An Efficient Chemical-Defined Condition for Generation of Human Induced Pluripotent Stem Cells

Xu Jinhong

Fudan University

Fang Shi

Xiamen University

Wang Naweng

Xiamen University

Li Bo

Xiamen University

Huang Yongheng

Xiamen University

Fan Qi

Fudan University

Shi Jingyi

Fudan University

Liu Huihui

Fudan University

shao zhicheng (✉ zcshao@fudan.edu.cn)

Fudan University <https://orcid.org/0000-0003-4383-119X>

Research

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Abstract

Background: Human induced pluripotent stem cells (hiPSCs) hold great potential in disease modeling, drug screening and cell therapy. However, efficiency and costs of hiPSCs preparation still need to be improved.

Methods: We screened the compounds that target signaling pathways, epigenetic modifications or metabolic-process regulation to replace the growth factors. After small molecules treatment, TRA-1-60 staining was performed to quantify the efficiency of somatic cell reprogramming. Next, small molecule cocktail induced ESCs or iPSCs were examined with pluripotent markers expression. Finally, Genome-wide gene expression profile was then analyzed by RNA-seq to illustrate the mechanism of human somatic cell reprogramming.

Result: Here, we found that a dual-specificity tyrosine phosphorylation-regulated kinase inhibitor ID-8 robustly enhanced human somatic cell reprogramming by upregulation of PDK4 and activation of glycolysis. Furthermore, we identified a novel growth-factor-free hiPSC generation system using small molecules ID-8/Kartogenin (IK). Finally, we developed IK medium combined with Low-dose bFGF to support the long-term expansion of human pluripotent stem cells. IK-iPSCs showed pluripotency and normal karyotype.

Conclusions: Our studies may provide a novel growth-factor-free culture system to facilitate the generation of hiPSCs for multiple application in regenerative medicine.

Background

Human induced pluripotent stem cells (hiPSCs)^{1,2}, which have the potentials of self-renewal and differentiation into three germ layers, can be used for an excellent model system for development biology, disease modeling and drug discovery, will have a wide variety of uses in regenerative medicine and cell therapy^{3,4}. However, hiPSCs generated from integration approach of transgenes pose the risk of tumor formation, disrupting the donor cellular genome and limiting their application in regenerative medicine. Therefore, integration-free hiPSCs have been established using several methods, including non-integrating viral, DNA-based and non-viral including episomal vectors, minicircle vectors and transposon vectors to increase the safety of therapeutic application⁵⁻⁸. However, low efficiency and mechanism of reprogramming on integration-free system are still unresolved issues.

Previous studies have developed approaches that conquered the threat of a random integration of viruses into the genome, however, they are technically challenging and less efficient than viral transduction. Therefore, to further facilitate hiPSC applications, numerous small molecules have been established that can be substitutes for transgenic DNA delivery and improve the efficiency of inducing pluripotency⁹⁻¹³. It has been reported that small molecular compounds could not only replace the Yamanaka reprogramming factors, but also promotes the reprogramming efficiency¹⁴⁻¹⁷. Different kinds of hiPSC induction

methods involved in various reprogramming factors or compounds may have distinctive mechanisms of reprogramming^{6,18–20}. Thus, only some of small molecules may work on specific reprogramming approaches. The method to generate virus-free, integration-free hiPSCs using episomal vectors has been widely used in pre-clinical studies on disease modeling and cell therapy^{8,20–23}. Accordingly, we focused on this method to further improve the human reprogramming efficiency and costs for accelerating the medical applications of hiPSCs in the future.

In this study, we found a dual-specificity tyrosine phosphorylation-regulated kinase inhibitor ID-8 robustly enhanced human somatic cell reprogramming by upregulation of PDK4 expression and activation of glycolysis. We also successfully established a safe and efficient reprogramming system using chemical compounds ID-8 and Kartogenin (K) without growth factors TGF β and bFGF. In addition, using ID-8, Kartogenin and Low-dose bFGF (IKB) supported the long-term expansion of human pluripotent stem cells. This novel culture system of hiPSCs preparation may hold more potentials for clinical applications.

Results

Chemical Compound Screening for Non-integrative Reprogramming system

Non-integrative reprogramming method using episomal vectors have the wide range of applications in fundamental researches and pre-clinical studies⁸. Chemical compounds that target signaling pathways, epigenetic modifications or metabolic-process regulation were applied to promote the induction efficiency of iPSCs^{9,11,13,14,16,17,24}. Firstly, to confirm this method, the transfection efficiency was tested by EGFP plasmid. We found that the most of transfected cells showed high expression of EGFP at 48 h, even over 57% cells still expressing EGFP after 7 days (**Supplementary Fig. 1a-d**). Then, we performed the transfection of human fibroblasts with reprogramming factors including OCT4, SOX2, KLF4, L-MYC, LIN28 and p53 shRNA in the Essential 8 (E8) culture system⁸. After 21 days, the colonies were formed with human ESC-like morphology, and showed TRA-1-60 positive indicating this method is available for compound screening (**Supplementary Fig. 1e-f**). Thus, we mainly selected 14 small molecules that have reported for increasing efficiency in other reprogramming systems, and targeting histone modification, Wnt signaling pathway, TGF β signaling pathway and kinase-related inhibitors et al. Under this well-defined medium and non-integrated reprogramming system, we designed the protocol and treated human fibroblasts with 14 small molecular compounds for 7 days at initial reprogramming stage (**Fig. 1a**). The results showed that CHIR99021, 1-azakenpaullone, XAV-939, VPA, SB431542 and RepSox slightly increased the efficiency of reprogramming. Importantly, we found that ID-8 significantly improved the efficiency of reprogramming after 7 days treatment (**Fig. 1b, c**). TRA-1-60 positive colonies induced by ID-8 showed human ESC-like morphology with clear edge and homogeneous cells (**Fig. 1c**). Together, our results revealed that ID-8 strikingly promote human fibroblast reprogramming into hiPSCs.

ID-8 Enhanced Reprogramming of Human fibroblasts and Astrocytes

To further determine the reliability of the initial screening results, we conducted dose-dependent and time-course ID-8 treatment. We found that ID-8 doses from 100 nM to 2 μ M notably improved the reprogramming efficiency. While, ID-8 at 250 nM concentration led to the optimal effect (**Fig. 2a and Supplementary Fig. 2a**). And then we investigated the time course by adding 250 nM ID-8 at different reprogramming stages. The results demonstrated that 3 days treatment of ID-8 was sufficient to accelerate the reprogramming of fibroblast cells. Whereas, by adjusting to 11 days treatment, the induction efficiency of iPSCs was noticeably increased (**Fig. 2b and Supplementary Fig. 2b**). During the long-term treatment, no toxic effect of ID-8 was detected on cells. Interestingly, we observed ID-8 enhanced the proliferation of ESC-like cell, and almost all of the colonies displayed human ESC-like morphology with few partially reprogramed cells as comparing with E8 culture system (**Supplementary Fig. 2c, d**). TRA-1-60 staining showed that ID-8 induced colonies were much denser than E8 culture system (**Supplementary Fig. 2d**). These results indicated that the similar function of promoting self-renewal treated with ID-8 have been reported by previous studies²⁵. After dose-dependent and time-course experiments, we determined 250 nM ID-8 treatment for 11 days as the final optimal protocol. Using this protocol, our results showed that the efficiency of reprogramming was notably increased by about 13 folds (**Fig. 2c, d**). Next, to explore whether ID-8 may work on other somatic cell reprogramming, we further examined ID-8 reprogramming in human primary astrocytes. Consistently, our results suggested that under same condition, ID-8 dramatically facilitated the conversion of astrocytes into iPSCs. Surprisingly, it took only 14 days to form large ESC-like colonies, which were about 17 times more efficient than the control group (**Fig. 2e, f**). In summary, these results demonstrated that ID-8 dramatically increased the generation of hiPSCs under this non-integrative reprogramming system.

Establishment of the Growth-Factor-free Reprogramming System Using Small Molecular Compounds

Under the aforementioned screening process, the reprogramming medium is E8 culture system. However, E8 medium containing growth factor bFGF and TGF β are expensive and unstable, which, to a great extent, impedes the construction of iPSC lines^{26,27}. Therefore, we propose to replace bFGF and TGF β in E8 component by using small molecular compounds in later reprogramming stage at day 7 to day 21. Previous studies have confirmed that Wnt and TGF β signaling pathways have great impacts on the self-renewal and differentiation of pluripotent stem cells^{24,28}. Thus, we screened small molecules that can maintain iPSCs expansion with the presence of ID-8 (**Fig. 3a**). We conducted several candidate compounds Kartogenin (K), CHIR99021 (C), 1-azakenpaullone (AK), and tacrolimus (T) during later reprogramming stage. The results showed that ID-8/C (IC) failed to maintain the growth of hiPSCs, only a few cells sustained the pluripotency through TRA-1-60 staining (**Supplementary Fig. 3a**). The combination of ID-8/T (IT) and ID-8/AK (IAK) can maintain the pluripotent state, but the clonal proliferation was slow and unable to generate hiPSC lines (**Supplementary Fig. 3b, c**). However, ID-

8/Kartogenin (IK) can maintain and promote the formation of TRA-1-60 positive colonies with ESC-like morphology. The colonies were significantly larger than the combination of IAK and IT (**Supplementary Fig. 3b-d**). In order to optimize the concentration of Kartogenin, we conducted a dose-dependent experiments. Our results showed that different doses of Kartogenin could enhance reprogramming efficiency (**Fig. 3c and Supplementary Fig. 3e**). However, high (2 μ M) concentrations of Kartogenin caused cell differentiation (**Supplementary Fig. 3f**), the optimal concentration (500 nM) can maintain the colonies growth and the pluripotency (**Fig. 3c and Supplementary Fig. 3e**).

Next, we further designed the IK reprogramming system (**Fig. 3a**). We noted that IK reprogramming system have generated the small colonies at day 7, but only a few clustered cells at control group (**Fig. 3b**). Most of iPSC colonies with IK treatment had ESC-like morphology with clear border and homogeneous cell population at day 14. Besides, the growth rate of colonies was remarkably higher than the control group (**Fig. 3b**). Those results revealed that IK robustly increased the reprogramming efficiency and accelerated the growth of colonies (**Fig. 3d, e**). To further confirm this IK reprogramming system, we induced human astrocytes into iPSCs using the same protocol. We found IK cocktail consistently promoted human astrocyte reprogramming and accelerated the generation of hiPSCs, only need for 14 days (**Fig. 3f, g and Supplementary Fig. 3g**). In summary, those results demonstrated that small molecule IK cocktail replacing growth factors strikingly improved the reprogramming efficiency and facilitated the induction of high-quality colonies.

IK-iPSCs Are Pluripotency

Although the IK cocktail had successfully maintained colonies with ESC-like morphology and showed TRA-1-60⁺ during the reprogramming process, we still needed to confirm the pluripotency of the IK-iPSCs. We picked out ESC-like colonies at day 20 and continued to culture in E8 and E6-IK medium respectively (**Fig. 4a-c**). We observed that colonies expanded faster in E8 medium, without differentiated cells (**Fig. 4a**). IK-iPS cell line can maintain over 6 passages in E8 medium (> one month) (**Fig. 4b**). Through immunofluorescence staining of pluripotent stem cell markers, OCT4, SOX2, NANOG, TRA-1-60, SSEA4 were all highly expressed in IK-iPSCs (**Fig. 4d**). The karyotypes of IK-iPSCs was normal (**Fig. 4e**). Those results showed that IK-iPSCs were fully pluripotent. Unfortunately, IK-iPSCs gradually differentiated in E6-IK culture medium, which indicated that IK were not a complete substitute for bFGF and TGF β in long term culture (**Fig. 4c**).

IKB medium maintain the long-term expansion of human pluripotent stem cells

For the long-term expansion of IK-iPSCs without growth-factors, we tested whether other compounds in combination with IK could maintain the pluripotency of human pluripotent stem cells. Firstly, we further tested small molecules with SB203580 (SB20), Go6983 (Go), SP600125 (SP), Doramapimod (Dora), Tacrolimus (T) and 1-azakenpaullone (AK) involved in sustaining pluripotency. Unfortunately, these combinations cannot completely replace the roles of growth factors for supporting pluripotency (**Supplementary Fig. 4**). Therefore, we presumed the low concentration of growth factor bFGF could help

to sustain the normal proliferation of colonies. Our results showed that the differentiated cells appeared in human embryonic stem cells (hESCs) after two passages cultured with 10 ng/ml or 20 ng/ml bFGF (**Fig. 5a-g**). However, under the dose of 50 ng/ml bFGF, the pluripotency of the cells could be maintained, and colonies could be cultured for over 6 passages (**Fig. 5h**), indicating that IK only replace TGF β . IK-iPS cell line could keep an ESC-like morphology in IK + 50 ng/ml bFGF (IKB) medium culture (**Fig. 5i**). The 24h survival index of H9 human embryonic stem cells cultured with IKB medium was about 200% and there were no significant differences as compared with E8 medium (**Fig. 5j**). The proliferation rate between IKB and E8 medium was similar (**Fig. 5k**). The pluripotency of hESCs in IKB medium were confirmed by the expression of pluripotent stem cell markers (**Fig. 5l**). Further, hPSCs cultured with IKB medium were successfully induced into dorsal cortical organoids and ventral brain organoids, indicating that IKB-hPSCs have normal capability of cell differentiation (**Fig. 5m-o**). In summary, these findings suggested that IKB medium without TGF β can maintain human pluripotent stem cells.

ID-8 Promotes Reprogramming by activating PDK4 Expression

Next, to investigate the mechanisms, we performed RNA-seq analysis on reprogrammed cells after ID-8 treatment. The statistics showed that there were 1669 differential genes (DGs) comparing with the E8 control (**Fig. 6a**), with 60.9% DGs upregulated in ID-8 group. In contrast, merely 96 DGs up-regulated compared with the E6 control (**Fig. 6b**). 88.5% of these DGs demonstrated higher expression treated by ID-8 (**Fig. 6c**). Accordingly, these results indicated that the ID-8 reprogramming pathways may be quite different with the E8 system. Through GO analysis in ID-8/E6 vs E6 group, we found that genes associated with metabolic pathways were significantly up-regulated (**Fig. 6d**). Interestingly, comparing with both E6 and E8 control groups, ID-8 significantly up-regulated genes linked to glycolytic metabolism pathways including PDK4, NMNAT3, GATM, ETNPPL, NAT8L, KCNJ11 and KHK (**Fig. 6e**). These results indicated that ID-8 may promote reprogramming by activating glycolytic metabolism. Previous studies showed PDK4 may be involved in cell reprogramming by regulation of glycolysis. To confirm this, we used small molecules Thiazolidinediones (TZDs) to activate PDK4 during reprogramming^{29,30}. Encouragingly, TZDs notably increased the efficiency of reprogramming, which can be blocked by Sodium dichloroacetate (DCA), the inhibitor of PDK4 (**Fig. 6f-g and Supplementary Fig. 5a-d**). Meanwhile, 2-Deoxyglucose (2-DG) as an inhibitor of glycolysis totally blocked the generation of iPSCs (**Supplementary Fig. 5e**). Taken together, our results demonstrated that ID-8 could up-regulated PDK4 to activate glycolytic metabolism for enhancing hiPSCs generation (**Fig. 6h**).

Discussion

No-virus infection and integration-free hiPSCs using episomal vectors have widely been used in pre-clinical studies on disease modeling and cell therapy^{4,20,23}. It is important to improve the efficiency and reduce costs of this system for hiPSCs translational medical application in the future. Here, we found that small molecule ID-8 robustly promoted human somatic cell reprogramming via upregulation of PDK4 expression and activation of glycolysis. Further, we identified IK medium as a novel growth-factor-free

system to efficiently induce hiPSCs generation. Finally, we successfully developed IKB medium to support the long-term expansion of human pluripotent stem cells.

Previous studies reported that ID-8 in combination with Wnt signaling pathway agonist Wnt3a could facilitate human embryonic stem cell expansion, which indicated ID-8 may maintain Wnt-mediated hESC self-renewal²⁵. The other studies revealed that combination with ID-8 and 1-azakenpauillone, GSK3 β inhibitor, as well as the calcineurin/NFAT pathway inhibitors Tacrolimus, termed as AKIT could support the long-term propagation of hPSCs¹². These researches indicated that ID-8 maintained hESC self-renewal via dependent on Wnt pathway. Here, we found that ID-8 combined with CHIR99021 or 1-azakenpauillone, which activated the canonical Wnt pathway, cannot maintain the colony growth during late stage of somatic cell reprogramming, and may lead to the differentiation of hPSCs for long-term culture. Our results may suggest that the effect of ID-8 was independent on activation of Wnt pathway during reprogramming. Comparing with the E7 and E8 culture system, AKIT system exhibited the higher efficiency of hiPSCs generation from human fibroblasts, which might be due to 1-azakenpauillone treatment¹². But here, our findings may suggest that ID-8 itself robustly enhanced somatic cell reprogramming into hiPSCs. Accordingly, we speculated that ID-8 may mainly contribute to the improvement of reprogramming in AKIT and the IKB culture system.

According to reported literatures, TGF β signaling is crucial for maintaining hPSCs^{31,32}. Previous studies indicated that Kartogenin may activate TGF β signaling and stimulates collagen synthesis in fibroblasts³³. But the role of Kartogenin in fibroblast cell reprogramming is unknown. Here, we found that Kartogenin further promoted ID-8 reprogramming and supported hiPSCs growth without TGF α and bFGF at late stage of reprogramming. However, we found that hPSCs failed to expand and gradually differentiated in IK medium, which indicated that IK was not sufficient to replace TGF α and bFGF for the maintenance of hPSCs. In contrast, IK treatment with low-dose bFGF medium notably promoted hPSCs growth and maintain their pluripotency. IKB medium exhibited the similar properties comparing with commercial E8 medium. Thus, as an E8 alternative our research offered a novel IKB culture system for expansion of hPSCs^{12,27,34}.

Metabolic transition involved in somatic cell reprogramming had been examined in previous studies^{35–38}. Accordingly, successful cell reprogramming requires a major metabolic shift from mitochondrial oxidative phosphorylation to glycolysis. Previous researches reported that pyruvate dehydrogenase kinase (PDK) can regulate glycolytic shift involved in cell fate reprogramming^{39–41}. Here, we demonstrated that ID-8 significantly upregulated PDK4 by activating glycolytic metabolism during reprogramming which may explained that ID-8 reprogramming pathways may differ from mechanism of E8 system. Also, small molecule TZDs as an activator of PDK4 increased the efficiency of hiPSCs induction^{29,30}, that can be blocked by DCA, the inhibitor of PDK4. Small molecular compound such as PS48, a small-molecule 3'-phosphoinositide-dependent kinase-1 activator also increased glycolytic gene expression to promote cell reprogramming¹⁷. Thus, these findings may suggested that small molecules targeting glycolysis alteration could be used for further promoting IK reprogramming efficiency.

Conclusions

In summary, our studies found that through upregulating PDK4 and activating glycolysis, a dual-specificity tyrosine phosphorylation-regulated kinase inhibitor ID-8 robustly enhances human somatic cell reprogramming. Subsequently, we established a novel growth-factor-free IK culture system for hiPSC generation and IKB medium supporting the hPSCs expansion. Furthermore, IK-iPSCs showed high pluripotency and normal karyotype. Our studies provided a virus-free, xeno-free, growth factor-free culture system for the generation of hiPSCs, which may support multiple applications in regenerative medicine in the future.

Materials And Methods

Cell culture

Human fibroblasts (ATCC, CRL-2097) were grown in Fibroblast Medium (FM) including DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (NATOCOR), 1% GlutaMAX (Thermo Fisher Scientific), 1% non-essential amino acids (Thermo Fisher Scientific), 10 ng/ml bFGF (Peprotech), 1% penicillin-streptomycin (Thermo Fisher Scientific), 0.1% β -mercaptoethanol. Human astrocytes (HA1800, Sciencell) were cultured with AM medium (Catalog #1801 Sciencell). Human ESCs and iPSCs were cultured on Matrigel (Catalog # 365230, Corning) coated plate with E8 medium including 100 ng/ml bFGF (Peprotech), 1.74 ng/ml TGF β and E6 medium which contains DMEM/F12 (Catalog # 11330032 Gibco) supplement with 13.6 μ g/ml sodium selenium (Sigma), 1 mg/ml sodium chloride (Sigma), 64 μ g/ml L-Ascorbic acid 2-phosphate (Sigma), 20 μ g/ml recombinant human insulin (Sigma) and 10 μ g/ml human holo-transferrin (Sigma), 1% penicillin-streptomycin (Thermo Fisher Scientific).

Small molecule reprogramming of human fibroblasts and astrocyte

Human fibroblasts and human astrocytes (Catalog #1800, Sciencell) were expanded and maintained with FM medium and AM medium respectively. The following episomal plasmids for iPSCs derivation were used for transfection at a 1:1:1 ratio: pCXLE-hOCT3/4-shp53-F (Addgene, 27077), pCXLE-hSK (Addgene, 27078), pCXLE-hUL (Addgene, 27080), and pCXLE-EGFP (Addgene, 27082). Total 4.5 μ g of the plasmid mixture was electroporated into $1-2 \times 10^6$ fibroblast cells or astrocytes according to the manual of Nucleofector kit (NHDF, Lonza) and Nucleofector 2b (Amaza, Lonza), and selected U-020 and T-020 system respectively. The transfected cells were seeded in Matrigel-coated 35 mm plates and cultured with FM medium containing 15% FBS (NATOCOR) for 2 days. The next day, Cells were re-seeded at a $1-2 \times 10^4$ cells/well into 12-well plates with FM medium. Day 0, the medium was changed to E6 and treated with different small molecules for 7 days, and then cultured in E8 medium for 21 days until hiPSCs colonies formed. We tested the compounds including: CHIR99021 (3 μ M, Tocris), RepSox (1 μ M, Tocris), XAV-939 (10 μ M, Tocris), SB431542 (10 μ M, MCE), ID-8 (0.5 μ M, MCE), VPA (0.5 mM, Cayman), 1-azakenpaullone (0.5 μ M, MCE), SAG (100 nM, EMD Millipore), LDN193189 (50 nM, Stemolecule), DAPT (2.5 μ M, Sigma), Y27632 (10 μ M, Tocris), Forskolin (10 μ M, Cayman), JQ1 (50 nM, Sigma) and SB203580 (1 μ M, MCE).

Chemical defined culture for hPSCs

Culture plates were coated with Matrigel for 30–60 min at 37°C. For the subculture, hESCs were rinsed twice with DPBS (Gibco), and detached as single cells with TrypLE (Catalog # 12563, Invitrogen) at 37°C for 1–2 min and replated on Matrigel-coated plates. Change the fresh medium daily and cells are passaged every four to six days. In the primary screen, we added a drug cocktail ID-8 (0.5 μ M), Kartogenin (0.5 μ M, MCE) combined with other small molecules including Go6983 (1 μ M, Santa Cruz), SP600125 (5 mM, Santa Cruz), Doramapimod (2 μ M, Cayman), Tacrolimus (20 pM, Cayman), 1-azakenpauillone (0.5 μ M) and SB203580 (1 μ M) for maintaining the pluripotency of human pluripotent stem cells for over 5 passages.

TRA-1-60 staining

In order to evaluate the efficiency of small molecular compounds on somatic cell reprogramming at 21 days, we used TRA-1-60 staining as below. Fix the cell with 4% PFA for 20 min at room temperature. And then wash five times per 2 minutes with PBS. Add biotin conjugated anti-human TRA-1-60 (catalog # 2174197, Invitrogen) diluted 1:250 in staining buffer with 3% BSA and 0.3% Triton X-100 in PBS and incubated at 4 °C overnight. The next day, wash five times with PBS. Add Streptavidin-HRP (Catalog # 405210, Biolegend) secondary antibody diluted 1:500 in staining buffer, incubate at RT for 2 hours. Wash 5 times with PBS. Develop staining with vector labs DAB kit until the colonies turn to purple and then wash 3 times with PBS.

Immunofluorescence

hiPSCs cultured on slice were washed twice with PBS. The cells were then fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature and then washed three times in PBS. Cells were blocked with 3% BSA and 0.3% Triton X-100 in PBS for 30 min at room temperature. Primary antibody: OCT4 (mouse 1:2000, 561465, BD Pharmingen), SOX2 (mouse 1:50, 561469, BD Pharmingen), NANOG (mouse 1:100, 560109, BD Pharmingen), SSEA4 (mouse 1:100, 3094649, EMD Millipore) and TRA-1-60 (mouse 1:100, 2906382, EMD Millipore) was added into 3% BSA and 0.3% Triton X-100 solution at 4°C overnight. The cells were washed three times in PBS. Secondary antibody (anti-mouse, Alexa 488, Life Technologies) and 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Labs) was added at a 500 fold dilution in 3% BSA and 0.3% Triton X-100 solution at room temperature for 1 hour. Wash three times with PBS and sealed for taking pictures.

Cortical organoids and ventral organoids were fixed in 4% PFA overnight at 4°C. And then transferred organoids into 30% sucrose solution after washing with PBS and kept them at 4°C for 48–72 h. After the organoid sunk into the bottom of solution then embedded in OCT medium (Tissue-Tek OCT Compound 4583, Sakura Finetek), snap-frozen on dry ice and stored at – 80°C. For immunofluorescence, 25 μ m-thick sections were made using a cryostat.

RNA-Seq

For the RNA-seq using three biological repeats with ID-8 treated human fibroblasts and E8 and E6 cultured control groups. The three groups of total RNA was extracted using the RNAiso Plus (9109, Takara) according to the manufacturer's protocol at day 14 of reprogramming. RNA purity, quantity and integrity were evaluated by RNA assessment using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then the cDNA libraries were established by using the recommended TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the recommended instructions. The RNA sequencing and analysis was completed by OE Biotech Co., Ltd (Shanghai, China). The cDNA libraries were sequenced on an Illumina HiSeq X Ten platform, with 150 bp paired-end reads generated. Raw data (raw reads) of fastq format were firstly processed using Trimmomatic. Obtained 46-57M clean reads were used for downstream analysis. The clean reads were mapped to the human genome using HISAT2. Cufflinks method was used to calculate FPKM value of each gene. Differentially expressed genes (DEGs) analyzed by Hierarchical cluster, and GO enrichment and KEGG pathway enrichment analysis of DEGs was used to investigate gene expression profiles.

Karyotype

hPSCs were dissociated with TrypLE and resuspended cells in chromosome medium (Gibco) and then incubated in incubator at 37°C and 7% CO₂ for 71 hours. Add Colcemid (KaryoMAX, Life Technologies) to incubate cells for 1 h at 37°C and 7% CO₂. Centrifuge cells and slowly add pre-warmed 75 mM KCl to the resuspended cells incubated for 12–20 min at 37°C, and then add chilled methanol-acetic acid fixative. Centrifuge cells and modestly resuspend the cell pellet in the remaining supernatant and add pre-cooled (-20 °C) methanol-acetic acid fixative for at least 30 min at -20 °C and repeat fixture for five times. The G-banding karyotype of 15 randomly selected mitotic metaphase nuclei were analyzed by kayoutaipu company.

Cortical organoid and ventral organoid induction

Human embryonic stem cells H9 (WA09, Wicell) or iPSCs were cultured in IKB medium on Matrigel-coated plate. Cells were trypsinized to single cells and grown as spheres in low-attachment plate (corning), added ROCK inhibitor Y27632 (10 µM) on the first day. Cortical organoid induction: for neural induction (from day 0 - day 7), spheroids were cultured in KSR medium (DMEM, 20% knockout serum replacement, 2 mM L-glutamine, 10 µM β-mercaptoethanol) with LDN193189 (100 nM) and SB431542 (10 µM). For neural progenitor cell (NPC) proliferation (from day 8 - day 21), the medium was B27 medium (Neurobasal, 2% B27, 100X NEAA, 100X GlutaMax) supplemented with 20 ng/ml bFGF and 20 ng/ml EGF. For neural maturation (from day 22), the medium was changed into B27 medium added with 20 ng/ml GDNF, 20 ng/ml BDNF and 200 µM ascorbic acid. For Ventral organoid induction: from day 0 to day 6, spheroids were floated in KSR medium supplemented with cocktail LDN193189 (100 nM), SAG (0.1 µM), SB431542 (10 µM) and IWP2 (5 µM). From day 7 to day 13, spheroids were cultured in KSR medium containing LDN193189 (100 nM), SAG (0.1 µM). From day 14 to day 21, the medium was changed into B27 medium into which SAG (0.1 µM), 100 ng/ml FGF8 were added. From day 21, to promote differentiation of NPC into interneurons, were replaced with B27 medium added with 200 µM ascorbic acid, 20 ng/ml GDNF and 20 ng/ml BDNF. Mediums were changed every two days.

Statistics

Data are showed as the mean $\pm$ SD of at least three independent experiments, significance level as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The number of independent experiments (n) is demonstrated in the figure legends. Statistics was used a two-tailed, unpaired t-test for statistical analysis.

Accession codes

The RNA-seq data were deposited at the GEO <https://www.ncbi.nlm.nih.gov/geo/> and the accession number is GSE166624.

Abbreviations

hiPSCs

human induced pluripotent stem cells; IKB:ID-8, Kartogenin and Low-dose bFGF; E8:Essential 8; E6:E8 without bFGF and TGF β ; I:ID-8; K:Kartogenin; C:CHIR9902; AK:1-azakenpaullone; T:tacrolimus; IK-

iPSCs:iPSC cultured in E6 with ID-8 and Kartogenin medium; Go:Go6983; SP:SP600125,

Dora:Doramapimod; DGs:differential genes; TZDs:Thiazolidinediones; DCA:Sodium dichloroacetate; 2-DG:2-Deoxyglucose; PDK:pyruvate dehydrogenase kinase

Declarations

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Authors' contributions

Z.S and J.X. designed the experiments. J.X., S.F., N.W., B.L., Y.H., Q.F., J.S. and H.L. performed fibroblast and astrocyte reprogramming, screening of small molecules for promoting reprogramming efficiency and maintaining pluripotency stem cells. Z.S and J.X. did RNA-seq analysis. Z.S., J.X. and N.W. wrote the manuscript and data interpretation. Z.S. supported this study financially.

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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.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing of interests

The authors declare no conflict of interest.

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Figures

Figure 1

Chemical Compound Screening for Non-integrative Reprogramming system. a, Schematic for reprogramming process. b, Fold change of TRA-1-60+ colonies for 14 small molecular compounds as compared with DMSO control group, the 14 small molecules including: CHIR99021 (3 μ M), AK (1-azakenpauillone, 500 nM), XAV939 (1 μ M), VPA (0.5 mM), SB431542 (1 μ M), RepSox (1 mM), DAPT (2.5 μ M), LDN193189 (50 nM), SAG (100 nM), Y27632 (10 μ M), Forskolin (10 mM), JQ-1 (50 nM), SB203580

(1 μ M), ID-8 (500 nM). n=3, means $\pm$ s.d., *** p<0.001. c, Representative images of TRA-1-60 staining at day 21 after the induction of reprogramming in human fibroblasts.

Figure 2

ID-8 Enhances Reprogramming of Human fibroblasts and Astrocytes. a, The number of TRA-1-60+ colonies treated with ID-8 at different concentrations. b, The number of TRA-1-60+ colonies of 250 nM ID-8 treated primary fibroblasts at different reprogramming stages. c, Representative images of TRA-1-60

staining for DMSO and ID-8 (250 nM) treatment in human fibroblasts. d, Fold change of ID-8 treatment as compared with E8 control. e, Representative images of TRA-1-60 staining of DMSO and ID-8 (250 nM) treatment of human primary astrocytes. f, TRA-1-60+ colonies of ID-8 treatment as compared with E8 control. n=3, means $\pm$ s.d., * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3

Establishing the Growth-Factor-free Reprogramming System Using Small Molecular Compounds. a, Schematic for growth-factor-free reprogramming system. b, Reprogrammed cells at different stages, scale bar, day 0 and day 7: 50 μm , day14 and day 21: 200 μm . c, TRA-1-60 positive colonies of kartogenin treated with different doses in combination with ID-8 at day 7 to day 21. d, Representative images of TRA-1-60 staining of human fibroblast reprogramming in ID-8 (I) and kartogenin (K) cocktail culture. e, Statistical results of human fibroblast reprogramming from (d). f, Representative images of TRA-1-60 staining of human astrocytes reprogramming in IK culture. g, Statistical results of human astrocyte reprogramming from (f). ID-8/kartogenin (IK). n=3, means $\pm$ s.d., ** p<0.01, *** p<0.001.

Figure 4

IK-iPSCs Are Pluripotent. a, IK-iPSCs colonies cultured in E8 medium, scale bar, 500 μ m. b, IK-iPSCs passage after 6 times in E8 medium, scale bar, 50 μ m. c, IK-iPSCs cultured in E6-IK medium scale bar, 100 μ m. d, Pluripotent marker staining with OCT4, SOX2, NANOG, TRA-1-60, SSEA4, scale bar, 50 μ m. e, Karyotyping of IK-iPSCs derived from human fibroblasts.

Figure 5

IKB medium maintains the long-term expansion of human pluripotent stem cells. a-g, hESCs cultured in E8, IK and different doses of bFGF combined with IK, scale bar, 50 μ m. h, hESCs cultured in IKB medium for 6 passages, scale bar, 50 μ m. i, IK-iPSCs cultured in IKB medium, scale bar, 50 μ m. j, 24 hours survival index of hESCs in E8 and IKB medium, n=3. k, Fold expansion of hESCs in E8 and IKB medium, n=3. l, Pluripotent marker staining of hESCs cultured in IKB medium for 6 passages, scale bar, 50 μ m. m and n,

Dorsal cortical organoids and ventral organoids differentiation from hESCs cultured in IKB medium, scale bar, 50 μ m. o and p, 3 weeks-old cortical organoids differentiation from hESCs cultured in IKB medium showed PAX6+/TUJ1+ neuronal progenitors, scale bar, 50 μ m. q, Ventral organoids showed NKX2.1+/TUJ1+ neuronal progenitors, scale bar, 100 μ m.

Figure 6

ID-8 Promotes human somatic cell Reprogramming by Activating PDK4 Expression. a, Differential gene Heatmap of ID-8 treated groups compared with E8, n=3. b, Differential gene Heatmap of ID-8 treatment compared with E6, n=3. c, Different expression gene number of ID-8 treatment compared with E8 and E6, n=3. d, Percentage of genes in biological process, n=3. e, FPKM of genes associated with metabolic processes, n=3. f, Representative images of TRA-1-60 staining of E8, E6, ID-8, ID-8+TZDs and ID-8+DCA treatment. g, Statistics of TRA-1-60+ colonies from (f), n=3. h, Working model of ID-8 reprogramming. n=3, means $\pm$ s.d., * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$.

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