invitrogen) using oligo(dT) primers according to the manufacturer’s instructions. For amplifications of human OCT4, mouse Sox2, and Gapdh cDNAs, PCR was performed with sets of primers listed in supplemental Table 1.

Chromatin Immunoprecipitation Assay—2 × 10⁶ HEK293T cells were cross-linked with 1% (w/v) formaldehyde (Sigma Aldrich) for 15 min at room temperature, and then formaldehyde was inactivated by the addition of 125 mM glycine (Sigma Aldrich). Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using anti-H3K9me3 (Upstate) and anti-H3K4me3 (Upstate) antibodies. Precipitated DNA was purified with PCR purification kit (Geneall) and analyzed by quantitative PCR with primers (supplemental Table 1).

MicroRNA Microarray and Real-time PCR—Plasmids based on eGFP expression vector were transfected into HEK293T cells using Lipofectamine™ according to the manufacturer’s instructions. Forty-eight hours after transfection, the transfected cells were treated with 0.25% trypsin-EDTA (invitrogen) and washed with PBS. 5 × 10⁵ GFP-positive cells were collected by FACSaria cell sorter (BD Biosciences) and sent for requesting microRNA expression analysis in a microarray planting 124 probes of stem cell related microRNAs (PANAarray™ miRNA expression profiling kit). To obtain small RNA for real-time PCR analysis, total RNA was sieved through RNasey mini column (Qiagen). Flow-through including small RNA was precipitated by 10 mM ammonium acetate and 100% ethanol and eluted by RNase-free water. Collected small RNA was converted into cDNA using miScript reverse transcription kit and miScript SYBR Green PCR kit (Qiagen) according to the manufacturer’s instructions. Real-time quantitative PCR was performed using
standard protocols on an Applied Biosystems 7900HT with 2× SYBR Green PCR Master Mix (Applied Biosystems). The reactions were 40 cycles of 94 °C for 15 s, 60–62 °C for 30 s, and 72 °C for 30 s with 10× miScript Universal Primer (Qiagen) and microRNA forward primer. The microRNA-specific primers used in this work were 5'-GTAAACATCCTACACTCTCAGC-3' for miR-30c, 5'-CAGCATTGTACAGGGCTATCA-3' for miR-107, 5'-TTAAGGCACGCGGTGAATGCCA-3' for miR-124a, 5'-CCTGAGACCCTTTAACCTGTG-3' for miR-125a, 5'-TTGGTCCCCTTCAACCAGCTA-3' for miR-133b, and 5'-CATTCATTGTGTGGGTGGGTGTT-3' for miR-181d. Reactions are typically run in duplicate. To ensure the specificity and integrity of the PCR products, melting curve analyses were performed on all amplified products. The MicroRNA copy numbers were normalized using RNU6b small nuclear RNA. Fold changes were calculated according to the $2^{-\Delta\Delta CT}$ method (19).

RESULTS AND DISCUSSION

We constructed FLAG- and GFP-tagged JHDM3A expression vectors (JHDM3A<sub>FLAG</sub> and JHDM3A<sub>GFP</sub>, respectively). Transient expression of JHDM3A<sub>FLAG</sub> in NIH3T3 cells revealed that FLAG signals coincided with the signals obtained using the anti-JHDM3A antibody (Fig. 1A, a–c). We confirmed that JHDM3A<sub>FLAG</sub> demethylated H3K9me3 but not H3K9me2 or H3K9me1 (Fig. 1, A, d, g, and j, respectively) as reported pre-
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FIGURE 2. HP1\textsuperscript{\textalpha}-induced shift in JHDM3A localization. In A, JHDM3A\textsubscript{\textalpha} (a and \textgamma) was fused with either \textalpha HP1\textsubscript{\textbeta} (JHDM3A\textsubscript{\textalpha}–HP1\textbeta; c and c'), or \textgamma HP1 (JHDM3A\textsubscript{\textalpha}–HP1\textgamma; d and d') and transiently expressed in NIH3T3 cells before immunostaining with anti-FLAG antibody (green). Panels a–d show fluorescence intensity profiles for JHDM3A\textsubscript{\textalpha} backbone proteins (green lines) and DAPI (blue lines). Lines over the merged images in a–d indicate arbitrarily chosen positions for reading fluorescence intensity profiles. Dotted circles in red denote pericentric heterochromatin regions. In B, the boxed parts of a and e are enlarged in b–d and f–h, respectively. Arrows in g and h indicate DAPI-dense heterochromatin regions.

Firstly, we expressed GFP-tagged JHDM3A\textsubscript{\textalpha} in early mouse embryos by microinjecting the expression construct into the pronucleus of mouse zygotes (data not shown). We expressed GFP-tagged JHDM3A\textsubscript{\textalpha} in early mouse embryos by microinjecting the expression construct into the pronucleus of mouse zygotes. We then examined Jhdm3a expression during early mouse development. RT-PCR results showed that the Jhdm3a transcript was detected at a low level in mature oocytes and was almost undetectable in cleavage stage embryos, including blastocysts (Fig. 1B). Immunostaining did not reveal clear signals positive for endogenous JHDM3A proteins in mouse embryos (data not shown). We expressed GFP-tagged JHDM3A (JHDM3A\textsubscript{\textalpha}–GFP) in early mouse embryos by microinjecting the expression construct into the pronucleus of mouse zygotes. When we delved into the resulting transgenic embryos (n = 134) at the four- and eight-cell stages, we were unable to detect any clue for the occurrence of global demethylation in JHDM3A\textsubscript{\textalpha}–GFP expressing nuclei (Fig. 1C). This result contrasted with the one for the cultured cells above, suggesting that JHDM3A alone is insufficient for global demethylation of H3K9me3 and that some unknown factor(s) is needed to assist the enzymatic action of JHDM3A in early mouse embryos.

The diffuse JHDM3A signal in the nucleus (Fig. 1A) suggests that JHDM3A preferentially acts on euchromatin, as noted previously (20). We tested whether JHDM3A could be localized to heterochromatin. To do so, we tagged JHDM3A\textsubscript{\textalpha} with heterochromatin protein-1\textalpha (HP1\textalpha), HP1\textbeta, or HP1\textgamma and expressed it in NIH3T3 cells. JHDM3A\textsubscript{\textalpha}–HP1\textalpha was mainly localized to euchromatin (Fig. 2, A, d), similar to JHDM3A\textsubscript{\textalpha} (Fig. 2, A, a). In contrast, signals for JHDM3A\textsubscript{\textalpha}–HP1\textalpha and JHDM3A\textsubscript{\textalpha}–HP1\textbeta expanded to heterochromatin regions (Fig. 2, A, b and c), as confirmed by the fluorescence intensity profiles (Fig. 2, A, \textalpha–\textgamma). The induced localization to pericentric regions was clearly visualized by the expression of GFP-fused JHDM3A–HP1\textalpha (JHDM3A\textsubscript{\textalpha}–GFP–HP1\textalpha; Fig. 2A). These results indicate that the HP1\textalpha or HP1\textbeta tag can steer the JHDM3A protein to heterochromatin.

H3K9me3 signals remained detectable in some JHDM3A\textsubscript{\textalpha}–GFP\textsuperscript{\textalpha} expressing cells, suggesting that they somehow avoided JHDM3A-induced demethylation (Fig. 1, A, d, arrow). This led us to assess the degree and frequency of demethylation in JHDM3A-overexpressing cells. We first categorized the cells expressing JHDM3A\textsubscript{\textalpha} into three subgroups depending on the extent of H3K9me3 demethylation (Fig. 3A): group 1-type cells, no visible pericentric H3K9me3 speckles due to severe demethylation (Fig. 3, A, a); group 2-type cells, diminished H3K9me3 speckles (Fig. 3, A, b); and group 3-type cells, an almost normal level of H3K9me3 speckles (Fig. 3, A, c). As shown in Fig. 3B, group 1-type cells comprised only 32.6% of JHDM3A\textsubscript{\textalpha}–GFP\textsuperscript{\textalpha} expressing cells 24 h after transfection (hptx; see also Table 1). This value increased to 80.1% when cells expressing JHDM3A\textsubscript{\textalpha}–GFP–HP1\textalpha were observed, suggesting that the inefficient chromatin accessibility of JHDM3A alone limits full-scale demethylation. An HP1\textalpha-mediated increase in the demethylation rate was also observed in JHDM3A\textsubscript{\textalpha}–HP1\textalpha- and JHDM3A\textsubscript{\textalpha}–HP1\textbeta-expressing cells (supplemental Fig. S2). These results suggest that JHDM3A demethylates H3K9me3 more efficiently and rapidly when it is tagged with HP1, most likely because of enhanced access to otherwise inaccessible chromatin regions. Meanwhile, the extent of demethylation was not linked with the expression levels of exogenous JHDM3A. When the fluorescence signal intensity for GFP, H3K9me3, and DAPI was measured for...
comparison in individual cells, the levels of H3K9me3 relative to DAPI signals did not differ between the cells with lower GFP levels and those with higher GFP levels (p < 0.001; Fig. 3C).

We attempted to make the best use of JHDM3A as an effector molecule or a “demethylating warhead,” by decreasing its size and moderating its rather indiscriminate activity toward chromatin. We made shorter JHDM3A proteins, JHDM3AGFP$_{406}$ and JHDM3AGFP$_{701}$, by removing the C-terminal regulatory domain (Fig. 4A); JHDM3AGFP$_{701}$ contained the region encompassing the coiled-coil domain, which was absent from JHDM3AGFP$_{406}$.

When we assessed the demethylation frequency in cells transfected with either construct, the proportion of group 1-type cells was significantly higher at 24 hptx among the JHDM3AGFP$_{406}$ transfectants than among the JHDM3AGFP$_{701}$ transfectants (p < 0.005; Fig. 4C); the demethylation frequency in JHDM3AGFP$_{701}$-transfected cells was lower than that in cells expressing JHDM3AGFP$_{406}$ whole protein (Fig. 3B).

Meanwhile, the overall level of H3K9me2 was not diminished in JHDM3AGFP$_{701}$-expressing cells (Fig. 4, B, m-p).

We next addressed whether the low demethylation frequency that resulted when the JHDM3AGFP$_{701}$ module was used could be modulated by a guide protein. We found that when
cells were transfected with JHDM3A\textsubscript{406}GFP OCT4 and the demethylation frequency was 24 hptx, group 1-type cells made up 4.3% of JHDM3A\textsubscript{701} OCT4-positive cells (Fig. 5B). The value was lower than the percentage of group 1-type cells in cell cultures transfected with the OCT4-untagged version (JHDM3A\textsubscript{701}, 20.8%; Fig. 4C and Table 1). Expression of both fusion proteins, JHDM3A\textsubscript{406}GFP OCT4 and JHDM3A\textsubscript{701}GFP OCT4, was exclusively nuclear (Fig. 5, A, a and e). As shown in Fig. 4, B (e and i), when the modules alone were expressed, the GFP signals were not confined to the nucleus, probably because of the lack of the rear nuclear localization signal inside the plant homeodomain (Fig. 4A). This suggests that the OCT4 tag causes the JHDM3A\textsubscript{406} and JHDM3A\textsubscript{701} modules to be confined to the nucleus by providing an effective nuclear localization signal of its own.

We also tested whether CTCF, a widely expressed 11-zinc finger nuclear protein that serves as a chromatin insulator protein with an unusual ability to recognize multiple target sites (21), could be used as a guide protein. CTCF tagging greatly increased the demethylation frequency in cells trans-

*FIGURE 4. Demethylation efficiency of JHDM3A\textsubscript{406} and JHDM3A\textsubscript{701} modules. A, truncated JHDM3A proteins. The carboxyl-terminal regulatory domain of JHDM3A was removed to make JHDM3A\textsubscript{406} and JHDM3A\textsubscript{701}, the former contained the region spanning amino acids 1–406, and the latter contained amino acids 1–701. JN, Jumonji N domain; JC, Jumonji C domain; NLS, nuclear localization signal; CC, coiled-coil domain; PHD, plant homeodomain. B, expression of truncated JHDM3A proteins. JHDM3A\textsubscript{406} (e–h) and JHDM3A\textsubscript{701} (i–l) proteins were simultaneously present in the cytoplasm and the nucleus, differing from JHDM3A\textsubscript{GFP} proteins shown in Fig. 2B. Panels of GFP control (a–d) show the cells transfected with empty GFP vector. Dotted circles denote nuclear boundaries of GFP-positive cells. Cells were stained for either H3K9me3 (b, f, and j) or H3K9me2 (n). C, demethylation frequency. The extent of H3K9me3 demethylation was measured 24 h after cells were transfected with either JHDM3A\textsubscript{406}GFP or JHDM3A\textsubscript{701}GFP expression plasmids. See Table 1 for detailed statistics.

*FIGURE 5. Modulation of JHDM3A\textsubscript{701} demethylation frequency by guide proteins. A, immunostaining for H3K9me3 in cells transiently expressing JHDM3A\textsubscript{406}GFP OCT4 (a–d), JHDM3A\textsubscript{701}GFP OCT4 (e–h), JHDM3A\textsubscript{406}GFP CTCF (i–l), and JHDM3A\textsubscript{701}GFP CTCF (m–p). Dotted circles denote nuclear boundaries of GFP-positive cells. B, demethylation frequency. Demethylation frequency was assessed 24 h after transfection in the cells transiently expressing JHDM3A\textsubscript{406}, JHDM3A\textsubscript{701}, JHDM3A\textsubscript{406}GFP OCT4, JHDM3A\textsubscript{701}GFP OCT4, JHDM3A\textsubscript{406}GFP CTCF, JHDM3A\textsubscript{701}GFP CTCF, JHDM3A\textsubscript{406}GFP HP1\alpha, and JHDM3A\textsubscript{701}GFP HP1\alpha. See Table 1 for detailed statistics.
affected with the JHDM3A^{701}_{\text{GFP}} module: 80.2% of cells were of the group 1 type (Fig. 5B). We then investigated the utility of using HP1α as a tag for JHDM3A^{701}_{\text{GFP}} and found that the frequency of group 1-type cells was 51.9%. Thus, the guide protein tag was used. In contrast, the use of the JHDM3A^{701}_{\text{GFP}} module resulted in relatively uniform frequencies of demethylation, ranging from 83 to 88%, regardless of the guide protein tag used (Fig. 5B). These values were equivalent to the demethylation frequency (79.5%) caused by the untagged version (JHDM3A^{406}_{\text{GFP}}, a negative control). The expression of OCT4 target genes was examined by RT-PCR of GFP-positive NIH3T3 cells that had been enriched by FACS. Of 124 stem cell-related microRNA probes planted on a microRNA microarray, 35 and 50 were positively expressed in GFP-OCT4- and JHDM3A^{701}_{\text{GFP}}-expressing cells, and GFP-positive cells were found to be enriched by FACS. Of 124 stem cell-related microRNA probes planted on a microRNA microarray, 35 and 50 were positively expressed in GFP-OCT4- and JHDM3A^{701}_{\text{GFP}}-expressing cells. This supports the ability of the JHDM3A^{701}_{\text{GFP}} module to act as an effector molecule in combination with various vehicle molecules and thereby alter the chromatin environment of gene expression. However, we failed to detect expression of other OCT4 target genes, including Nanog; so it is possible that the relatively short time of 40 hptx limited the number of OCT4 regulatory genes that could be activated by the module.

It has been shown that master transcription factors such as OCT4 turn microRNAs on and off (23). We examined whether the JHDM3A^{701}_{\text{GFP}} fused OCT4 could induce expression of stem cell-related microRNAs. GFP-OCT4 and JHDM3A^{701}_{\text{GFP}}-OCT4 were transiently expressed in HEK293 cells, and GFP-positive cells were found to be enriched by FACS. The expression levels of the microRNAs in JHDM3A^{701}_{\text{GFP}}-OCT4-expressing cells were mostly higher than those in GFP-OCT4-expressing cells (Fig. 7A); of 53 microRNAs that were expressed either in GFP-OCT4 or JHDM3A^{701}_{\text{GFP}}-OCT4 cells, 33 were expressed in the JHDM3A^{701}_{\text{GFP}}-OCT4 cells by 20% or more. The results of quantitative real-time PCR for the microRNAs showing the differential expression levels between the groups of cells conformed to the microarray data (Fig. 7B). This result suggests that these microRNAs are brought to derepression more rapidly and efficiently by the expression of JHDM3A^{701}_{\text{GFP}}-fused OCT4 than by the expression of OCT4 alone. Further study is necessary to determine whether individual microRNAs are directly regulated by OCT4, but the present result indicates that an H3K9-specific demethylating enzyme such as JHDM3A is clearly required to facilitate the OCT4-mediated derepression of these stem cell-related microRNAs.
In this study, we tested whether JHDM3A modules could be utilized as demethylating warheads when they were fused with guide proteins with delivery function to specific chromatin targets. Used alone, JHDM3A can indiscriminately demethylate chromatin as a whole. We observed this in JHDM3A-expressing cells, in which there was universal demethylation. Such a drastic change in the histone methylation level may threaten the integrity of the chromatin structure and lead to disastrous consequences for the cell. Here, we investigated candidate guide proteins to curb the random access of JHDM3A to chromatin and direct the module to predefined target chromatin loci. HP1α and HP1β directed the otherwise euchromatin-posted JHDM3A to also localize to pericentric heterochromatin (Fig. 2A). Moreover, OCT4-tagged JHDM3A701 induced demethylation of loci regulated by OCT4 (Fig. 6A) and derepressed the SOX2 gene (Fig. 6B). Furthermore, JHDM3A701-guided OCT4 more efficiently turned on stem cell-related microRNAs than did OCT4 itself (Fig. 7).

When we structurally dissected the JHDM3AGFP protein by generating the JHDM3AGFP406 and JHDM3AGFP701 constructs, we found that their demethylation activities toward H3K9me3 were markedly different (Fig. 4). Compared with JHDM3AGFP, the JHDM3A406701 module had stronger demethylation activity; conversely, JHDM3A701 had weaker demethylation activity than the full-length JHDM3A. It is possible that the coiled-coil domain or cryptic domain(s) in the 407–701-amino acid stretch present in JHDM3A701 could interfere with its access to chromatin. This suggested that the JHDM3A701 module was suitable for further modification and had potential as a tool for target-specific demethylation. We found that the efficiency of JHDM3A701-catalyzed demethylation varied depending on the guide proteins attached. As shown in Fig. 5B, cells expressing JHDM3A701CTCF701 reached 80%. The low demethylation frequency observed in JHDM3A701OCT4 cells was likely because of stringent guidance by OCT4, which directs the catalytic module to only a handful of target loci. Unlike OCT4, CTCF is well known for its unusual ability to bind to multiple target sites (21); this could account for the high demethylation frequency of
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JHDM3A\textsuperscript{701}_{\text{GFP}}-CTCF even though it had the same catalytic module as JHDM3A\textsuperscript{701}_{\text{GFP}}-OCT4. According to these results, JHDM3A\textsuperscript{701}_{\text{GFP}} shows promise as an effector module that can be delivered to target loci by guide proteins.

Immunocytochemical screening showed that the overall levels of H3K9me3 varied among cells overexpressing the JHDM3A protein and that in a significant proportion of JHDM3A-positive cells, the H3K9me3 levels appeared not to be affected by demethylation. In JHDM3A\textsuperscript{701}_{\text{GFP}}-OCT4-transfected cells in particular, the percentage of group 3-type cells showing a normal level of H3K9me3 reached almost 80%. H3K9me3 levels of these group 3-type cells appeared unchanged on the surface, but we propose that demethylation indeed occurred in these cells, although the changes were too small to be visualized by immunostaining. This is supported by the ChIP results (Fig. 6A), which showed changes in H3K9me3 levels at OCT4 regulatory loci. If only a very limited portion of the cells had been demethylated, the ChIP results would not have been as robust.

Our guide-driven JHDM3A protein system would be particularly useful in the research fields of somatic cell nuclear transfer (24) and induced pluripotent stem cells (2). Despite their unquestionably promising future in the biomedical industry and regenerative medicine, the somatic nuclear transfer and iPSC fields have long been adversely affected by extremely low efficiency. There is universal agreement that the primary cause of this lies in inefficient dedifferentiation or reprogramming, by which differentiated cells can ultimately be brought back to undifferentiated states (25–27). Therefore, the greatest priority for these research areas is to facilitate the de-differentiation process in their donor cells or patient cells. In this regard, use of the guide JHDM3A fusion system, which aims to draw out efficient reprogramming in differentiated cells by inducing the demethylation of specific chromatin regions, would be a better choice of strategy for dedifferentiation. For example, expression of the JHDM3A module tagged by a stemness-related protein such as OCT4 and SOX2 in donor cells before or during the somatic nuclear transfer procedure would lead to an initial modification of target chromatin, which could make the donor cells primed and ready for further modification in the oocyte cytoplasm, consequently transfiguring the chromatin to be far more receptive to incoming reprogramming events. Such a pretreatment could be important, given that JHDM3A activity was hardly detectable in early stage embryos (Fig. 1B) and also that no demethylation was induced in embryonic nuclei in which JHDM3A was ectopically expressed (Fig. 1C). In a similar vein, we could expect that linking iPSC-inducing transcription factors or Yamanaka factors (2) as guide proteins to the JHDM3A module would increase the activation efficiency of iPSCs. The outcome would be the derepression of a group of target genes under the control of the guide protein and then the activation of their downstream-level genes. Such a target-based approach could demethylate chromatin regions in a highly controlled and selective fashion. By doing this, we can avoid a potentially chaotic epigenomic situation of a state of whole genome undermethylation that could inevitably emerge from employing chemical inhibitors to, for example, Suv39h1, which is implicated in the maintenance of global H3K9 methylation patterns (28).

In conclusion, we found that JHDM3A showed a degree of flexibility with regard to localization and demethylation frequency depending on the guide proteins to which it was fused. The two truncated JHDM3A modules, JHDM3A\textsuperscript{706}_{\text{GFP}} and JHDM3A\textsuperscript{701}_{\text{GFP}} exhibited different demethylation efficiencies; the former was associated with relatively high and constant demethylation frequencies (~80%) regardless of the guide protein, whereas the latter exhibited a broad range of demethylation rates (4–80%). ChIP analysis demonstrated a reduction in H3K9me3 levels at OCT4 regulatory gene loci in cells expressing JHDM3A\textsuperscript{701}_{\text{GFP}}-OCT4. Expression of the OCT4 target gene SOX2 was detected in JHDM3A\textsuperscript{701}_{\text{GFP}}-OCT4-expressing cells. JHDM3A\textsuperscript{701}-assisted OCT4 turned out to be more efficient in turning on the stem cell-related microRNAs than OCT4 alone. Our results suggest that the JHDM3A\textsuperscript{701}_{\text{GFP}} module is a promising effector molecule; when it is fused to a guide protein, it can effectively and specifically alter the histone methylation state of target loci.

REFERENCES

1. Baylin, S. B., and Ohm, J. E. (2006) Nat. Rev. Cancer 6, 107–116
2. Takahashi, K., and Yamanaka, S. (2006) Cell 126, 663–676
3. Jaenisch, R., and Young, R. (2008) Cell 132, 567–582
4. Grant, S. (2009) Clin. Cancer Res. 15, 7111–7113
5. Mikkelsen, T. S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B. E., Jaenisch, R., Lander, E. S., and Meissner, A. (2008) Nature 454, 49–55
6. Heller, G., Schmidt, W. M., Ziegler, B., Holzer, S., Müllauer, L., Bilban, M., Zielinski, C. C., Drach, J., and Zehnbauer-Müller, S. (2008) Cancer Res. 68, 44–54
7. Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Smitow, M., Chen, A. E., and Melton, D. A. (2008) Nat. Biotechnol. 26, 795–797
8. Wu, L. P., Wang, X., Li, L., Zhao, Y., Lu, S., Yu, Y., Zhou, W., Liu, X., Yang, I., Zheng, Z., Zhang, H., Feng, J., Yang, Y., Wang, H., and Zhu, W. G. (2008) Mol. Cell. Biol. 28, 3219–3235
9. Enright, B. P., Kubota, C., Yang, X., and Tian, X. C. (2003) Biol. Reprod. 69, 896–901
10. Shi, Y., Do, J. T., Desponts, C., Hahm, H. S., Schöler, H. R., and Ding, S. (2008) Cell Stem. Cell 2, 525–528
11. Huang, Y., Stewart, T. M., Wu, Y., Baylin, S. B., Marion, L. J., Perkins, B., Jones, R. J., Woster, P. M., and Casero, R. A., Jr. (2009) Clin. Cancer Res. 15, 7217–7228
12. Klose, R. J., and Zhang, Y. (2007) Nat. Rev. Mol. Cell. Biol. 8, 307–318
13. Yang, Z. Q., Imoto, I., Fukuda, Y., Pimkhaokham, A., Shimada, Y., Imamura, M., Sugano, S., Nakamura, Y., and Inazawa, J. (2000) Cancer Res. 60, 4735–4739
14. Ma, D. K., Chiang, C. H., Ponnusamy, K., Ming, G. L., and Song, H. (2008) Stem Cells 26, 2131–2141
15. Chen, Z., Zang, I., Whestine, J., Hong, X., Davrazou, F., Kutateladze, T. G., Simpson, M., Mao, Q., Pan, C. H., Dai, S., Hagman, J., Hansen, K., Shi, Y., and Zhang, G. (2006) Cell 125, 691–702
16. Whestine, J. R., Nottke, A., Lan, F., Huaere, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacovo, M., and Shi, Y. (2006) Cell 125, 467–481
17. Woo, S. M., Kim, J., Han, H. W., Chae, J. I., Son, M. Y., Cho, S., Chung, H. M., Han, Y. M., and Kang, Y. (2009) BMC. Neurosci. 10, 97
18. Chae, J. I., Kim, J., Woo, S. M., Han, H. W., Cho, Y. K., Oh, K. B., Nam, K. H., and Kang, Y. K. (2009) Proteomics 9, 1128–1141
19. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
20. Klose, R. J., Yamane, K., Bae, Y., Zhang, D., Erdjument-Bromage, H., Tempst, P., Wong, J., and Zhang, Y. (2006) Nature 442, 312–316
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21. Wallace, J. A., and Felsenfeld, G. (2007) Curr. Opin. Genet. Dev. 17, 400–407
22. Chavez, L., Bais, A. S., Vingron, M., Lehrach, H., Adjaye, J., and Herwig, R. (2009) BMC. Genomics 10, 314
23. Marson, A., Levine, S. S., Cole, M. F., Frampton, G. M., Brambrink, T., Johnstone, S., Guenther, M. G., Johnston, W. K., Wernig, M., Newman, J., Calabrese, J. M., Dennis, L. M., Volkert, T. L., Gupta, S., Love, J., Hannett, N., Sharp, P. A., Bartel, D. P., Jaenisch, R., and Young, R. A. (2008) Cell 134, 521–533
24. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. (1997) Nature 385, 810–813
25. Reik, W., Dean, W., and Walter, J. (2001) Science 293, 1089–1093
26. Rideout, W. M., 3rd, Eggan, K., and Jaenisch, R. (2001) Science 293, 1093–1098
27. Surani, M. A. (2001) Nature 414, 122–128
28. Peters, A. H., O’Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schöfer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., and Jenuwein, T. (2001) Cell 107, 323–337
29. Liu, H., Kim, J. M., and Aoki, F. (2004) Development 131, 2269–2280
30. Yeo, S., Lee, K. K., Han, Y. M., and Kang, Y. K. (2005) Mol. Cells 20, 423–428
31. Kang, Y. K., Park, J. S., Lee, C. S., Yeom, Y. I., Chung, A. S., and Lee, K. K. (1999) J. Biol. Chem. 274, 36585–36591