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ORIGINAL ARTICLE

BMPs functionally replace Klf4 and support efficient reprogramming of mouse fibroblasts by Oct4 alone

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Generation of induced pluripotent stem cells by defined factors has become a useful model to investigate the mechanism of reprogramming and cell fate determination. However, the precise mechanism of factor-based reprogramming remains unclear. Here, we show that Klf4 mainly acts at the initial phase of reprogramming to initiate mesenchymal-to-epithelial transition and can be functionally replaced by bone morphogenetic proteins (BMPs). BMPs boosted the efficiency of Oct4/Sox2-mediated reprogramming of mouse embryonic fibroblasts (MEFs) to ~1%. BMPs also promoted single-factor Oct4-based reprogramming of MEFs and tail tibial fibroblasts. Our studies clarify the contribution of Klf4 in reprogramming and establish Oct4 as a singular setter of pluripotency in differentiated cells.

***Keywords*:** Oct4; iPSCs; reprogramming; stem cell; BMPs; MET

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Introduction

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by ectopic expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* [1-6]. However, the Yamanaka factors included *Klf4* and *Myc* both not directly implicated in pluripotency, but did not have the pluripotency regulator *Nanog*, suggesting that there is a mechanistic difference between induction and maintenance of pluripotency [1]. We recently reported that mouse fibroblasts undergo a mesenchymal-to-epithelial transition (MET) orchestrated by Oct4, Sox2 and Myc to suppress *snail* expression and blunt TGF-β signaling, and Klf4 to activate expression of epithelial markers, such as *Cdh1*, during the initial phase of reprogramming [7], thus, defining a concrete molecular and cellular step towards pluripotency [7, 8]. The division of labor among the Yamanaka factors suggests that each factor plays distinct roles during the reprogramming process. Taken together, one would expect that Oct4 alone can reprogram somatic cells such as mouse fibroblasts into pluripotency as Oct4 governs embryonic cell fate [9, 10]. In this study, we analyzed the relative contribution of Sox2 and Klf4 towards reprogramming under a chemically defined system optimized for robust reprogramming. We showed that Klf4 plays an important role in triggering MET, which can also be initiated by bone morphogenetic proteins (BMPs) [8, 11]. Then, we demonstrated that Oct4/Sox2 (OS) and Oct4 alone can reprogram mouse embryonic fibroblasts (MEFs) and tail tibial fibroblasts (TTFs) efficiently in the presence of BMPs.

Results

Klf4 mainly acts at the initial phase of reprogramming

Multifactorial requirement is a great barrier for mechanistic investigation of factor-based reprogramming. However, the function of each factor employed in reprogramming is poorly understood and at least two factors are required (including ectopic and endogenous expression) in the literature [7, 12-16]. Among the four Yamanaka factors, c-Myc was proven dispensable and can be replaced by various methods [17-20]. Oct4, Sox2

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and Klf4 are currently considered as indispensable factors, as replacement of them would greatly reduce reprogramming efficiency. OK-mediated reprogramming of mouse fibroblasts was reported with moderate efficiency, by using compounds targeting various pathways such as Bayk8644, CHIR99021, RepSox and A83-01 [7, 12, 13, 16, 21]. By contrast, OS-mediated reprogramming was reported only with p53-null fibroblasts [14]. We previously reported that OKS-mediated reprogramming can be enhanced by the optimized serum-free medium iSF1 [19]. Further optimization led to improved efficiency of OKS-mediated reprogramming and we found that iPSCs can be generated by either OK or OS with very low efficiency (Supplementary information, Figure S1A) in a newly formulated medium iCD1. The resulting OS or OK iPSCs were similar to ESCs and could be differentiated into three germ layers in teratoma assay as expected (Supplementary information, Figure S1B). Remarkably, OK-mediated reprogramming appeared to be more efficient and more rapid than OS (Supplementary information, Figure S1A).

We then examined the requirement of Klf4 or Sox2 in a doxycycline-inducible system. We found that Klf4 is required mostly during the initial phase of reprogramming (Figure 1A). We performed transcriptomic analysis and showed that Klf4 upregulated genes important for epithelial development (Figure 1B). Consistent with this analysis, OK stimulates robust expression of epithelial markers (such as *Cdh1* and *Occludin*), while OS has little effect on them (Figure 1C). As MET is an early requisite step during the reprogramming of MEFs and as Klf4 participates in MET by activating the epithelial program [7], we conclude that MET is a rate-limiting step in OS-mediated reprogramming (Figure 1D).

BMPs functionally replace Klf4 and support efficient reprogramming with OS

BMPs have been implicated in MET by reversing TGF-β-induced EMT in NP1 cells and reported to enhance MET during reprogramming [8, 11], and thus may functionally substitute Klf4. To test this possibility, we showed that BMP4 and BMP7 can enhance the expression of epithelial genes (*Cdh1*, *EpCAM*, etc.) and inhibit the expression of mesenchymal genes (*Zeb1*, *Twist1*, etc.) in OS-infected MEFs, but cannot promote epithelial markers further in OK-infected cultures (Figure 2A and 2B and Supplementary information, Figure S2A and S2B). These results indicate that BMPs can trigger MET in OS-induced reprogramming, but cannot further enhance the strong MET activated by Klf4 in OK reprogramming. Consistently, BMP4 greatly improved the kinetics and efficiency of OS reprogramming (up to ~0.8% at day 15, a 400-fold plus 9 days improvement), but had no effect on OK reprogramming and even had inhibitory effect on OKS reprogramming (Figure 2C and 2D and Supplementary information, Figure S2C). We also showed that BMP2, BMP6, BMP7 and BMP9, similar to BMP4, significantly enhanced the OS-mediated reprogramming (Figure 2E, and Supplementary information, Figure S2D). We also examined the treatment windows of BMP4 in OS-mediated reprogramming and showed that exposure of BMP4 at days 3-9 is most effective (Figure 2F).

We then investigated the role of Cdh1 in OS-mediated reprogramming and showed that knockdown of Cdh1 decreased reprogramming efficiency significantly (Supplementary information, Figure S2E), suggesting that the observed enhancement by BMP4 is Cdh1 dependent. We also tested other compounds including Kenpaullone [22] and showed that none of them can enhance OS-mediated reprogramming (Supplementary information, Figure S2F). As expected, OS-iPSC clones derived with BMP4 expressed pluripotent markers such as Oct4, Nanog, Rex1 and SSEA-1 (Figure 2G). We then showed that these OS iPSCs contributed to the generation of chimeric mice, which underwent germline transmission (Figure 2H), indicating that the OS iPSCs were fully reprogrammed.

BMPs induce efficient Oct4-mediated reprogramming by promoting MET

We then hypothesized that BMPs should enhance Oct4-mediated reprogramming by inducing MET. As in OS-mediated reprogramming, BMP4 and BMP7 improved the expression of Cdh1 and EpCAM in Oct4-transduced MEFs (Figure 3A and Supplementary information, Figure S3A). We then showed that Oct4 alone was able to convert MEFs into iPSCs efficiently in iCD1+BMP4 with reasonable kinetics (~0.05% at D24 post-infection; Figure 3B). Under the same culture condition, we showed that adult mouse TTFs can also be reprogrammed by Oct4 alone reproducibly, demonstrating that Oct4 is sufficient to induce adult somatic cells to pluripotency (Figure 3C-3E). We also showed that BMPs has no effect on the expression level of p53 and p21 (Supplementary information, Figure S3B) and further showed that no other tested chemical compounds can support Oct4-mediated reprogramming (Supplementary information, Figure S3C).

We then characterized the Oct4-iPSC clones isolated with iCD1+BMP4 and showed that they expressed pluripotent markers Oct4, Nanog, Rex1, SSEA-1, Dppa3 and Dnmt3l (Figure 4A and 4B, and Supplementary information, Figure S3D). All Oct4-iPSC clones that we obtained

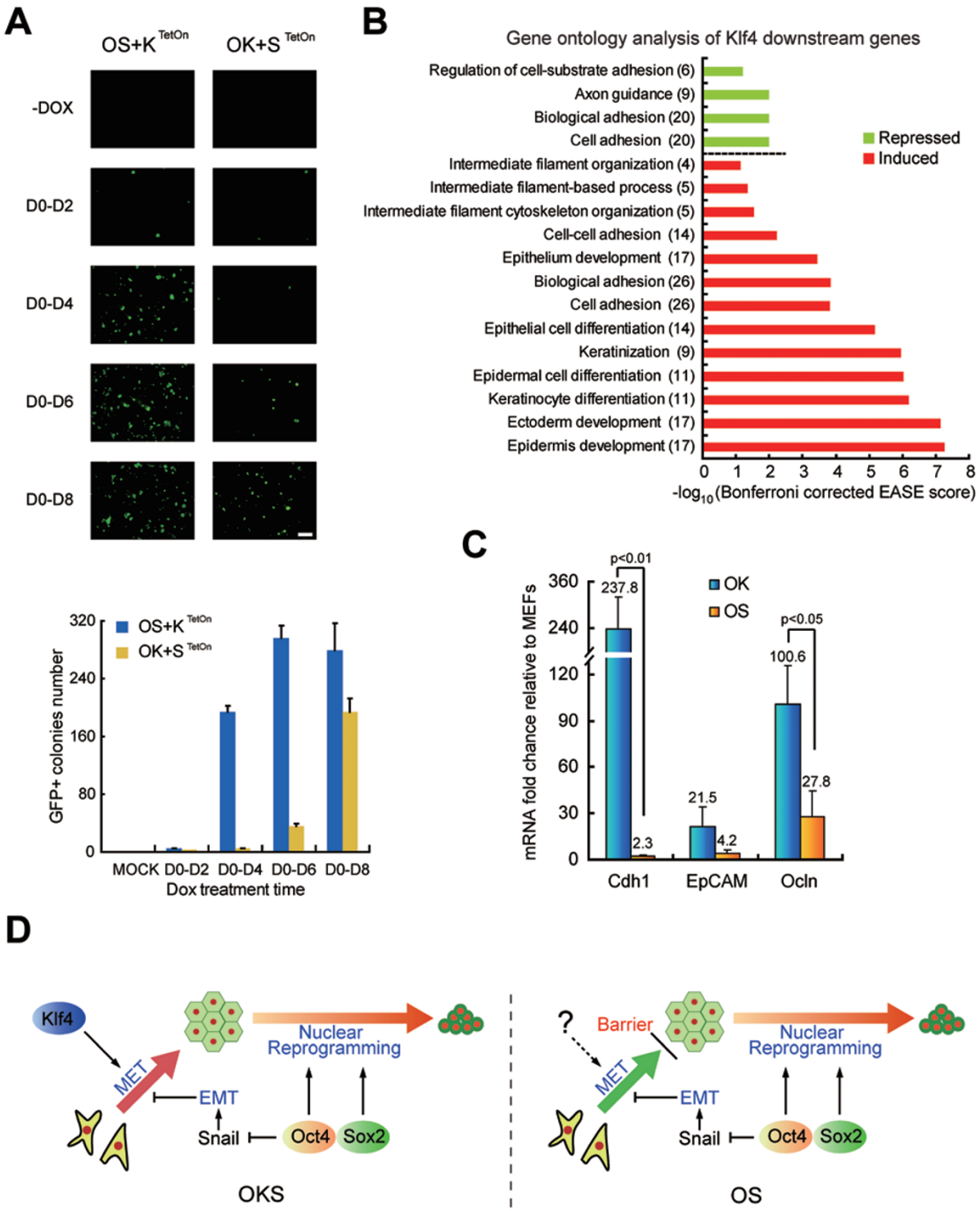

Figure 1 Klf4 mainly acts at early MET phase of reprogramming. **(A)** Klf4 is critical in the initial stage of reprogramming. By using inducible system, ectopic expression of Klf4 or Sox2 in OKS-mediated reprogramming was terminated in different days. Representative photos were taken and reprogramming efficiency was scored at day 12. *n* = 3. Scale bar, 2 mm. **(B)** Klf4 mainly regulated cell-cell adhesion and induced epithelial markers. MEFs transduced by retroviral OS and lentiviral TetOn-*Klf4* were cultured in iCD1 with or without doxycycline for 3 days and analyzed by DNA microarray. Gene ontology analysis (Bonferroni corrected EASE scores were presented) based on the DE list (expression change > three folds), number in brackets indicated the number of genes including in DE list. **(C)** Expression of epithelial marks in OK- or OS-infected MEFs at post-infection day 3. *n* = 3. **(D)** Hypothesis of Klf4 and Sox2's function in the course of reprogramming.

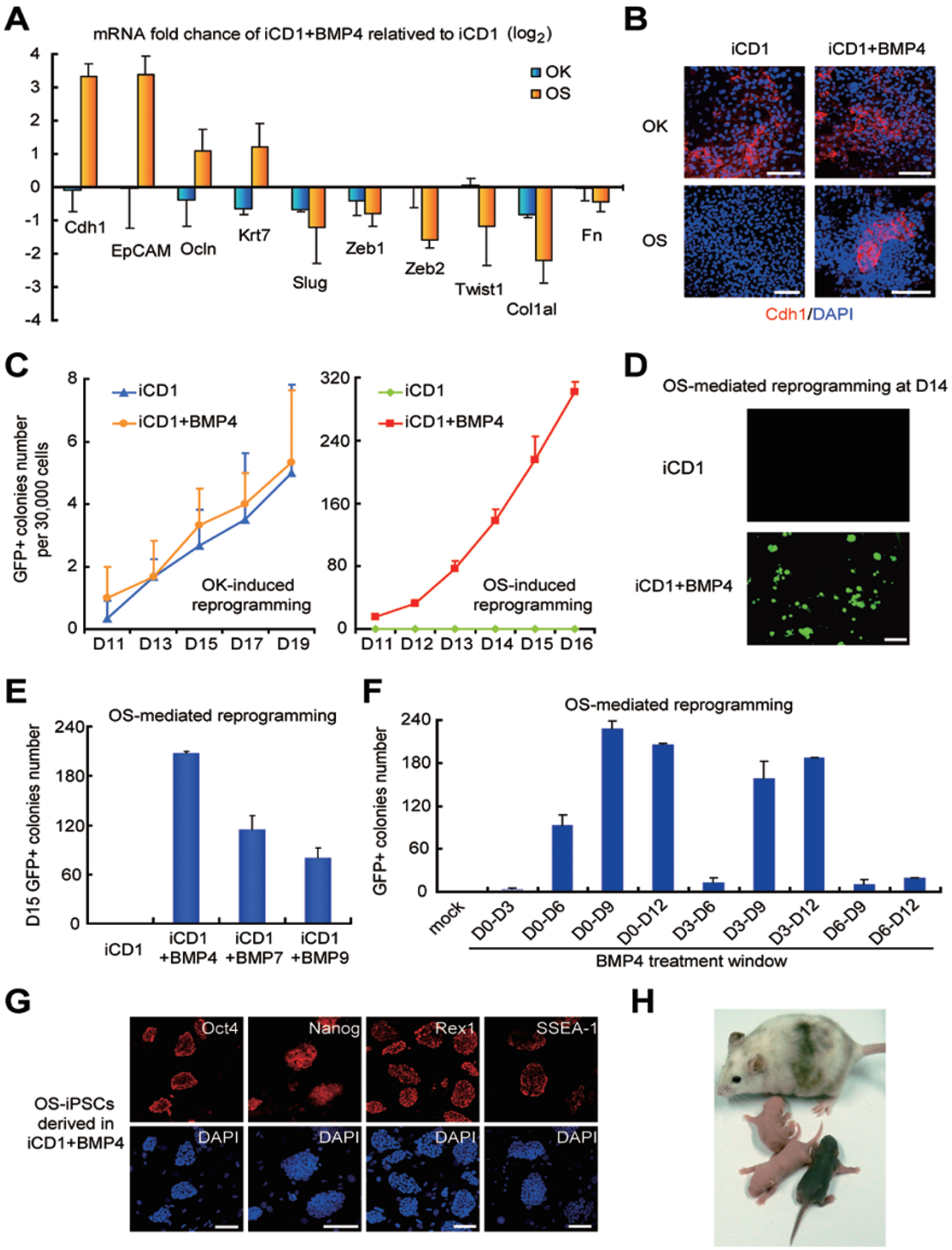

Figure 2 BMPs functionally replace Klf4 and support efficient reprogramming with OS. **(A)** Expression of indicated genes were analyzed by qRT-PCR in OK- or OS-infected MEFs at day 5 post-treatment in iCD1+BMP4 or iCD1. *n* = 3. **(B)** BMP4 induces Cdh1 expression in OS-infected MEFs. Scale bar, 100 μm. **(C)** BMP4 enhances the reprogramming mediated by OS, but not OK. MEFs infected with OK and OS were cultured in iCD1 or iCD1+BMP4. Oct4-GFP-positive colonies were scored from day 11 post-treatment and are shown. *n* = 3. **(D)** Representative fields of OS-infected MEFs at day 14 post-treatment in iCD1 or iCD1+BMP4 are shown. Scale bar, 2 mm. **(E)** BMP7 and BMP9, similar to BMP4, enhance reprogramming mediated by OS. *n* = 3. **(F)** BMP4 treatment window experiment show that D0-D9 is more sensitive to time. *n* = 3. **(G)** OS iPSCs colonies express Oct4, Nanog, Rex1 and SSEA-1. Scale bar, 100 μm. **(H)** Germline transmission of chimeric mice generated from OS iPSCs.

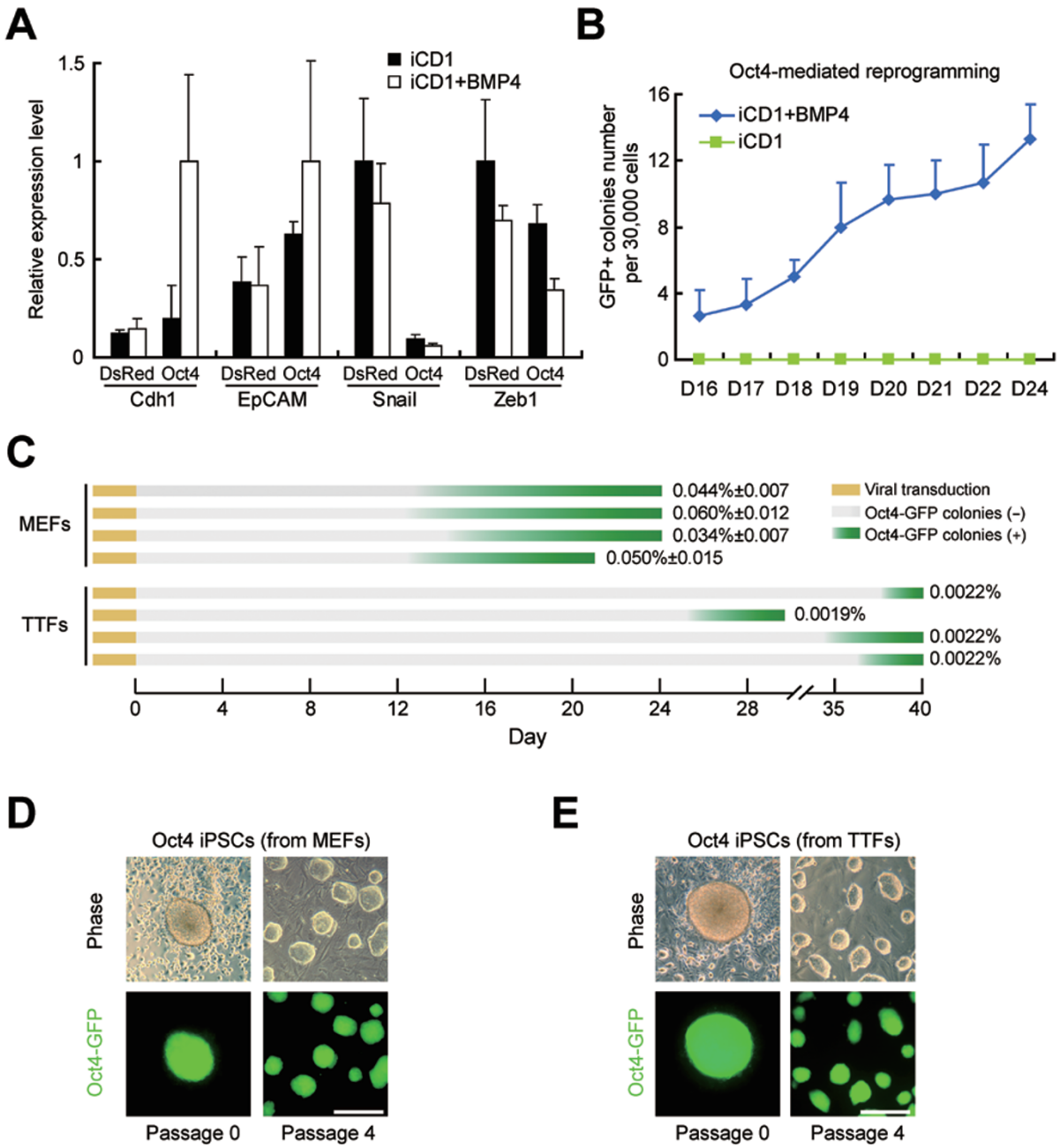

Figure 3 BMPs promote Oct4-mediated reprogramming by inducing MET. **(A)** BMP4 induces MET during Oct4-mediated reprogramming. MEFs infected with *Oct4* or *DsRed* were cultured in iCD1 or iCD1+BMP4. Expression levels of *Cdh1*, *EpCAM*, *Snail* and *Zeb1* were analyzed by qRT-PCR and the highest values of each gene were set to 1. *n* = 3. **(B)** BMP4 enhances Oct4-mediated reprogramming. MEFs infected with *Oct4* were cultured in iCD1 or iCD1+BMP4. Oct4-GFP-positive colonies were scored from day 16 to day 24 post-treatment. *n* = 3. **(C)** BMP4 enhances Oct4-mediated reprogramming. MEFs infected with Oct4 were cultured in iCD1 or iCD1+BMP4. Oct4-GFP-positive colonies were scored from day 16 to day 24 post-treatment. *n* = 3. **(D)** The morphology of one iPSC clone derived from MEFs by Oct4 in iCD1+BMP4. Scale bar, 250 μm. **(E)** The morphology of one iPSC clone derived from TTFs by Oct4 in iCD1+BMP4. Scale bar, 250 μm.

showed normal karyotypes (Figure 4C). We then characterized the integration of the Oct4 transgene by PCR and confirmed that only the Oct4 transgene was present in all Oct4-iPSC clones examined (Figure 4D). The ectopic

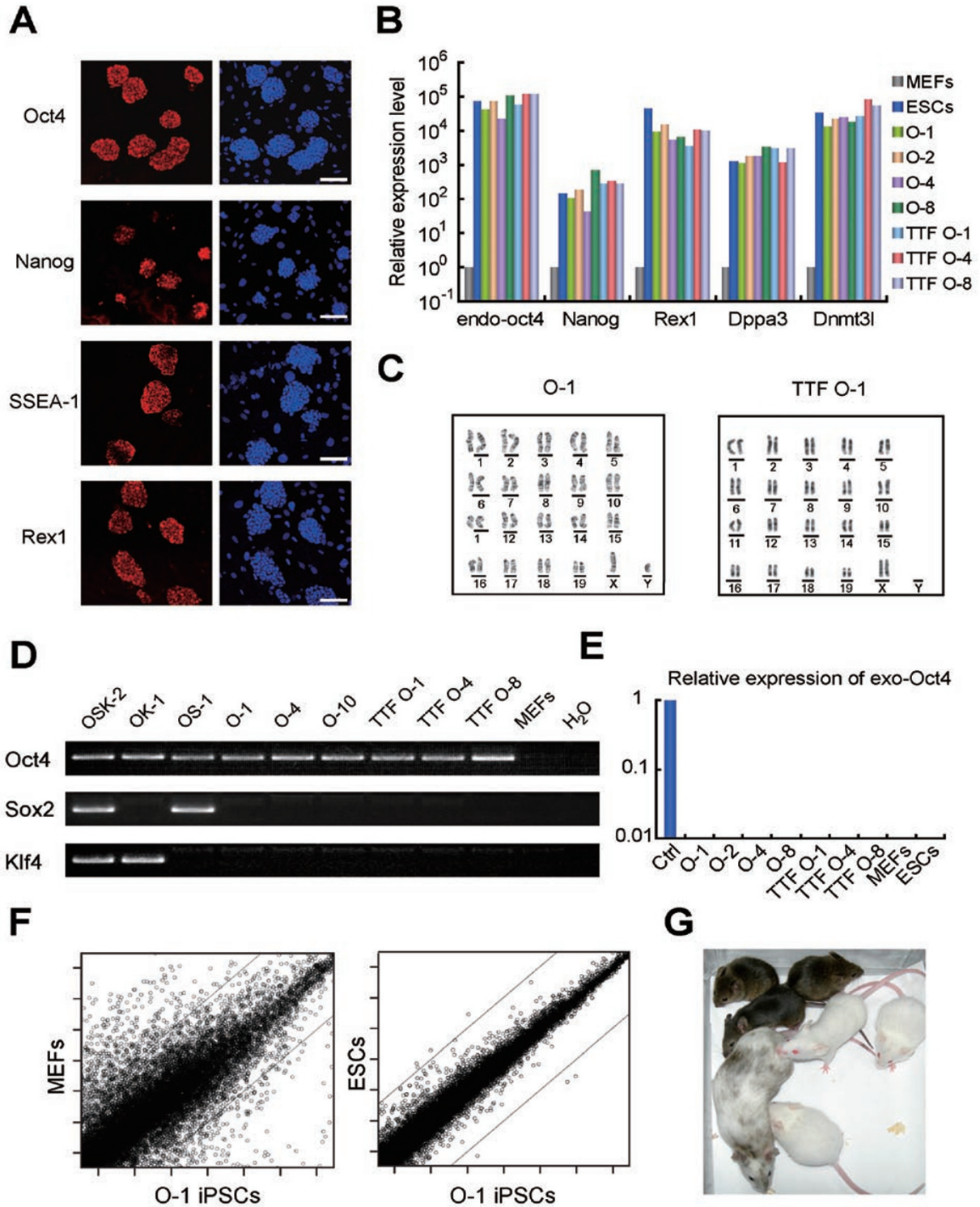

Figure 4 Oct4 iPSCs are pluripotent. **(A)** Oct4 iPSCs express Oct4, Nanog, SSEA-1 and Rex1 (left panels). DAPI stating was served as control (right panels). Scale bar, 100 μm. **(B)** Oct4 iPSCs express pluripotent markers. The endogenous *Oct4*, *Nanog*, *Rex1*, *Dppa3* and *Dnmt3l* expression of selected iPSC clones were analyzed by qRT-PCR. Expression values were relative to that of MEFs. **(C)** The Oct4 iPSC clones have normal karyotype. **(D)** Integration analysis confirms the derivation of Oct4 iPSC clones. The presence of the retroviral transgene was examined by PCR. **(E)** Retroviral expression was silenced in iPSC clones. The expression of exogenous Oct4 was analyzed by qRT-PCR in selected iPSC clones. MEFs infected with OKS for 4 days were used as positive controls. **(F)** Global gene expression of Oct4 iPSCs is similar as that of ESCs. **(G)** Germline transmission of chimeric mice generated from Oct4 iPSCs.

retroviruses were silenced in these iPSCs, indicating that they maintain pluripotency without transgene expression (Figure 4E). By global transcriptomic analysis, we showed that Oct4-iPSCs resemble ESCs and are different from MEFs (Figure 4F). The Oct4-iPSCs contributed to chimera mice when injected into blastocysts (Figure 4G and Supplementary information, Figure S3E). Furthermore, we obtained germline transmission of Oct4-iPSCs by breeding the chimeras (Figure 4G). These results demonstrate that the iPSCs generated from MEFs or TTFs with Oct4 alone are fully pluripotent.

We further examined whether the replacement of Klf4 by BMP4 is mediated by the activation of endogenous Klf4 expression and showed that BMP4 does not increase the endogenous Klf4 expression in O-, OK- or OS-transduced MEFs (Supplementary information, Figure S4A). In fact, BMPs did not stimulate endogenous expression of other reprogramming factors (including OKSM and Esrrb [23]) during reprogramming (Supplementary information, Figure S4B-S4D). We then examined the expression of TGF-β pathway members during reprogramming and showed that BMPs had inhibitory effect on Tgfbr2 and Tgfbr3, while Klf4 repressed Tgfb1, suggesting that BMPs and Klf4 inhibited the TGF-β pathway through different mechanisms (Supplementary information, Figure S4E). So, we concluded that BMPs replace Klf4 functionally by inducing MET through a mechanism different from activating endogenous reprogramming factors.

Discussion

We showed here that Klf4 works at a rate-limiting MET step in the early phase of reprogramming by inducing epithelial markers, while Oct4 and Sox2 contribute little to this step. Therefore, Klf4 appears to play a more important role than Sox2 during the reprogramming of mouse fibroblasts. We showed that BMPs were able to induce MET in Oct4- or OS-transduced fibroblasts through a different mechanism from Klf4 and can replace Klf4 functionally (Figure 5). Therefore, the effect of BMPs is context dependent on the reprogramming factors used and is beneficial only in the absence of Klf4, suggesting that BMPs are functionally redundant with Klf4 on inducing MET, or Klf4 could inhibit the function of BMPs, similar to the Wnt pathway that has a different effect on reprogramming with or without c-Myc [24]. We also showed that although Sox2 is a core transcription factor to maintain pluripotency, it appears to only play an auxiliary role in reprogramming. Since KS could not support the generation of iPSCs (data not shown), the central role of Oct4 became apparent in the reprogramming paradigm (Figure 5). Finally, we showed that Oct4 is sufficient to reprogram MEFs and TTFs at efficiencies similar to that originally reported for Oct4, Sox2, Klf4 and Myc. Therefore, one may speculate that Oct4-mediated reprogramming can be further improved or used for screening small chemicals that can replace Oct4.

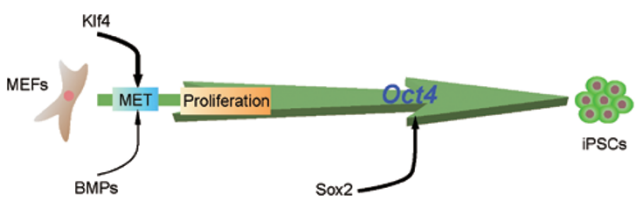

Figure 5 Relative contributions of individual reprogramming factors to reprogramming. Oct4 is the core factor sufficient for the establishment of pluripotency, and Sox2 participates to assist Oct4 in reprogramming. Klf4, which can be substituted by BMPs, is responsible for triggering MET, an early step of reprogramming.

Materials and Methods

Cell culture

MEFs were cultured with DMEM+10% FBS and ESCs/iPSCs were cultured with either mES medium (DMEM supplemented with 15% FBS, LIF and other components) or KSR medium (replace the FBS in mES medium with Knockout Serum Replacement). MEF feeder cells were inactivated by mitomycin C. BMP4, BMP7, BMP2, BMP6 and BMP9 were used at final concentrations of 10 ng/ml, 100 ng/ml, 30 ng/ml, 30 ng/ml and 10 ng/ml, respectively. Vitamin C was purchased from Sigma (Cat No. 49752), BMPs from R&D and CHIR99021 was synthesized at GIBH. Rest of the chemicals and reagents were purchased from Invitrogen.

Generation of iPSCs

Plasmids carrying murine *Oct4*, *Klf4*, *Sox2* and *c-Myc* cDNA were purchased from Addgene. For direct reprogramming, Oct4-GFP transgenic MEFs were plated at 4 000 cells/cm^2 (for OKS combination) or 7 500 cells/cm^2 (for other combinations), and then infected with retrovirus packed by plat-E cells for two rounds. After 48 hours, the tested media were added and the day is defined as day 0 post-treatment. iPSCs were induced with iCD1, which is based on DMEM+vitamin C [25] supplemented with bFGF, CHIR99021 and other chemically defined components to support growth. The photos taken by SteREO Lumar.V12 (Zeiss) show the entire well. Reprogramming efficiency was determined by directly scoring the number of Oct4-GFP-positive colonies under microscope.

Blastocyst injection

Resulting iPSCs or ESCs were cultured in mES (containing 15% FBS) or KSR (containing 15% serum replacement). For generation of chimaeras, iPSCs were injected into ICR blastocysts using Piezo Micro Manipulator. Injected blastocysts were transplanted into pseudopregnant ICR females. Germline transmission of resulted chimeric mice was determined by breeding F2 mouse

with ICR mouse.

Plasmid construction

The pWP-TetOn vector was constructed by replacing the EF-1α promoter of the pWPXLd vector with the TRE promoter of pTRE-Tight vector (Clontech). M2rtTA from pTet-On Advanced vector (Clontech) was cloned into the pWPXLd vector.

Quantitative RT-PCR

Total mRNA was isolated using TRIzol and then was converted to cDNA. Quantitative PCR was performed and analyzed with ABI 7300. All qRT-PCR results presented in this study were from at least two independent experiments with independent viral preparations.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)