

Tight Junction-Mediated Morphologic and Adherent Diversities Act on Hematopoietic Fate Induced by OCT4 in human hair follicle mesenchymal stem cells

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Research

Keywords: tight junction pathway, human hair follicle mesenchymal stem cells, OCT4, hematopoiesis

Posted Date: July 28th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-48698/v1>

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Version of Record: A version of this preprint was published on October 27th, 2020. See the published version at <https://doi.org/10.1186/s13287-020-01976-1>.

Abstract

Background: Human hair follicle mesenchymal stem cells (hHFMSCs) isolated from hair follicles possess multilineage differentiation potential. OCT4 is a gene critically associated with pluripotency properties. The cell morphology and adhesion of hHFMSCs significantly changed after transduction of OCT4 and two subpopulations emerged, including adherent cells and floating cell. Floating cells cultured in hematopoietic induction medium and stimulated with erythropoietic growth factors could transdifferentiate into mature erythrocytes, whereas adherent cells formed negligible hematopoietic colonies. The aim of this study was to reveal the role of cell morphology and adhesion on erythropoiesis induced by OCT4 in hHFMSCs and to characterize the molecular mechanisms involved.

Methods: Floating cell were separated from adherent cell by centrifugation of the upper medium during cell culture. Cell size was observed through flow cytometry and cell adhesion was tested by disassociation and adhesion assays. RNA sequencing was performed to detect genome-wide transcriptomes and identify differentially expressed genes. GO enrichment analysis and KEGG pathway analysis were performed to analysis the functions and pathways enriched by differentially expressed genes. The expression of tight junction core members was verified by qPCR and Western blot.

Results: The overexpression of OCT4 influenced the morphology and adhesion of hHFMSCs. Transcripts in floating cells and adherent cells are quite different. Data analysis showed that upregulated genes in floating cells were mainly related to the pluripotency, germ layer development (including hematopoiesis lineage development), and downregulated genes were mainly related to cell adhesion, cell junctions and the cytoskeleton. Most molecules of the tight junction (TJ) pathway were downregulated and molecular homeostasis of the TJ was disturbed, as CLDNs were disrupted, and JAMs and TJPs were upregulated. The dynamic expression of cell adhesion-related gene E-cadherin and cytoskeleton-related gene ACTN2 might cause different morphology and adhesion. Finally, a regulatory network centered to OCT4 was constructed, which elucidated the TJ pathway critically bridges pluripotency and hematopoiesis in a TJP1-dependent way.

Conclusions: Regulations of cell morphology and adhesion via the TJ pathway conducted by OCT4 might modulate hematopoiesis in hHFMSCs, thus developing potential mechanism of erythropoiesis in vitro.

Introduction

Erythropoiesis is a stepwise process through which red blood cells (RBCs, erythrocytes) are generated from hematopoietic stem and progenitor cells (HSPCs) and is controlled by multiple elements. Inducing erythrocyte production in vitro provides a model system for exploring the mechanisms of erythropoiesis. Previously, a population of small round floating cells with subtle expression of hematopoietic stem cell (HSC) marker CD45, gradually emerged from OCT4(POU5F1)-reprogrammed human hair follicle mesenchymal stem cells (hHFMSCs^{OCT4}), and could transdifferentiate into mature enucleated RBCs when stimulated with a combination of hematopoietic cytokines (1). This prompted us to consider an

association between this particular cell morphology and adhesion with possible erythropoiesis mechanisms, that is, low adhesion and round-like cell morphology conferring higher hematopoietic capacity to hHFMSCs^{OCT4} when treated with cytokines, thus promoting transduction of cellular signals and subsequently initiating the process of erythropoiesis.

It is well known that blood cells grow in suspension, and the process of erythropoiesis is accompanied by great changes in cell morphology. The cell size gradually increases as hematopoietic progenitor cells (HPCs) differentiate into precursors (2), and then decreases during erythroblast maturation accompanied by cytoskeleton remodeling and loss of cytoplasmic-nuclear connections (2, 3). Moreover, some studies have revealed that the self-renewal and differentiation of HSCs are affected by cell morphology and adhesion. Rho kinases controlling the cytoskeleton are required for the biological functions of HSCs, and are particularly significant for enucleation during erythropoiesis (4–6). Cell morphology and adhesion affect the polarity and proliferation of HSPCs (7–9). Platelet factor 4 binds to HPCs, strengthens the adhesion of HPCs to the extracellular matrix, and ultimately modulates hematopoiesis (10). However, whether the characteristics of a specific cell morphology and low adhesion are crucial factors in erythropoiesis and how they facilitate RBC development are still obscured.

Cell junction molecules are a type of cell adhesion molecules, and cell adhesion and cell junction systems dynamically and mutually interact (11). Furthermore, tight junctions (TJs) play a role in recruiting various cytoskeleton and signaling molecules on their cytoplasmic surface, and linking extracellular proteins with intracellular signaling pathways to the cytoskeleton (12). TJ members could affect the biological functions of HSCs. For example, intimate intercellular contact mediated by JAMs is required for efficient transduction of Notch signaling in HSCs, and deficiency of JAM1 results in impaired HSC specification (13, 14). JAM2 regulates the maintenance of HSCs through heterotypic interactions with JAM3 (15, 16), and depletion of JAM3 leads to a sharp decrease in the frequency of myeloid progenitors in the bone marrow (17). It is worth noting that the master hematopoietic regulator RUNX1 can respectively bind to TJP1, OCLN and CLDN5 via the "TGGGGT" DNA sequence in the promoter region (18). Overall, the overexpression of OCT4, the most important pluripotent transcription factor (TF), alters the morphology and adhesion of hHFMSCs, making the cells more prone to cytokine stimulation and differentiate towards the hematopoietic lineage.

In this study, we utilized next-generation sequencing combined with bioinformatics analysis, qPCR, and Western blotting, to investigate the interactions between cell adhesion, cytoskeleton, hematopoiesis and pluripotency modulated by OCT4 in hHFMSCs. Ideally, elucidating the molecular mechanisms of OCT4-reprogrammed hHFMSCs differentiation into erythrocytes will not only help to understand the mechanism of RBC production, but also provide an experimental basis for hematopoiesis in vitro for future clinical applications.

Materials And Methods

Cell Culture

hHFMSCs were isolated from root tissue of hair follicles cultured in 96-well plates (19), hHFMSCs^{OCT4} were obtained by lentivirus transduction of OCT4 into hHFMSCs, and the cells were seeded onto Matrigel-coated culture plates. Floating cells (floating hHFMSCs^{OCT4}) were sorted from the upper medium of adherent cells (adherent hHFMSCs^{OCT4}) by centrifugation during culture. All cells were maintained in H-DMEM/F12 medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin-streptomycin (HyClone) and 10 ng/mL fibroblast growth factor-basic (Acro Biosystems) at 37 °C and 5% CO₂ in a cell culture incubator.

Flow Cytometry Analysis

A total of 10⁷ cells/mL were prepared for flow cytometry analysis. A 200µL aliquot was added to 96-well plates per well, and then the light-scattering properties of the cells were measured by flow cytometry. In the flow cytometry assay, the value of forward scatter (FSC) is proportional to the size of the cells, so the FSC can be used to compare the relative size of the cells. FSC distribution histogram plots were generated by a computer with raw flow cytometry data.

Dissociation Assay

Cell dissociation assays were performed as previously described (20). Cells were treated with dispase II (2.4 U/mL, Sigma Aldrich) to disassociate cells from culture plates with minimal destruction of intercellular junctions. The numbers of single and total cells were counted with a hemocytometer. The percentage of individual cells to the total number of cells was inversely proportional to cell-to-cell adhesion. Each sample was tested three times.

Cell Adhesion Assay

Cell-extracellular matrix adhesion was assayed according to Codogno et al with minor modifications (21). The same number of cells was seeded onto Matrigel-coated 24-well plates and incubated for 2 hours. The plates were washed three times to remove unattached cells and the cell number was counted using a hemocytometer. The percentage of remaining cells to the total number of cells was proportional to cell-extracellular matrix adhesion.

RNA-seq and DEG Analysis

Three independent biological replicates of the same sample were sent to Shanghai Oe-biotechnology (Shanghai, China) for RNA-seq analysis. Total RNA was extracted using the mirVana miRNA Isolation Kit and RNA integrity number (RIN) was evaluated by an Agilent 2100 Bioanalyzer. Samples with RIN greater than or equal to 7 were sequenced by an Illumina HiSeq X Ten sequencer (Illumina). The filtered clean reads were mapped to the reference genome database (accession number: GCF_000001405.38) by hisat2, v2.2.1.0 (<http://ccb.jhu.edu/software/hisat2/index.shtml>). Each transcript was normalized by FPKM to eliminate the influence of gene length and sequencing depth. The counts of each sample were mapped to the annotated genome after standardization and normalization. Finally, fold change (FC) and difference significance were used to screen the differentially expressed genes (DEGs). DEGs with FC value greater than 2 or lower than -2, and a *P*-value lower than 0.05 were considered significant.

GO and KEGG Enrichment and Network Analysis

GO term and KEGG pathway enrichment analyses were carried out using the tool for Function Annotation in the DAVID (<https://david.ncifcrf.gov/>). The KEGG pathway maps were obtained from the KEGG database (<http://www.kegg.jp/>). Significant genes were visualized by the STRING database (<http://string-db.org/>), and a network was constructed using Cytoscape software (<https://cytoscape.org/>).

Expression Validation using qPCR

Total RNA was extracted from 5×10^6 cells treated with 1 mL TRIzol (Sparkjade, Shandong, China), and the purity and concentration were determined by a NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesized with the PrimeScript RT reagent Kit (+ gDNA Eraser) and then subjected to qPCR using TB Green® Premix Ex Taq™ II (Takara). The gene mRNA levels were determined using 50 ng of cDNA on an Applied Biosystems 7300. All template amplifications were conducted in triplicate with a three-step PCR process, which included one cycle of 95 °C for 30 seconds, 40 cycles of 95 °C for five seconds and 60 °C for 31 seconds and one final cycle of 95 °C for 15 seconds, 60 °C for one minute and 95 °C for 15 seconds. Using GAPDH expression as a normalization control, the relative expression was calculated as $2^{-\Delta\Delta C_t}$. The primer sequences are provided in Table 1.

Table 1
qPCR primer sequences.

Gene	Forward primer	Reverse primer
TJP1	CAACATACAGTGACGCTTCACA	CACTATTGACGTTTCCCCACTC
TJP2	TTTCCCGACACCCCAAAGTAG	CACCCTGCTTCCTTGAGACC
TJP3	AGCTACAAGCCTCGGGTTCC	GCTGTTCCAGATGGTCAGC
JAM1	ATGGGGACAAAGGCGCAAG	CAATGCCAGGGAGCACAACA
JAM3	TCCAGCAATCGAACCCAG	CTTGTCTGCGAATCCGTAATGAT
CLDN5	CTCTGCTGGTTCGCCAACAT	CAGCTCGTACTTCTGCGACA
CLDN6	TGTTCCGGCTTGCTGGTCTAC	CGGGGATTAGCGTCAGGAC
CLDN7	AGTTAGGAGCCTTGATGCCG	GCACAGGGAGTAGGATACGC
CLDN11	CATTGCCAGTTGACTGCCTG	ATCCGGATGCAGGGAAGAAC
ACTN2	GACATCGTGAACACCCCTAAC	CCGCAAAGCGTGGTAGAA
E-cadherin	ACCACGGGCTTGATTTTGA	GGAGGTGGTGAGAGAGACCT
ROCK1	AAGTGAGGTTAGGGCGAAATG	AAGGTAGTTGATTGCCAACGAA
RUNX1	GTGGGTACGAAGGAAATGACTCAA	GCAGCGTGGTAAAAGAAATCATTGAG

Western Blot Analysis

Total proteins were isolated with Radio Immunoprecipitation Assay buffer (Solarbio) supplemented with 1% Phenylmethylsulfonyl fluoride (Solarbio), followed by centrifugation to remove cell debris. Then protein extracts were subjected to protein estimation using a BCA protein assay kit (Solarbio). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried with equal amounts of proteins, and then proteins were transferred to a polyvinylidene fluoride membrane (Millipore). Upon blocking with 5% nonfat milk, the membrane was incubated with primary antibody overnight at 4 °C. After washing three times with TBST, the membrane was incubated with HRP-conjugated secondary antibody at room temperature for 2 h. Finally, the blots were developed with Immobilon Western Chemilum HRP Substrate (Millipore). Differential protein expression was calculated as a ratio normalized to β -actin protein expression.

Statistical Analysis

Data comparing two groups were statistically analyzed by Student's *t*-test. GraphPad Prism 8.0.2 was used to generate histograms. *P*-values inferior to 0.05 were considered significant.

Results

Overexpression of OCT4 Causes Changes in Morphology and Adhesion in hHFMSCs

The morphology of OCT4-reprogrammed hHFMSCs greatly changed in the process of erythropoietic differentiation, whereby the spindle-shaped cells became polygonal, and a population of small floating round or quasi-round cells emerged from the adherent polygonal cells (Fig. 1a). We then determined the relative size of these cells by flow cytometry. The data showed that the size of adherent hHFMSCs^{OCT4} was notably smaller than that of hHFMSCs, while floating hHFMSCs^{OCT4} were smaller than the adherent cells (Fig. 1b), which was consistent with what we observed under an optical microscope. At the same time, cell adhesion changed as a portion of the cells gradually became suspended in the medium, so dissociation and adhesion assays were carried out to detect cell adhesion. The percentage of single cells was higher in adherent hHFMSCs^{OCT4} (47.5%) than in hHFMSCs (9.4%), and the percentage was higher in floating hHFMSCs^{OCT4} (85%) than in adherent hHFMSCs^{OCT4} (Fig. 1c). In the adhesion assay, the percentage of remaining cells was lower in adherent cells (18.4%) than in hHFMSCs (70.9%), while it was lower in floating hHFMSCs^{OCT4} (2.3%) than in adherent hHFMSCs^{OCT4} (Fig. 1d). The above results validated that the morphology of OCT4-reprogrammed hHFMSCs changed and adhesion decreased. It is worth noting that it was the population of floating hHFMSCs^{OCT4} with low-adhesion prone to transdifferentiate towards erythroid lineage after stepwise stimulation by cocktails of hematopoietic cytokines. Accordingly, cell morphology and adhesion might be the negative factors affecting erythropoiesis.

Transcripts in Cells with Diverse Morphology and Adhesion are Quite Different

To investigate the role of cell morphology and adhesion during erythrocyte differentiation from hHFMSCs^{OCT4}, RNA-seq was performed and the DEGs were sorted. First, to compare the similarity of samples within a group and the diversity of each group, the principle components were analyzed. As shown in the three-dimensional distribution (Fig. 2a), the closer distances of cells within each group implied good repetitiveness, and the distances between every two groups were significantly greater, especially when adherent hHFMSCs^{OCT4} and floating hHFMSCs^{OCT4} were compared with hHFMSCs, respectively. Based on these data combined with the correlation coefficient and clustering of correlation provided in Additional file 1, OCT4 induced hHFMSCs to derive *novo* cell populations, remarkably, cells with different morphology and adhesion might possess distinct gene transcripts.

Next, DEGs were sorted among the three groups. When we compared adherent hHFMSCs^{OCT4} with hHFMSCs, 2401 upregulated genes and 1882 downregulated genes were identified (Fig. 2b), and 3107 upregulated genes and 2999 downregulated genes were determined in floating hHFMSCs^{OCT4} compared to hHFMSCs (Fig. 2c), indicating that OCT4 conferred considerable changes of transcriptome in the whole genome to hHFMSCs. Importantly, 833 upregulated genes and 1107 downregulated genes were also identified when floating hHFMSCs^{OCT4} were compared with adherent hHFMSCs^{OCT4} (Fig. 2d). Although the number is smaller, it would definitely play a considerable role in floating cells. Venn diagram analysis revealed a total of 612 and 388 group-specific DEGs for adherent hHFMSCs^{OCT4} vs. hHFMSCs and floating hHFMSCs^{OCT4} vs. adherent hHFMSCs^{OCT4}, respectively (Fig. 2e). In particular, 1785 group-specific DEGs were identified in floating hHFMSCs^{OCT4} vs. hHFMSCs, which was a much larger number than that in the other two comparison groups. This considerable number of DEGs probably bring about significant changes in biological function to OCT4-reprogrammed hHFMSCs. Especially, the group-specific DEGs may yield tremendous changes to floating hHFMSCs^{OCT4} when compared with hHFMSCs. In the DEGs analysis, it is likely that the common and group-specific DEGs collectively affected the morphological characteristics and subsequent transdifferentiation.

Floating Cells Lose Part of Pluripotency and Gain Hematopoietic Differentiation Potential

Transduction of the key pluripotent TF OCT4 would definitely influence the pluripotency of hHFMSCs. Consequently, we focused our analysis on the expression of related genes in cells with different morphology and adhesion. hHFMSCs expressed negligible levels of OCT4, LEFTY2, SOX18, POU3F2 and SEMA4D (Additional file 2: Table S1), and both adherent hHFMSCs^{OCT4} and floating hHFMSCs^{OCT4} expressed higher levels of pluripotent genes, including LEFTY2, KLF4, MYC, POU, SEMAs and SOXs, than hHFMSCs (Fig. 3a). Some of the pluripotent genes, such as LEFTY2, SOX4, and SEMA6C, however, were downregulated in floating hHFMSCs^{OCT4} compared with adherent hHFMSCs^{OCT4} (Fig. 3a), which was validated by KEGG enrichment analysis as downregulated genes were enriched in the term signaling

pathways regulating pluripotency of stem cells in floating hHFMSCs^{OCT4} vs. adherent hHFMSCs^{OCT4} (Fig. 3b). These results suggested that OCT4-reprogrammed hHFMSCs acquired pluripotency but lost some of it after adherent cells transformed into the floating subset. The DEGs were then clustered according to their GO terms using DAVID, and the top 10 GO terms related to differentiation and development enriched with upregulated genes and downregulated genes were separately analyzed. Upregulated DEGs were involved in the terms of germ layer differentiation in adherent hHFMSCs^{OCT4} and floating hHFMSCs^{OCT4} when compared with hHFMSCs (Table 2 and Additional file 2: Table S2, S3), and the upregulated genes in floating cells were specially enriched in the terms T-helper 1 cell differentiation and regulation of erythrocyte differentiation (Table 2), implying a potential for erythropoietic differentiation.

Table 2
Differentiation- and development-related GO terms enriched of upregulated DEGs in floating hHFMSCs^{OCT4} versus hHFMSCs.

id	Term	P-value	Enrichment score
GO:0046548	retinal rod cell development	0	6.255870445
GO:0045063	T-helper 1 cell differentiation	0	6.255870445
GO:0060351	cartilage development involved in endochondral bone morphogenesis	0	6.255870445
GO:1903225	negative regulation of endodermal cell differentiation	0	6.255870445
GO:0060538	skeletal muscle organ development	0	6.255870445
GO:0061153	trachea gland development	0	6.255870445
GO:1902871	positive regulation of amacrine cell differentiation	0	6.255870445
GO:0045646	regulation of erythrocyte differentiation	0	6.255870445
GO:0003431	growth plate cartilage chondrocyte development	0	6.255870445
GO:0045605	negative regulation of epidermal cell differentiation	0	6.255870445

Downregulation of the Tight Junction Pathway in Floating hHFMSCs^{OCT4}

To further explore the role of cell morphology and adhesion during erythropoiesis in hHFMSCs^{OCT4}, the top 10 GO terms, covering biological process, molecule function and cellular component, are displayed in Fig. 4a. Downregulated genes were obviously enriched in relevant terms, such as cell adhesion, focal adhesion, cytoskeleton and cell-cell junction in adherent hHFMSCs^{OCT4} compared to hHFMSCs. In addition, downregulated genes were significantly enriched in the term cell adhesion in floating hHFMSCs^{OCT4} relative to adherent hHFMSCs^{OCT4}, suggesting the sharp decrease in adhesion of floating hHFMSCs^{OCT4}. Besides, KEGG analysis revealed that downregulated genes were enriched in cell signaling

pathways including regulation of actin cytoskeleton, cell adhesion molecules and focal adhesion in adherent hHFMSCs^{OCT4} vs. hHFMSCs, as well as pathways of regulation of actin cytoskeleton, gap junction, adherens junction and focal adhesion in floating hHFMSCs^{OCT4} vs. adherent hHFMSCs^{OCT4} (Fig. 3b). These results verified the changes in cell morphology and adhesion of hHFMSCs^{OCT4}, which were consistent with our observations. Therefore, the morphology- and adhesion-related genes aroused corresponding alterations in OCT4-reprogrammed hHFMSCs and facilitated the switch between adherent and floating subpopulations.

Tight junctions, generally known for their fence function controlling cellular matter diffusion, can also modulate cell adhesion and the cytoskeleton (12, 22). The TJ pathway was found to be downregulated by KEGG analysis in these three comparison groups, and the TJ pathway was annotated through the KEGG database and the DEGs were annotated (Fig. 4b). There were 12 upregulated genes and 20 downregulated genes in the TJ pathway. The results clearly showed that TJ genes were dynamically expressed, and several programs, such as cell proliferation, adhesion, cytoskeleton, cell polarity, paracellular permeability and most importantly cell differentiation, were involved. Furthermore, fluctuation of one member in the TJ pathway would inevitably affect other member molecules and thereby have an impact on the biological functions of hHFMSCs^{OCT4} and initiate the switch between the two states characterized by different morphology and adhesion.

Gene Expression Validation by qPCR and Western blot

Cell junction molecules, including TJ members are involved in cell adhesion and could directly affect cell adhesion (11). Therefore, we performed qPCR to detect the mRNA expression levels of selected genes associated with TJs, adhesion or cytoskeleton (Fig. 5a). As expected, the expression level of the TJ member gene CLDN11 was significantly decreased in adherent hHFMSCs^{OCT4} and floating hHFMSCs^{OCT4} relative to hHFMSCs, but both CLDN6 and CLDN7 were increased. Especially, CLDN5 was downregulated in adherent hHFMSCs^{OCT4} and then upregulated in floating hHFMSCs^{OCT4}. The expression levels of JAM1 and JAM3, which play an important role in the commitment of lineage specification and cellular signaling transduction in HSCs, were respectively decreased and increased in floating hHFMSCs^{OCT4} vs. hHFMSCs. Moreover, the expression levels of TJP1, TJP2 and TJP3, core members associated with the cytoskeleton and intracellular signaling transduction, were remarkably upregulated 5.4-fold, 59.4-fold and 7.6-fold in floating hHFMSCs^{OCT4}. These results indicated disrupted molecular homeostasis of the TJ pathway upon OCT4 transduction. We also found that the cytoskeleton gene ACTN2 increased more than 20-fold in floating cells, and the expression of E-cadherin increased in adherent hHFMSCs^{OCT4} and then decreased in floating hHFMSCs^{OCT4}, implying that the changes in adhesion of these cells might be related to calcium signals.

Changes in cell morphology and adhesion in the process of erythropoiesis could influence the biological functions of hematopoietic cells, including proliferation, self-renewal, differentiation and etc. (8), we also detected the expression levels of the terminal erythroid differentiation-related gene ROCK1 and the essential hematopoietic development gene RUNX1. Expression of these two genes were found decreased

in floating cells, indicating that the state of hematopoietic program was not yet triggered, although OCT4 conferred pluripotency in this group of cells to some degree as other pluripotency genes MYC, KLF4, etc. were upregulated as shown in previous sequencing data.

Next, Western Blotting was carried out to validate the protein expression levels of TJP1, JAM1, CLDN5, CLDN11 and RUNX1. As shown in Fig. 5b, the expression tendency of these proteins was consistent with the mRNA levels. The above results corresponded to our sequencing data, except for TJP1, which was remarkably increased in adherent cells and floating cells compared to hHFMSCs but was not identified by sorting DEGs.

A Putative Regulatory Network in Floating hHFMSCs^{OCT4}

Although pluripotency-, cell morphology- and cell adhesion-related genes have been identified, the internal correlations between these factors are still unknown. Therefore, we visualized the significant DEGs in floating hHFMSCs^{OCT4} vs. hHFMSCs and constructed a network (Fig. 6a). This network suggested that pluripotency-related genes in hHFMSCs, such as OCT4, SOX2, c-MYC (MYC) and KLF4, were regulated by genes related to cell adhesion, cell junction, and cytoskeleton, including TJP1, TJP2, FN1 (fibronectin 1), CTNNB1 (β -catenin), CDH1 (E-cadherin), ACTB (β -actin) and ACTG1 (γ -actin 1). In addition, there were interactions within the TJ pathway, as well as interplay between the TJ pathway, cell adhesion- and cytoskeleton-related molecules, such as PTK2 (FAK), CDH1, CTNNB1, ACTB, and ACTG1. Hematopoietic genes, such as, CD44, CD117 (KIT), RUNX1 and ROCK1 could similarly bind to or interact with cell adhesion and junction molecules. These results strongly imply that dynamic expression of genes related to adhesion, cytoskeleton and junctions results in the low-adhesion and different morphology of hHFMSCs^{OCT4}, whereas floating cells lost some of the pluripotency and became more prone to differentiation into blood cells. Furthermore, TJP1 uniquely links pluripotency, hematopoiesis and cytoskeleton with the TJ pathway through KLF4, RUNX1, ACTB and ACTG1. Fluctuation of one member in TJ pathway would inevitably affect other member molecules, thereby impacting the biological functions of hHFMSCs^{OCT4} and initiating the transform between the two states of different morphology and adhesion characteristics.

In summary, transduction of OCT4 brought about great differences in morphology and adhesion to hHFMSCs, whereby two subsets of cells appeared and gained pluripotency, with floating cells losing some of their pluripotency. The dynamically expressed TJ pathway before erythropoietic inducement might act as a pivotal point of changes in cell morphology and adhesion, resulting in damaged pluripotency in floating cells and probable constructed hematopoietic capacity in a TJP1-dependent way.

Discussion

It was reported that floating hHFMSCs^{OCT4} treated with hematopoietic cytokines could transdifferentiate into RBCs (1). Here, we investigated the correlation between cell morphology, adhesion, pluripotency and hematopoiesis to elucidate the mechanisms of erythropoiesis in OCT4-reprogrammed hHFMSCs. The

results verified the alterations in morphology and adhesion in hHFMSCs^{OCT4}, and the corresponding changes in gene expression were detected by RNA-seq, qPCR and Western blot. In-depth analysis of sequencing data showed that the expression of genes related to pluripotency changed in hHFMSCs^{OCT4} with diverse adhesion and morphology, and floating cells displayed hematopoietic differentiation potential. All these results suggest the possible effects of changes in cell adhesion, junctions, and cytoskeleton on pluripotency and hematopoietic differentiation in OCT4-reprogrammed hHFMSCs.

Pluripotency-related DEGs dynamically expressed in two subpopulations of hHFMSCs^{OCT4}, and pluripotency was presumably reduced in floating cells compared with adherent cells. In humans, direct conversions of mature somatic cells to another type of cells using the single factor OCT4, a key pluripotency TF, have been widely developed (23, 24). Although not shown to be involved in physiological hematopoiesis, OCT4 is capable of promoting the expression of essential hematopoietic regulators in supportive culture conditions (23). hHFMSCs are easily available and nonimmunogenic, making them a potential alternative stem cell source for patient-specific applications. Transduction of the individual factor OCT4 allows hHFMSCs to transdifferentiate into RBCs under multiple hematopoietic induction conditions, providing an optional way to generate RBCs in vitro for transfusion (1). Evolutionary conservation analysis was performed to identify a relative subset of targets of OCT4 and other POU proteins that link the regulation of cell-cell adhesion to differentiation (25). Whether OCT4 regulates pluripotency through adhesion or regulates both in parallel is difficult to determine, however, analysis of the conserved OCT4 network indicates that the regulation of differentiation and adhesion is inseparable (25). This may partially explain the relatively decreased pluripotency in floating hHFMSCs^{OCT4}.

Attenuated pluripotency prompts cells to be more likely to differentiate into a certain cell type. Blood cells are well known to grow in suspension and erythropoietic precursors show a progressive reduction in cell and nuclear size during erythropoiesis (26), extremely similar to the sequencing results and our observations upon floating hHFMSCs^{OCT4}. A number of molecules associated with cell adhesion and the cytoskeleton in hematopoietic cells are proved essential for erythropoiesis under homeostasis and stress (27–31). Embryonic hematopoiesis involves activation of a hematopoietic transcriptional program, followed by major morphological changes and breakage of the tight junctions (32). Tight junction members, TJPs, CLDNs and JAMs control self-renewal, proliferation and recovery from stress in hematopoietic cells, including ESCs, HSCs and pluripotent stem cells (17, 18, 33–37). Our results of sequencing analysis, qPCR and Western blot clearly demonstrate dynamically expressed TJ members in hHFMSCs^{OCT4} with divergent morphology and cell adhesion. Mutual conversion between adherent hHFMSCs^{OCT4} and floating hHFMSCs^{OCT4} seems to account for the dynamic TJ pathway, causing disparate morphology and adhesion, and ultimately regulating the expression of genes related to pluripotency and hematopoiesis (Fig. 6b). However, it is still unclear whether these morphological alterations initiate the process of erythropoiesis, or are the inevitable phenomenon accompanying erythroid hematopoiesis since erythropoiesis requires membrane biogenesis, establishment of cell polarity and cytoskeleton assembly during differentiation and enucleation (38–40). More research should be conducted to address this problem.

In mammals, RUNX1 is identified as a core regulator of hematopoiesis that is essential for the initiation of the hematopoietic program in embryonic hematopoiesis (41–43), and there is bidirectional negative regulation between TJP1 and RUNX1 (18, 44). Therefore, TJP1 suppressed the expression of RUNX1 in floating cells, implying insignificant hematopoietic differentiation tendency, while the expression of TJPs (TJP1, TJP2 and TJP3) was upregulated in floating cells. Consequently, the morphological changes potentially confer a certain degree of pluripotency to floating hHFMSCs^{OCT4}, accompanied by probable enhanced sensitivity to hematopoietic cytokines and thus trigger the hematopoietic program.

Herein we present a hypothetical model (Fig. 6b), in which the TJ pathway might regulate hematopoiesis in OCT4-reprogrammed hHFMSCs by transforming cell morphology from adherent polygonal or fusiform to floating small and round. Although further experiments should be followed up, such as knock-out of TJP1, investigations of pluripotency and hematopoietic capacity, study of morphological changes effect on intercellular signal transduction and detection of sensitivity to hematopoietic cytokines.

Conclusions

We have developed potential mechanisms of erythropoiesis in OCT4-reprogrammed hHFMSCs, where changes in cell morphology and adhesion were involved through the TJ pathway. This study characterized possible hematopoietic molecular mechanisms in hHFMSCs^{OCT4} in vitro, providing comprehensive insight into the potential role of the TJ pathway during erythropoiesis.

Abbreviations

RBCs
red blood cells; HSPCs:hematopoietic stem and progenitor cells; HSCs:hematopoietic stem cell; hHFMSCs:human hair follicle mesenchymal stem cells; hHFMSCs^{OCT4}:HPCs:hematopoietic progenitor cells; TJs:tight junctions; TF:transcription factor; FSC:forward scatter; RIN:RNA integrity number; FC:fold change; DEGs:differentially expressed genes; KEGG:Kyoto Encyclopedia of Genes and Genomes; GO:Gene Ontology.

Declarations

Acknowledgments

Not applicable.

Funding

This work was supported by Qingdao Science and Technology Bureau [grant number 18-2-2-66-jch].

Availability of Data and Materials

The datasets supporting the conclusions of this article are available in the SRA database, with unique accession code PRJNA615033 and hyperlink to dataset(s) in <https://dataview.ncbi.nlm.nih.gov/object/PRJNA615033?reviewer=oru002jv1ibpkdhnj42usqa1o>. All other data are concluded in this article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

- XY conceived and designed the experiments, performed the experiments, wrote the paper, prepared figures and/or tables.
- ZL conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- PS and XH contributed reagents/materials/analysis tools.
- HC, WJ and YR analyzed the data, reviewed drafts of the paper.
- WH and XT analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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References

1. Liu Z, Lu S-J, Lu Y, Tan X, Zhang X, Yang M, et al. Transdifferentiation of Human Hair Follicle Mesenchymal Stem Cells into Red Blood Cells by OCT4. *Stem cells international*. 2015;2015:389628.
2. Nigra AD, Casale CH, Santander VS. Human erythrocytes: cytoskeleton and its origin. *Cell Mol Life Sci*. 2020;77(9):1681–94.
3. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. 2008;112(3):470–478.
4. Mulloy JC, Cancelas JA, Filippi MD, Kalfa TA, Guo F, Zheng Y. Rho GTPases in hematopoiesis and hemopathies. *Blood*. 2010;115(5):936–47.
5. Nayak RC, Chang KH, Vaitinadin NS, Cancelas JA. Rho GTPases control specific cytoskeleton-dependent functions of hematopoietic stem cells. *Immunol Rev*. 2013;256(1):255–68.
6. Ji P, Jayapal SR, Lodish HF. Eucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol*. 2008;10(3):314–21.
7. Reichert D, Friedrichs J, Ritter S, Käubler T, Werner C, Bornhäuser M, et al. Phenotypic, Morphological and Adhesive Differences of Human Hematopoietic Progenitor Cells Cultured on Murine versus Human Mesenchymal Stromal Cells. *Scientific reports*. 2015;5:15680.
8. Walenda T, Bork S, Horn P, et al. Co-culture with mesenchymal stromal cells increases proliferation and maintenance of haematopoietic progenitor cells. *J Cell Mol Med*. 2010;14(1–2):337–50.
9. Wein F, Pietsch L, Saffrich R, et al. N-cadherin is expressed on human hematopoietic progenitor cells and mediates interaction with human mesenchymal stromal cells. *Stem Cell Res*. 2010;4(2):129–39.
10. Dudek AZ, Nesselova I, Mayo K, Verfaillie CM, Pitchford S, Slungaard A. Platelet factor 4 promotes adhesion of hematopoietic progenitor cells and binds IL-8: novel mechanisms for modulation of hematopoiesis. *Blood*. 2003;101(12):4687–94.
11. Steinbacher T, Ebnet K. The regulation of junctional actin dynamics by cell adhesion receptors. *Histochem Cell Biol*. 2018;150(4):341–50.
12. Matter K, Aijaz S, Tsapara A, Balda MS. Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. *Curr Opin Cell Biol*. 2005;17(5):453–8.
13. Ebnet K. Junctional Adhesion Molecules (JAMs): Cell Adhesion Receptors With Pleiotropic Functions in Cell Physiology and Development. *Physiol Rev*. 2017;97(4):1529–54.
14. Kobayashi I, Kobayashi-Sun J, Kim AD, et al. Jam1a-Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling. *Nature*. 2014;512(7514):319–23.
15. Arcangeli ML, Bardin F, Frontera V, et al. Function of Jam-B/Jam-C interaction in homing and mobilization of human and mouse hematopoietic stem and progenitor cells. *Stem cells*. 2014;32(4):1043–54.
16. Arcangeli ML, Frontera V, Bardin F, et al. JAM-B regulates maintenance of hematopoietic stem cells in the bone marrow. *Blood*. 2011;118(17):4609–19.
17. Praetor A, McBride JM, Chiu H, et al. Genetic deletion of JAM-C reveals a role in myeloid progenitor generation. *Blood*. 2009;113(9):1919–28.

18. Miao YS, Zhao YY, Zhao LN, et al. MiR-18a increased the permeability of BTB via RUNX1 mediated down-regulation of ZO-1, occludin and claudin-5. *Cell Signal*. 2015;27(1):156–67.
19. Wang Y, Liu J, Tan X, et al. Induced pluripotent stem cells from human hair follicle mesenchymal stem cells. *Stem Cell Rev Rep*. 2013;9(4):451–60.
20. Hobbs RP, Amargo EV, Somasundaram A, et al. The calcium ATPase SERCA2 regulates desmoplakin dynamics and intercellular adhesive strength through modulation of PKC α signaling. *FASEB J*. 2011;25(3):990–1001.
21. Codogno P, Doyennette-Moyne MA, Aubery M. Evidence for a dual mechanism of chick embryo fibroblast adhesion on fibronectin and laminin substrata. *Exp Cell Res*. 1987;169(2):478–89.
22. Mattagajasingh SN, Huang SC, Hartenstein JS, Benz EJ Jr. Characterization of the interaction between protein 4.1R and ZO-2. A possible link between the tight junction and the actin cytoskeleton. *J Biol Chem*. 2000;275(39):30573–85.
23. Mitchell R, Szabo E, Shapovalova Z, Aslostovar L, Makondo K, Bhatia M. Molecular evidence for OCT4-induced plasticity in adult human fibroblasts required for direct cell fate conversion to lineage specific progenitors. *Stem Cells*. 2014;32(8):2178–87.
24. Sancho-Martinez I, Baek SH, Izpisua Belmonte JC. Lineage conversion methodologies meet the reprogramming toolbox. *Nat Cell Biol*. 2012;14(9):892–9.
25. Livigni A, Peradziryi H, Sharov AA, et al. A conserved Oct4/POUV-dependent network links adhesion and migration to progenitor maintenance. *Curr Biol*. 2013;23(22):2233–44.
26. Nandakumar SK, Ulirsch JC, Sankaran VG. Advances in understanding erythropoiesis: evolving perspectives. *Br J Haematol*. 2016;173(2):206–18.
27. Jacobsen RN, Perkins AC, Levesque JP. Macrophages and regulation of erythropoiesis. *Curr Opin Hematol*. 2015;22(3):212–9.
28. Ueda T, Yokota T, Okuzaki D, et al. Endothelial Cell-Selective Adhesion Molecule Contributes to the Development of Definitive Hematopoiesis in the Fetal Liver. *Stem Cell Reports*. 2019;13(6):992–1005.
29. Anselmo A, Lauranzano E, Soldani C, et al. Identification of a novel agrin-dependent pathway in cell signaling and adhesion within the erythroid niche. *Cell Death Differ*. 2016;23(8):1322–30.
30. Sudo T, Yokota T, Okuzaki D, et al. Endothelial Cell-Selective Adhesion Molecule Expression in Hematopoietic Stem/Progenitor Cells Is Essential for Erythropoiesis Recovery after Bone Marrow Injury. *PLoS One*. 2016;11(4):e0154189.
31. Sudo T, Yokota T, Oritani K, et al. The endothelial antigen ESAM monitors hematopoietic stem cell status between quiescence and self-renewal. *J Immunol*. 2012;189(1):200–10.
32. Ottersbach K. Endothelial-to-haematopoietic transition: an update on the process of making blood. *Biochem Soc Trans*. 2019;47(2):591–601.
33. Sugano Y, Takeuchi M, Hirata A, Matsushita H, Miyajima A. Junctional adhesion molecule-A, JAM-A, is a novel cell-surface marker for long-term repopulating hematopoietic stem cells. *Blood*.

- 2008;111(3):1167–72.
34. Thompson PD, Tipney H, Brass A, et al. Claudin 13, a member of the claudin family regulated in mouse stress induced erythropoiesis. *PLoS One*. 2010;5(9):e12667.
 35. Lapierre LA. The molecular structure of the tight junction. *Adv Drug Deliv Rev*. 2000;41(3):255–64.
 36. Lie-A-Ling M, Marinopoulou E, Li Y, et al. RUNX1 positively regulates a cell adhesion and migration program in murine hemogenic endothelium prior to blood emergence. *Blood*. 2014;124(11):e11–20.
 37. Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, Dejana E. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *J Biol Chem*. 2000;275(27):20520–6.
 38. Konstantinidis DG, Pushkaran S, Johnson JF, Cancelas JA, Kalfa TA. Signaling and cytoskeletal requirements in erythroblast enucleation. *Blood*. 2012;119(25):6118–27.
 39. Keerthivasan G, Wickrema A, Crispino JD. Erythroblast enucleation. *Stem Cells Int*. 2011;2011:139851.
 40. Lazarides E, Woods C. Biogenesis of the red blood cell membrane-skeleton and the control of erythroid morphogenesis. *Annu Rev Cell Biol*. 1989;5:427–52.
 41. Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the *Cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A*. 1996;93(8):3444–9.
 42. Lacaud G, Gore L, Kennedy M, Kouskoff V, Kingsley P, Hogan C, et al. Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro. *Blood*. 2002;100(2):458–66.
 43. Okuda T, Deursen JV, Hiebert SW, Grosveld G, Downing JR. AML1, the Target of Multiple Chromosomal Translocations in Human Leukemia, Is Essential for Normal Fetal Liver Hematopoiesis. *Cell*. 1996;84(2):321–30.
 44. Remue E, Meerschaert K, Oka T, et al. TAZ interacts with zonula occludens-1 and – 2 proteins in a PDZ-1 dependent manner. *FEBS Lett*. 2010;584(19):4175–80.

Figures

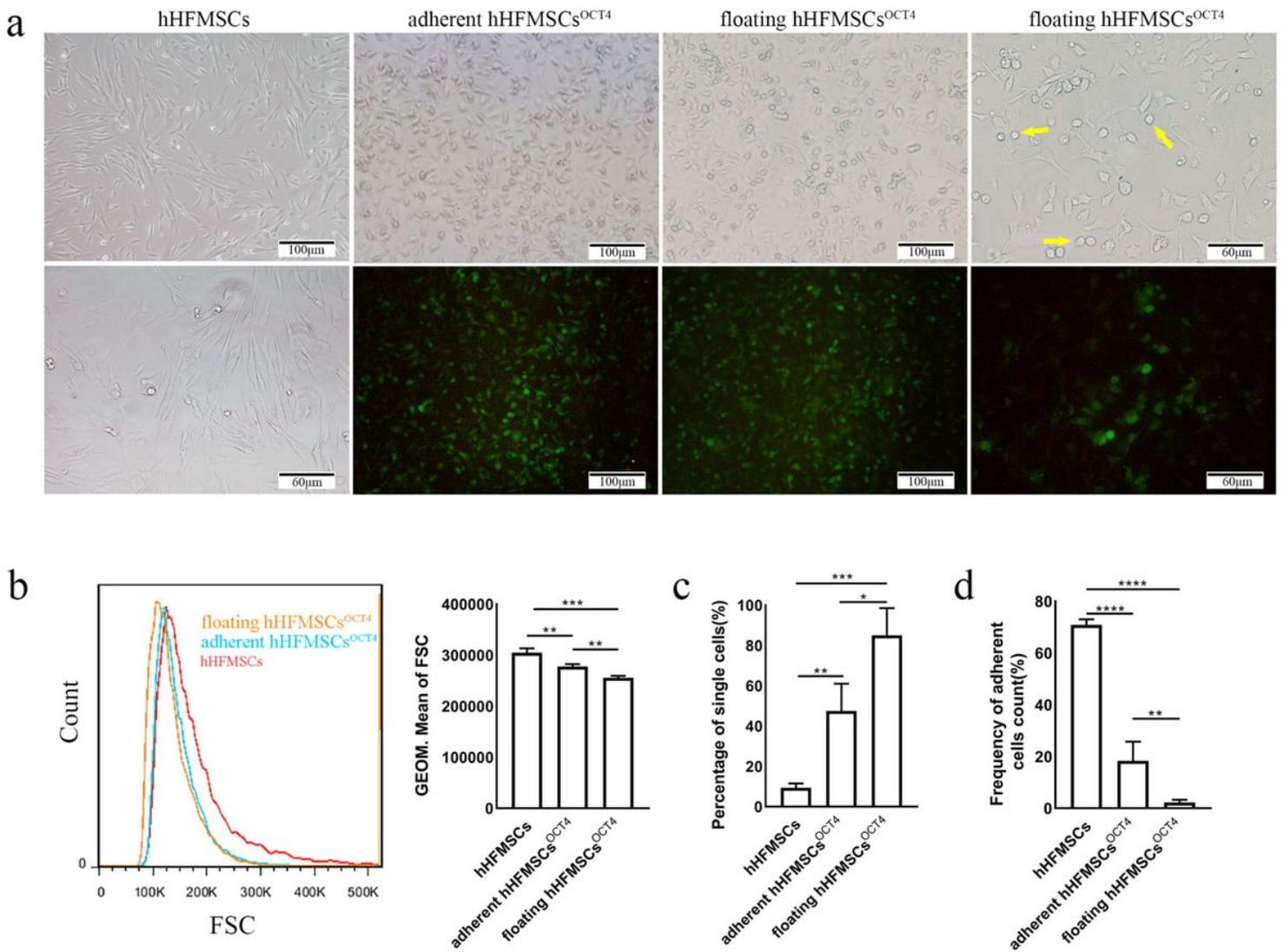


Figure 1

Cell morphology and adhesion alterations. (a) The morphology of hHFMSCs was gradually altered after the transduction of OCT4 (hHFMSCsOCT4). Yellow arrows indicate round floating hHFMSCsOCT4. (b) Flow cytometric plot of three groups of hHFMSCs and geometric mean of the FSC; n=3. (c) Percentage of single cells adhered to the plates after treatment with dispase; n=3. (d) Percentage of cells adhered to the Matrigel-coated plates after rinsing with PBS three times; n=3. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

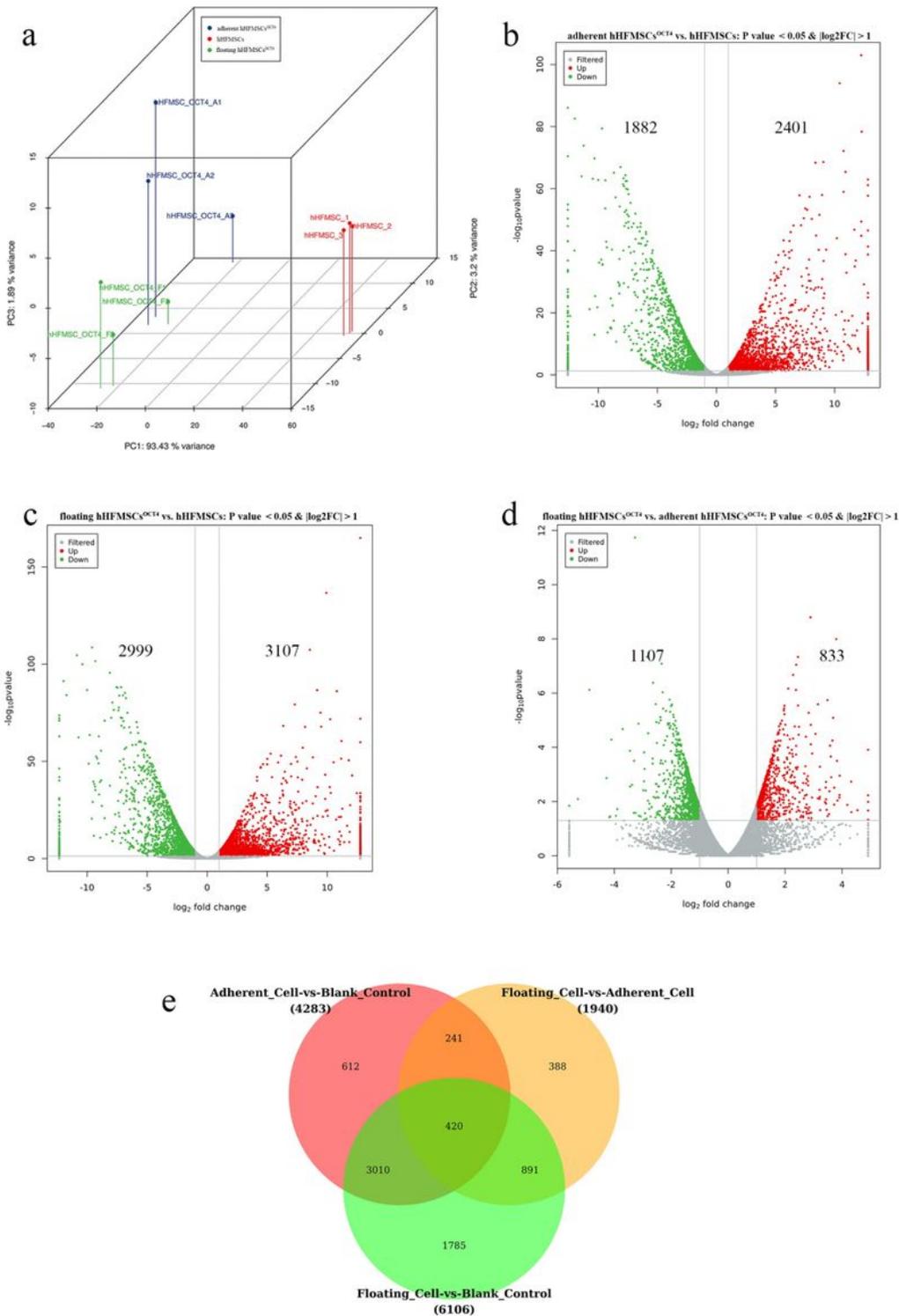


Figure 2

Comparisons of the transcripts and DEG analysis. (a) Principal component of samples distributed in three dimensions; n=3. (b), (c) and (d) Differential expression of genes in the three different comparison groups represented by volcano plots; n=3. Colors are representative by $P \leq 0.05$ and $|FC| \geq 2$. (e) Venn diagram is used to represent common and specific DEGs between the different comparison groups; n=3.

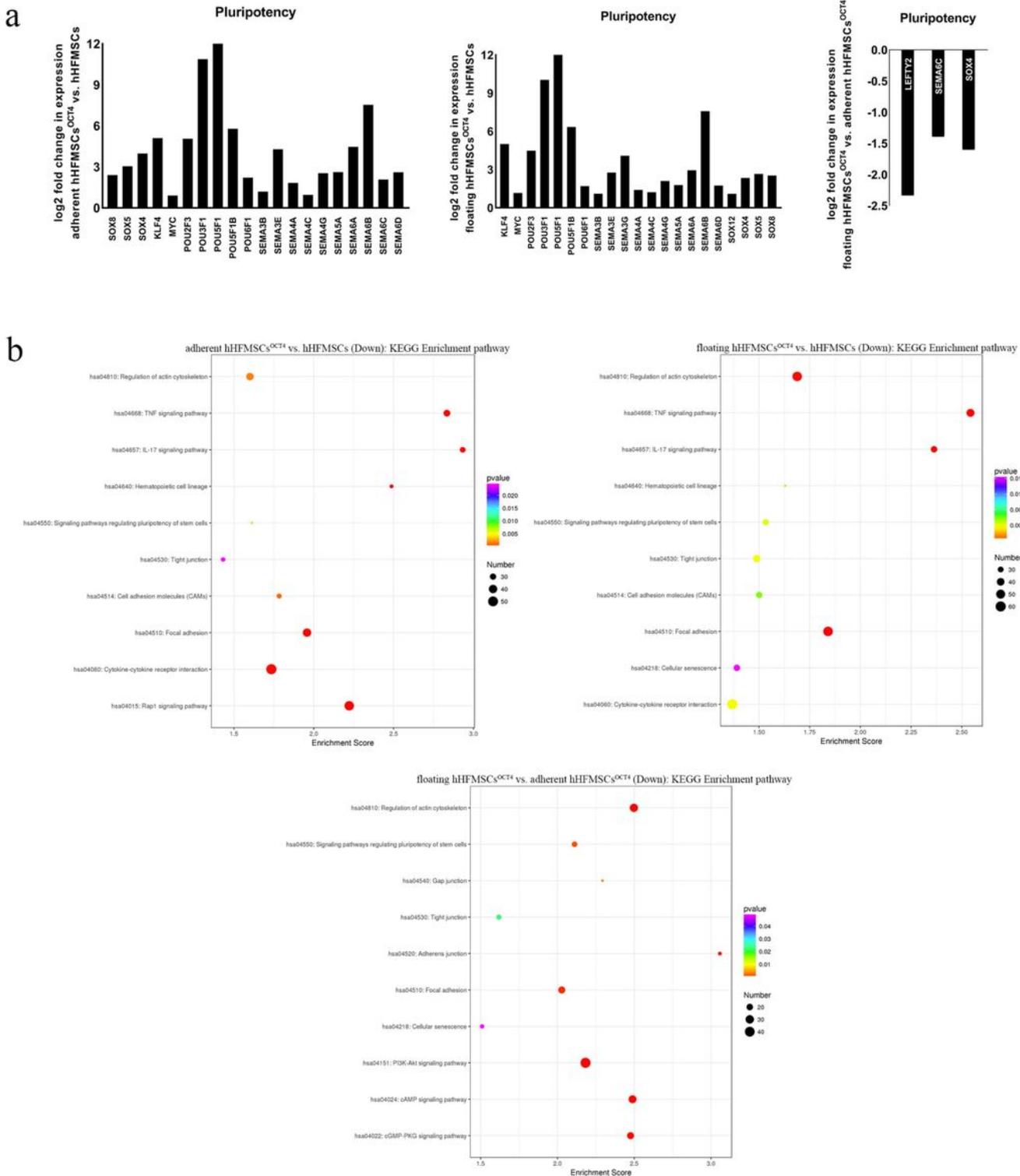


Figure 3

Expression of pluripotent genes and KEGG enrichment analysis. (a) Pluripotency-related gene expression in hHFMSCs, adherent hHFMSCsOCT4 and floating hHFMSCsOCT4. (b) Bubble chart representing the significantly enriched pathways from the KEGG analysis. The color of dots in the bubble chart indicates the significance of the enriched category, and the size represents the scale of enriched genes in the terms.

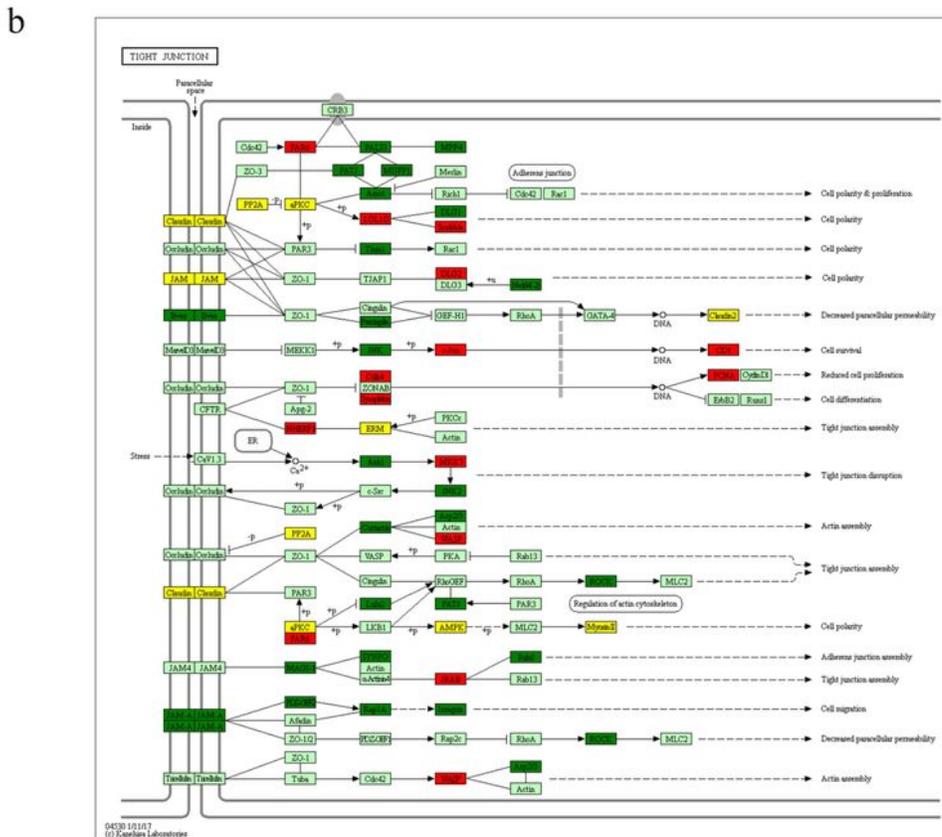
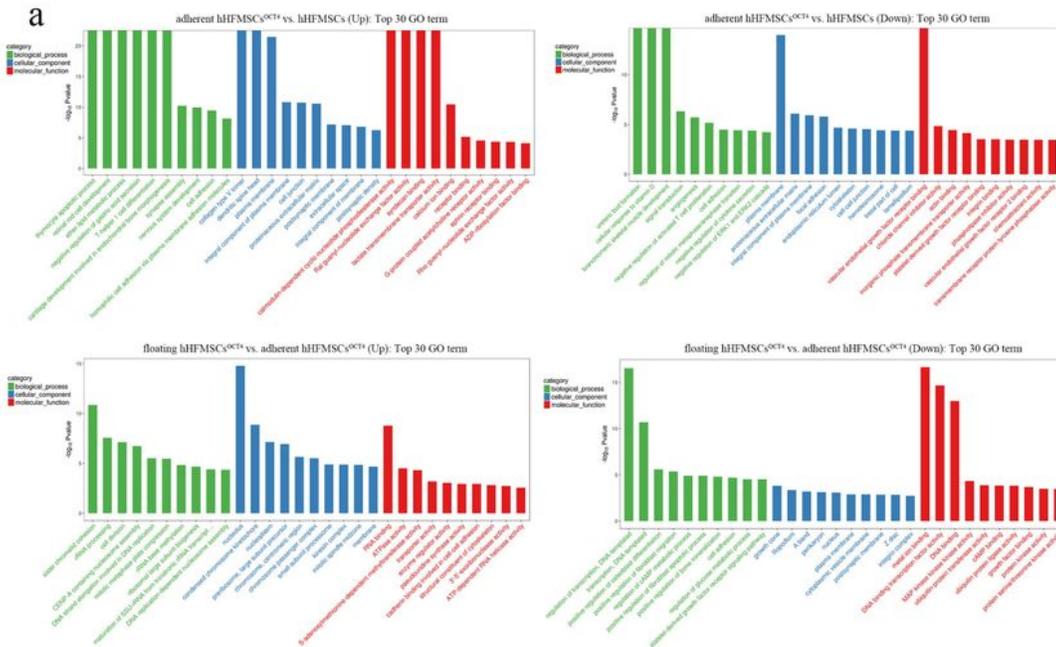


Figure 4

GO analysis of DEGs and TJ pathway annotation. (a) Top 10 enriched GO terms covering biological process, cellular component and molecule function in adherent hHFMSCs^{OCT4} vs. hHFMSCs, floating hHFMSCs^{OCT4} vs. hHFMSCs and floating hHFMSCs^{OCT4} vs. adherent hHFMSCs^{OCT4} for upregulated genes and downregulated genes, respectively. (b) Annotated TJ pathway according to the KEGG

database. Red boxes indicate upregulated genes, green boxes indicate downregulated genes and yellow boxes indicate both upregulated and downregulated genes and paralogs.

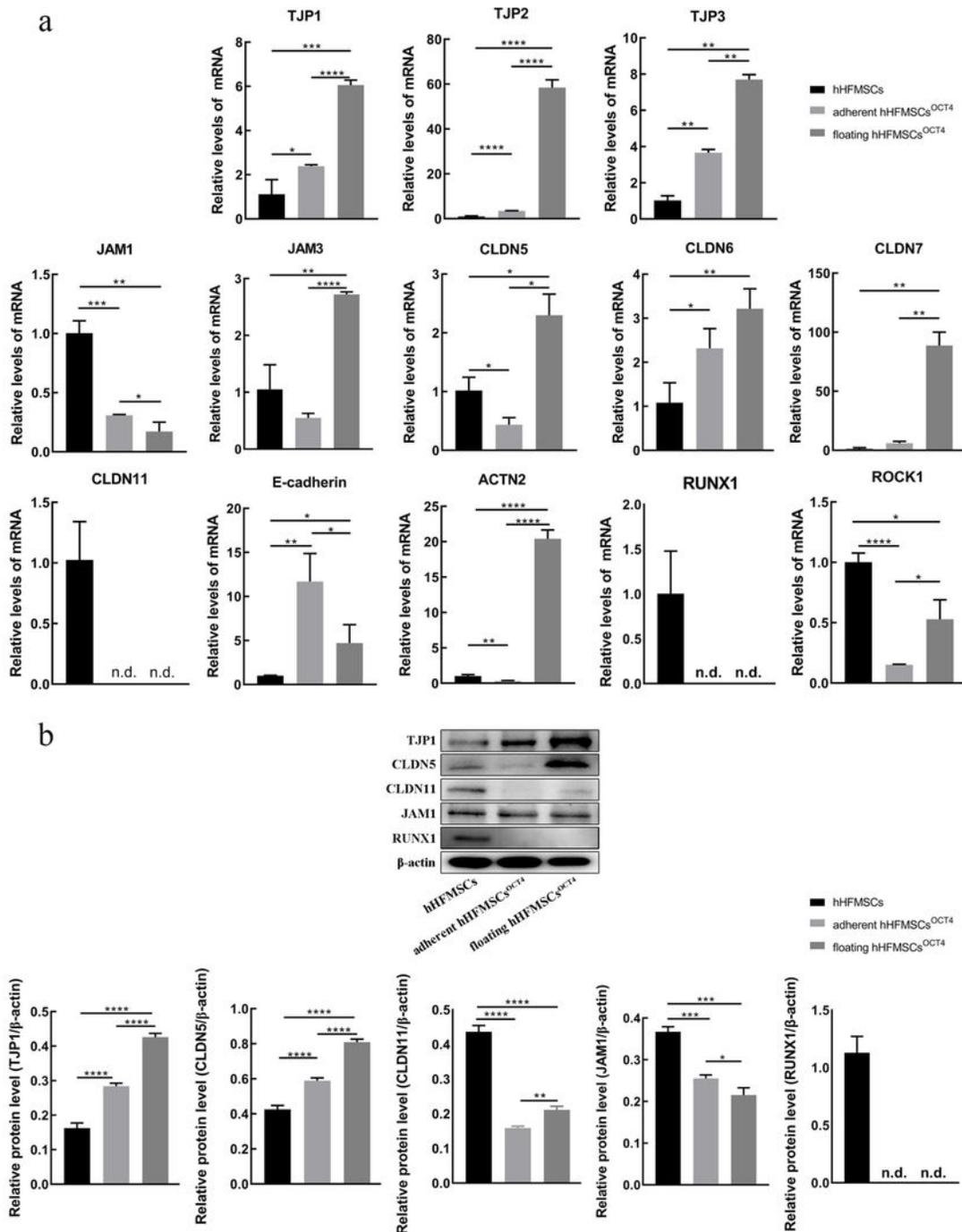


Figure 5

Gene expression validation by qPCR and Western blot. (a) and (b) Histograms are mapped to validate the expression of the DEGs by qPCR and Western blot, respectively; $n=3$. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. n.d., undetected.

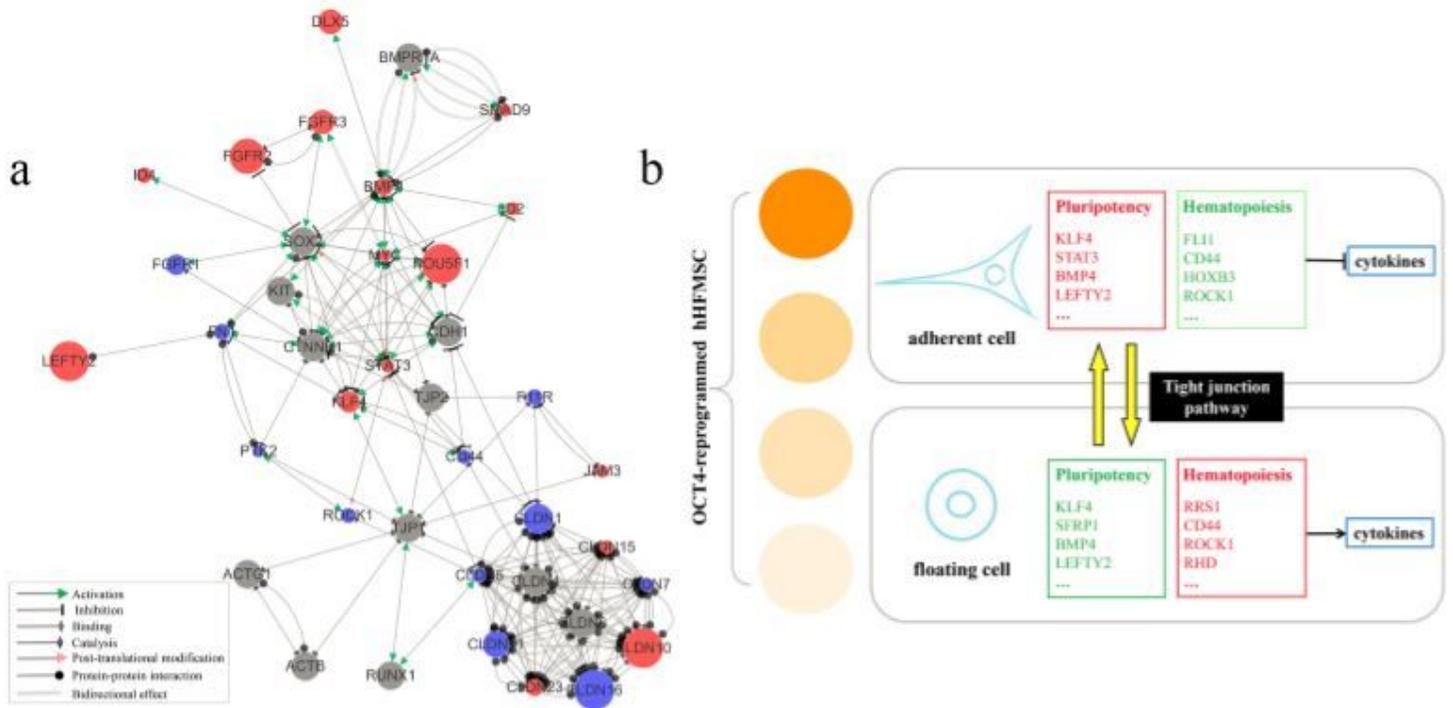


Figure 6

Network analysis and hypothetical model construction. (a) A pluripotency regulatory network of floating hHFMSCsOCT4. Red dots indicate genes that are upregulated, blue dots are downregulated genes, and undifferentially expressed genes are shown in gray. The size of the dot is proportional to the absolute value of the log₂-fold change value. (b) Hypothetical model in which OCT4-reprogrammed hHFMSCs modulate pluripotency and hematopoietic capacity by regulating morphology and adhesion via the TJ pathway. The red box represents genes upregulated genes in cells, and green one represents downregulated genes. Circles with different shades of color represent changes in cell adhesion, and light to dark represent low to high.

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