Supplementary data

Material and methods

Preparation of feeders and mESCs culture

MEFs were cultured on 10cm dish in DMEM containing 10% fetal bovine serum supplemented with 1% P/S. 5ug/ml mitomycin C (Sigma, Cat.M0503) was added to inactivate the 3rd passage of MEFs (P3 MEFs) after the they were spreading over the dish. R1 ESCs were cultured on the pretreated MEFs at about $10^6/cm^2$ in DMEM containing 15% embryonic stem cell-qualified fetal bovine serum in the presence of 1000U/ml leukemia inhibitory factor (LIF) (Millipo, Cat.ESG1107) with supplement with 1mM sodium pyruvate (Gibco, Cat.11360), 0.1mM non-essential amino acid (Gibco, Cat.11140-050), 1% P/S (Sigma, Cat.M0503), and 0.1mM beta-mercaptoethanol (Gibco, Cat.21985-023). For transfection of mESCs with plasmids or siRNA, R1 cells were cultured without feeders in 6-well plates pre-coated by gelatin for 1h at final concentration of 0.2% (w/v). The re-plate of R1 cells in 6-well plates were as following: Firstly, the R1 cells were subject to digestion for 3min by 0.25% trypsin (Gibco, Cat.25200072). After centrifuge, add fresh medium to suspend the mESCs and then static-cultivate the mESCs in incubator for 40-50 min to remove MEFs. And then the upper suspension was plated into the pre-treated 6-well plate at $5X10^4$ cells/well and cultured overnight in normal mESCs medium.

Construction of St14 over-expression vector

The Control plasmid was pcDNA3.0 (+). The target CDS sequence of St14 was amplified using LA Taq DNA Polymerase (Takara, Cat. RR002A) with the following primers: forward sequence: 5'-GGACCGCCAAAACCAT; reverse sequence: 5'-CTATACCCCAGTGTGCTCTT. The CDS sequence was amplified as the following conditions: 94 ℃ for 1min, 32 cycles of 94 ℃ for 30s, 60 ℃ for 30s and 68 ℃ for 3min. After purification using PCR Product Isolation Kit (Tiangen, Cat. #DP214-03), the PCR product was ligated to pGM-T vector (Tiangen, Cat. VT302) according to the manufacture of T4 ligase (Ferrmentas, Cat. #EL0014). Then the T vector with target CDS sequence and pcDNA3.0 (+) plasmids were cleaved respectively by *Bam*HI (NEB, Cat. R0136S) together with *Eco*RI (NEB, Cat. R0101S). At the end of double digestion, Calf Intestinal Alkaline Phosphatase (NEB, Cat.M0290S) was added to dephosphorylate the pcDNA3.0 (+) for 30 mins at 37 ℃. Ligation of the target sequence with pcDNA3.0 (+) by T4 ligase was performed after purification. The mixture of ligation was then transduced into DH5α and subjected to blue-white selection. The positive clones were identified by endonuclease digestion and sequencing, and then the target expressional plasmids were isolated by purification Kit (Roche, Cat. 11754785001).

Transfection of target plasmids and HAIs siRNA

After replacing the normal mESC medium for P/S-free mESCs medium, 6ug/well of the target and control plasmids were transfected into mESCs by X-tremeGENE HP

DNA transfection reagent respectively(Roche, Cat.06366236001). HAI-1 siRNA and HAI-2 siRNA were transfected by Lipotamine 2000 (Invitrogen, Cat.11668-027). HAI-1 siRNA was 5'-AACUGCAACUUGGCGCUAGUdTdT-3' and referred to (Oberst et al., 2005); and HAI-2 siRNA was 5'-GGCAGCCUUUAUAGAUGAAUUdTdT-3' and the target sequence was 5'-GCTTCATCTACGGCGGGTGCA-3'. The scrambled siRNA was transfected as negative control. To identify interaction of St14 with HGF and/or proHGF, HGF and proHGF were added into the medium respectively with final concentration of 50ng/ml after transfection for 48hs. For RNA isolation, the cells were lysed by 1ml Trizol after washing with PBS. For immunoblotting, mESCs were lysed in 60ul RIPA lysis buffer in which protease inhibitor (0.1% PMSF) and 0.1% Sodium orthovanadate (Beyotime, Cat.S1873) were added before use.

RNA isolation and cDNA synthesis

200 ul Pheno/Chloroform was add into the samples and the mixture was shaken violently. Then the supernatant was transferred to clean eppendorf tubes after centrifuge at 1200g for 11 min at 4 ℃. Equal volume of isopropanol was added and the mix was centrifuged under the same condition. The segment was washed twice with 80% ethanol pre-treated with DEPC. To dissolve the RNA precipitate, DEPC-water was added after volatilization of residual ethanol. Reverse transcription was carried on by the commercial First Strand cDNA Synthesis Kit (Invitrogen, Cat.C02010A) according to the manufacture with random primers.

Quantitative real-time RT-PCR

The cDNAs were subjected to real-time PCR by FastStart SYBR Green Master Mix (Roche, Cat. 04673484001) according to the manufacture using 7900 HT Fast Real Time PCR system (ABI). The qRT-PCR assay was performed in triplicate including GAPDH as inner control. The amplification conditions were set as following: 94 ℃ for 5min, 40 cycles of 94 ℃ for 15s, 60 ℃ for 15 and 72 ℃ for 40s. The primers for specific genes were listed in Supplementary Data. The output data was analyzed by comparative Ct method. All the primers used for PCR and qRT-PCR were shown in Table S1 and Table S2 respectively.

Immunoblotting

The cellular lysis was boiled for 5 min at 95 ℃ and subject to electroblotting to transfer the protein onto polyvinlidene difluoride membrane (Millipore Cat. No. ISEQ00010) after electrophoresis on 12% SDS-polyacrylamide gel. 5% BSA was used to block the PVDF membrane for overnight at 4 ℃. The primary and HRP-conjugated secondary antibodies which were referred to Supplementary data were incubated with PVDF membrane for 1h after diluted by 1:1000 and 1:3000 respectively. And then ECL detection Kit (Millipore, Cat. WBKLS0500) was used to detect the protein abundance.

Identification of proHGF and HGF in mESCs medium by ELISA

The medium of different passages of feeders with or without mitomycin treatment

was collected respectively and then subjected to enzyme-linked immunosorbent assay (ELISA). The medium of P1 MEF, P2MEF, P3MEF, P3MEF_C_D1 and P3MEF_C_D2 were collected respectively and subjected to ELISA according to the manufacture of Mouse/Rat HGF Quantikine ELISA Kit (Cat.MHG00, R&D).

Supplementary figures

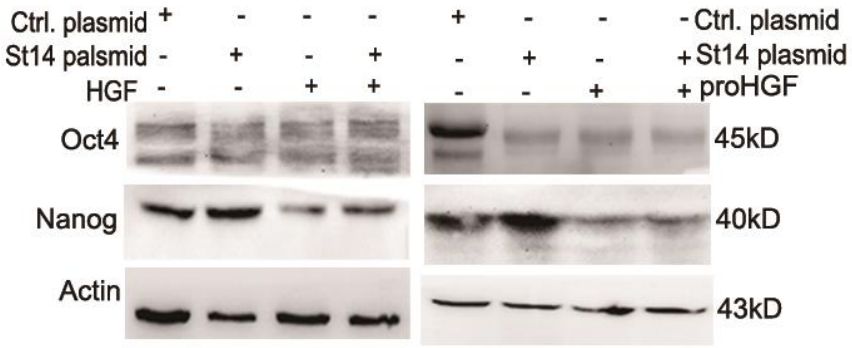

Figure S1. Immunoblotting of Oct4 and Nanog from the four group treatments mentioned in the text.

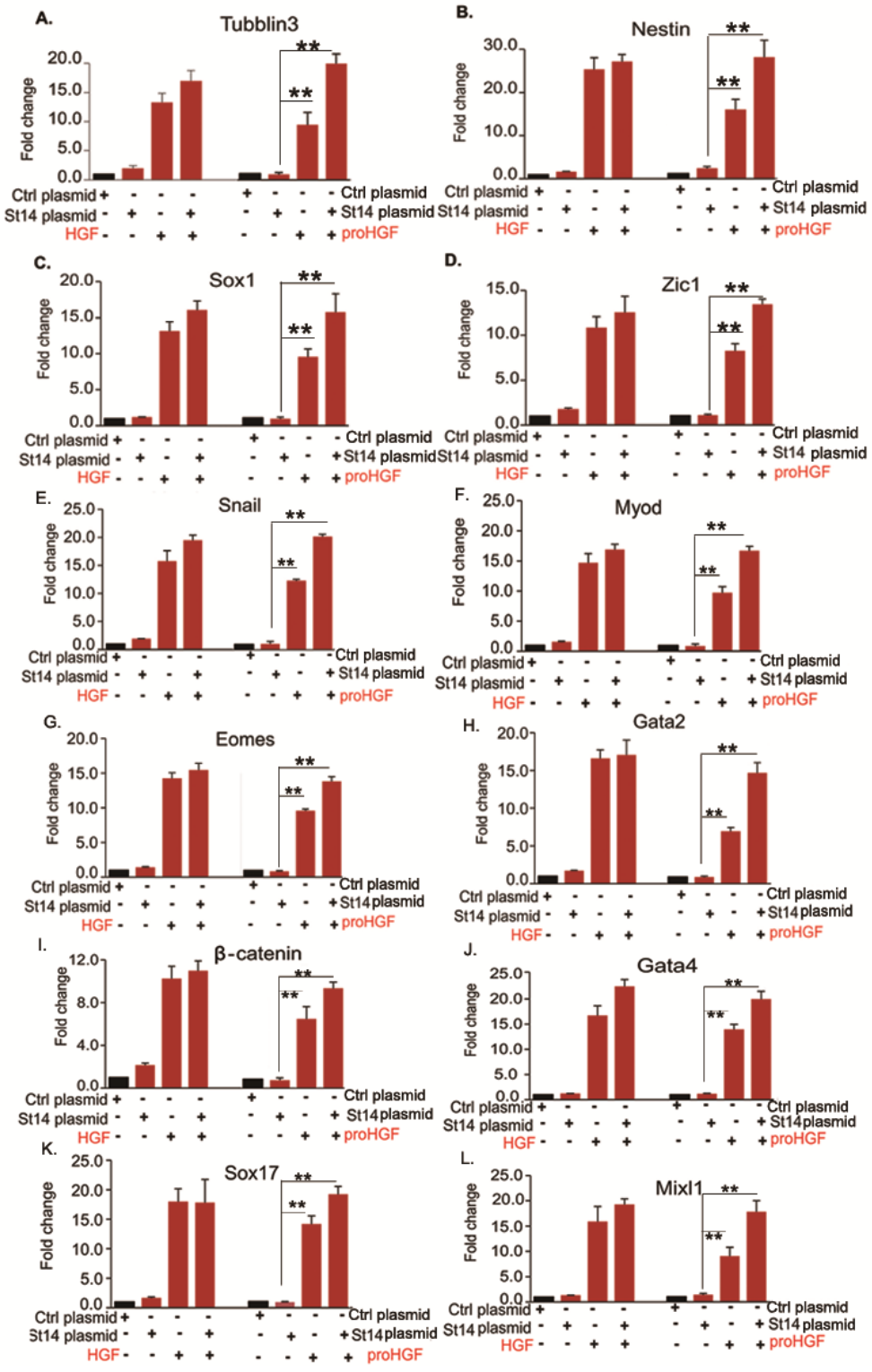

Figure S2. The increase of differentiation genes was more significant in simultaneous treatment of St14 & proHCF (Group 4) than that either treatment alone (Groups 2 and 3). qRT-PCR was performed to detect the expression of genes specific to three germ

layers (ectoderm: A-D; mesoderm: E-H; endoderm: I-L) after over-expression of St14 at the presence of proHGF (left columns) and HGF (right columns), respectively. All the experiments were repeated three times. * 0.05>p-value >0.01, **p-value< 0.01.

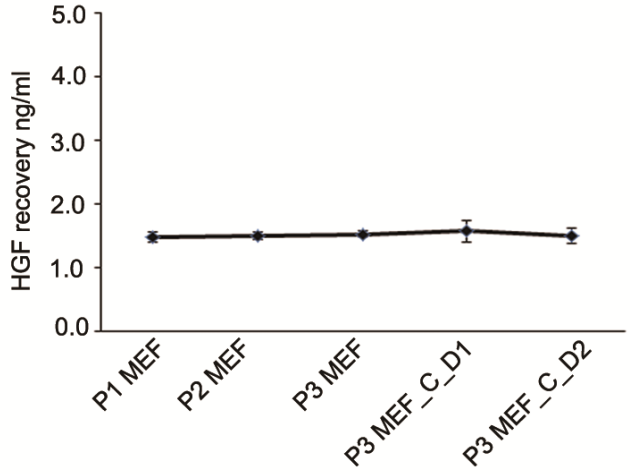

Figure S3. Detection of baseline level of murine HGF and proHGF derived from the mESCs medium by ELISA. P1-P3 MEF represented the medium of MEFs from passage 1 to passage 3; P3 MEF_C_D1 represent the medium of P3 MEFs with treatment by cytomycin C for 1 day; P3 MEF_C_D2 represented the medium of P3 MEF with treatment by cytomycin C for 2 days, all results were repeated three times.

Notes: In this supplementary Figure 3, the total abundance of proHGF and HGF in the medium was about 1.5ng/ml. This was much less than the Kd of native HGF binding to c-Met, which was reported 50-70pM (4.15-5.81ng/ml) (Lokker et al., 1992, EMBO J), indicating that the basal level of proHGF and HGF in mESCs medium was far less than the threshold that necessary for the activation of HGF/c-Met pathway in mESCs. Therefore, the effect of proHGF and HGF derived from the medium could be omitted.

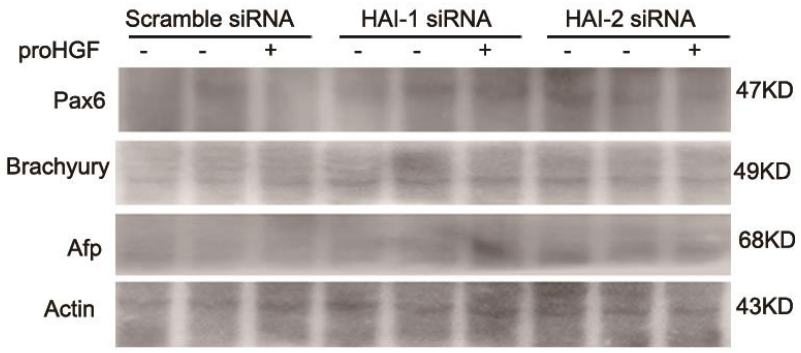

Figure S4. Immunoblotting of differentiation genes after KD of HAIs at the presence and absence of proHGF.

Table S1. Primer sequences used for PCR

Gene	Primer sequences	Product
St14_ for T vector_forward	5'-GGACCGCCAAAACCAT	2568bp
St14_for T vector_reverse	5'-CTATACCCCAGTGTGCTCTT	
Gapdh_forward	5'-GCAAATTCAACGGCACAGTC	399bp
Gapdh_reverse	5'-TCTTCTGGGTGGCAGTGATG	
St14_forward	5'-ATCCTTTACCAAACAGGCTCG	371bp
St14_reverse	5'-AGATGGGGCGCACGAC	
HAI-1_forward	5'-CATCTCTGCCTGCTTCCTCAT	226bp
HAI-1_reverse	5'-TGTGTTGTCTGCCTCGTTCA	
HAI-2_forward	5'-TTTGTTTATGGAGGCTGTGAAGG	100bp
HAI-2_reverse	5'-TGTCATCAGTGGTGTTCTCAGT	

Table S2. Primer sequences used for qPCR

	Gene	Primer sequences	Product
Pluripotent genes	St14 forward	5'-TCCTACGACTCCAACGACCC-3'	131bp
	St14 reverse	5'-TTGCATCGGCAGTAACGCT-3'	
	Nanog forward	5'-GCTATCTGGTGAACGCATCTGGAAG-3'	196bp
	Nanog reverse	5'-AAGTTATGGAGCGGAGCAGCATTC-3'	
	Oct4 forward	5'-TCTTTCCACCAGGCCCCCGGCTC-3'	224bp
	Oct4 reverse	5'-TGCGGGCGGACATGGGGAGATCC-3'	
	Fbx15 forward	5'-ATGGAGGAGTCGGAATTGGAG-3'	114bp
	Fbx15 reverse	5'-GATGGAGGAAGAGCAACGCT-3'	
	Sox2 forward	5'-TAGAGCTAGACTCCGGGCGATGA-3'	297bp
	Sox2 reverse	5'-TTGCCTTAAACAAGACCACGAAA-3'	
Ectoderm	Tubb3 forward	5'-CGGCAACTATGTAGGGGACT-3'	195bp
	Tubb3 reverse	5'-CCAGCACCACTCTGACCAA-3'	
	Nestin forward	5'-GGACAGGACCAAGAGGAACA-3'	599bp
	Nestin reverse	5'-TCCCACCTCTGTTGACTTCC-3'	
	Zic1 forward	5'-GCGATCCGAGCACTATGCT-3'	570bp
	Zic1 reverse	5'-GGGTGCGTGTAGGACTTATCG-3'	
	Pax6 forward	5'-AGTACCAGTGTCTACCAGCCAAT-3'	195bp
	Pax6 reverse	5'-GCACGAGTATGAGGAGGTCTGA-3'	
	Sox1 forward	5'-GCGAGGCGATGCCAACT-3'	182bp
	Sox1 reverse	5'-CCCAAAAGAGCGGTAACAACTA-3'	
Mesoderm	Brychuary forward	5'-GCTCATCGGAACAGCTCTCCAACC-3'	320bp
	Brychuary reverse	5'-GGAGAACCAGAAGACGAGGACGTG-3'	
	Gata2 forward	5'-GCCGGGAGTGTGTCAACTG-3'	201bp
	Gata2 reverse	5'-AGGTGGTGGTTGTCGTCTGA-3'	
	Eomes forward	5'-ATCTCCCACGGATTCCCCTA-3'	233bp
	Eomes reverse	5'-GCTTGTTGGTCACAGGTTGC-3'	
	Myod1 forward	5'-GCTCCAACTGCTCTGATG-3'	273bp
	Myod1 reverse	5'-CCTGTTCTGTGTCGCTTAG-3'	
	Snail forward	5'-CCGATGAGGACAGTGGCA-3'	365bp
	Snail reverse	5'-GCAGTGGGAGCAGGAGAAT-3'	
Endoderm	Foxa2 forward	5'-TGCTGGGAGCCGTGAAG-3'	548bp
	Foxa2 reverse	5'-GCTCAGCGTCAGCATCTTGT-3'	
	Gata4 forward	5'-CTCCTACTCCAGCCCCTACC-3'	591bp
	Gata4 reverse	5'-GTGGCATTGCTGGAGTTACC-3'	
	Mixl1 forward	5'-CCGACAGACCATGTACCCAG-3'	146bp
	Mixl1 reverse	5'-GAGGATAAGGGCTGAAATGACT-3'	
	Sox17 forward	5'-TGCGGGATACGCCAGTG-3'	128bp
	Sox17 reverse	5'-CCTCGCCTTTCACCTTTACAT-3'	
	Afp forward	5'-AGTGCGTGACGGAGAAGAAT-3'	494bp
	Afp reverse	5'-TGTCTGGAAGCACTCCTCCT-3'	
	ß-catenin forward	5'-CATCTTAAGCCCTCGCTCGG-3'	226bp
	ß-catenin reverse	5'-CAGGTCAGCTTGAGTAGCCAT-3'	