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DSG2 Is a Functional Cell Surface Marker for Identification and Isolation of Human Pluripotent Stem Cells

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SUMMARY

Pluripotent stem cells (PSCs) represent the most promising clinical source for regenerative medicine. However, given the cellular heterogeneity within cultivation and safety concerns, the development of specific and efficient tools to isolate a pure population and eliminate all residual undifferentiated PSCs from differentiated derivatives is a prerequisite for clinical applications. In this study, we raised a monoclonal antibody and identified its target antigen as desmoglein-2 (DSG2). DSG2 co-localized with human PSC (hPSC)-specific cell surface markers, and its expression was rapidly downregulated upon differentiation. The depletion of DSG2 markedly decreased hPSC proliferation and pluripotency marker expression. In addition, DSG2-negative population in hPSCs exhibited a notable suppression in embryonic body and teratoma formation. The actions of DSG2 in regulating the self-renewal and pluripotency of hPSCs were predominantly exerted through the regulation of β-catenin/Slug-mediated epithelial-to-mesenchymal transition. Our results demonstrate that DSG2 is a valuable PSC surface marker that is essential for the maintenance of PSC self-renewal.

INTRODUCTION

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) can self-renew and give rise to the differentiated progeny of all three germ layers while maintaining pluripotency. Therefore, they are promising sources for clinical applications in cell therapy, tissue repair, and drug development (Yamanaka, 2012). However, given the cellular heterogeneity observed during the cultivation of PSCs *in vitro* and safety concerns related to teratoma development *in vivo*, the characterization of putatively therapeutic PSC populations and the isolation of pure populations according to molecular biomarker expression are prerequisites for clinical applications (Ben-David and Benvenisty, 2011). In this respect, cell surface molecules of PSCs represent useful tools for identifying unique cell populations with self-renewal capacity and eliminating residual undifferentiated PSCs from differentiated derivatives. However, the cell surface molecules conventionally used to identify undifferentiated PSCs are restricted to stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA-1-81, L1 cell adhesion molecule (L1CAM), and epithelial cell adhesion molecule (EpCAM) (Choi et al., 2014). Unfortunately, one or two surface molecule-based cell classification is insufficient to define and eliminate undifferentiated cells. Therefore, having a variety of PSC-specific cell surface molecules will improve the isolation and effective targeting of undifferentiated cells. In addition, the development of monoclonal antibodies (mAbs) against PSC-specific cell surface molecules will facilitate efficient separation of unique populations with self-renewal and pluripotency from cultured PSCs, as well as the complete elimination of residual undifferentiated PSCs from differentiated derivatives.

PSCs depend on extracellular signals to maintain their self-renewal and pluripotency and initiate their differentiation, and some PSC surface molecules regulate extracellular signals through interactions with extracellular matrix proteins and receptors on the PSC surface (Gu et al., 2010). We recently demonstrated that L1CAM supports the self-renewal of hESCs through the activation of fibroblast growth factor receptor-1 signaling in a ligand-independent

manner (Son et al., 2011). In addition, a recent study reported the involvement of cell surface glycans in the maintenance of ESC pluripotency and undifferentiation (Alisson-Silva et al., 2014). However, the roles in the maintenance of the self-renewal and pluripotency of PSCs have been elucidated only for a few surface molecules (Lu et al., 2010; Son et al., 2011). Therefore, identifying a variety of PSC-specific cell surface molecules is essential for both clarifying the relationship between the pluripotency and phenotype of PSCs and understanding the mechanisms governing their self-renewal and pluripotency.

Desmoglein-2 (DSG2) is an adhesion molecule. Its extracellular domain mediates adhesion, and its cytoplasmic tail associates with desmosomal plaque proteins (Johnson et al., 2014). Desmosomes are considered the most important junctional complex for intercellular adhesion in epithelial and some non-epithelial tissues. In humans, four DSG isoforms (DSG1–DSG4) and three desmocollin isoforms (DSC1–DSC3) have been identified, which are temporospatially expressed (Brooke et al., 2012). DSG2 is expressed in all desmosome-bearing tissues, including cardiac muscle, whereas the distribution of other isoforms is confined primarily to epithelial tissue (Johnson et al., 2014). Desmosomes were not found in undifferentiated hESCs, and their existence indicates that these cells have differentiated into epithelial cells (Oh et al., 2005). In mice, it has been reported that DSG2 is expressed in desmoplakin-negative mouse ESCs (mESCs) in non-desmosomal junctions and is needed for ESC proliferation and survival (Eshkind et al., 2002). Previous studies have also reported that DSG2 is expressed on the hESC membrane by a proteomics and membrane-polysome translation state array analysis (Dormeyer et al., 2008; Kolle et al., 2009). However, the role of DSG2 in hPSCs has not yet been studied.

Previously, we generated hybridomas by immunizing mice with hESC clumps and isolated mAbs that bound to hESCs but not to mESCs (Son et al., 2011). Here, we selected and characterized a new mAb, K6-1 (IgG1,κ), which binds specifically to DSG2 in hPSCs. This study focused on whether DSG2 can be used as a surface marker to identify, isolate, and characterize undifferentiated hPSCs. In addition, we investigated its roles and the underlying signaling mechanism in the maintenance of self-renewal and acquisition of pluripotency during reprogramming. Finally, our results illustrated that DSG2 is a valuable cell surface marker that is highly enriched in undifferentiated PSCs compared with its levels in their differentiated derivatives. Moreover, we demonstrated that DSG2 plays an important role in the maintenance of self-renewal and pluripotency in PSCs through the regulation of β-catenin/Slug-mediated epithelial-to-mesenchymal transition (EMT).

RESULTS

Identification of a mAb Binding to the hPSC Surface and Elucidation of Its Antigen

To identify PSC surface markers, we generated hybridomas raised against undifferentiated hESCs and screened mAbs that bound to cell surface proteins on hPSCs. Among them, we selected an applicable mAb, K6-1, and characterized its utility as a surface marker for hPSC determination. The K6-1 mAb bound to the surface of H9 hPSCs in a similar manner as SSEA-3 and SSEA-4 bound to H9 cells (Figure 1A, top panel). Furthermore, K6-1 bound to the surfaces of three hPSC lines, namely H1, iPSC#1, and iPSC#2, as well as the surface of NTERA-2 cells, a human embryonic carcinoma cell line (Figure 1A, bottom panel). Subsequently, immunofluorescence analysis revealed that the K6-1 antigen co-localized with EpCAM, a well-known surface marker, in the membranes of undifferentiated PSCs (Figure 1B). Phenotyping analysis of K6-1 antigen-positive cells illustrated that 93.28% ± 4.68% of hPSCs were positive for K6-1 antigen expression (Figure 1A). To elucidate the target antigen of K6-1, we biotinylated the surface of H9 hESCs and immunoprecipitated the biotinylated whole-cell lysates with K6-1, followed by western blot analysis with streptavidin-horseradish peroxidase. We found approximately 160- and 100-kDa (a cleavage form) bands (Figure 1C), and performed target identification via mass spectrometry. Peptide sequence analysis revealed that the antigen was DSG2 (Figure S1A), and the specificity of K6-1 for DSG2 was confirmed using 6D8, a commercial antibody against DSG2 (Figure 1C). Next, to determine whether DSG2 is also expressed on the surface of mouse PSCs (mPSCs), another antibody (ab150372) with cross-reactivity to both hDSG2 and mDSG2 was applied to evaluate Dsg2 expression in the mESC line J1, as K6-1 and 6D8 did not recognize mDSG2 (Figure S1B). As shown in Figures 1D–1F, DSG2 was highly expressed on the surface of mPSCs compared with that of mouse embryonic fibroblasts (MEFs; a mouse primary fibroblast line), indicating that DSG2 is a surface marker for hPSCs and mPSCs.

DSG2 Is Rapidly Downregulated during the Differentiation of hPSCs

Next, to determine whether DSG2 can be a useful surface marker to specifically identify, isolate, and qualify undifferentiated hPSCs and clarify whether DSG2 expression is downregulated upon differentiation, we compared the expression levels of DSG2 in undifferentiated and differentiated hPSCs. hPSCs were differentiated by two methods: embryonic body (EB) formation and retinoic acid (RA) treatment. DSG2 surface expression was markedly downregulated upon EB formation, and its reduction was substantially faster than that of well-known surface markers, such

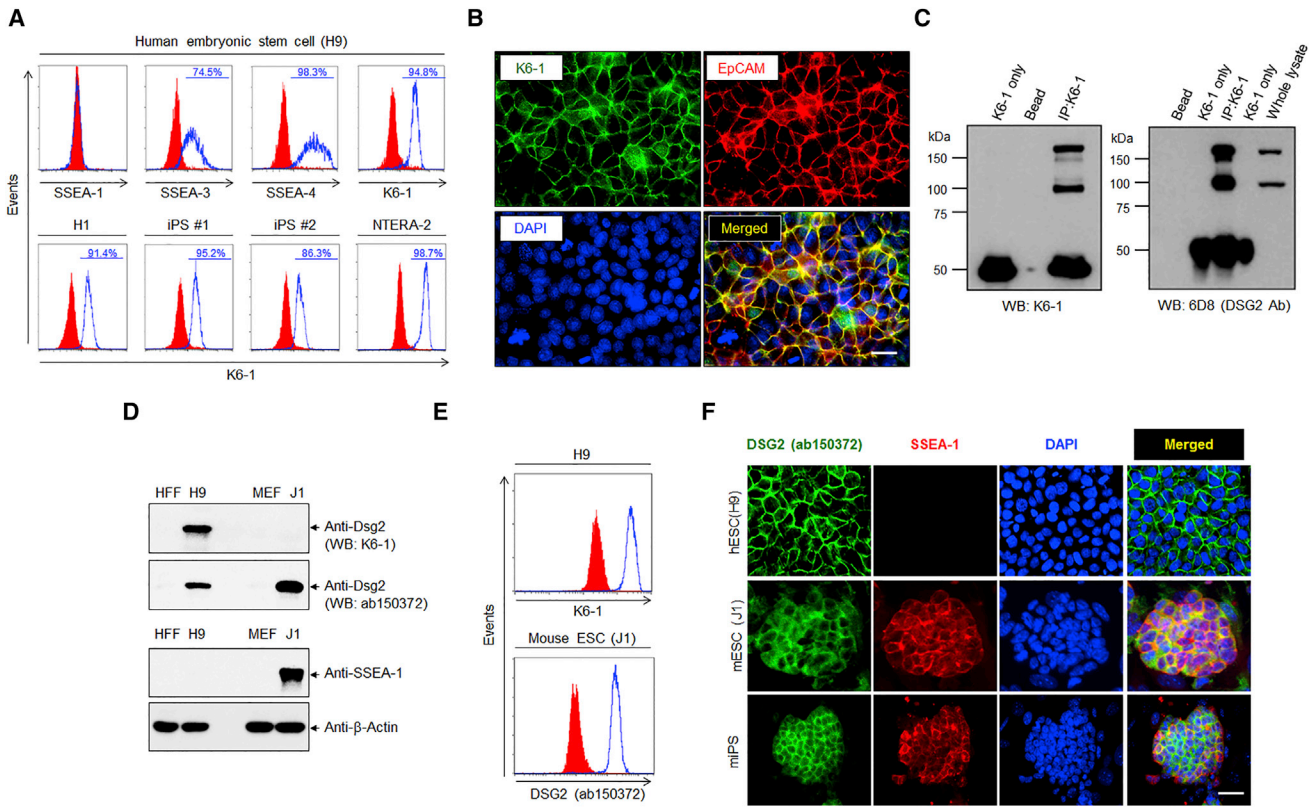

Figure 1. Identification of an mAb Binding to the hPSC Surface and Elucidation of Its Antigen

(A) The expression levels of various surface markers of hESCs, including SSEA-1 (for differentiation), SSEA-3, and SSEA-4 (for undifferentiation), on human H9 cells were compared by fluorescence-activated cell sorting (FACS) using K6-1 mAb (upper panel). Representative FACS plots demonstrate K6-1 binding to the human PSC surface (bottom panel).

(B) K6-1 antibody co-localized with EpCAM. Immunofluorescence staining of H9 cells with K6-1 and EpCAM. Scale bar, 20 μm.

(C) Immunoblotting analysis revealed that the K6-1–targeted antigen identified 160- and 100-kDa proteins (left panel). Immunoprecipitation (IP) and subsequent immunoblotting were performed to confirm DSG2 using an anti-Dsg2 antibody, 6D8 (right panel).

(D and E) The DSG2 expression level in human and mouse ESCs was determined by immunoblotting (D) and FACS (E) using K6-1 antibody for hESCs or a commercial DSG2 antibody (ab150372) for hESCs and mESCs.

(F) DSG2 co-localized with SSEA-1 on undifferentiated mouse PSCs. Immunofluorescence staining of cells with DSG2 and SSEA-1. Scale bar, 30 μm.

as TRA-1-60, SSEA-4, E-cadherin, and EpCAM (Figures 2A and 2B), or even transcription factors, such as OCT4 and NANOG, in whole-cell lysates (Figure 2B), although its expression was subsequently increased after 9 days of EB differentiation.

We also observed that the reduction of *DSG2* expression was regulated at the transcriptional level (Figure 2C). In addition, we analyzed the expression level of DSG2 in RA-treated hPSCs and in comparison with that of the three germ-layer markers, namely glial fibrillary acidic protein (ectoderm), α-fetoprotein (endoderm), and α-smooth muscle actin (mesoderm). DSG2 expression was also markedly downregulated upon RA treatment, whereas that of the three germ-layer markers was increased after RA treatment (Figure 2D). To determine whether DSG2 expression is also downregulated upon mESC differentiation, we next examined the expression of mDSG2 in mESC-derived EBs and evaluated the differentiation status, followed by an analysis of SSEA-1 cell surface expression and differentiation-related gene expression (Figures S2A and S2B). Consistent with the hPSC results, mDSG2 expression was also downregulated upon EB formation (Figures S2C and S2D). To further clarify the specificity of DSG2 expression in the undifferentiated hPSCs, we compared the expression of DSG2 between fibroblasts and iPSCs during reprogramming. As shown in Figures 2E and S2E, unlike the hPSC surface markers E-cadherin, EpCAM, and TRA-1-60, DSG2 expression was rapidly increased at the early stage of reprogramming in human foreskin fibroblasts (HFFs). These results suggest that DSG2 takes precedence over conventional

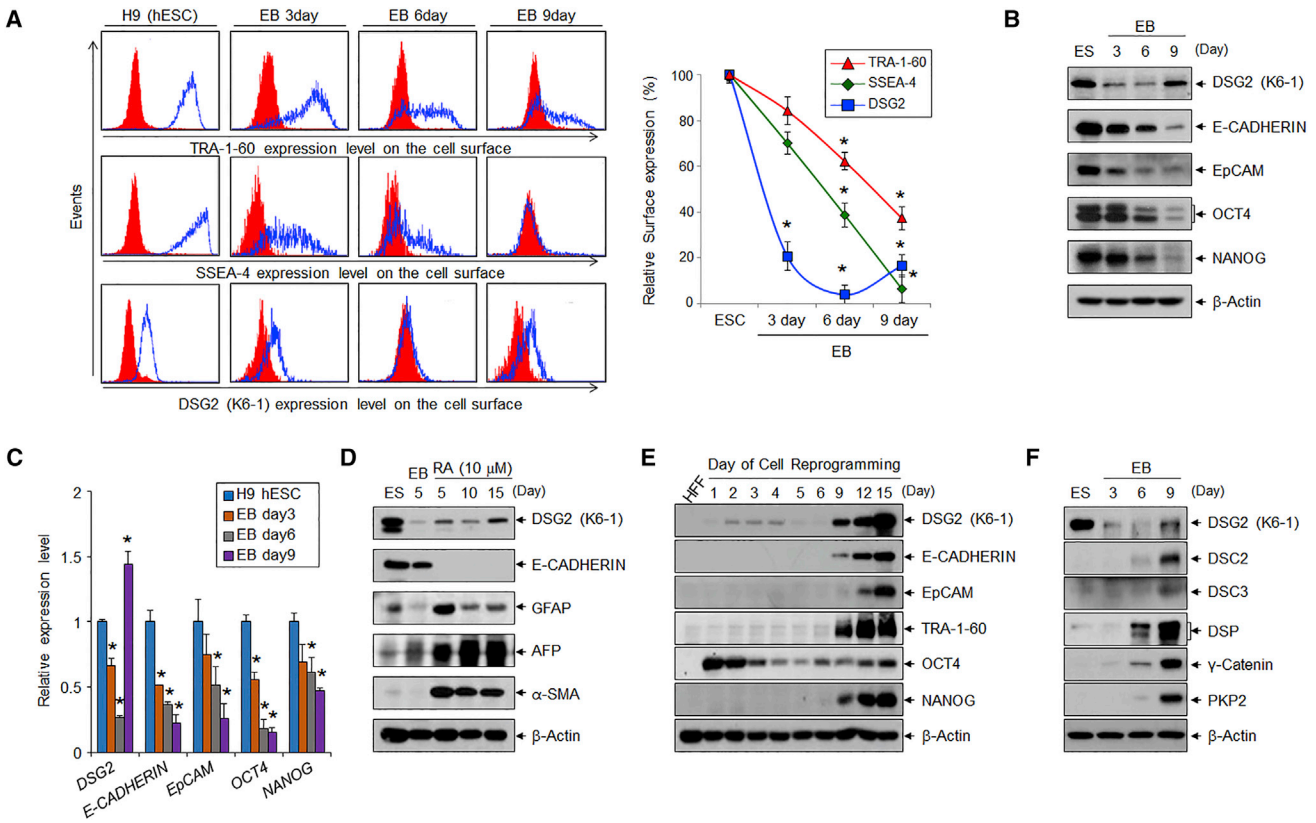

Figure 2. DSG2 Is Rapidly Downregulated During hPSC Differentiation

(A) DSG2 expression levels in undifferentiated H9 cells and in differentiated 3-, 6-, and 9-day EBs were examined using FACS and compared with other ESC surface markers. *$p < 0.05$ versus H9 hESCs.

(B and C) The DSG2 expression level in hESCs and differentiated EBs was determined by immunoblotting (B) and qPCR (C) compared with pluripotency markers and other hESC surface markers. *$p < 0.05$ versus H9 hESCs.

(D) Differentiation of H9 cells significantly reduced the DSG2 expression level. After EB formation on day 5, EB cells were attached to Matrigel-coated dishes and treated with 10 μM retinoic acid (RA) for the indicated times.

(E) HFFs were transfected by electroporation using the reprogramming episomal plasmid set. Cell lysates were collected and analyzed by immunoblotting using the indicated antibodies.

(F) The expression levels of desmosome components excluding DSG2 increased during ESC differentiation. Desmosome component expression levels were examined by immunoblotting.

surface markers in determining whether PSCs are differentiated or undifferentiated.

DSG2 is an adhesion molecule of desmosome complexes. Therefore, we next compared the expression of different members of desmosome between differentiated and undifferentiated cells. As shown in Figures 2F and S3A, DSG2 was highly expressed in undifferentiated PSCs and rapidly downregulated upon EB formation, whereas the expression of different desmosome components was reversely increased in differentiating cells. To further evaluate DSG2 as a highly specific surface marker of undifferentiated PSCs among the desmosome components, we examined its expression in all human cell types by querying the Amazonia expression atlas (Assou et al., 2007). *DSG2* is indeed highly expressed in various hESC and human iPSC (hiPSC) lines, as well as in human embryonic carcinoma cell lines, but is absent in more than 250 samples of somatic tissues (Figure S3B). Together, these results clearly demonstrate that DSG2 is a unique surface marker for undifferentiated hPSCs and is only pluripotent specific among desmosome components.

DSG2 Is Essential for Self-Renewal and Suppressing Differentiation

Self-renewal involves proliferation with a concomitant suppression of differentiation (Thomson et al., 1998). To elucidate the role of DSG2 in the self-renewal of undifferentiated hPSCs, we generated stable DSG2-depleted hESC lines via transduction with lentiviral particles harboring short hairpin RNA (shRNA) plasmids targeting DSG2.

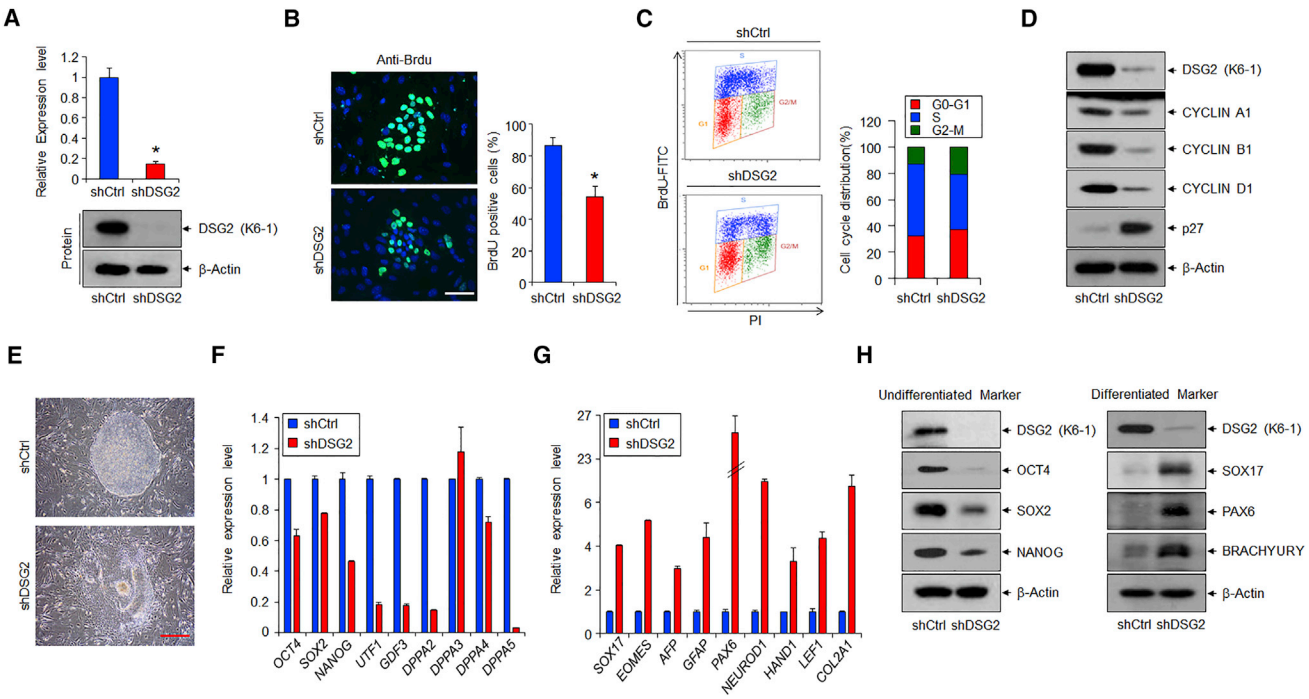

Figure 3. DSG2 Is Essential for Self-Renewal and Suppressing Differentiation

(A) Expression level of DSG2 in shCtrl and shDSG2 cells was determined by qPCR (top panel) and immunoblotting (bottom panel). Relative expression levels of *DSG2* analyzed by qPCR are shown on the graph. *$p < 0.05$ versus shCtrl.

(B) Cell proliferation was assessed using the BrdU incorporation assay. The graph represents the findings of five independent experiments in which 10 colonies of BrdU-positive cells were counted under each condition. *$p < 0.01$ versus shCtrl. Scale bar, 50 μm.

(C) Cell-cycle distribution was analyzed by FACS using BrdU and propidium iodide incorporation.

(D) The expression level of cell-cycle regulators was analyzed by immunoblotting.

(E) DSG2-KD hESCs spontaneously differentiated. Phase-contrast images showing shCtrl and shDSG2 ESCs. Scale bar, 200 μm.

(F and G) The gene expression level of core pluripotency transcription factors (F) and three germ-layer markers (G) was assessed by qPCR.

(H) Cell lysates were collected and analyzed by immunoblotting using the indicated antibodies. The K6-1 antibody was used for DSG2 detection.

hESC lines stably exhibiting >85% and >96% downregulation at the mRNA and protein levels, respectively, were selected (Figure 3A), and the effect of DSG2 on the proliferation of hESCs was evaluated by bromodeoxyuridine (BrdU) incorporation and cell-cycle analysis. As shown in Figure 3B, BrdU-positive cells accounted for approximately 86% of the total control shRNA-transfected hESC population. Interestingly, stable depletion of DSG2 decreased the BrdU-positive cell population compared with that in the control cells (Figure 3B). In addition, cell-cycle analysis revealed that DSG2 downregulation resulted in a smaller S-phase population (Figure 3C). Consistently, cyclin A1, B1, and D1 expression was markedly downregulated in DSG2-depleted hESCs, whereas the cell-cycle inhibitor p27 was markedly upregulated (Figure 3D). Together, these results indicate that DSG2 has an essential role in the proliferation of undifferentiated hPSCs.

Undifferentiated PSC colonies typically have clear borders from the feeders and contain small round cells, with spaces between them, and large nuclei with notable nucleoli. In general, any colony with a change in its typical morphology can be considered to be differentiating (Thomson et al., 1998). Accordingly, DSG2-depleted hESCs did not exhibit a flattened morphology with clearly defined and separated cell borders, whereas they displayed a translucent appearance and a large detached clump with a brown color at the center (Figure 3E), indicating that spontaneous differentiation or apoptotic processing had occurred in DSG2-depleted hESCs. In this respect, to determine whether DSG2 is associated with the maintenance of an undifferentiated state in hPSCs, we compared the expression of undifferentiated PSC markers and the three germ-layer markers between DSG2-depleted and control hESCs. DSG2-depleted hESCs exhibited significantly decreased expression of the undifferentiated PSC markers compared with the control cells (Figure 3F). By contrast, the expression of the three germ-layer markers was markedly enhanced in DSG2-depleted hESCs compared with

that in control cells (Figure 3G). We also confirmed this gene expression signature via RNA sequencing (data not shown). Moreover, the expression of major transcription factors, including OCT4, SOX2, and NANOG, was dramatically downregulated, whereas three germ-layer markers were upregulated in DSG2-depleted hESCs (Figure 3H). These results suggest that DSG2 plays an important role in the maintenance of the undifferentiated state of PSCs.

DSG2 Is Essential for the Maintenance of hPSC Pluripotency and Acquisition of Pluripotency during Somatic Cell Reprogramming

ESCs and iPSCs are characterized by their pluripotency, which describes the ability of a cell to develop into the three primary germ cell layers of the early embryo. In general, EB and teratoma formation is an essential criterion in determining the pluripotency of hPSCs (Pera, 2010). Next, to evaluate the role of DSG2 in the pluripotency of hPSCs, we sorted DSG2-positive or -negative cells in hESCs (Figure 4A) and determined the expression of key molecular markers for pluripotency. Consistent with the observations in Figure 3H, the expression of OCT4, NANOG, and SOX2 was also downregulated in DSG2-negative cells compared with that in DSG2-positive cells (Figures 4B and 4C). Note that DSG2-negative cells exhibited a marked suppression in EB formation (Figure 4D). To further confirm the role of DSG2 in the pluripotency of hPSCs *in vivo*, we also injected each population into each side of the testis of severely immunosuppressed NSG mice. As indicated in Figures 4E and 4F, all mice with DSG2-positive cells yielded differentiated teratomas containing terminally differentiated cell types from all three germ layers, whereas DSG2-negative cells did not. Single-cell clonogenic ability also characterizes the pluripotency and self-renewal of hPSCs (Stewart et al., 2006). To determine whether DSG2 can affect the single-cell-derived colony-forming ability of PSCs, we completely dissociated cells sorted according to DSG2 expression and seeded them onto Matrigel-coated plates. Notably, only DSG2-positive cells could develop into compact alkaline phosphatase (AP)-positive colonies (Figure 4G). Interestingly, although DSG2-negative cells lost their pluripotency, specific PSC surface markers, such as SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, were still highly expressed in DSG2-negative cells (Figure 4H). These results suggest that compared with other surface markers, DSG2 may be a reliable indicator for determining the pluripotency of PSCs.

Because our results illustrated that DSG2 was highly expressed in both ESCs and iPSCs and activated during the course of pluripotency reprogramming (Figures 1A and 2E), it was of interest to determine whether disrupting DSG2 expression during somatic cell reprogramming would affect the efficiency of reprogramming. To this end, we introduced DSG2 shRNA into HFFs using lentiviral vectors (Figure S4A) and induced HFF reprogramming via ectopic reprogramming factor expression. As shown in Figure S4B, the morphology of DSG2-depleted colonies was irregular and loose, dissimilar to the typical compacted dome-like structure of hPSC colonies formed by control cells. In addition, the introduction of *DSG2* shRNA significantly reduced the reprogramming efficiency of HFFs according to the number of AP-positive colonies on day 15 (Figure S4C). Consistent with AP staining, the TRA-1-60 expression level was reduced at the late phase of somatic reprogramming in DSG2-depleted HFFs (Figures S4D and S4E). We also observed a marked repression of reprogramming surface marker signatures, as well as the elicitation of the pluripotency-associated genes *OCT4*, *NANOG*, *UTF1*, *REX1*, *DPPA2*, and *GDF3* (Figures S4F and S4G), during somatic cell reprogramming as a consequence of DSG2 depletion. Notably, DSG2-knockout PSCs also did not form AP-positive colonies (Figure S5A), nor did these cells express PSC-related markers (Figures S5B and S5C). Taken together, these results suggest that DSG2 plays an essential role in the acquisition of pluripotency during somatic cell reprogramming.

DSG2 Regulates the EMT/Mesenchymal-to-Epithelial Transition Process in PSCs through the Regulation of Slug Expression

EMT is an important process for embryonic development and is involved in maintaining self-renewal or inducing differentiation in PSCs (Thiery et al., 2009). Therefore, we hypothesized that DSG2 maintains self-renewal and pluripotency in hPSCs through regulating the EMT process. First, we examined the relationship between DSG2 loss and E-cadherin expression. Interestingly, the depletion of DSG2 in hESCs resulted in a noticeable decrease in E-cadherin expression, whereas vimentin was upregulated (Figure 5A). Consistently, E-cadherin expression was reduced in the DSG2 low-PSC subpopulation, whereas vimentin and Slug expressions were significantly increased (Figures S6A and S6B). Moreover, immunofluorescence analysis illustrated that E-cadherin expression was markedly downregulated in shDSG2 cells (Figure 5B), suggesting that EMT was induced in DSG2-depleted hESCs. Several transcription regulators have been associated with controlling EMT, including the Snail family proteins Snail and Slug as well as Twist (Lee et al., 2015; Thiery, 2002). Among them, Snail family proteins have been associated with gastrulation, the earliest EMT within the developing embryo. They are also expressed during ESC differentiation and are capable of inducing EMT (Thiery et al., 2009). Accordingly, we determined whether the loss of DSG2 enhances the expression of Snail family proteins. As shown in Figures 5C and 5E, Slug expression was dominantly

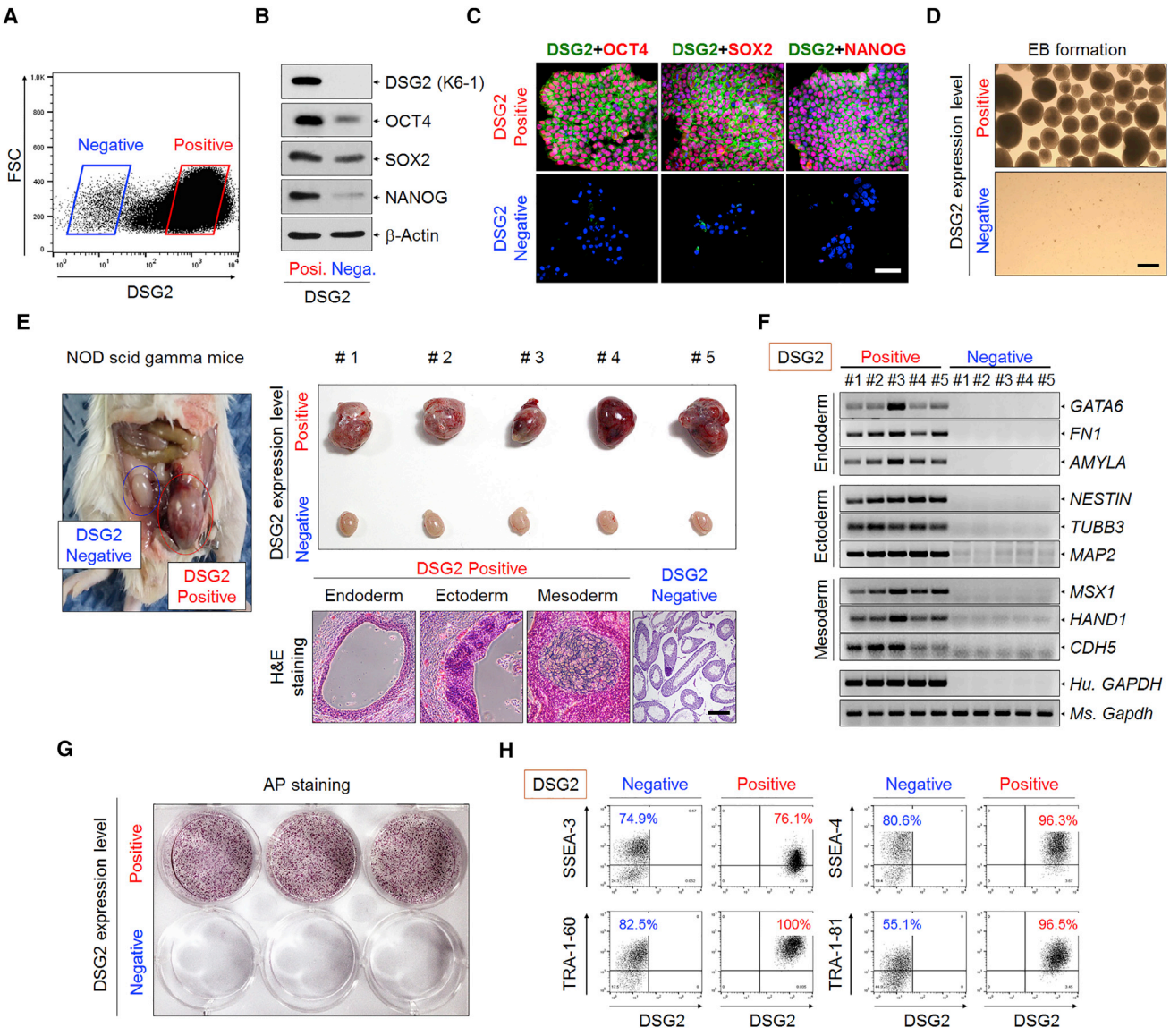

Figure 4. DSG2 Is Essential for the Maintenance of hESC Pluripotency

(A–C) To evaluate the role of DSG2 in pluripotency, we (A) isolated PSC with DSG2 expression dependent on flow cytometry and then analyzed the PSC marker expression level within DSG2-positive and -negative populations using immunoblotting (B) and immunofluorescence staining (C). Scale bar, 50 μm.

(D) Phase-contrast images indicated EB formation in DSG2-positive and -negative populations. After FACS sorting, each population was forced to aggregate using the Aggrewell plate and was then transferred into a low-attachment culture dish followed by a 7-day culture period. Scale bar, 20 μm.

(E) Depending on the DSG2 expression, isolated cells were injected into each side of mouse testes. Teratoma formation was observed 8 weeks after the injection. Images of five teratomas were derived from DSG2-positive and -negative populations formed in NSG mice. Representative H&E staining indicated the three germ-layer tissues (endoderm, gut-like epithelium; ectoderm, neural epithelium; mesoderm, cartilage). Scale bar, 100 μm.

(F) Expression of the three representative germ-layer markers in each teratoma was compared using PCR.

(G) Representative images indicate AP activity in hESCs. Pluripotent colonies were recovered 5 days after sorting cells from DSG2-positive populations, but not from DSG2-negative populations.

(H) After PSC sorting depending on DSG2 expression level, DSG2-positive and -negative populations were stained with SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, and subsequently drawn with a dot plot.

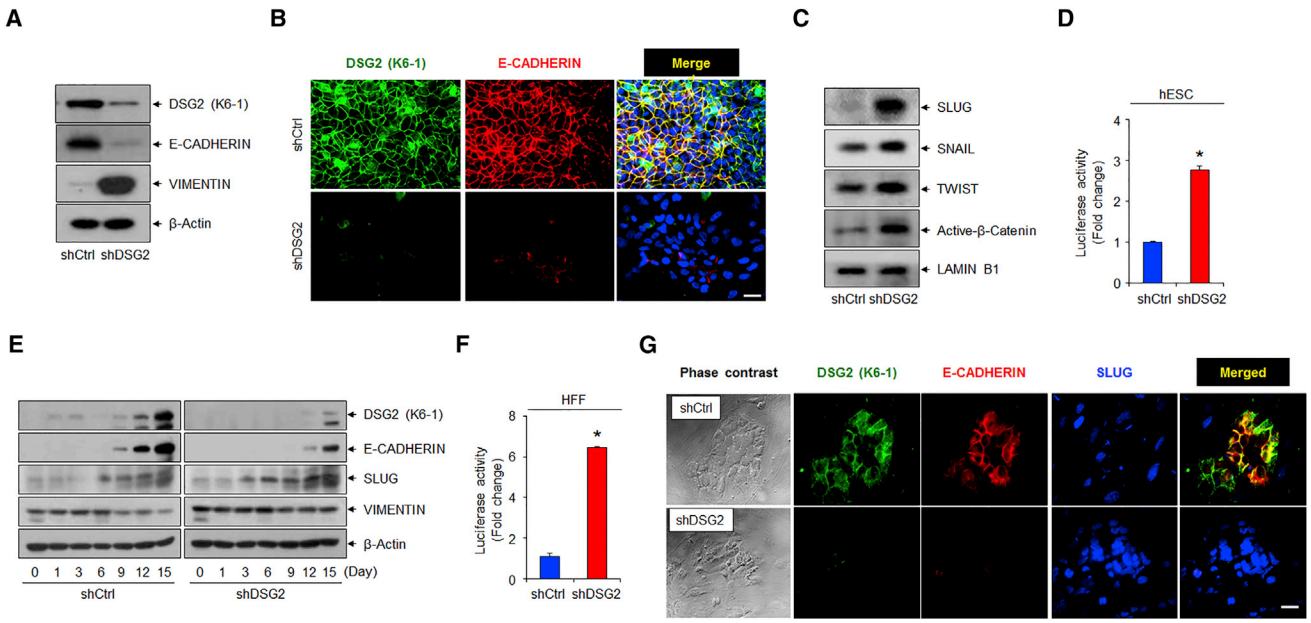

Figure 5. DSG2 Involves EMT/MET in hESCs through the Regulation of SLUG Expression

(A) E-cadherin and vimentin expression levels were determined by immunoblotting.

(B) Double immunofluorescence staining of DSG2 and E-cadherin in shCtrl and shDSG2 hESC. Scale bar, 30 μm.

(C) Expression levels of EMT-inducing transcription factors and active β-catenin were determined by immunoblotting.

(D) *Slug* transcriptional activity in shCtrl and shDSG2 hESCs was measured using a dual-luciferase reporter assay. Quantification from three independent assays is shown on the graph. Error bars indicate ±SEM. *$p < 0.01$ versus shCtrl.

(E) The expression level of EMT-related markers was determined by immunoblotting in shCtrl- and shDSG2-reprogrammed HFFs.

(F) Dual-luciferase reporter assay indicated that *Slug* promoter activity was significantly increased in shDSG2 HFFs compared with that in shCtrl HFFs. Quantification from five independent assays is shown on the graph. Error bars indicate ±SEM. *$p < 0.05$ versus shCtrl.

(G) shCtrl and shDSG2 HFFs were fixed 9 days after HFFs were reprogrammed and then stained with the indicated antibodies. The K6-1 antibody was used for Dsg2 detection. Scale bar, 30 μm.

elevated in DSG2-depleted hESCs and HFFs compared with that in control cells. Based on this observation, we postulated that increased Slug expression or decreased E-cadherin expression is a consequence of the DSG2 depletion-mediated spontaneous differentiation of PSCs or that the loss of DSG2 activates Slug transcription, which leads to the suppression of E-cadherin expression and the induction of EMT. To address this issue, we performed a promoter activity assay using a plasmid constructed by linking the *Slug* promoter (from −981 to +172) to a luciferase gene. As shown in Figure 5D, the Slug promoter displayed an approximately 3-fold increase in luciferase expression in DSG2-depleted hESCs, suggesting that the loss of Dsg2 increases the transcriptional activation of *Slug*.

The reprogramming of somatic cells to iPSCs also requires a reverse process, mesenchymal-to-epithelial transition (MET), for the acquisition of pluripotency (Li et al., 2010). In this context, we further determined whether the reduced reprogramming efficiency in DSG2 shRNA-expressing HFFs is a consequence of MET inhibition through the sustained expression of Slug via DSG2 depletion. As shown in Figure 5E, introducing DSG2 shRNA into HFFs resulted in delayed and decreased induction of E-cadherin during reprogramming. Moreover, a significant reduction of vimentin expression was observed after 9–15 days in DSG2 shRNA-expressing HFFs, and the promoter activity of *Slug* was markedly increased (Figure 5F). Specifically, co-localization experiments indicated that DSG2-negative HFFs did not express E-cadherin, whereas Slug expression was definitely increased in the nuclei (Figure 5G). Taken together, these results demonstrate that DSG2 regulates the EMT/MET process through the regulation of Slug expression in hPSCs or somatic cells.

DSG2 Sequesters β-Catenin at the Cell Membrane to Suppress EMT Induction in Undifferentiated PSCs

We next scrutinized the mechanism by which the loss of DSG2 activates the transcription of Slug in hPSCs. β-Catenin is considered the most potent regulator of Slug transcription in many cell types (Nelson and Nusse, 2004). To determine whether DSG2 expression influences

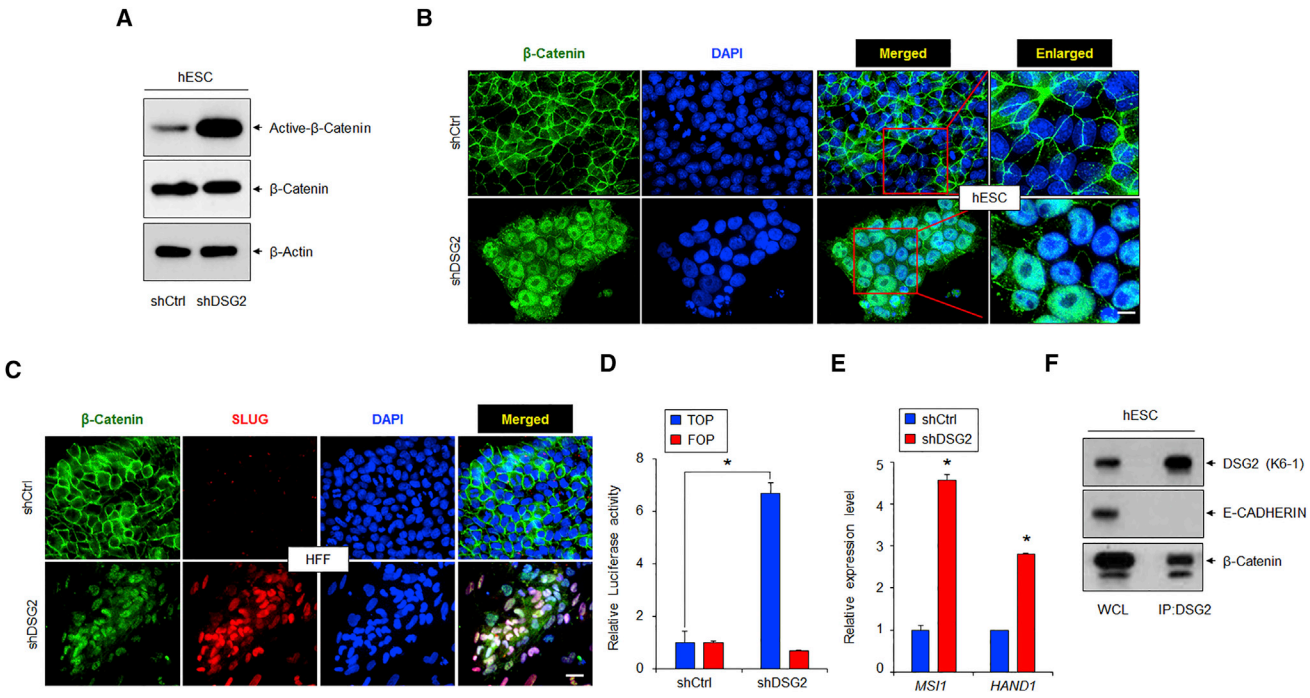

Figure 6. DSG2 Sequesters β-Catenin at the Cell Membrane to Suppress EMT Induction in Undifferentiated PSCs

(A) shCtrl and shDSG2 cell lysates were collected and analyzed by immunoblotting.

(B) The translocation of β-catenin to the nucleus was significantly increased in shDSG2 cells. shCtrl and shDSG2 hESCs were fixed and then stained with anti-β-catenin antibody. Right panels show magnification of boxed areas in merged images. Scale bar, 5 μm.

(C) shCtrl and shDSG2 reprogrammed HFFs were fixed on day 15 of reprogramming and stained with anti-β-catenin and anti-Slug antibodies. Scale bar, 50 μm.

(D) β-Catenin/TCF reporter assay in shCtrl and shDSG2 hESCs. Measurements are reported as the average luciferase units of experiments performed in triplicate for the TOP and FOP reporter constructs. Quantification from three independent assays is shown on the graph. *$p < 0.01$ versus shCtrl. Error bars indicate ±SEM.

(E) Expression levels of β-catenin target genes were determined by qPCR. *$p < 0.01$ versus shCtrl. Error bars indicate ±SEM.

(F) Co-immunoprecipitation of DSG2 and β-catenin in hESCs. DSG2 was immunoprecipitated with K6-1 antibody, and co-immunoprecipitated E-cadherin or β-catenin was detected by western blotting using the appropriate antibody.

β-catenin signaling in hPSCs, we first examined the total protein level of β-catenin via immunoblotting. Depletion of DSG2 did not alter the total levels of β-catenin. However, active nuclear β-catenin expression was significantly increased in DSG2-depleted hESCs (Figure 6A). Consistently, immunofluorescence analysis revealed that β-catenin accumulated in the nuclei of DSG2-depleted hESCs (Figure 6B). In addition, co-localization experiments illustrated the increased localization of β-catenin and Slug expression in the nuclei of DSG2-depleted HFFs compared with that in control cells during reprogramming (Figure 6C). To confirm whether the loss of DSG2 promotes β-catenin/T cell factor (TCF) signaling, we also used a luciferase-based transcriptional reporter assay (TOP-Flash reporter) to measure *β-catenin*-dependent transcription in Dsg2 shRNA-expressing HFFs. As shown in Figure 6D, *β-catenin*/TCF signaling was increased by approximately 6-fold in DSG2 shRNA-expressing HFFs relative to that in control cells. To further verify the increased β-catenin/TCF transcriptional activity, we analyzed the mRNA levels of the *β-catenin*/TCF target genes (Herbst et al., 2014) *MSI1* and *HAND1* in DSG2-depleted hESCs. In line with the reporter assay results, we observed upregulation of these target genes (Figure 6E), indicating that the loss of DSG2 promotes β-catenin nuclear localization and *β-catenin*/TCF-dependent transcription in hPSCs. Because DSG2 loss enhanced the nuclear localization of β-catenin, we next investigated whether β-catenin can be associated with DSG2 in a protein complex. Co-immunoprecipitation and co-localization experiments with DSG2 and β-catenin clearly demonstrated that DSG2 associates with β-catenin. However, we did not observe an interaction between DSG2 and E-cadherin in hPSCs (Figure 6F). Collectively, these results suggest that DSG2 sequesters β-catenin at cell-cell junctions to suppress its nuclear localization and transcriptional activity and that its loss leads to the differentiation

of PSCs through the induction of β-catenin/Slug axis-mediated EMT.

DISCUSSION

Recent advances in stem cell biology have resulted in the successful generation of iPSCs, which might overcome many scientific and ethical problems associated with cell-based therapies for human diseases (Robinton and Daley, 2012). Therefore, hiPSCs can serve as a promising source of cells for regenerative medicine, which aims to replace or restore tissue damaged by disease or injury through the transplantation of differentiated and functional hiPSCs (Trounson and DeWitt, 2016). However, given the cellular heterogeneity of cultivation, in which PSC lines are morphologically and phenotypically heterogeneous *in vitro*, and safety concerns regarding tumor formation *in vivo*, the development of specific and efficient tools to isolate pure populations and eliminate all residual undifferentiated PSCs from differentiated derivatives is a prerequisite for clinical applications. In the present study, we identified DSG2 as a valuable specific PSC surface marker that maintains self-renewal in the undifferentiated state and regulates the acquisition of pluripotency during somatic reprogramming. During PSC differentiation, DSG2 is rapidly downregulated and its loss promotes EMT via the β-catenin-mediated transcriptional activation of *Slug*, resulting in spontaneous differentiation. Therefore, we expect that DSG2 and antibodies against it can be useful for characterizing and isolating putatively therapeutic PSC populations and removing teratoma-forming cells.

A limited number of cell surface markers have been reported to be indicative of undifferentiated hPSCs, including carbohydrate-associated molecules such as TRA-1-60, TRA-1-81, SSEA-3, -4, and -5 and cell adhesion molecules, and claudin-6 (Ben-David et al., 2013; Choi et al., 2014; Son et al., 2011). However, most of them are directed against carbohydrate antigens and their exact functions are not fully elucidated. Moreover, some protein markers are also immunoreactive in differentiated somatic cells from PSCs, and thus are useful only within a limited lineage differentiation of PSCs (Ben-David et al., 2013; Ng et al., 2010; Son et al., 2011). Therefore, the identification of additional markers distinguishing undifferentiated PSCs from heterogeneous populations and differentiated derivatives can further assist in increasing the differentiation potential of pure PSCs and depleting teratoma-initiating cells. In addition, the development of antibodies against these markers will facilitate the efficient purification of PSCs from mixed populations of cultured PSCs. Furthermore, understanding the mechanisms maintaining the undifferentiated pluripotent nature of ESCs and iPSCs can help to resolve the heterogeneity and differentiation barriers.

DSG2 has been found to be expressed in the inner cell mass of mouse blastocysts and is involved in non-desmosomal adhesion (Eshkind et al., 2002; Johnson et al., 2014). Given the positive expression and downregulation of Dsg2 in hPSCs and differentiating EBs, respectively, we presented its expression during somatic cell reprogramming and its crucial role in the maintenance of self-renewal and pluripotency in PSCs. DSG2 surface expression was rapidly downregulated upon EB formation and RA treatment, and its suppression was substantially faster than that of well-known surface markers and transcription factors in hPSCs and mESCs; conversely, its induction occurred earlier than that of other hPSC surface markers, suggesting that DSG2 is more useful for determining the undifferentiation status and pluripotency of hPSCs than conventional surface markers in common use and that it is the definitive indicator for clarifying the reprogramming status of somatic cells. We also demonstrated that depletion of DSG2 resulted in significant decreases in hPSC proliferation and spontaneous differentiation. Furthermore, the expression of OCT4, NANOG, and SOX2 was markedly downregulated in DSG2-depleted hESCs, and a similar result was found in the DSG2-negative population compared with the findings in the Dsg2-positive population. DSG2-depleted hPSCs exhibited upregulated gene expression for various lineage markers, even in the undifferentiated state. By contrast, the expression of undifferentiated PSC markers was significantly decreased in DSG2-depleted hPSCs compared with that in control cells, indicating that DSG2 is essential for the maintenance of self-renewal and pluripotency in PSCs. Consequently, we observed that all mice with DSG2-positive cells yielded differentiated teratomas containing terminally differentiated cell types from all three germ layers, whereas DSG2-negative cells did not. Our results strongly suggest that DSG2 and its antibodies are useful for developing various strategies for eliminating residual undifferentiated PSCs from differentiated derivatives.

In somatic cell reprogramming, some technical issues such as low derivation efficiency, genome integration, and difficulties in selecting completely reprogrammed iPSCs remain unresolved problems (Yamanaka, 2009). Among these issues, identifying reliable surface markers is a prerequisite for isolating functional iPSCs. At present, EpCAM and E-cadherin are the most commonly used surface markers for characterizing iPSCs. However, these markers are useful for characterization only at the late stage of reprogramming. Thus, new optimal surface markers that are expressed at the early stage of reprogramming and are associated with the acquisition of pluripotency are essential for the determination and enrichment of

reprogramming cells. In this respect, our results revealed that DSG2 can be a useful marker for the identification and/or isolation of reprogramming hiPSCs. DSG2 is induced earlier than EpCAM and E-cadherin in fibroblasts during reprogramming. Interestingly, the introduction of DSG2 shRNA-expressing lentivirus resulted in a marked reduction in reprogramming efficiency. In addition, the expression of reprogramming surface marker signatures such as E-cadherin and EpCAM, as well as the elicitation of the pluripotency-associated genes, was significantly reduced in DSG2 shRNA-expressing HFFs. The present study reveals that DSG2 plays an important role in the acquisition of pluripotency in somatic cells during reprogramming and that DSG2 is a valuable surface marker for monitoring and distinguishing the stable reprogramming status of somatic cells.

This study provides further insight into the mechanism by which DSG2 regulates self-renewal and pluripotency in hPSCs. Recently, the expression of desmosome components has been demonstrated to be significantly associated with tumor progression and metastasis in several malignant tumors (Dusek and Attardi, 2011). The most extensively studied model suggests that desmosome dysfunction can provoke the release of specific desmosomal constituents such as plakoglobin, which is highly homologous to β-catenin (Al-Jassar et al., 2013). The cadherin-catenin adhesion complex is the key component of intercellular adherens junctions. When not bound to cadherins, β-catenin can translocate to the nucleus to stimulate the transcription of lymphoid enhancer factor (LEF)/TCF-dependent target genes (Behrens et al., 1996). Plakoglobin can also substitute for β-catenin in adherens junctions and localize to the nucleus, where it can regulate the transcription of LEF/TCF target genes (Butz et al., 1992). However, we did not observe plakoglobin expression in undifferentiated PSCs. Thus, we hypothesized that β-catenin replaces plakoglobin at intercellular adherens junctions in undifferentiated PSCs by binding to DSG2, leading to the sequestration of β-catenin at the cell membrane and, in turn, suppression of the transcription of LEF/TCF-dependent target genes. Indeed, we clearly observed that DSG2 directly associated with β-catenin at the PSC membrane. Loss of DSG2 induced β-catenin nuclear localization and activated LEF/TCF-dependent transcription. Our findings provide evidence that DSG2 maintains self-renewal and pluripotency in hPSCs through the regulation of β-catenin localization.

We further investigated the mechanism by which DSG2 loss-mediated β-catenin activation impairs the maintenance of self-renewal in undifferentiated PSCs and the acquisition of pluripotency during somatic cell reprogramming. The nuclear transactivation of β-catenin has been illustrated to upregulate the expression of the E-cadherin suppressor genes, Snail and Slug, to promote EMT in other cell types (Polyak and Weinberg, 2009). Recent studies have uncovered the dual function of Wnt/β-catenin signaling, which regulates the self-renewal and/or differentiation of hESCs (Huang et al., 2015; ten Berge et al., 2011). Short-term activation of Wnt/β-catenin signaling temporally enhances the self-renewal of hESCs by upregulating E-cadherin expression. However, its long-term activation increases free cytoplasmic β-catenin, leading to the translocation of β-catenin into the nucleus and upregulation of Slug expression. Subsequently, the upregulated Slug inhibits E-cadherin expression, and in turn leads to EMT, resulting in the definitive differentiation of hESCs (Huang et al., 2015). Noticeable Slug upregulation was observed in DSG2-depleted hESCs and the $DSG2^{low}$ hESC subpopulation. Consistent with this finding, E-cadherin expression was markedly downregulated in DSG2-depleted hESCs, whereas vimentin was upregulated, indicating that β-catenin-mediated Slug upregulation enforces hPSC differentiation through the induction of EMT in DSG2-depleted hPSCs.

It is also well established that MET is an essential event occurring in the early stage of reprogramming of MEFs as well as other cells (Shu and Pei, 2014). A recent study provided evidence that a sequential EMT-MET event can enhance the reprogramming of MEFs. This study demonstrated that the initial EMT event is necessary for the homogenization of heterogeneous MEFs or conversion of these cells into an optimal mesenchymal state that can be more efficiently reprogrammed into iPSCs (Liu et al., 2013; Shu and Pei, 2014). Thus, the initial introduction of reprogramming factors can enhance the reprogramming efficiency of MEFs due to Slug activation at the early phase of reprogramming, whereas prolonged activation of these factors represses the reprogramming of MEFs by Slug, leading to the inhibition of MET. Indeed, sustained Slug expression was observed in DSG2 shRNA-expressing HFFs compared with that in control HFFs. Conversely, we could not find noticeable E-cadherin expression in DSG2 shRNA-expressing HFFs or in Slug-expressing control HFFs, suggesting that Slug expression is necessary for the initial EMT event in the early phase of reprogramming, but sustained Slug expression during reprogramming precludes MET, resulting in incomplete reprogramming. These results explain why DSG2 is initially expressed at the early reprogramming stage and then is temporally suppressed at the middle reprogramming stage in HFFs.

In conclusion, we clearly showed that DSG2 is a valuable surface marker for determining the undifferentiation status of hPSCs and a stable reprogramming status of somatic cells, and is likely useful in combination with already known markers in characterizing and purifying undifferentiated hPSCs. Our results also demonstrate that DSG2 is essential for the maintenance of PSC self-renewal and

acquisition of pluripotency during somatic cell reprogramming through controlling β-catenin/Slug-mediated EMT. We believe that our results will help understand the heterogeneity and reprogramming barriers of somatic cells into iPSCs. In addition, antibodies against DSG2 and additional PSC-specific markers will be useful in the development of various strategies for eliminating residual undifferentiated PSCs and ensuring the safety of patients undergoing clinical trials using hPSC derivatives.

EXPERIMENTAL PROCEDURES

Dual-Luciferase Reporter Gene Assay

To evaluate Slug and TCF promoter activity following the loss of Dsg2 in hESCs and HFFs, we dissociated stable cells into single cells using Accutase or 0.05% trypsin/EDTA treatment. See Supplemental Experimental Procedures for additional experimental information.

Teratoma Formation

NOD scid gamma mice were obtained from The Jackson Laboratory. All mice used in the study were 6 weeks of age. Mouse care followed the guidelines of the Animal Care Committee of the Korea Research Institute of Bioscience and Biotechnology. The Dsg2-positive or -negative cells were dissociated using Accutase and the pellets were suspended in 1:1 (v/v) DMEM/F12 and Matrigel. Approximately 1 cm of abdominal skin was incised with fine scissors under isoflurane inhalation anesthesia. The testes were removed from the abdominal cavity using sterilized forceps, and cells were injected into each side of the testes using a 31-gauge Ultra-Fine syringe. See Supplemental Experimental Procedures for additional experimental information and further procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.05.009.

AUTHOR CONTRIBUTIONS

J.P. and Y.S. designed and performed experiments, analyzed the data, and wrote the manuscript. N.G.L., D.G.L., J.S., Jaemin Lee, and S.K. supplied the materials and performed experiments. M.J.C., J.-H.J., K.L., Jungwoon Lee, J.-G.P., and Y.-G.K. analyzed the data. Jungwoon Lee and Y.S.C. performed experiments and supplied cells. J.-S.K., Y.-J.P., B.S.H., and K.-H.B. supported the work with helpful comments and data analysis. S. Han, B.K., and S. Haam analyzed data and provided helpful comments. S.-H.L., S.C.L., and J.-K.M. supervised the overall project, analyzed the data, and wrote the manuscript.

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