Dextran sulfate prevents excess aggregation of human pluripotent stem cells in 3D culture by inhibiting ICAM1 expression coupled with down-regulating E-cadherin through activating the Wnt signaling pathway

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Abstract

Background: Human pluripotent stem cells (hPSCs) have great potential in applications for regenerative medicine and drug development. However, 3D suspension culture systems for clinical-grade hPSC large-scale production have been a major challenge. Accumulating evidence has demonstrated that the addition of dextran sulfate (DS) could prevent excessive adhesion of hPSCs from forming larger aggregates in 3D suspension culture. However, the signaling and molecular mechanisms underlying this phenomenon remain elusive.

Methods: By using a cell aggregate culture assay and separating big and small aggregates in suspension culture systems, the potential mechanism and downstream target genes of DS were investigated by mRNA sequence analysis, qRT-PCR validation, colony formation assay, and interference assay.

Results: Since cellular adhesion molecules (CAMs) play important roles in hPSC adhesion and aggregation, we assumed that DS might prevent excess adhesion through affecting the expression of CAMs in hPSCs. As expected, after DS treatment, we found that the expression of CAMs was significantly down-regulated, especially E-cadherin (E-cad) and intercellular adhesion molecule 1 (ICAM1), two highly expressed CAMs in hPSCs. The role of E-cad in the adhesion of hPSCs has been widely investigated, but the function of ICAM1 in hPSCs is hardly understood. In the present study, we demonstrated that ICAM1 exhibited the capacity to promote the adhesion in hPSCs, and this adhesion was suppressed by the treatment with DS. Furthermore, transcriptomic analysis of RNA-seq revealed that...
Background

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) [1] and human-induced pluripotent stem cells (hiPSCs) [2], have great potential as a robust cell source in regenerative medicine due to their capacity for self-renewal and multi-lineage differentiation potential [3]. In most previous studies, hPSCs were conventionally cultured in a two-dimensional (2D) adherent condition, allowing not only long-term expansion but also maintenance of hPSCs with high quality. However, this approach is highly dependent on feeder cells or commercial matrices [4, 5]. Moreover, 2D culture of hPSCs occupies a large space and consumes too much effort for scaling up production, for example, by using a multilayered cell factory [6], thus impeding the application of hPSCs in clinical treatment because of the difficulty in obtaining a large number of high-quality stem cells. For example, in clinical applications of cell-based therapies, at least one billion functional cells are required for each patient to restore the function of a damaged organ such as the liver [7], pancreas [8], or heart [9]. In order to solve this problem, three-dimensional (3D) suspension culture combined with bioreactors has been developed for large-scale production and integrated differentiation of hPSCs.

There are several kinds of 3D culture methods that have been established for large-scale expansion of hPSCs, including cell aggregates [10, 11], cells on microcarriers [12], and cells embedded in microcapsules [13]. Among these approaches, expansion of hPSCs in an aggregate form has been widely employed, because it is not only independent of biological materials or matrices (e.g., Matrigel) from animal origins, but it is also much easier to retrieve cells, making it more convenient than microcarrier/microcapsule-based approaches. By using cell aggregate approaches, researchers have achieved a yield of up to $1.5 \times 10^6$ hPSCs per milliliter with good maintenance of pluripotency, and the ability for long-term, good manufacturing practice (GMP) grade serial expansion of hPSCs [14]. Moreover, the expansion of hPSCs as aggregates under 3D suspension culture systems is also adaptive to different bioreactors, such as static bag [14], dynamic spinner [15], horizontal stirred bioreactor [16], and vertical-wheel bioreactor [17]. This suggests that the use of a suspension culture system with aggregates may be a powerful and promising method for the large-scale production of hPSCs.

However, the size of aggregates is a crucial parameter for hPSC doubling, retrieving and the integrated differentiation process into functional somatic cells [18]. The excessive aggregation of hPSCs in 3D suspension culture conditions hampers the differentiation process. Fazaneh et al. investigated the size of hPSC aggregates on their capacity for differentiating into definitive endoderm spheres, and they found that bigger aggregates resulted in a lower SOX17 positivity rate [19]. In addition, apoptosis is more likely to happen in cell aggregates with bigger sizes rather than those with smaller sizes in our study and others [20]. This may be due to the limited ability of nutrients and oxygen to diffuse toward the interior of the bigger hPSC aggregates, leading to the generation of hypoxia and necrosis in the central of aggregates [21–23]. Therefore, it is important to control the size of aggregates in developing repeatable and high-performance hPSC large-scale production.

Researchers have attempted several physical and biochemical approaches to produce size-controlled and homogeneous hPSC aggregates under suspension culture conditions. One simple approach to obtain the size-controlled hPSC aggregates was by seeding a determined number of cells into microwell culture plates [24]; however, this method could not be combined with bioreactor suspension culture systems and was limited for hPSC large-scale expansion. The most reported approach was utilizing different agitation rates of spinner flasks or bioreactors to suppress excessive aggregation of hPSCs [25, 26]. Employing impeller stirring ensured the homogeneous distribution of nutrients and gases in suspension culture. The stirred-type bioreactor has been extensively used in manufacturing traditional biological products, which emphasized the increased production of the proteins, such as recombination proteins generated by Chinese hamster ovary (CHO) cells, rather than the quality of cells [27, 28]. However, the purpose of hPSC
large-scale expansion is to harvest high-quantity and high-quality stem cells and overly high shear stress has a negative effect on cell viability and differentiation [29–31]. Thus, bioreactors which control aggregate size by operating at a high rotational speed may not constitute the best solution for hPSC suspension culture. A better approach may be to avoid excessive adhesion between aggregates by adding chemical reagents, such as methylcellulose [14], knockout serum replacement (KSR) [32], lysophosphatidic acid [33], sphingosine-1-phosphate [33], or dextran sulfate (DS) [34]. Among these chemical reagents, DS, a polysulfated compound, has been widely employed in various cell systems of the biopharmaceutical industry to reduce cell aggregation [35, 36]. Previous studies, including ours, have demonstrated that DS exhibited excellent performance in preventing excessive aggregation under hPSC suspension culture conditions without compromising cell viability and cellular pluripotency [23, 34, 37]. However, the signaling and molecular mechanisms underlying this phenomenon remain elusive. For hPSCs, cellular adhesion molecules (CAMs) play an important role in cell adhesion, attachment, and cell aggregation [38, 39], suggesting that the expression of CAMs may affect aggregation of hPSCs under suspension culture conditions. Therefore, we speculated that DS prevents hPSC aggregate adhesion through affecting the expression of CAMs. To test this hypothesis, we evaluated the expression of CAMs after DS treatment and determined the potential contribution of this signaling pathway.

Materials and methods

hPSC culture and maintenance
The hESC line, H9, was obtained from the WiCell Research Institute (Madison, WI, USA) under a Materials Transfer Agreement (No. 19-W0512) and was experimented during the 38th–52nd passages. The hiPSC line was provided as a gift by Dr. Liangxue Lai and was experimented during the 24th–29th passages. The hiPSC line was obtained from the University of Wisconsin-Madison Research Institute (Madison, WI, USA) under a Material Transfer Agreement (No. 19-W0512) and was experimented during the 38th–52nd passages. The hiPSC line was obtained from the University of Wisconsin-Madison Research Institute (Madison, WI, USA) under a Material Transfer Agreement (No. 19-W0512) and was experimented during the 38th–52nd passages. The hiPSC line was provided as a gift by Dr. Liangxue Lai and was experimented during the 24th–29th passages. The hiPSC line was provided as a gift by Dr. Liangxue Lai and was experimented during the 24th–29th passages.

Aggregate suspension culture of hPSCs
To initiate suspension culture with aggregates, hPSC colonies cultured on hESC-qualified Matrigel-supported adherent systems were dissociated into single cells by Gentle Cell Dissociation Reagent (GCDR, STEMCELL Technologies, 07174), and then cells were counted by hemocytometer using trypan blue (Solarbio, C0040) staining and seeded into ultra-low attachment 6-well plates (Corning, 3471) at a cell density of 2 × 10⁵ cells/ml, and cultured in mTeSR1 medium with 10 µM Y-27632 (Selleck, S1049) in normoxic (21% O₂) conditions for static suspension culture to form the aggregates. The medium was exchanged daily by angling the plates at 45° to allow aggregates to settle onto the bottom edge, and the DS compound was mixed with culture medium throughout the entire culture process at a final concentration of 100 µg/ml. Five days later, the morphology of hPSC aggregates was observed and photographed by phase contrast microscope. Cell counting evaluation was performed using a trypan blue staining after dissociation of aggregates into single cells by TrypLE (Thermo Fisher, 12604021). The diameter of aggregates was measured by ImageJ software.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Total RNA was extracted using RNAiso Plus kit (Takara, 9109) according to the manufacturer’s manual. Following quantification in a NanoDrop microspectrophotometer (Thermo Fisher), 1 µg of RNA was used to synthesize cDNA using the PrimeScript™ RT Master Mix (Takara, RR036B). qRT-PCR was performed in triplicate using PowerUp™ SYBR™ Green (Thermo Fisher, A25742) on the Quant Studio™ 1 Real-Time PCR system (ABI, ABI7500). CT values for each sample were normalized against the corresponding expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression levels were quantified using the 2−ΔΔCT method. The primer sequences for qRT-PCR used in the present study are listed in Additional file 1: Table S1.

Flow cytometry (FC) analysis
hPSC colonies were dissociated into single cells by treatment with TrypLE, and cells were fluorescently labeled by incubation with PE antihuman OCT4 (OCT3) antibody (STEM CELL, 60093PE), PE antihuman TRA-1-81 antibody (STEM CELL, 60065PE), PE antihuman SSEA-4 antibody (STEMCELL, 60062PE), or PE mouse isotype-controlled antibody (BD, 556650).
Fluorescence-positive cells were then detected using a BD FACS Celesta flow cytometer.

**Immunofluorescence (IF) staining**

Aggregates were collected, washed with PBS, and fixed overnight in 4% paraformaldehyde at 4 °C, and then permeabilized with 0.5% Triton X-100 for 20 min at 4 °C. Three washes with PBS were included between each step. Following washing, the samples were incubated in blocking buffer containing goat serum for 30–60 min at room temperature, then incubated in PBS containing primary antibodies such as rabbit anti-beta catenin antibody (Bioss, bs-23663R, 1:500) overnight at 4 °C followed by rewarming to room temperature and incubation in PBS containing secondary antibodies such as Alexa Fluor 594-conjugated goat anti-rabbit IgG (Cell Signaling Technology, 8889S, 1:800) for 1 h in the dark at room temperature. Nuclei were counterstained by incubation with DAPI (Solarbio, C0065) for 5 min, and the fluorescence signal was imaged on the single photon confocal microscopy (Ti-E A1, Nikon). The details of all antibodies used in the present study are listed in Additional file 2: Table S2.

**Western blot (WB)**

Cells were lysed in RIPA lysis buffer (Solarbio, R0020) supplemented with PMSF (Solarbio, P0100) on ice. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, C0037). CCK-8 reagent was added to each well and incubated for 1 h, and the absorbance was read at 450 nm and recorded using a microplate spectrophotometer.

**Single-cell cloning assay**

Ten thousand single cells of the H9 cells were inoculated on hESC-qualified Matrigel in a 6-well plate and cultured in mTeSR1 with 10 μM Y27632 on day 1; 100 μg/ml DS, 10 μM A-205804, DS plus 5 μM IWR-1-endo (Selleck, S7086), or 5 μM IWP2 (Selleck, S7085) were added in the experimental groups. Five days after seeding, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and colonies were visualized by alkaline phosphatase (AP) staining kit (Beyotime, C3206) according to the manufacturer’s instructions. The efficiency of cloning by single cell was assessed by counting the percentage of colony number versus cell number inoculated, and the area of colonies was calculated by ImageJ software. To count the cell number 24 h after inoculation, 1*10^5 single cells of H9 were inoculated on hESC-qualified Matrigel in a 12-well culture plate and cultured in mTeSR1 supplemented with 10 μM Y27632, 100 μg/ml DS, 10 μM A-205804, DS plus 5 μM IWR-1-endo, or 5 μM IWP2. Y27632 was removed by freshing the medium 12 h after seeding. The representative images of each group were observed and photographed by phase contrast microscope, and cell numbers were calculated by hemocytometer using trypan blue 24 h after culture.

**Integrated 3D hepatic differentiation of hPSC aggregates**

The derivation of hepatic spheres from hPSC aggregates was performed as previously described [19]. In brief, after 5 days of culture, aggregates cultured with or without DS were collected, washed with PBS and then transferred to differentiation medium directly in an ultra-low 6-well plate. For the induction of endodermal cells, the basal medium consisted of RPMI 1640 (Thermo Fisher, 61870036), 1 × B27 (Gibco, 17504044) and 0.1% bovine serum albumin (BSA, Sigma, SRE0098), 6 μM CHIR99021 (Selleckchem, S1263) was added on day 1, and then 10 ng/ml Activin A (Peprotech, 120-14) was added for 2–3 days. For hepatic differentiation, aggregates were treated in DMEM/F12 (Gibco, 11330032) supplemented with 2% KSR, 10 ng/ml fibroblast growth factor 4 (FGF-4) (Peprotech, 100–31), and 10 ng/ml hepatocyte growth factor (HGF) (Peprotech, 100–39) for 6 days. Finally, for hepatocyte maturation, they were treated in the same medium plus 50% hepatocyte culture media without EGF (HCM, Lonza, CC-3198), 10 ng/ml oncostatin M (Peprotech, 300–10), and 10⁻⁷ M (ICAM1 inhibitor, Selleck, S2885). The cell viability was measured using a Cell Counting Kit-8 (CCK-8, Beyotime, C0037). CCK-8 reagent was added to each well and incubated for 1 h, and the absorbance was read at 450 nm and recorded using a microplate spectrophotometer.
dexamethasone (Sigma, D4902) for another 12 days. On
day 21, the hepatic spheres were collected and analyzed.

ICAM1 knockdown
The doxycycline (Dox)-inducible short hairpin RNA (shRNA) targeting the human intercellular adhesion
molecule 1 (ICAM1) gene and non-targeting scrambled
shRNA (shNT), conjugated with GFP cloned in a lenti-
virus vector, were purchased from GeneCopoeia. The
target sequence of shNT was GCT TCGCCCCGTAGT
CTTA, the target sequences of shRNAs included four
groups designated as a, b, c, and d, and their sequences
were GCT GAC GTG TGC AGT AAT ACT, GCC AGC TTA
CTT A, the target sequences of shRNAs included four
select the most efficient shRNA for further knockdown
efficiency of knockdown of ICAM1 by qRT-PCR. The fluorescence images were observed and photographed by JuLI Stage Real-Time Cell History
Recorder (NanoEntek, JS1000S).

Subcellular fractionation
The H9 aggregates with different treatments were col-
lected, washed with PBS, then harvested in 350 μl of
hypotonic buffer (10 mM Hepes, pH 7.4, 42 mM KCl,
5 mM MgCl₂), and incubated for 30 min on ice. The
cells were lysed by passing through a 25-gauge needle 10
times. The cytosolic fraction (supernatant) was collected
by centrifugation at 200 g for 10 min at 4 °C. The nuclear
pellet was washed twice by resuspending in 500 ml of
hypotonic buffer and passed through a 25-gauge need-
le10 times. The nuclei were pelleted by centrifugation at
3000 g for 10 min at 4 °C. Isolated cytosolic and nuclear
fractions were resuspended in lysis buffer, respectively.
The loading buffer was added, followed by boiling for
5 min, and then subjected to immunoblot analysis.

Statistical analysis
Data are expressed as the mean ± standard deviation
(n = 3). Statistical analysis was performed using Graph-
Pad Prism 6; the unpaired Student’s t test and one-way
ANOVA (Tukey correction for multiple comparisons)
were used to evaluate statistical significance. Differences
were considered statistically significant at P < 0.05.

Results
DS treatment prevents excessive aggregation
and promotes hepatic differentiation of the H9 cells in 3D
description culture
In line with previous studies, including ours [23, 34],
the addition of 100 μg/ml DS significantly decreased the
diameter of H9 aggregates on day 5 in 3D static suspen-
sion culture conditions (Fig. 1A–C). More small-sized
and homogeneous aggregates with reduced standard
deviations were observed in the presence of DS. The

Fig. 1 The effect of DS on H9 aggregate sizes and further integrated hepatic differentiation in 3D suspension culture conditions. A Representative images of the H9 aggregates on day 5 after treatment with 100 μg/ml DS. Scale bar = 200 μm. B Comparison of average diameter of the H9 aggregates treated with or without DS on day 5. C Comparison of the cell numbers after 5 days of culture. D Representative images of hepatic spheres on day 15 after hepatic differentiation induction, the morphology of hepatic spheres in the control group was big and hollow, and it was relatively small and dense in DS group. Scale bar = 200 μm. E Comparison of average diameter of the hepatic aggregates from control and DS group at day 21 of hepatic differentiation. F Gene expression analysis by qRT-PCR for hepatic marker genes AFP, ALB, HNF4A, and A1AT from different treatment on the H9 aggregates. (H) Gene expression analysis by qRT-PCR for CYP450 including CYP3A4, CYP1A1, CYP1B1, CYP2C9, and CYP2E1. Relative gene expression represents data normalized to GADPH and expressed relative to undifferentiated H9. Data represent the mean ± SD. **P < 0.05, ***P < 0.01, and ****P < 0.001. Abbreviations: AFP, α-fetoprotein; ALB, albumin; HNF4A, hepatocyte nuclear factor 4 alpha; A1AT, alpha-1 antitrypsin; CYP, cytochrome P450 family.
Fig. 1 (See legend on previous page.)
average diameter of aggregates reached $403 \pm 182 \ \mu m$ in the absence of DS, which was 50% bigger than that of aggregates in DS group (Fig. 1B). Less than 5% of the aggregates had an average diameter more than 400 μm after DS treatment, as opposed to 30% in the control group (Fig. 1C). In addition, DS treatment did not reduce the proliferation capacity of H9 aggregates (Fig. 1D). In addition, these results were repeated with hiPSCs and obtained similar tendency (Additional file 4: Fig. S2A-D).

To estimate the effect of DS on further 3D differentiation of H9 aggregates, we collected the aggregates with or without DS treatment after 5 days of static suspension culture and transferred them into hepatic differentiation medium [19]. After 15 days of differentiation, the hepatic spheres were collected for analysis. Similar to the situation in mTeSR1, DS-treated small-sized aggregates differentiated into smaller and relatively dense hepatic spheres ($208 \pm 57 \ \mu m$). Without DS treatment,
Fig. 3 (See legend on previous page.)
bigger aggregates became cystic and bigger-sized hepatic spheres (412 ± 133 μm) after the differentiation process (Fig. 1E, F). Furthermore, the hepatic spheres in the DS-treated group expressed greater levels of mature hepatocyte markers like albumin (ALB), HNF4A and α1-antitrypsin (A1AT), and lower levels of the immature hepatocyte marker, alpha fetoprotein (AFP) (Fig. 1G), indicating that DS treatment promoted the hepatic differentiation of hPSC aggregates in suspension culture due to a relative smaller size of aggregates. Moreover, the expression of several important phase I drug-metabolizing enzymes, including CYP3A4, CYP1A1, CYP1B1, CYP2C9, and CYP2E1, were also significantly higher than those in the control group (Fig. 1H). These results demonstrated that DS treatment not only enabled the formation of uniform and small-sized aggregates, but it also was beneficial for 3D hepatocyte differentiation [23].

**Distinct CAMs expression after DS treatment**

Previous studies suggested that, for hPSCs, the differently adhesive ability of aggregates in suspension culture correlated with the expression of CAMs [40]. Therefore, we speculated that the prevention of excessive aggregation by DS might be caused by differentially expressed CAMs. To verify this hypothesis, we first compared the expressions of a panel of adhesion-related genes and pluripotency-related genes between DS groups and control groups by qRT-PCR. Notably, genes related to extracellular matrix (e.g., vitronectin (VTN) and extracellular matrix protein 1 (ECM1)), a variety of integrin molecules (e.g., integrin subunit alpha 5 (ITGA5), integrin subunit alpha 7 (ITGA7) and integrin subunit beta 2 (ITGB2)), genes directly associated with cell adhesion (e.g., cadherin 3 (P-cad) and platelet/endothelial cell adhesion molecule 1 (PECAM)), and natural inhibitors of the matrix metalloproteinases (e.g., tissue inhibitors metalloproteinases2/3 (TIMP2/3)) were down-regulated after treatment with DS (Fig. 2A, B). In addition, the expression of transforming growth factor beta induced (TGFβi) was up-regulated after DS treatment, which would result in inhibiting cell adhesion (Fig. 2B) [41]. However, the expressions of pluripotent genes OCT4, SOX2, and NANOG did not exhibit significant differences (Fig. 2C). In terms of genes related to CAMs, the expression of E-cadherin (E-cad) and ICAM1 which are highly expressed in H9 cells was also significantly down-regulated after DS treatment (Fig. 2D). E-cad is highly correlated with hPSC adhesion and attachment [38, 42]. For various tumor cell lines, ICAM1 plays an important role in cell aggregation under suspension cultures [39, 43]; however, to the best of our knowledge, there are no reports about the roles of ICAM1 in hPSC adhesion. Therefore, we focused on the roles of E-cad and ICAM1 to determine whether DS functioned through these two CAMs.

**Distinct expressions of ICAM1 between big and small aggregates**

By using repeated natural sedimentation, we separated H9 aggregates which were expanded under static suspension culture conditions for 5 days into big and small aggregates, respectively (Fig. 3A). The average diameter of big aggregate group was 400 ± 153 μm which is nearly twofold of that of small aggregate group (Fig. 3B), and more than 60% of the aggregates were bigger than 400 μm in the diameter. Approximately 90% of the aggregates were smaller than 250 μm in diameter in the small aggregates group (Fig. 3C). However, qRT-PCR results revealed that there was no obvious difference on the expression of pluripotent genes and E-cad between the two groups (Fig. 3D, E). Notably, among the CAMs detected, only ICAM1 was expressed differentially, which was fivefold higher in the big aggregate group than in the small one (Fig. 3F). The difference of ICAM1 between two groups was further confirmed by protein expression through western blot analysis, while the protein level of E-cad remained comparable (Fig. 3G–I). These results indicated that ICAM1 might play an important role in affecting the size of aggregates or adhesion ability of aggregates in 3D suspension cultures.

(See figure on next page.)

**Fig. 4** Controlling the size of the aggregates of the H9 cells by ICAM1 inhibitor A-205804. **A** Representative images of the aggregates of the H9 cells after treatment with various concentrations of A-205804 on day 5. Scale bar = 200 μm. **B** Comparison of average diameter of the aggregates of the H9 cells after treatment with various concentrations of A-205804 on day 5. **C** Diameter distribution of the aggregates of the H9 cells after treatment with various concentrations of A-205804 on day 5. **D** Cell viability of the H9 cells assayed by CCK-8 after treatment with various concentrations of A-205804. **E** The protein levels of E-cad and ICAM1 were determined by western blotting in aggregates of the H9 cells after treatment with various concentrations of A-205804. **F, G** The densitometry for the protein levels of E-cad and ICAM1 were quantitated in E; GAPDH was used as a loading control. **H** Representative images show the addition of 50 μM A-205804 to the inoculum abolished aggregate formation completely, Scale bar = 200 μm. **I** AP staining of the H9 colonies with or without adding A-205804, single cells were seeded in six-well culture plates coated with Matrigel, 10 μM Y-27632 was added on day 1 in both groups. Representative images are shown. Scale bar = 200 μm. **J** Quantification of the efficiency of AP positive colonies in I. (K) Quantification of the average colony area in I. Data represent the mean ± SD. *P < 0.05 and **P < 0.01 and ***P < 0.001
Fig. 4 (See legend on previous page.)
Modulation of the size of aggregates by regulating the expression of ICAM1

To assess whether regulation of ICAM1 expression could modulate the size of aggregates in 3D suspension cultures, we utilized the ICAM1 specific inhibitor, A-205804 to test this hypothesis. After treatment with various concentrations of A-205804 under 3D suspension culture condition, we found that the size of aggregates exhibited a tendency to decrease in a dose-dependent manner (Fig. 4A–C). 67% of the aggregates were smaller than 250 μm in diameter, and only 3% of the aggregates were bigger than 400 μm in diameter in the group treated with 10 μM A-205804, while only 30% of the aggregates were smaller than 250 μm in diameter, and 25% of the aggregates were bigger than 400 μm in diameter in the control group (Fig. 4C). Importantly, no obvious difference in cell viability was observed regardless of A-205804 concentrations investigated (Fig. 4D), indicating that the decrease in the aggregate size was not the results of toxicity caused by the higher dose of the inhibitors. As expected, the expression level of ICAM1 protein also was decreased with the increased dose of A-205804 (Fig. 4E–G), but E-cad levels remained unchanged (Fig. 4E, F), consistent with aforementioned results. In addition, by adding 50 μM A-205804 into medium at the time point of seeding, we found that high-dosed ICAM1 inhibitor would impede the aggregation of the H9 cells, whereas formation of aggregates was observed in the control group as expected (Fig. 4H). This concentration of A-205804 did not affect cell viability (Fig. 4D), indicating that the expression of ICAM1 is important for aggregate formation, and it appears that the use of the inhibitor was an effective approach to decrease the size of aggregates through interfering with ICAM1 expression in 3D suspension cultures. To further investigate whether ICAM1 has an effect on extracellular adhesion during the proliferation of hPSCs, we performed a single-cell cloning assay with or without treatment with 10 μM A-205804. After 5 days of culture, the formation efficiency of the alkaline phosphatase (AP)-positive colonies and the size of the average colony area in the control group were significantly higher than in the conditioned group (Fig. 4I–K), demonstrating that the expression of ICAM1 was crucial to extracellular adhesion even in 2D adhesion culture conditions.

Next, knockdown of endogenous ICAM1 by shRNA was performed to further determine the function of ICAM1 expression on extracellular adhesion. A homogenous population of EGFP-positive colonies was observed (Fig. 5A), indicating a high transduction rate of a lentiviral vector co-expressing shNT/shRNAs and EGFP in the H9 cells. Through screening the knockdown efficiency of ICAM1-shRNAs (Fig. 5A, B), shRNA-b which significantly down-regulated ICAM1 was selected for further 3D suspension culture experiments. Dox was added to both groups, shNT and shRNA-b, to a final concentration of 1 μg/ml to induce the expression of shRNA. On day 5 after culture, cells transduced with shRNA-b exhibited significant smaller-sized aggregates when compared to those in the shNT group (Fig. 5C, D), further demonstrating that ICAM1 was an important factor in the induction of cellular adhesion. Although the expression of ICAM1 was significantly down-regulated, the expression of OCT4, one of the most important pluripotent genes, remained unchanged (Fig. 5E), indicating that ICAM1-shRNA only interfered with the expression of ICAM1 rather than affecting the pluripotency of the H9 cells. The expression of ICAM1 protein was further confirmed by western blot analysis (Fig. 5F, G). These results together showed the importance of the expression of ICAM in hPSCs for cell adhesion and for controlling aggregate sizes in 3D suspension culture.

Disturbance of aggregate adhesion by DS-mediated E-CAD reduction acted through the activation of the canonical Wnt signaling pathway

Previous studies have revealed that in 3D suspension cultures of hPSCs, the secreted Wnt antagonists were significantly up-regulated and led to the down-regulation of canonical Wnt-targeted genes when compared with 2D adherent cultures, and they further enhanced the expression of the central adherens junction components, such as E-cad [44]. In our previous study, we
performed RNA-seq analyses to investigate the mechanisms by which DS treatment controlled the size of the H9 cell aggregates [23]. In the present study, as indicated by KEGG analysis, we determined that the Wnt signaling pathway was significantly up-regulated after DS treatment (Fig. 6A). These results were further verified by qRT-PCR and western blot analysis. Several Wnt ligands (WNT4, WNT7B, WNT8A, and WNT10B), receptors (FZD5 and FZD8), and a gene involved in the Wnt signaling pathway (LEF1) were highly expressed, associated with the decrease in E-CAD after DS treatment (Fig. 6B). Furthermore, with the up-regulation of Wnt signaling, we observed a higher translocation of β-catenin into the nucleus by immunofluorescence staining and western blot analysis (Fig. 6C, D), indicating the activation of the canonical Wnt signaling pathway [45]. In addition, there are several transcription factors (TFs) which are Wnt target genes down-regulating the expression of E-cad, including the twist family bHLH transcription factor (Twist), snail family transcriptional repressor (Snai), and the matrix metallopeptidase (MMP) families [46, 47]. We detected the gene expression patterns of these three families by qRT-PCR and found that the expressions of Twist1, Snai2, MMP3, and MMP7 were significantly up-regulated after DS treatment in the H9 aggregates under 3D suspension culture conditions (Fig. 6E), and the protein expression levels of these TFs were further confirmed by western blot analysis, which was consistent with the qRT-PCR analysis (Fig. 6B, E, F). These results indicated that DS treatment could suppress the expression of E-cad through activating the canonical Wnt signaling pathway.

The expression of E-cad in hPSCs is indispensable for cell adhesion and aggregation under 3D culture conditions [38, 42]. Thus, we further performed single-cell colony assay to confirm whether DS treatment could disturb the adhesion of hPSCs and to verify whether Wnt signaling inhibitors could rescue this phenomenon under 2D culture conditions. As expected, the formation efficiency of the single-cell colony and the average area of colonies were decreased after DS treatment (Fig. 7A–C). However, the colony-forming efficiency and the average area of colonies could be rescued partially by adding Wnt inhibitors, 5 μM IWR-1 and 5 μM IWP2, respectively (Fig. 7A–C). In order to decouple the effect of proliferation on colony formation assay, we also counted the cell number 24 h after inoculation. As expected, the cell number was decreased prominently after DS treatment and could be rescued by Wnt inhibitors (Additional file: Fig. S3A–B). Moreover, we also conducted rescue assay with Wnt inhibitors under 3D suspension culture condition. By adding 5 μM IWR-1 or 5 μM IWP2 with DS into mTeSR1, the average size of aggregates was notably bigger than those treated with DS only, but still smaller than aggregates in control group (Additional file: Fig. S4A–C) after 5 days of culture. Interestingly, the expression of E-cad was also up-regulated after the treatment with Wnt inhibitors (Additional file: Fig. S4E). In addition, there is still no obvious difference on the cell number between each group (Additional file: Fig. S4D). These results further demonstrated that DS functioned through the Wnt signaling pathway.

In order to determine whether the functions of DS on hPSC aggregates could be replaced by ICAM1 inhibitor and Wnt agonist directly, further investigation was performed. To address this issue, we replaced DS with 10 μM A-205804 plus 3 μM CHIR99021 or 10 ng/ml Wnt3A (both are Wnt agonist) and cultured H9 aggregates for 5 days. After the treatment with ICAM1 inhibitor and WNT agonist, the sizes of aggregates were not uniform and exhibited heterogeneity (Additional file: Fig. S5A) when compared to those in control; moreover, these H9 aggregates no longer maintained the stable expression of three core pluripotent genes, OCT4, SOX2, and NANOG (Additional file: Fig. S5B). Surprisingly, 5 days after the aforementioned treatment, H9 aggregates spontaneously differentiated into three germ layers (mesoderm, endoderm, and ectoderm) determined by qRT-PCR analysis (Additional file: Fig. S5C). It appears that the effect of DS could not be replaced by WNT agonist.
Fig. 6 (See legend on previous page.)
WNT signaling pathway is a more complicated system and plays a large number of functions in cell; DS treatment most likely induced a specific pathway indicated in our study; however, WNT agonist might induce more pathways which might inhibit the pluripotency and trigger the differentiation of hPSC. Thus, the mechanism of DS treatment on hPSCs still needs future work to investigate thoroughly.

Taken together, our results revealed that DS treatment interfered with the expression of E-cad through activation of the canonical Wnt signaling pathway and further co-modulated the adhesion of hPSC aggregates with DS-mediated ICAM1 reduction in 3D suspension culture conditions (Fig. 7D).

Discussion

The unlimited self-renewal ability and multi-lineage differentiation potential of hPSCs emphasize their potential use in clinical treatment and in the pharmaceutical industry [48, 49]. In order to take full advantage of hPSCs, large-scale expansion is indispensable in order to obtain enough cells for further applications. The bottleneck for large-scale production of clinical quantities of hPSCs currently is the lack of an appropriate scalable bioprocess protocol [50].

By utilizing stirred-tank bioreactors, several laboratories have established expandable protocols for hPSC aggregates using 3D suspension culture systems [10, 11, 17, 25, 26, 51]; the best of these protocols obtained 70-fold cell expansion in 7 days and achieved a density of $3.5 \times 10^7$ cells/ml [26]. However, the major limitation still unsolved was the complex hydrodynamics and high shear stress at the tip of impeller, which could be injurious to stem cell viability and differentiation [29–31]. Furthermore, it was not as easy as reported to control aggregate sizes by different agitation rates in stirred-tank bioreactors [17]. Borys and colleagues demonstrated that they failed to generate consistent hPSC aggregates in all tested agitation rates in horizontal-blade stirred-tank bioreactors, most likely because aggregates were limited from moving throughout the entire volume of the bioreactor [17]. Actually, controlling hPSC aggregate sizes in 3D suspension culture systems is the most difficult and critical process. To date, there has not been a good solution for preventing hPSC excess adhesion and aggregation formation, thus limiting the production of clinical quantities of hPSCs. In general, properly combining the physical approaches with biochemical approaches may be the best solution to control aggregate sizes of hPSCs. For example, by stirring the impellers at a low agitation rate, it not only avoids generating harmful high shear stress and enabling the distribution of gas and nutrients, but supplementing with DS further prevents adhesion among aggregates [23]. This combination should lead to the harvesting of high-quality and high-quality hPSCs with homogeneous-sized hPSC aggregates, thus limiting various differentiation trends caused by a variety of the sizes of aggregates [18, 19]. Therefore, it is necessary to understand the molecular mechanism of DS in 3D suspension culture of hPSCs.

We and others have shown that DS is effective in controlling the size of the hPSC aggregates in 3D suspension culture conditions [23, 34, 37]. However, the molecular changes after DS treating on hPSCs are hardly understood. In our previous research, we have compared the transcriptome of hPSCs after treatment with DS or control groups, and many genes and pathways related to cell adhesion were found to be expressed differentially [23]; this guided us to further explore the molecular mechanisms. In the present study, we revealed the regulatory mechanism of DS treatment in controlling the aggregate size of hPSCs in 3D suspension culture conditions. First, the expression of CAMs such as ICAM1 was down-regulated after DS treatment. In addition, DS stimulated hPSCs to activate the expression of Wnt ligands and receptors, further promoting the expression of canonical Wnt signaling pathway target genes such as Twist1, SnaI2, MMP3, and MMP7, consequently suppressing the expression of E-cad (Fig. 7D).

A number of reports revealed that the CAMs-mediated cohesive interaction among cells contributed significantly to the self-renewal and the pluripotent state of hPSCs [52, 53]. Moreover, the strong expression of CAMs such
Fig. 7 (See legend on previous page.)
as E-cad is also related to somatic cell reprogramming of iPSCs. The enhancement of E-cad could promote the reprogramming efficiency of iPSCs and even replace the demand for OCT4 during the iPSC reprogramming [54, 55]. E-cad has also been used as an undifferentiated marker to identify pluripotent stem cells [56], indicating the importance of CAMs in regulating the pluripotency and stemness of hPSCs [57]. The expression of E-cad directly affects the differentiation potential and proliferation rate of hPSCs [58]. However, after DS treatment, the multi-lineage differentiation ability and proliferative capacity of hPSC aggregates have not been obviously altered [23], suggesting that the regulation of E-cad and other CAMs by DS impacts on aggregate adhesion rather than the stemness of hPSCs. The expression of TIMP2/3 is also bound up with E-cad. They complex with metalloproteinases such as MMP families (e.g., MMP3/7) and irreversibly inactivate MMP3/7 by binding to their catalytic zinc cofactor. However, the expression of TIMP2/3 was down-regulated significantly with DS treatment, resulting in the over-expression of MMP3/7 genes, and further interfering with the expression of E-cad on hPSCs [59].

ICAM1 is a transmembrane glycoprotein in the immunoglobulin superfamily, regulating signal transduction and cell–cell adhesion [60]. ICAM1 has been reported to mediate adhesion-dependent cell–cell interactions and to play an important role in regulating the size of tumors and the spherical sizes of cancer cell lines in vivo and in vitro [43, 61, 62]. Furthermore, ICAM1 is also expressed on stem cells, including bone marrow mesenchymal stem cells, periodontal ligament stem cells, and adipose stem cells [63, 64]. However, the function of ICAM1 in hPSCs has not been investigated.

In this study, we first revealed that the expression of ICAM1 was important for the adhesion of hPSCs during the culture. Furthermore, the expression of ICAM1 was suppressed by DS treatment of hPSCs in 3D suspension conditions and the inhibition of ICAM1 prevented the adhesion among the aggregates of hPSCs, and finally reduced the heterogeneity of aggregate diameters.

The insights acquired in the current study increased our understanding of how DS prevented the adhesion among the aggregates of hPSCs. Previous studies suggested that the surface charge of CHO cells was altered by DS [35] and thus prevented the adhesion between CHO cells. Taken together, our results suggest that DS treatment prevented the adhesion through suppressing the expression of CAMs coupled with activating the canonical Wnt signaling pathway to suppress the expression of E-cad, thus co-regulating the size formation of the aggregates of hPSCs in 3D culture system.

Conclusion

In the present study, we demonstrated that DS controlled the size of hPSC aggregates in 3D suspension culture through co-regulating the expression of CAMs, E-cad and ICAM1. By suppressing the expression of ICAM1, together with activating the canonical Wnt signaling pathway for further suppressing the expression of E-cad, the cell adhesion ability of hPSCs was attenuated and the sizes of aggregates were decreased. This in turn will allow for the retrieval of high-quality hPSCs and for enhancing the integrated differentiation process. The aggregate large-scale expansion strategy of hPSCs with the addition of DS is highly promising, and the results of this study may be helpful for improving the use of DS in 3D suspension cultures of hPSC in the future.
Additional file 7: Fig. S5. Simulation of the effect of DS by replacing DS with ICAM1 inhibitor and Wnt agonist. (A) Representative images of the aggregates treated with or without 10 μM A-205804 plus 3 μM CHIR99021 or 10 ng/ml WNT3A on day 5 after the treatment. Scale bar = 200 μm. (B) Gene expression analysis by qRT-PCR for pluripotent genes, OCT4, SOX2, and NANOG. (C) Gene expression analysis by qRT-PCR for genes SOX17, BRACHYURY, and GATA4 of mesendoderm germ layers.

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Author contributions
HBW conceived and designed the experiments, collected and analyzed the data, and prepared the manuscript; XLT was responsible for the experiment, figures, and data analysis. YYW, NW, QCC, JHX, SPL, ZYZ, YQQ, and PST contributed to the collection of the data; MAZ reviewed and revised the manuscript. YYD, HLC, and JW conceived and designed the experiments and provided financial supporting.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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