Knockdown of *Brm* and *Baf170*, Components of Chromatin Remodeling Complex, Facilitates Reprogramming of Somatic Cells

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The SWI/SNF (SWItch/Sucose NonFermentable or BAF, Brg/Brahma-associated factors) complexes are epigenetic modifiers of chromatin structure and undergo progressive changes in subunit composition during cellular differentiation. For example, in embryonic stem cells, esBAF contains Brg1 and Baf155, while their homologs, Brm and Baf170, are present in BAF of somatic cells. In this study, we sought to determine whether Brm and Baf170 play any roles in induced pluripotent stem cell (iPSC) reprogramming by using shRNA-mediated knockdown studies in the mouse model. We found that knocking down Brm during early, mid, and late stages (days 3, 6, and 9 after initial iPSC induction) and knocking down Baf170 during late-stage (day 9) reprogramming improve the numbers of iPSC colonies formed. We further showed that inhibition of these somatic BAF components also promotes complete reprogramming of partially reprogrammed somatic cells (pre-iPSCs). Finally, we found that the expression of Brm and Baf170 during reprogramming was regulated by Jak/Stat3 activity. Taken together, these data suggest that inhibiting somatic BAF improves complete reprogramming by facilitating the activation of the pluripotency circuitry.

Introduction

INDUCED PLURIPOTENT STEM CELLS (iPSCs) are embryonic stem cell (ESC)-like cells reprogrammed using ectopic transcription factors, *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (OKSM) [1,2]. However, transcription factor-mediated reprogramming is a slow and inefficient process achieved by overcoming a series of epigenetic barriers [3]. Acquisition of induced pluripotency requires an intricate interplay among specialized transcriptional circuitries, signaling pathways, and chromatin remodeling. In addition to DNA and histone modifications, ATP-dependent enzymes that remodel chromatin are important controllers of chromatin structure and assembly and are major contributors to regulations of gene expression [4,5].

The SWI/SNF (SWItch/Sucose NonFermentable) [also known as BAF (Brg/Brahma-associated factors)] complex consists of at least 15 core subunits and has ATP-dependent chromatin remodeling activity. It is essential for the formation of totipotent and pluripotent cells of early embryos [6]. In addition, the BAF complex is the most frequently mutated chromatin regulatory complex in human cancers and thus their manipulation constitutes a major strategy for

tumor suppression [7]. The BAF complex participates in numerous developmental transitions by changing its subunit composition. For example, the BAF complex in ESCs, esBAF, has a unique subunit composition defined by the presence of *Brg*, *Baf155*, and *Baf60a* and the absence of their somatic cell homologs *Brm*, *Baf170*, and *Baf60c* [8]. Altering this subunit composition caused a reduction in self-renewal and pluripotency in mouse ESCs (mESCs) [8]. In addition, *Baf250a* is also essential for self-renewal and pluripotency in mESCs [9,10]. It has been shown that the mechanisms of maintaining ESC pluripotency by esBAF are mediated by conditioning the genome for LIF/STAT3 signaling and by regulating the functions of the polycomb complex [11]. Conversely, adding esBAF components to fibroblasts facilitates their reprogramming to pluripotent cells. For example, *Brg1* and *Baf155*, combined with *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, synergistically increased reprogramming efficiency by enhancing the binding of *Oct4* to target promoters [12]. These data also suggest that specific components of the BAF complex serve to facilitate the activation of the pluripotency circuitry.

Given the influence of epigenetic factors over reprogramming fate and the documented role of SWI/SNF

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complexes in pluripotency, we sought to test the roles of somatic *Brm* and *Baf170* in mouse iPSC generation through shRNA-mediated knockdown studies. Using mouse embryonic fibroblasts (MEFs) harboring the green fluorescence protein (GFP) driven by the Oct4 promoter (OG-MEFs), we found that inhibiting components of the somatic BAF improve complete reprogramming by facilitating the activation of the pluripotency circuitry.

Materials and Methods

Chemicals and protein expression constructs

Jak inhibitor I (Jaki) and doxycycline were purchased from EMD Millipore. Erk inhibitor PD0329501 and GSK3 β inhibitor CHIR99021 (CHIR) were obtained from SelleckChem. The vectors for *pMXs-Oct4*, *Klf4*, *Sox2*, and *c-Myc* [1], pLKO.1-puro, pLKO.1-scramble shRNA control [13], and retro- and lentiviral packaging constructs, *pUMVC*, *pCMV-VSV-G*, and *psPAX2* [14], were all purchased from Addgene. DNA oligos designed against the mouse *Brm* and *Baf170* cDNA (shBrm_1, shBrm_2, and shBaf170_1, shBaf170_2) and scramble sequence (shCtl) (Supplementary Table S1; Supplementary materials are available online at <http://www.liebertpub.com/scd>) were subcloned into pLKO.1-puro vector. All DNA subcloning was performed using the standard restriction enzyme digestion or Infusion PCR Cloning Kit (Clontech) and expression constructs of shBrm and shBaf170 were verified by DNA sequencing. The human embryonic kidney cell line, 293T, for viral packaging was obtained from Invitrogen.

Cell culture, viral preparation, and reprogramming assay

OG-MEFs as well as MEFs from CD1 mice were generated from E13.5 embryos as described [15]. OG-MEFs up to passage 4 were used for reprogramming. Briefly, *pMXs*, *pMCs*-, or *pLKO.1*-constructs, together with packaging vectors, *pUMVC* (for retrovirus), *psPAX2* (for lentivirus), and *pCMV-VSV-G* plasmids, were cotransfected into 293T cells according to Addgene protocols. Retrovirus OKSM and lentiviral short hairpin RNA were collected 48 and 72 h after transfection. The iPSC induction from OG-MEFs using viral OKSM and reprogramming medium was conducted as described [15]. Briefly, OG-MEFs were plated on six-well plates and transduced (day 0) with retroviral OKSM with 10 μ g/mL polybrene (American Bioanalytical). After 24 h of viral transduction (day 1), cells were trypsinized and passaged onto two two-well plates preseeded with mitomycin C-treated CD1 MEF feeders. Lentiviral shBrm and shBaf170 constructs were then transduced to reprogrammed cells at different stages after OKSM transduction according to the experiment design (Supplementary Fig. S1A). Dox was added after lentiviral shBrm and shBaf170 infection, and GFP⁺ colonies were counted 3 weeks after OKSM transduction under a Nikon fluorescence microscope. GFP⁺ iPSC colonies were picked 3 weeks after retroviral transduction and expanded in 2i/LIF medium until passage 5 when further characterization was conducted. The average size of colonies (areas; mean $\pm$ SD) was measured by using the ImageJ software (<http://imagej.nih.gov/ij/index.html>).

Somatic cells partially reprogrammed by transfection of OKM (pre-iPSCs) were generated from our previous study [16]. These cells have ESC colony morphology, but are GFP negative. For complete reprogramming of pre-iPSCs to iPSCs, shBrm and shBaf170 were transduced (day 0) and GFP⁺ colonies were counted 10 days after transfection (Supplementary Fig. S1B), and mRNA of these reprogrammed cells was also subjected for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis. shCtl constructs were transduced to reprogrammed cells and pre-iPSCs as controls.

For Jak inhibition, OG-MEFs were reprogrammed by OKSM and cultured with either DMSO control or a low dosage (1 μ M) of Jaki, starting on day 3 of reprogramming (Supplementary Fig. S1A). mRNA of these cells was then collected for gene expression analysis at day 18.

FACS analyses

Cells reprogrammed from pre-iPSCs were trypsinized and flow cytometry analysis was performed using BD FACS ARIA III. All data analyses were carried out using the FlowJo software.

Embryoid body formation

Established iPSC lines at passage 6 were grown on CD1 MEF feeders. After 2 days, the cells were trypsinized and replated to the original plate for 2 h to allow MEFs to attach. The iPSCs that remained in the medium were collected and subsequently plated onto Petri dishes containing DMEM + 10% FBS (Invitrogen) without LIF. Upon 7 days of differentiation, embryoid bodies (EBs) formed and were transferred to 0.1% gelatin-coated cell culture dishes (Invitrogen). The cells were allowed to reattach and to continue differentiation for another 7 days before proceeding for RNA extraction, qRT-PCR, or immunostaining as described below.

Alkaline phosphatase staining and immunostaining

Alkaline phosphatase (AP) staining was performed using a Vector Red Alkaline Phosphate Substrate Kit I (Vector Laboratories) according to the manufacturer's instruction. For immunostaining, cells were grown on 12-mm glass coverslips (Fisher Scientific) in six-well plates containing CD1 MEFs as feeders. Cells were fixed in 4% paraformaldehyde with 1% sucrose in PBS for 15 min at room temperature. The cell membranes were permeabilized with 0.5% TX-100 in PBS-T, then incubated for 2 h at 37°C in 5% donkey or goat serum with mouse anti-SSEA1 IgM (1:100), rabbit anti-Sox2 IgG (1:100), or rabbit anti-Nanog IgG (1:100) (all from Millipore), or rabbit anti-Oct4 antibody (1:100; Santa Cruz), washed in PBS-T, and then incubated with Alexa Fluor 594-conjugated donkey anti-rabbit or goat anti-mouse secondary antibodies (1:500; Invitrogen). Cells incubated in serum without primary antibodies were kept as negative controls and R1-ESCs were stained as positive controls. After the washes, cells were counterstained with DAPI and mounted under coverslips. Fluorescence images were taken using a Nikon fluorescence microscope.

Western blot analyses

OG-MEFs were transduced with lentiviral vector, shCtl, shBrm, or shBaf170 and cultured in medium containing 10% FBS for 4 days. OG-MEFs, pre-iPSCs, iPSCs, and

R1-ESCs were cultured as described above. Total cellular proteins were extracted using RIPA buffer (Thermo Scientific) with 1× proteinase and phosphatase inhibitors (Thermo Scientific). Proteins were quantified with the Pierce™ BCA Protein Assay Kit (Thermo Scientific) and subjected to 10% SDS-PAGE gel electrophoresis using the BioRad minigel system and subsequently transferred to PVDF membranes.

The blotted membranes were then blocked with 5% nonfat dry milk in TBS-T and incubated with primary antibodies at 4°C overnight. The antibodies used were as follows: anti-*Brm* (1:1,000; Cell Signaling), anti-*Baf170* (1:1,000; Cell Signaling), and anti-*GAPDH* (1:2,000; Abcam, Inc.). Membranes were then washed and blotted with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:5,000; Santa Cruz). Blotting signals were detected by chemiluminescence using Pierce ECL western blot substrate (Thermo Scientific) and quantified by ChemiDoc XRS+ imaging system with Image Lab™ software (BIO-RAD).

Quantitative real-time reverse transcription–polymerase chain reaction

Total RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed using the SuperScript III Reverse Transcription Kit (Invitrogen). qRT-PCR was performed with specific primers (Supplementary Table S2) using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI 7500 Fast instrument. Combined and endogenous levels of *Oct4*, *Sox2*, and *Klf4* were distinguished by primers designed at different regions. Data were analyzed using the 7500 software version 2.0.2 provided with the instrument. Quantification was normalized to the endogenous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal control and relative mRNA expression was calculated using R1-ESC as the reference.

Statistical analysis

Data were analyzed using one-way ANOVA with Tukey's multiple comparisons or Student's *t*-test. All experiments were performed at least twice ($N \geq 2$). Values in figures are presented as mean ± standard deviation (SD). A *P* value < 0.05 (*) or < 0.01 (**) was considered statistically significant.

Results

Knockdown of *Brm* or *Baf170* at different stages promotes reprogramming

To investigate the roles of *Brm* and *Baf170* in reprogramming and differentiation, we first measured their mRNA levels in OG-MEFs, OG-MEF-derived iPSC clones, OKM-reprogrammed pre-iPSCs, and R1-ESCs. The levels of mRNAs for *Brm* and *Baf170* in OG-MEFs and in pre-iPSCs were five to eight times higher than those in iPSCs and R1-ESCs (Fig. 1A). We further found that subunits of esBAF (*Baf60a*, *Baf200*, *Baf250a*, *Brg1*, and *Baf155*) were upregulated in iPSCs and R1-ESCs compared with OG-MEFs and pre-iPSCs (Supplementary Fig. S2), suggesting that the BAF complex undergoes subunit changes while cells transit from differentiated to pluripotent states. Wes-

tern blot analyses also revealed a dramatic higher level of proteins for *Brm* and *Baf170* in OG-MEFs and pre-iPSCs than those in iPSCs and R1-ESCs (Fig. 1D). The inverse correlation between *Brm*, *Baf170*, and the state of differentiation, especially in partially reprogrammed pre-iPSCs, suggests that *Brm* and *Baf170* may interfere with somatic reprogramming. We then asked whether knocking down *Brm* or *Baf170* would improve reprogramming. Using lentiviral shRNA constructs designed specifically against either *Brm* or *Baf170*, we were able to knock down *Brm* and *Baf170* mRNA levels in OG-MEFs by more than 95% (Fig. 1B, C, and Supplementary Fig. S3A, B), which were also confirmed at protein levels by western blot analyses (Fig. 1E, F). We then reprogrammed OG-MEFs with OKSM (day 0) in the presence of shBrm or shBaf170 or shCtl on day 3 and cultured the cells in 2i/LIF medium. Two weeks after OKSM induction, dome-shaped ESC-like GFP⁺ colonies started to appear in all treatment groups. Knocking down of *Brm* resulted in a significant increase in GFP⁺ colonies compared with controls (Fig. 1G). On the contrary, knocking down *Baf170* had minimal impact on GFP⁺ colony formation (Fig. 1G). To further dissect the effect of somatic BAF components on reprogramming, we knocked down *Brm* and *Baf170* at later stages, that is, on day 6 or 9. Interestingly, similar results were obtained when treatments were given on day 6 (Fig. 1H), while knocking down *Baf170* on day 9 produced a significant gain on reprogramming efficiency (Fig. 1I). Collectively, these observations demonstrate that *Baf170* inhibits reprogramming at a later stage, while removal of *Brm* at any stage improves reprogramming (Fig. 1G–I).

Subsequently, we characterized the GFP⁺ colonies (Fig. 2A) for their reprogramming status. Interestingly, we found that the colonies induced by shBrm were significantly smaller than those treated with shBaf170 or shCtl (Fig. 2B). Furthermore, we observed that GFP⁺ colonies induced by shBrm and shBaf170 shared the following properties with the control ESC line, R1: (1) formation of tight compact colonies with strong AP activity (Fig. 2C); (2) propagation in 2i/LIF medium and positive immunostaining for pluripotent genes and surface markers, including *Oct4*, *Sox2*, and SSEA1 (Fig. 2D); and (3) formation of EBs in vitro (Fig. 2E) and differentiation to cells expressing markers of the three germ layers (data not shown). Of note, no differences were found in the numbers of AP, *Sox2*, *Oct4*, and SSEA1-positive colonies or EBs between shBrm or shBaf170 and shCtl-treated cells except for a slightly smaller EB size in shBrm and shBaf170-treated cells. The reprogramming was also relatively complete because the retroviral transgenes were virtually silenced in the induced iPS colonies (Fig. 2F). Taken together, the above data demonstrate that inhibition of *Brm* or *Baf170* is critical for efficient reprogramming in a stage-specific manner.

Knockdown of *Brm* or *Baf170* promotes complete reprogramming of pre-iPSCs

Because we had determined that knockdown of *Brm* and *Baf170* is critical for efficient late-stage reprogramming of OG-MEFs, and also *Brm* and *Baf170* were highly expressed in pre-iPSCs (Fig. 1A, D), we asked whether their inhibition is also required for the complete reprogramming of these

FIG. 1. Knockdown of *Brm* or *Baf170* promotes reprogramming in a stage-specific manner. (A) The levels (mean $\pm$ SD) of *Brm* and *Baf170* mRNAs are low in induced pluripotent stem cells (iPSCs) and murine embryonic stem cells (R1-ESCs), but high in OG-MEFs, pre-iPSCs induced by ectopic *Oct4/Klf4/c-Myc* (OKM) infection ($n=3$). The OG-MEFs are mouse embryonic fibroblasts harboring the green fluorescence protein (GFP) driven by the Oct4 promoter. Transfection of OG-MEFs with lentiviruses expressing different small hairpin RNAs against *Brm* (shBrm; B) and *Baf170* (shBaf170; C) reduced *Baf170* and *Brm* by 95% and 96%, respectively. (D) Western blots showing the protein levels of *Baf170* and *Brm* in OG-MEFs, Pre-iPSCs, iPSCs, and R1-ESCs. Both *Baf170* and *Brm* are present in OG-MEFs and Pre-iPSCs, while nearly absent in iPSCs and R1-ESCs. *GAPDH* was used as the loading control. Transfection of OG-MEFs with shBrm (E) and shBaf170 (F) efficiently reduced the protein levels of *Brm* and *Baf170*, respectively. Knocking down *Brm* on day 3 (G), day 6 (H), and day 9 (I) and knocking down *Baf170* only on day 9 (I) significantly increased the numbers of GFP⁺ colonies (mean $\pm$ SD) induced by OKSM (** P values <0.01 ; $n=3$).

cells. We knocked down *Brm* and *Baf170* in two stable pre-iPS cell lines from our previous study [16] and found that suppressing *Brm* and *Baf170* increased the numbers of GFP⁺ colonies by 5.5- and 11-fold, respectively, compared with controls (Fig. 3A). Similarly, FACS analysis showed that reduced *Brm* and *Baf170* resulted in 49.1% and 70.6% GFP⁺ cells, respectively, compared with controls (5.57%; Fig. 3B). Characterization of GFP⁺ colonies from the treated pre-iPSCs demonstrated that these colonies grew readily in 2i/LIF medium, maintained bright GFP expression and ESC-like colony morphology under repeated passaging, stained positive for AP activity, and expressed ESC-specific gene markers, such as *Oct4*, *Sox2*, *Nanog*, and SSEA1 (data not shown). These data corroborated with those in OG-MEF reprogramming that inhibition of either *Brm* or *Baf170* efficiently converted pre-iPSCs to iPSCs, while *Baf170* knockdown at this late stage of reprogramming is more effective than *Brm* knockdown.

Knockdown of *Brm* or *Baf170* promotes reprogramming by activating the pluripotency circuitry

As depletion of *Brm* or *Baf170* enhanced reprogramming efficiency and promoted complete late-stage reprogramming, we sought to investigate the mechanisms of these effects by examining the expression of ESC/iPSC-specific genes. We found that early removal of *Baf170* (days 3 and 6) did not affect the expression of core pluripotency genes (Fig. 4A, B), while knockdown of *Baf170* on day 9 resulted in significant upregulation of *Sox2*, *Nanog*, *Esrrb*, and *Tbx3* (Fig. 4C). On the contrary, inhibiting *Brm* at any stage of reprogramming significantly increased the expression of *Nanog*, *Esrrb*, and *Tbx3* (Fig. 4A–C). *Brm* inhibition also elicited stage-specific upregulation of pluripotent genes. For example, *Oct4* and *Rex1* were induced on day 3, *Sox2* on day 6, and *Sox2* and *Klf4* on day 9. Additionally, we also

FIG. 2. Cells reprogrammed by OKSM and *Brm*- or *Baf170*-knockdown are pluripotent. (A) Representative iPSC colonies (passage 5) induced by OKSM plus shBrm or shBaf170 and cultured in 2i/LIF medium. The GFP⁺ colonies indicate activation of the internal *Oct4* gene (scale bar = 250 μm). The average size of colonies (areas; mean $\pm$ SD) induced by shBrm is significantly smaller than that by shBaf170 and shCtl (B) (***P* values < 0.01; *n* = 3). The induced colonies stained strongly for AP (C; passage 5) and pluripotency markers, including SSEA1, *Oct4*, and *Sox2* (D; scale bar = 25 μm ; passage 6). The induced colonies also formed EBs after 7 days of differentiation (E; scale bar = 250 μm). Nuclei of the cells were counterstained with DAPI. All GFP⁺ colonies induced by shBrm and shBaf170 shared similar properties with the control R1-ESCs. (F) Similar levels of combined and endogenously (*Oct4**, *Sox2**, and *Klf4**) expressed transcription factors in OKSM-infected OG-MEFs also transfected with shBaf170 or shBrm were observed in the reprogrammed cells, suggesting that the exogenous induction factors were silenced. Values were normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and relative to R1-ESCs (*n* = 3).

FIG. 3. Knocking down *Brm* or *Baf170* promotes complete reprogramming of pre-iPSCs. The numbers of GFP⁺ colonies (mean $\pm$ SD; A) significantly increased 10 days after pre-iPSCs were infected with shBrm or shBaf170. Mean values of three independent experiments from two pre-iPS cell lines are shown (***P* values < 0.01; *n* = 3). The reprogrammed colonies contained different percentages of GFP⁺ cells as analyzed by fluorescence-activated cell sorting (B; *n* = 2).

FIG. 4. Knocking down *Brm* or *Baf170* promotes reprogramming by activating the pluripotency circuitry. Relative levels of mRNA for selected ESC-specific genes in OG-MEFs transfected with shBaf170, shBrm, or shCtl on day 3 (A), day 6 (B), and day 9 (C) of OKSM reprogramming. *Oct4**, *Sox2**, and *Klf4** represent mRNA expressed endogenously. Values were normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and relative to shCtl (**P* values < 0.05; *n* = 3). (D) Relative levels of mRNA for selected ESC-specific genes in pre-iPSCs 10 days after transfection with shBaf170, shBrm, or shCtl (*n* = 3). Results were normalized with *GAPDH* and relative to shCtl.

showed that *Sox2*, *Nanog*, *Rex1*, *Esrrb*, and *Tbx3* were significantly upregulated when *Brm* and *Baf170* were inhibited in pre-iPSCs (Fig. 4D). Taken together, these results demonstrated that inhibition of *Brm* or *Baf170* promotes reprogramming efficiency by activating the pluripotency circuitry in a stage-specific manner.

Inhibition of Brm or Baf170 promotes Jak/Stat3 signaling during complete reprogramming

It has been reported that esBAF facilitates pluripotency by conditioning the genome for *LIF/Stat3* signaling [11]. We have also shown that *Jak/Stat3* signaling plays an essential role in epigenetic regulation of late-stage somatic cell reprogramming [16]. In this study, we sought to determine if somatic BAF components are involved in the intricate interplay of *Stat3* signaling during reprogramming. We analyzed *Stat3* mRNA transcripts 10 days after shBrm or shBaf170 transduction of pre-iPSCs. Interestingly, we found that expression of *Stat3* was significantly increased by inhibiting *Brm* or *Baf170* in pre-iPSCs (Fig. 5A). We further revealed that knockdown of *Brm* or *Baf170* significantly stimulated the expression of *Socs3*—the direct target of *Stat3* (Fig. 5A). These results suggest that the presence of *Brm* and *Baf170* inhibited the *Stat3* pathway during reprogramming.

Retroviral silencing is a prerequisite for pluripotency establishment in retroviral transgene-mediated reprogramming and is achieved by de novo DNA methylation through DNA methyltransferases (*Dnmts*) 3a, 3b, and *Dnmt3L* [16–20]. We have also shown previously that *Jak/Stat3* activity facilitates *Dnmt3L* expression. This, in turn, stimulates de novo DNA methylation, which silences retroviral transgenes and possibly lineage commitment genes [16]. Examining the expression of *Dnmts* here revealed that *Brm* and *Baf170* knockdown drastically increased the expression of de novo methyltransferase *Dnmt3a* and *3L* (Fig. 5B). These results suggested that reducing somatic *Brm* or *Baf170* stimulates de novo DNA methylation, which is correlated with the elevated *Stat3* pathway. Therefore, somatic BAF reduction may coordinate with the *Stat3*-regulated epigenetic network for pluripotency establishment.

To further dissect the intricate interplay of *Brm/Baf170* and *Jak/Stat3* signaling, we examined whether inhibiting *Jak/Stat3* would affect *Brm* and *Baf170*. We reprogrammed OG-MEFs and cultured them with either DMSO control or a low dosage (1 μ M) of the reversible *Jak* inhibitor I (*Jaki*), starting on day 3 of reprogramming. *Jaki* treatment virtually blocked the formation of all GFP⁺ colonies; however, this did not change the number of GFP-negative colonies formed (data not shown). Furthermore, mRNAs for components of esBAF, *Baf60a*, *Baf250a*, *Brg1*, *Baf155*, *Baf47*, *Baf200*,

FIG. 5. Inhibition of *Brm* or *Baf170* promotes Jak/Stat3 signaling during complete reprogramming. Relative levels of mRNA for (A) *Stat3* and its direct target, *Socs3*, as well as *do novo* *Dnmts* (B) in pre-iPSCs 10 days after being transfected with shBaf170 or shBrm. Results were normalized with *GAPDH* and related to shCtl (***P* values < 0.01; *n* = 3). (C) Relative levels of mRNA for different subunits of the BAF complex in OG-MEFs, R1-ESCs, and OKSM-transduced OG-MEFs 18 days after initial OKSM viral transduction. The reprogrammed cells were treated with either DMSO or 1 μ M Jaki. Results were normalized with *GAPDH* and relative to those in R1-ESCs (**P* values < 0.05; *n* = 3). (D) Relative levels of mRNA for *Brm* and *Baf170* in OG-MEFs, R1-ESCs, and passage two (P2) of reprogrammed cells. The reprogrammed cells were treated with either DMSO or 1 μ M Jaki. Results were normalized with *GAPDH* and relative to those in R1-ESCs (**P* values < 0.05; *n* = 3).

Baf60b, and *Baf57*, were consistently expressed and unchanged, while the somatic BAF components, *Brm* and *Baf170*, were significantly upregulated in Jaki-treated cells compared with DMSO-treated control cells (Fig. 5C). To minimize the heterogeneity of cells in the analysis, three typical colonies from each treatment were collected 3 weeks after viral transduction and analyzed for their gene expression at passage 2 (P2). A dramatic upregulation of mRNAs for *Brm* and *Baf170* was consistently observed in Jaki-treated cells compared with the DMSO controls (Fig. 5D). Thus, a positive feedback loop exists between *Jak/Stat3* and the inhibition of *Brm* and *Baf170* during ground-state pluripotency establishment.

Discussion

iPSCs can be generated from somatic cells by ectopic expression of transcription factors, such as *Oct4*, *Sox2*, *Klf4*, and *Myc* (OKSM) [1], but the efficiency remains low. A variety of chromatin modifiers, such as *Brg1/Baf155*, *Utx*, and *MBD3/NuRD*, have been implicated in facilitating epigenetic changes leading to authentic iPSC reprogramming [12,21–24]. In this study, we identified a negative role by *Brm* and *Baf170* on OKSM-mediated reprogramming of OG-MEFs. We found that shRNA-mediated knockdown of *Brm* or *Baf170* led to an increase in reprogramming effi-

ciency. Specifically, *Baf170* seems to be more inhibitory to reprogramming at the late stage when the pluripotency network is becoming more stably established, while *Brm* inhibits reprogramming at all phases. In addition, it is interesting to note that colonies induced by *Brm* knockdown were significantly smaller than those from *Baf170* knockdown. Sizes of ES colonies and EBs have been shown to affect their differentiation trajectories [25] through different gradients of signaling molecules [26]. The size difference observed may reflect a mechanism that cells employ to regulate their fates upon changes of BAF compositions.

Components of esBAF have been shown to be important in both maintenance of mESCs and iPSC induction. Deficiency in *Brg1*, *Baf47*, *Baf155*, or *Baf250* impaired the ability of mESCs to proliferate and to maintain pluripotency [8–11,27,28]. *Brg1* and *Baf155*, combined with *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, can synergistically increase reprogramming efficiency [12]. Because iPSCs have similar properties with ESCs, it is logical to infer that homologs of *Brg1* and *Baf155* and *Brm* and *Baf170* obstruct reprogramming, which was proven in the current study. Our results are also in agreement with the recent finding that levels of *Brm* increase during differentiation of ESCs [10]. Moreover, the dynamic composition of BAF seems to correlate with the differentiation status of the cells, that is, *Baf170* is more important in differentiated cells, whereas *Baf155* is more important in

undifferentiated ES cells [8,10]. Competition between *Baf170* and *Baf155* subunits within the BAF complex was also observed during progression of neurogenesis and affected euchromatin structure and thereby modulated the binding efficiency of their targets [29]. Our results expanded this notion that the BAF complex undergoes subunit changes while cells transit from differentiated to pluripotent states, that is, acquisition of esBAF and disposition of the somatic BAF components (*Brm* and *Baf170*). Moreover, it suggested that somatic BAF inhibits the formation of esBAF and therefore serves as a barrier during reprogramming.

We discovered that removal of *Brm* and *Baf170* aids in full reprogramming. At selective loci, pre-iPSCs and somatic cells possess similar chromatin structures, which are condensed and arrested epigenetically during reprogramming [30,31]. Pre-iPSCs can be completely reprogrammed to pluripotency by overexpressing *Sox2* or *Nanog* [32]. The pre-iPSC stage is an epigenetically stable landmark along the journey of reprogramming and these cells can progress toward the authentic iPSCs through the removal of epigenetic determinants for the intermediate state [31]. In this study, we provide a new insight that the presence of the somatic *Brm* and *Baf170* is a potential epigenetic barrier during reprogramming. First, high levels of both *Brm* and *Baf170* are present in pre-iPSCs. Second, knockdown of *Brm* or *Baf170* upregulated the expression of pluripotency-related core transcription factors, such as *Sox2*, *Nanog*, *Rex1*, *Tbx3*, and *Esrrb*, suggesting that the somatic BAF complex may be inhibitory to the expression of pluripotency-related genes. Interestingly, during mouse somatic reprogramming, *Brg1* and *Baf155* were recruited by *Oct4* to relax chromatin structure and facilitate the binding of other transcription factors that enhance reprogramming [12]. Genome-wide colocalization of *Brg1* with *Oct4*, *Sox2*, and *Nanog* [27,33] further suggests that the somatic components of BAF are not compatible with reprogramming.

An important contribution of this study was to provide evidence that the suppressed *Brm* and *Baf170* may coordinate with the *Stat3*-regulated epigenetic network during pluripotency establishment. Activation of the Jak/Stat3 signal pathway is essential for maintaining mESC pluripotency [34] and for reactivating the endogenous pluripotency network in somatic cells [16,35]. Although the role of the SWI/SNF complex in *Stat3* signaling was discovered previously, the finding was only limited for *Brg1* [11,36,37]. More recently, binding sites of *Stat3* and esBAF had been shown to frequently overlap in the ESC genome [8,27]. Moreover, Ho et al. [11] provided evidence that esBAF is required to establish chromatin accessibility at *Stat3* binding targets and to prepare these sites to respond to LIF signaling, therefore allowing in vitro preservation of ground-state pluripotency. These studies established a strong connection between the esBAF complex and *Stat3* activities. For the first time, we demonstrated here a link between *Brm*, *Baf170*, and *Stat3* activities. We not only showed that knocking down *Brm* and *Baf170* stimulated *Stat3* activity but also inhibiting *Jak/Stat3*, in turn, increased the expression of *Brm* and *Baf170* during reprogramming, thus revealing an interplay between *Stat3* and somatic BAF during reprogramming. In addition, we demonstrated that reducing somatic *Brm* and *Baf170* drastically increased the expression of de novo methyltransferase *Dnmt3a* and *3L*. These

changes were also seen when the *Stat3* pathway was activated [16]. Therefore, *Brm* and *Baf170* inhibition integrates in the *Stat3*-regulated epigenetic network for pluripotency establishment. Our results provide new insights that *Stat3* may promote esBAF formation by inhibiting somatic BAF. As *Stat3* activation is key to ground-state pluripotency establishment during somatic cell reprogramming [16], our data here indicate that inhibiting *Brm* and *Baf170* coordinates with the determining effects of *Stat3* in pluripotency establishment, and a mutually suppressive interaction between *Stat3* and somatic BAF is one of the mechanisms involved.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Takahashi K and S Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
2. Okita K, T Ichisaka and S Yamanaka. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313–317.
3. Apostolou E and K Hochedlinger. (2013). Chromatin dynamics during cellular reprogramming. *Nature* 502:462–471.
4. Jaenisch R and R Young. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell* 132:567–582.
5. Ho L and GR Crabtree. (2010). Chromatin remodelling during development. *Nature* 463:474–484.
6. Wang W, Y Xue, S Zhou, A Kuo, BR Cairns and GR Crabtree. (1996). Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev* 10:2117–2130.
7. Kadoch C, DC Hargreaves, C Hodges, L Elias, L Ho, J Ranish and GR Crabtree. (2013). Proteomic and bioinformatic analysis of mammalian SWI/SNF complexes identifies extensive roles in human malignancy. *Nat Genet* 45:592–601.
8. Ho L, JL Ronan, J Wu, BT Staahl, L Chen, A Kuo, J Lessard, AI Nesvizhskii, J Ranish and GR Crabtree. (2009). An embryonic stem cell chromatin remodeling complex, esBAF, is essential for embryonic stem cell self-renewal and pluripotency. *Proc Natl Acad Sci U S A* 106:5181–5186.
9. Gao X, P Tate, P Hu, R Tjian, WC Skarnes and Z Wang. (2008). ES cell pluripotency and germ-layer formation require the SWI/SNF chromatin remodeling component BAF250a. *Proc Natl Acad Sci U S A* 105:6656–6661.
10. Yan Z, Z Wang, L Sharova, AA Sharov, C Ling, Y Piao, K Aiba, R Matoba, W Wang and MS Ko. (2008). BAF250B-associated SWI/SNF chromatin-remodeling complex is required to maintain undifferentiated mouse embryonic stem cells. *Stem Cells* 26:1155–1165.
11. Ho L, EL Miller, JL Ronan, WQ Ho, R Jothi and GR Crabtree. (2011). esBAF facilitates pluripotency by conditioning the genome for LIF/STAT3 signalling and by regulating polycomb function. *Nat Cell Biol* 13:903–913.

12. Singhal N, J Graumann, G Wu, MJ Arauzo-Bravo, DW Han, B Greber, L Gentile, M Mann and HR Scholer. (2010). Chromatin-remodeling components of the BAF complex facilitate reprogramming. *Cell* 141:943–955.
13. Buganim Y, DA Faddah, AW Cheng, E Itskovich, S Markoulaki, K Ganz, SL Klemm, A van Oudenaarden and R Jaenisch. (2012). Single-cell expression analyses during cellular reprogramming reveal an early stochastic and a late hierarchic phase. *Cell* 150:1209–1222.
14. Stewart SA, DM Dykxhoorn, D Palliser, H Mizuno, EY Yu, DS An, DM Sabatini, IS Chen, WC Hahn, et al. (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 9:493–501.
15. Tang Y, CJ Lin and XC Tian. (2011). Functionality and transduction condition evaluation of recombinant Klf4 for improved reprogramming of iPS cells. *Cell Reprogram* 13: 99–112.
16. Tang Y, Y Luo, Z Jiang, Y Ma, CJ Lin, C Kim, MG Carter, T Amano, J Park, S Kish and XC Tian. (2012). Jak/Stat3 signaling promotes somatic cell reprogramming by epigenetic regulation. *Stem Cells* 30:2645–2656.
17. Okano M, DW Bell, DA Haber and E Li. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99: 247–257.
18. Wernig M, A Meissner, R Foreman, T Brambrink, M Ku, K Hochedlinger, BE Bernstein and R Jaenisch. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448:318–324.
19. Ooi SK, D Wolf, O Hartung, S Agarwal, GQ Daley, SP Goff and TH Bestor. (2010). Dynamic instability of genomic methylation patterns in pluripotent stem cells. *Epigenetics Chromatin* 3:17.
20. Theunissen TW, AL van Oosten, G Castelo-Branco, J Hall, A Smith and JC Silva. (2011). Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. *Curr Biol* 21:65–71.
21. Mansour AA, O Gafni, L Weinberger, A Zviran, M Ayyash, Y Rais, V Krupalnik, M Zerbib, D Amann-Zalcenstein, et al. (2012). The H3K27 demethylase Utx regulates somatic and germ cell epigenetic reprogramming. *Nature* 488:409–413.
22. Rais Y, A Zviran, S Geula, O Gafni, E Chomsky, S Viukov, AA Mansour, I Caspi, V Krupalnik, et al. (2013). Deterministic direct reprogramming of somatic cells to pluripotency. *Nature* 502:65–70.
23. Gafni O, L Weinberger, AA Mansour, YS Manor, E Chomsky, D Ben-Yosef, Y Kalma, S Viukov, I Maza, et al. (2013). Derivation of novel human ground state naive pluripotent stem cells. *Nature* 504:282–286.
24. dos Santos RL, L Tosti, A Radziszewska, IM Caballero, K Kaji, B Hendrich and JC Silva. (2014). MBD3/NuRD facilitates induction of pluripotency in a context-dependent manner. *Cell Stem Cell* 15:102–110.
25. Bauwens CL, R Peerani, S Niebruegge, KA Woodhouse, E Kumacheva, M Husain and PW Zandstra. (2008). Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells* 26:2300–2310.
26. Warmflash A, B Sorre, F Etoc, ED Siggia and AH Brivanlou. (2014). A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. *Nat Methods* 11:847–854.
27. Ho L, R Jothi, JL Ronan, K Cui, K Zhao and GR Crabtree. (2009). An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network. *Proc Natl Acad Sci U S A* 106:5187–5191.
28. Schaniel C, YS Ang, K Ratnakumar, C Cormier, T James, E Bernstein, IR Lemischka and PJ Paddison. (2009). Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. *Stem Cells* 27:2979–2991.
29. Tuoc TC, S Boretius, SN Sansom, ME Pitulescu, J Frahm, FJ Livesey and A Stoykova. (2013). Chromatin regulation by BAF170 controls cerebral cortical size and thickness. *Dev Cell* 25:256–269.
30. Mattout A, A Biran and E Meshorer. (2011). Global epigenetic changes during somatic cell reprogramming to iPS cells. *J Mol Cell Biol* 3:341–350.
31. Chen J, H Liu, J Liu, J Qi, B Wei, J Yang, H Liang, Y Chen, J Chen, et al. (2013). H3K9 methylation is a barrier during somatic cell reprogramming into iPSCs. *Nat Genet* 45:34–42.
32. Ichida JK, J Blanchard, K Lam, EY Son, JE Chung, D Egli, KM Loh, AC Carter, FP Di Giorgio, et al. (2009). A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. *Cell Stem Cell* 5:491–503.
33. Kidder BL, S Palmer and JG Knott. (2009). SWI/SNF-Brg1 regulates self-renewal and occupies core pluripotency-related genes in embryonic stem cells. *Stem Cells* 27:317–328.
34. Niwa H, T Burdon, I Chambers and A Smith. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev* 12:2048–2060.
35. Yang J, AL van Oosten, TW Theunissen, G Guo, JC Silva and A Smith. (2010). Stat3 activation is limiting for reprogramming to ground state pluripotency. *Cell Stem Cell* 7:319–328.
36. Giraud S, A Hurlstone, S Avril and O Coqueret. (2004). Implication of BRG1 and cdk9 in the STAT3-mediated activation of the p21waf1 gene. *Oncogene* 23:7391–7398.
37. Ni Z and R Bremner. (2007). Brahma-related gene 1-dependent STAT3 recruitment at IL-6-inducible genes. *J Immunol* 178:345–351.

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