

# BMP-SMAD-ID promotes reprogramming to pluripotency by inhibiting p16/INK4A-dependent senescence

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**Fibrodysplasia ossificans progressiva (FOP) patients carry a missense mutation in *ACVR1* [617G > A (R206H)] that leads to hyperactivation of BMP-SMAD signaling. Contrary to a previous study, here we show that FOP fibroblasts showed an increased efficiency of induced pluripotent stem cell (iPSC) generation. This positive effect was attenuated by inhibitors of BMP-SMAD signaling (Dorsomorphin or LDN1931890) or transducing inhibitory SMADs (SMAD6 or SMAD7). In normal fibroblasts, the efficiency of iPSC generation was enhanced by transducing mutant *ACVR1* (617G > A) or *SMAD1* or adding BMP4 protein at early times during the reprogramming. In contrast, adding BMP4 at later times decreased iPSC generation. ID genes, transcriptional targets of BMP-SMAD signaling, were critical for iPSC generation. The BMP-SMAD-ID signaling axis suppressed p16/INK4A-mediated cell senescence, a major barrier to reprogramming. These results using patient cells carrying the *ACVR1* R206H mutation reveal how cellular signaling and gene expression change during the reprogramming processes.**

reprogramming | pluripotency | BMP | senescence | FOP

Reprogramming somatic cells into pluripotent stem cells is an exciting paradigm in biology and has critical implications for transplantation medicine and disease modeling. We developed a method to generate induced pluripotent stem cells (iPSCs) by transducing defined factors, such as *OCT4*, *SOX2*, *KLF4*, and *C-MYC* (OSKM), into somatic cells (1, 2). These transcription factors regulate the expression of genes important for self-renewal and pluripotency. However, only a small proportion of cells become iPSCs after the introducing these defined factors (3), and this is a major roadblock toward applying this technology for biomedicine. Cytokine- and chemical-induced cell signaling affect the efficiency of iPSC generation (4, 5), but the precise effects and mechanisms in reprogramming are unclear.

The BMP-SMAD signal has important roles in the induction and maintenance of pluripotency. BMP promotes the self-renewal of mouse embryonic stem cells (mESCs) (6, 7). In addition, BMP-SMAD signaling facilitates mouse iPSC (miPSC) generation (8). Thus, BMP signaling has positive effects on both the induction and self-renewal of mouse pluripotent stem cells. In contrast, BMPs inhibit self-renewal of human PSCs (9–13). Recently, Hamasaki et al. (15) tried to generate human iPSCs (hiPSCs) from the human dermal fibroblasts (HDFs) of patients with fibrodysplasia ossificans progressiva (FOP; Online Mendelian Inheritance in Man no. 135100) who carried a missense mutation in *ACVR1* (617G > A) that leads to hyperactivation of the BMP-SMAD signaling pathway (14), with little success; they obtained many differentiated colonies, but only a few undifferentiated ESC-like

colonies. These results indicated that BMP-SMAD signaling negatively affects hiPSC generation as well as their self-renewal.

In this study, we independently generated hiPSCs from FOP patients. Although our primary motivation was to establish in vitro disease models of FOP (16, 17), we unexpectedly found that the efficiency of hiPSC generation from FOP HDFs was much higher than that of control HDFs without any BMP inhibitors. Thus, we explored the roles of the BMP-SMAD signaling during reprogramming to hiPSCs. Our findings show that patient-derived hiPSCs of human genetic diseases, such as FOP, are useful to understand how specific gene mutations affect reprogramming processes, in addition to their utilities to model human diseases.

## Results

**Increased Efficiency of HiPSC Generation from FOP HDFs Under Low Cell Density.** We used episomal vector-mediated iPSC generation with HDFs from FOP1–3, as well as four additional control HDFs

### Significance

The development of iPSCs provides unprecedented opportunities for life sciences, drug discovery, and regenerative medicine. The efficiency of iPSC generation is quite low: typically less than 1% of human primary somatic cells that have received reprogramming factors turn into iPSCs. Previous studies revealed that cellular senescence was a major barrier to iPSC generation. In this study using human FOP mutant cells, we provide evidence that the BMP-SMAD-ID signaling suppressed p16/INK4A-mediated cellular senescence during the early phase in iPSC generation. These results are unexpected because BMP-SMAD signaling has negative effects on the self-renewal of human iPSCs. Here, we show that a human natural mutation increases the efficiency of iPSC generation.

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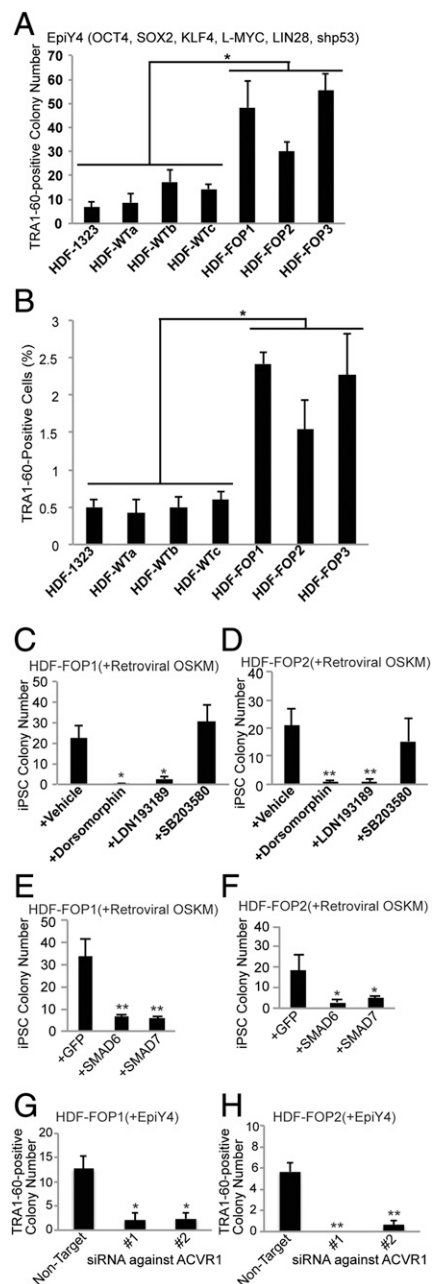
This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1603668113/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1603668113/-DCSupplemental).

(1323, WTa, WTb, and WTC). We determined the efficiency of hiPSCs by detecting colonies that were positive for a pluripotent stem cell marker, TRA-1-60 (18). After transfecting episomal plasmids containing *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and shRNA for *p53* (epiY4) and replating at 10,000 cells per well of six-well plate, all three FOP HDFs produced significantly more TRA-1-60–positive colonies than the four normal HDFs (Fig. 1A). The ratio of TRA-1-60–positive cells in reprogrammed FOP cells was also higher than that in normal HDFs detected with flow cytometry (Fig. 1B). We also generated iPSCs from normal and FOP fibroblasts using retroviral vectors and found that the increased efficiency of iPSC generation from FOP HDFs was observed when plated at low density (SI Text and Fig. S1). These results indicated that hiPSC generation was more efficient from FOP HDFs than from control HDFs, regardless of reprogramming methods and factors. We then established hiPSC lines from FOP HDFs and characterized them as they maintained self-renewal and pluripotency. In brief, these lines had normal karyotypes, expressed pluripotency markers, including TRA1-60 and NANOG, and were able to differentiate into various cells of the three germ layers both in vitro and in teratomas (16).

**Hyperactivated BMP-SMAD Signaling Contributes to Increased iPSC Generation.** We next determined if the increased efficiency of hiPSC generation from FOP HDFs could be attributed to the FOP mutation itself and its signaling effect. We examined the effects of chemical inhibitors of BMP signaling on hiPSC generation from FOP HDFs under retroviral OSKM conditions. BMP-SMAD signaling inhibitors [i.e., Dorsomorphin (19) and LDN-193189 (20)] markedly decreased hiPSC generation from FOP HDFs, but a BMP-P38 MAPK signaling inhibitor [i.e., SB203580 (21)] had little effect (Fig. 1C and D). However, Dorsomorphin and LDN-193189 are cytotoxic and/or inhibit protein kinases other than BMP-SMAD signaling (22). To confirm the direct effects of BMP-SMAD signaling on hiPSC generation from FOP HDFs, we overexpressed inhibitory SMADs (*SMAD6* or *SMAD7*) by retroviral infection together with OSKM in hiPSC generation (Fig. S2A). Overexpressing *SMAD6* or *SMAD7* decreased the efficiency of hiPSC generation from FOP HDFs (Fig. 1E and F). We also transfected siRNA against *ACVR1* (targeting both the wild-type and FOP mutant) during iPSC generation with episomal plasmids (Fig. S2B). Knockdown of *ACVR1* decreased the efficiency of hiPSC generation from FOP HDFs (Fig. 1G and H). These results indicated that inhibition of BMP-SMAD signaling decreased the efficiency of iPSC generation from FOP HDFs.

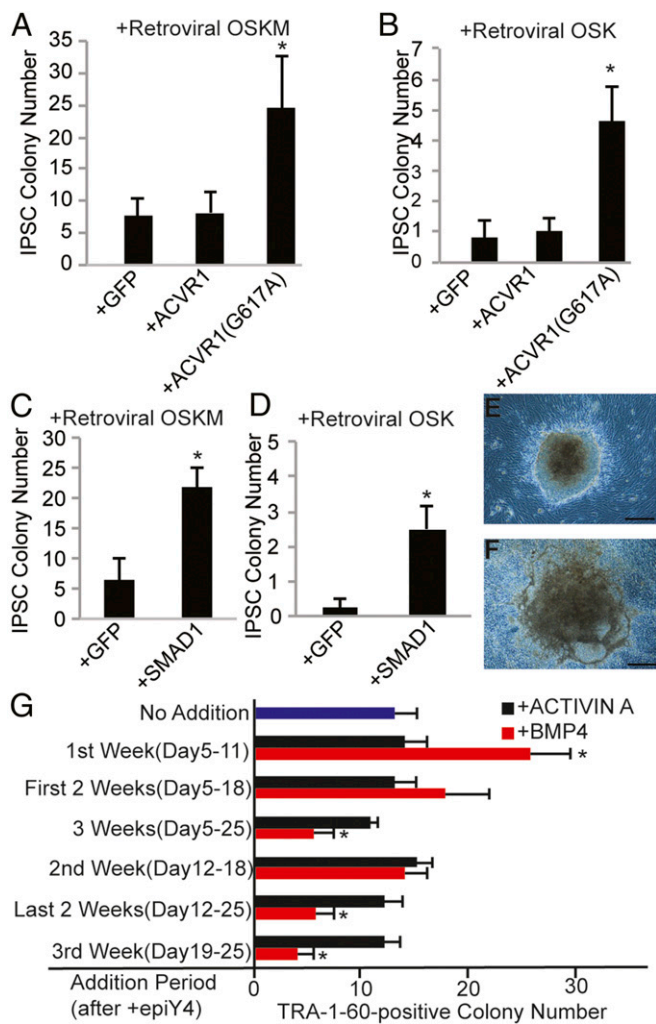
**Activating BMP-SMAD Signaling Promotes iPSC Generation in the Early Reprogramming Phase.** We asked whether activating BMP-SMAD signaling increases the efficiency of iPSC generation from normal HDFs. To examine the effect of the FOP mutation on iPSC generation from normal HDFs, we overexpressed wild-type or FOP mutant *ACVR1* (617G > A) by retroviral infection together with *OCT4*, *SOX2*, and *KLF4* (OSK) or OSKM for generating iPSCs. Overexpressing FOP mutant, but not wild-type, *ACVR1* has been reported to cause hyperactivation of BMP-SMAD signaling (23–25). The expression levels of wild-type and mutant *ACVR1* in HDFs were similar as assessed by RT-quantitative PCR (qPCR) and flow cytometry (Fig. S2C–F). Overexpressing FOP mutant, but not wild-type, *ACVR1* generated more hiPSCs from normal HDFs under both OSKM and OSK retroviral conditions (Fig. 2A and B).

Next, we determined the effects of elevated expression of *SMAD1* on hiPSC generation from normal HDFs by overexpressing *SMAD1* by retroviral infection with OSKM or OSK to generate iPSCs. *SMAD1* overexpression caused hyperactive BMP-SMAD signaling (26). *SMAD1* overexpression (Fig. S2A) increased the efficiency of hiPSC generation from normal HDFs under both OSKM and OSK retroviral conditions (Fig. 2C and D).



**Fig. 1.** Increased efficiency of hiPSC generation from FOP HDFs via the BMP-SMAD signaling pathway. (A and B) Number of TRA-1-60–positive colonies (A) or the ratio of TRA-1-60–positive cells (B) from reprogrammed FOP HDFs deposited in the Coriell Institute and collected at UCSF and four different normal HDF lines. TRA-1-60–positive cells were analyzed 25 d after transfection from 10,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$  ( $t$  test). (C and D) Effects of BMP-SMAD signal inhibitors, Dorsomorphin (1  $\mu$ M), LDN-193189 (1  $\mu$ M), P38 MAPK inhibitor, SB203580 (10  $\mu$ M), and vehicle (0.1% DMSO) on hiPSC generation from HDF-FOP1 (C) and HDF-FOP2 (D) with OSKM retroviral transduction. ESC-like colonies were counted 25 d after the transduction from 50,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\*\* $P < 0.01$  (Dunnett's test with vehicle conditions). (E and F) Effects of the overexpression of inhibitory SMADs SMAD6 or SMAD7 on hiPSC generation from HDF-FOP1 (E) and HDF-FOP2 (F) with OSKM retroviral transduction. ESC-like colonies were counted 25 d after transduction from 50,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$  (Dunnett's test with GFP conditions). (G and H) Effects of *ACVR1* knockdown on hiPSC generation from HDF-FOP1 (G) and HDF-FOP2 (H) with episomal plasmids. TRA-1-60–positive colonies were counted 25 d after transduction from 5,000 cells per 35-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$  (Dunnett's test with nontarget siRNA conditions).

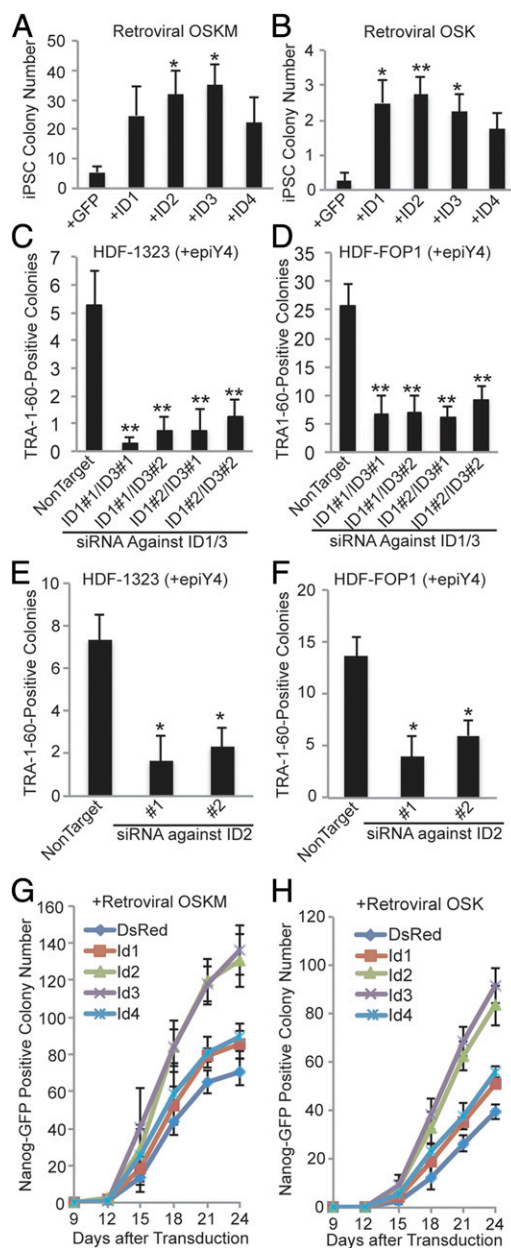




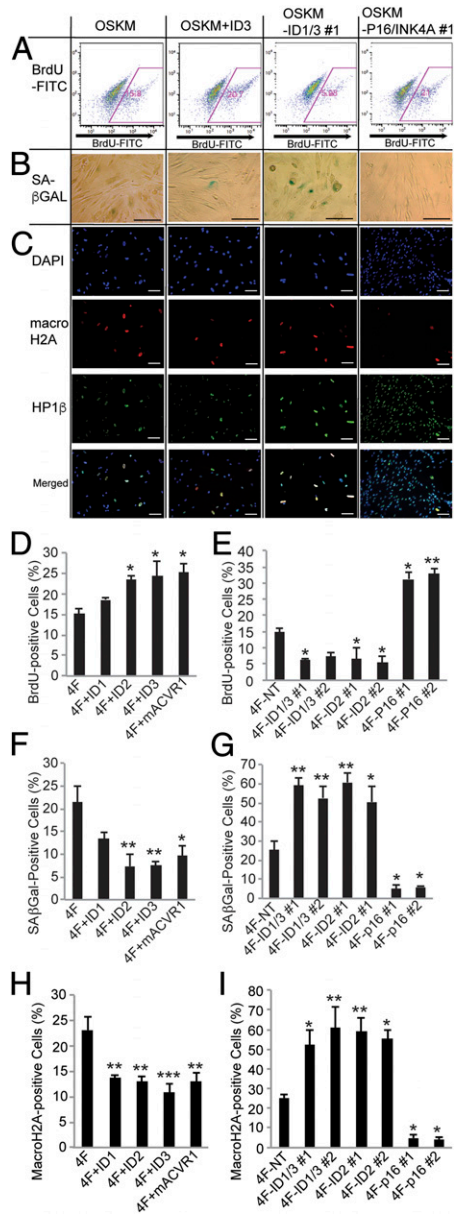
**Fig. 2.** BMP-SMAD signaling activation increases the efficiency of hiPSC generation from normal HDFs. (A and B) Effects of the overexpression of wild-type or mutant (G617A) ACVR1 on hiPSC generation from normal HDFs with OSKM (A) or OSK (B) retroviral transduction. ESC-like colonies were counted 25 d after transduction from 50,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 4$ . \* $P < 0.05$  (Dunnett's test with +GFP conditions). (C and D) Effects of the overexpression of SMAD1 on hiPSC generation from normal HDFs with OSKM (C) and OSK (D) retroviral transduction. ESC-like colonies were counted 25 d after transduction from 50,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$  ( $t$  test). (E and F) An ESC-like colony, epithelial with a round edge, made with BMP4 for first 1 wk (E) and a degenerated (or differentiated) colony with sac-like structure made with BMP4 for 3 wk (F). (Scale bars: 200  $\mu\text{m}$ .) (G) Effects of recombinant BMP4 and ACTIVIN A proteins on hiPSC generation from normal HDFs with episomal plasmids. TRA-1-60-positive colonies were counted 25 d after electroporation of epiY4 from 10,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 4$ . \* $P < 0.05$  (Dunnett's test with no addition conditions).

To examine how the timing of BMP-SMAD signaling affects hiPSC generation, we added recombinant BMP4 protein at specific times during iPSC generation from normal HDFs under epiY4 conditions. Recombinant ACTIVIN A protein, which only activates TGF $\beta$ -SMAD signaling, but not BMP-SMAD signaling, served as a control. BMP4 proteins (10 ng/mL) or ACTIVIN A proteins (10 ng/mL) were added in weeks 1, 2, and 3, and 1–2, 2–3, or 1–3 wk after the cells were replated in hiPSC culture conditions. Adding BMP4 early during reprogramming (until week 1) increased the efficiency of iPSC generation (Fig. 2E and F). However, late addition (week 3) of BMP4 decreased the efficiency of hiPSCs and produced degenerated or differentiated

colonies with sac-like morphologies (Fig. 2G). In contrast, adding ACTIVIN A in any periods had no effect. These results



**Fig. 3.** ID genes are crucial for iPSC generation. (A and B) Effects of overexpressed ID genes on hiPSC generation from normal HDFs with OSKM (A) and OSK (B) retroviral transduction. ESC-like colonies were counted 25 d after transduction from 50,000 cells per 100-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$  (Dunnett's test with +GFP conditions). (C and D) Effects of ID1/3 knockdown on hiPSC generation from HDF-1323 (C) and HDF-FOP1 (D) with episomal plasmids. TRA-1-60-positive colonies were counted 25 d after electroporation of epiY4 from 10,000 cells (of HDF-1323) or 5,000 cells (of HDF-FOP1)/35-mm dish at replating. Results are mean and SE,  $n = 3$ . \*\* $P < 0.01$  [Dunnett's test with nontarget (NT) conditions]. (E and F) Effects of ID2 knockdown on hiPSC generation from HDF-1323 (E) and HDF-FOP1 (F) with episomal plasmids. TRA-1-60-positive colonies were counted 25 d after electroporation of epiY4 from 10,000 cells (of HDF-1323) or 5,000 cells (of HDF-FOP1)/35-mm dish at replating. Results are mean and SE,  $n = 3$ . \* $P < 0.05$  (Dunnett's test with NT conditions). (G and H) Effects of overexpressed Id genes on generating miPSCs from Nanog-GFP MEF with OSKM (G) and OSK (H) retroviral transduction. Nanog-GFP-positive colonies were counted every 3 d after transduction from 10,000 cells (OSKM) or 50,000 cells (OSK)/35-mm dish at replating. Results are mean and SE,  $n = 4$ .



**Fig. 4.** BMP-SMAD-ID signaling axis regulates cell senescence during hiPSC generation. (A, D, and E) BrdU assay of normal HDFs transduced with OSKM and ID genes or mACVR1 or transfected with siRNA against ID genes or P16/INK4A. Three days after transduction, these cells were replated at 10,000 cells per 35-mm dish. The next day, the medium was changed to feeder cell-conditioned human ESC (hESC) medium. The cells were treated with siRNAs at day 2 and with BrdU at day 9. Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\*\* $P < 0.01$  (Dunnett's test with 4F conditions in D and with 4F – NT conditions in E). (B, F, and G) SA $\beta$ gal staining of normal HDFs transduced with OSKM and ID genes or mACVR1 or transfected siRNA against ID genes or P16/INK4A. The transduced cells were fixed to stain with SA $\beta$ gal at day 12. (Scale bars: 100  $\mu$ m.) Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$  (Dunnett's test with 4F + GFP conditions in F with 4F – NT conditions in G). (C, H, and I) Immunocytostaining of reprogramming human fetal lung fibroblast line (MRC-5) transduced with OSKM and ID genes or mACVR1 or transfected siRNA against ID genes or P16/INK4A. The transduced cells were fixed to stain with antibodies targeting for HP1 $\beta$  and macroH2A at day 12. Secondary antibodies for HP1 $\beta$  or macroH2A were labeled with Alexa Fluor 488 (green) or Alexa Fluor 555 (red), respectively. Nuclei were stained with DAPI (blue). (Scale bars: 100  $\mu$ m.) Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Dunnett's test with 4F conditions in H with 4F – NT conditions in I).

indicated that BMP-SMAD signaling promotes reprogramming into iPS cells in the early phase of reprogramming, but promote differentiation in the late phase. These hiPSC lines from normal fibroblasts reprogrammed with transient BMP4-SMAD activation maintain their self-renewal and pluripotency (SI Text and Fig. S3 A–G).

**Inhibitor of Differentiation Genes Regulated by BMP-SMAD Signaling Enhance iPSC Generation.** We asked whether inhibitor of differentiation (ID) genes regulated by BMP-SMAD signaling had a role during iPSC generation. ID genes are direct targets of BMP-SMAD signaling in various biological contexts (27–30), which maintain the pluripotency of mESCs (6, 31) and the stem cell identity of several somatic stem cells (32, 33). FOP-HDF alleviated the decrease of ID gene expression during iPSC generation (SI Text and Fig. S4B). To determine if exogenous ID genes promote iPSC generation, we overexpressed *ID1*, *ID2*, *ID3*, or *ID4* with OSKM or OSK by retroviral vectors during iPSC generation from normal HDFs (Fig. S5A). Overexpressing any ID gene increased the efficiency of iPSC generation from normal HDFs (Fig. 3 A and B). Next, we examined the role of endogenous expression of ID genes by knockdown experiments. We designed siRNAs against *ID1*, *ID2*, and *ID3* (*ID4* was not expressed in HDFs as shown in Fig. S5A) (Fig. S5B). Because the expression patterns and functions of *ID1* and *ID3* are highly redundant (34), we used mixed siRNA oligos against *ID1/ID3* during hiPSC generation from normal and FOP HDFs. Knockdown of *ID1/3* markedly decreased the efficiency of hiPSC generation from normal and FOP HDFs (Fig. 3 C and D). Knockdown of *ID2* also decreased the efficiency of iPSC generation from normal and FOP fibroblasts (Fig. 3 E and F). These results indicated that the expression of ID genes is critical for successful reprogramming into hiPSCs.

To confirm these findings in miPSC generation, we overexpressed mouse versions of *Id1*, *Id2*, *Id3*, or *Id4* by retroviral infection with OSKM or OSK in Nanog-GFP MEF to generate miPSCs (35) (Fig. S5C). The efficiency and speed of generating Nanog-GFP positive colonies were much higher with Id genes, especially with the +*Id2* and +*Id3* conditions (Fig. 3 G and H). The efficiency of generating alkaline phosphatase (ALP)-positive colonies detected at 12 d after transduction was also higher with Id genes (Fig. S6 A–C). These results indicated that overexpressing Id genes promoted miPSC generation in the early phase of reprogramming. We confirmed that miPSC lines generated with Id genes had full developmental potential, including germline transmission (SI Text and Fig. S6 D–L).

**BMP-SMAD-ID Signaling Axis Suppresses P16/INK4A-Mediated Cell Senescence During iPSC Generation.** We asked how the BMP-SMAD-ID signaling axis promotes iPSC generation. Activating the BMP-SMAD-ID signaling axis increases the cell proliferation rate and decreases cell senescence during iPSC generation (Fig. S5 G and H and Fig. 4 A–I). We determined if *p16/INK4A* (*CDKN2A*), which has a major role in cell senescence during iPSC generation (36, 37), was regulated by BMP-SMAD-ID signaling axis. ID genes suppress *p16/INK4A*-mediated cell senescence in other biological contexts (38–41). *p16/INK4A* protein expression was up-regulated in the samples transduced with retroviral OSKM for 5 d, but no or little expression could not be seen in established hiPSCs (Fig. S5 I and J). In contrast, FOP HDFs had lower protein levels of *p16/INK4A* than normal HDFs under these conditions. Overexpressing ID genes decreased the *p16/INK4A* protein levels and the knockdown of *ID2* or *ID1/3* increased the levels during iPSC generation (Fig. S5 K and L). These results indicated that BMP-SMAD-ID signaling axis negatively regulates *p16/INK4A* expression levels during iPSC generation.

We asked whether the BMP-SMAD-ID signaling axis was functionally epistatic to *p16/INK4A*-mediated cell senescence in iPSC generation. As reported (37), inhibiting *p16/INK4A* by siRNA (tested in Fig. S5 D–F) increased BrdU incorporation (Fig. 4 A and E) and decreased senescence associated  $\beta$ -galactosidase



(SA $\beta$ gal) activity (Fig. 4 *A* and *G*) and senescence associated heterochromatin foci (SAHF) formation (Fig. 4 *A* and *I*) during iPSC generation. Also as reported (36, 37), knockdown of p16/INK4A increased the efficiency of iPSC generation from normal HDFs; however, knockdown of p16/INK4A had no effect or only a slight increase in the efficiency of iPSC generation from FOP HDFs (Fig. 5*A*). Overexpressing ID genes in normal HDFs attenuated the increase of the efficiency of hiPSC generation by knocking down p16/INK4A (Fig. 5*B*). Furthermore, knockdown of p16/INK4A partially rescued the decrease of the efficiency of hiPSC generation from normal HDFs by the knockdown of ID2 or ID1/3 (Fig. 5*C*). These results indicated that BMP-SMAD-ID signaling was functionally epistatic to p16/INK4A-mediated cell senescence in iPSC generation.

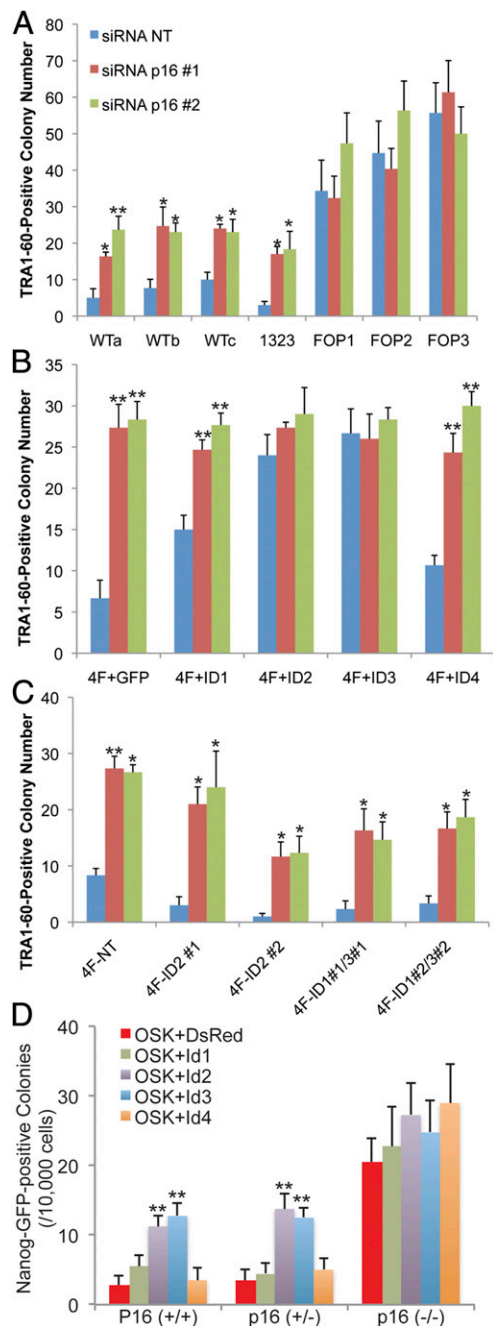
We also used a miPSC generation system by crossing Nanog-GFP mice (35) and p16/ink4a null mice (42). We obtained Nanog-GFP MEF with p16/ink4a<sup>+/+</sup>, <sup>+/-</sup>, or <sup>-/-</sup> and reprogrammed them to generate Nanog-GFP-positive iPSC colonies by transducing retroviral OSK and Id genes. As reported, the efficiency of Nanog-GFP colonies was higher in p16/ink4a null MEF than that in p16/ink4a<sup>+/+</sup> or <sup>+/-</sup> MEF, confirming that p16/ink4a-mediated cell senescence is important in mouse iPSC generation (36, 37). The efficiency of iPSC generation in p16/ink4a<sup>+/+</sup> or <sup>+/-</sup> MEF was increased by transducing Id2 or Id3, but not in p16/ink4a<sup>-/-</sup> (Fig. 5*D*). These results suggested that ID genes are epistatic to p16/ink4a-mediated cell senescence in iPSC generation.

## Discussion

In this study, we found a positive effect of BMP-SMAD signaling on human iPSC generation. However, the positive effect was easily masked by overgrowth of nonreprogrammed cells when initial cell densities were too high, or by alteration in the periods of BMP treatment because the positive effect requires BMP exposure in the early phases of iPSC generation. It is likely that these complex effects are responsible, at least in part, for the seemingly contradictory results from our study and those of Hamasaki et al. (15). Also, the differences of ingredients in the culture conditions might also modulate the results because recent studies showed that Activin A contributed the activation of BMP-SMAD signaling in FOP cells (43, 44).

We found that ID genes, induced by BMP-SMAD signaling or exogenous transduction, support successful reprogramming into iPSCs. In contrast, ID genes were down-regulated during iPSC generation, and further down-regulation by knockdown decreased the efficiency of iPSC generation. The expression of ID genes alleviates the p16/INK4A-mediated cell senescence barrier during iPSC generation. Conversely, the inhibition of ID genes during hiPSC generation induced p16/INK4A expression and increased cell senescence. Previous studies showed that, during iPSC generation, OSKM treatment up-regulates p16/INK4A expression, which causes cell senescence and inhibits reprogramming (37, 45); however, the mechanisms regulating p16/INK4A expression during iPSC generation remained unclear. Our results suggest that ID genes may be key factors that link transducing reprogramming factors and cell senescence.

We found that a genetic mutation found in FOP patients increased the efficiency of iPSC generation; this is contrary to the case of the *FANCA* or *FANCD2* mutation in Fanconi anemia patients, which makes hiPSC generation more difficult (46, 47). Also, although our study is not primarily intended to clarify FOP pathology, the mechanism of cell senescence mediated by p16/INK4A, which is counterbalanced by BMP-SMAD signaling and ID gene expression, could be relevant to FOP pathology. In FOP patients, the pool of multipotent and proliferating cells plays a major role in the preossification stage (48, 49). The suppression of cell senescence mediated by P16/INK4A caused by the ACVR1 mutation and the hyperactive BMP-SMAD signaling could positively contribute to this process. Collectively, iPSC generation from patient cells carrying genetic mutation are



**Fig. 5.** BMP-SMAD-ID signaling axis is functionally epistatic to p16/INK4A in iPSC generation. (A–C) Effects of p16/INK4A knockdown during reprogramming on hiPSC generation from FOP and normal HDFs (A), normal HDFs (HDF-WTc) transduced with retroviral OSKM and ID1–4 (B), or normal fibroblasts (HDF-WTc) transfected with ID2 or ID1/3 siRNA (C). TRA1-60-positive colonies were counted 25 d after electroporation of epiY4 from 10,000 cells per 100-mm dish at replating (A and C) or after transduction with retroviral OSKM and ID1–4 from 50,000 cells per 100-mm dish at replating (B). Results are mean and SE,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Dunnett's test with each siRNA NT condition). (D) Effects of overexpressed ID genes on generating miPSCs from p16<sup>+/+</sup>, p16<sup>+/-</sup> MEF, or p16<sup>-/-</sup> MEFs (crossed with Nanog-GFP) with OSK retroviral transduction. Nanog-GFP-positive colonies were counted at 16 d after transduction from 10,000 cells per 35-mm dish at replating. Results are mean and SE,  $n = 4$ . \*\*\* $P < 0.01$  (Dunnett's test with each +DsRed condition).

useful for clarifying the reprogramming processes as well as disease modeling.

## Materials and Methods

HiPSCs were generated with retrovirus or episomal plasmids as described (2) with some modifications, within six passages after receipt of fibroblasts (summarized in Table S1). Informed written consent was obtained from all donors. All procedures were approved by the Ethics Committee of the Department of Medicine and Graduate School of Medicine, Kyoto University, and the University of California, San Francisco, Institutional Review Board. All of the protocols of mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. See Table S2 for the siRNA sequences and Table S3 for DNA oligos and primers. Details of the materials, methods, and associated references are in *SI Materials and Methods*.

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