Laterally Confined Growth of Cells Induces Nuclear Reprogramming in the Absence of Exogenous Biochemical Factors

Bibhas Roy[1, 2], Saradha Venkatachalapathy[1], Prasuna Ratna[1], Yejun Wang[1], D. S. Jokhun[1], Mallika Nagarajan[1], and G. V. Shivashankar[1, 2, *]

AFFILIATIONS

[1]Mechanobiology Institute, National University of Singapore, Singapore 117411.

[2]Institute of Molecular Oncology, Italian Foundation for Cancer Research, 20139 Milan, Italy.

*Correspondence to: shiva.gvs@gmail.com.

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

ES cell culture: The ES cell line E14 was derived from the inbred mouse strain 129/Ola in 1985 by Dr. Martin Hooper in Edinburgh, Scotland (1). Cells were further expanded in different laboratories under a variety of conditions. As a positive control of stemness properties, the ES cells used in this study were cultured for at least ten passages under feeder free conditions. E14 cells were cultured on 1% gelatin coated plates in DMEM, high glucose, HEPES (Gibco, Thermo Fisher Scientific) supplemented with 15% FBS, ES cell-qualified, US origin (Gibco, Life Technologies), 0.1 mM nonessential amino acids (Gibco, Thermo Fisher Scientific), 1 mM sodium pyruvate (Gibco, Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol, 1500 U/ml mLIF (Millipore), 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific). The media was replaced with fresh culture media on every alternate day unless otherwise stated.

Chromosome spreads and karyotyping: To quantify the copy number variations during the induction process, metaphase spreads was performed in the 10 day old spheroid and NIH3T3 cells as shown in Fig. S9. Cells were incubated with 0.5 mg/ml nocodazole for 2–2.5 hours to arrest the cells in metaphase. The cells were exposed to hypotonic treatment with 75 mM KCl solution and fixed with methanol:glacial acetic acid (3:1) and spread onto clean slides.

Epitect (ChIP-qPCR) assay: *Fixing and preparation for immunostaining:* NIH3T3 cells were grown on rectangular patterns for 3 hr, 3 days, 6 days, and 10 days. The cells at the four-stated time-points were fixed with 2% formaldehyde for 5 min at room temperature (RT). Following fixation, cells were quenched with 127 mM glycine for 10 min at RT. Cells were then washed with Phosphate-buffered Saline (PBS). The cells were lysed and nuclei were prepared in lysis buffer (10 mM Tris-HCl (pH 8), 10 mM NaCl, 0.2% IGEPAL CA-630 (Sigma)) with protease inhibitor cocktail (Roche) for 30 min on ice with intermittent agitation. Nuclei were washed with 1x Fast Digest (FD) buffer (Thermo Fisher Scientific). 400 µl of 1 x FDbuffer and 6 µl of 20% SDS was added to the nuclei and incubated at 37˚C for 60 min with constant agitation.

40 µl of 20% Triton X-100 was added and incubated at 37˚C for 60 min with constant agitation. 30 µl of HindIII (50 U/µl; Thermo Fisher Scientific) was added and incubated at 37˚C for overnight with constant agitation. Nuclei were washed with PBS and blocked with 5% BSA for 1 hr at RT before immunostaining. Nuclei were then washed with 5% BSA, scraped and collected in a tube.

Coupling with beads: Dynabeads coupled with Anti-Rabbit (M-280; Thermo Fisher Scientific) were re-suspended in 1 ml of washing Buffer (Ca^{2+} and Mg^{2+} free PBS supplemented with 0.1% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.4). Dynabeads were washed using the DynaMagTM-2 Magnet. A solution of 5% BSA and ChIp grade Anti-Histone 3 (acetyl K9) Rabbit pAb (Abcam) was added to the beads and incubated with gentle tilting and rotation at RT for one hour. Unbound Anti-Histone 3 (acetyl K9) Rabbit pAb was removed using the DynaMagTM-2 Magnet. Dynabeads were then washed with 5% BSA to ensure there was not any unbound Anti-Histone 3 (acetyl K9) Rabbit pAb remaining. These Dynabeads were then resuspended in the nuclei in 5% BSA and incubated for over 12 hours at 4°C. The product obtained after the incubation was a tertiary complex comprised of Dynabeads coated with Anti-Rabbit, bound to Anti-Histone 3 (acetyl K9) Rabbit pAb, which was further bound to chromatin associated with Histone 3 (acetyl K9). The beads were washed with PBS to ensure that any chromatin not associated with Histone 3 (acetyl K9) was removed.

Reverse crosslinking: Reverse crosslinking was performed using 5ul of Proteinase K (PK; Thermo Fisher Scientific) in 200ul of PK buffer (30mM Tris (pH8.0), 10mM EDTA, 1% SDS) incubated at 65˚C for at least 90 min. Using the DynaMagTM-2 Magnet the supernatant was separated from the Dynabeads. 50 µL of PK buffer was added to the bead fraction to elute any remaining DNA. The supernatant collected was purified using Qiagen PCR clean-up to concentrate the DNA. This DNA was further amplified using the REPLI-g Single Cell Kit (Qiagen). The amplified DNA was analyzed using designed primers for EMT EpiTect ChIP qPCR array (Qiagen). The Mouse Epithelial to Mesenchymal Transition (EMT) Epitect ChIP qPCR Array was used to measure histone acetylation changes at certain promoters. This array profiles 84 genes representing epithelial genes, mesenchymal genes, proliferation genes, transcription factors *etc* involved in EMT.

Chromosome Fluorescence *in situ* hybridization (FISH): Cells were washed with 1× PBS to remove cell culture medium followed by incubation on ice for 5-8 minutes, with 0.25 % Triton in CSK buffer (100 mM NaCl, 300 mM Sucrose, 3 mM MgCl2, 10 mM PIPES with pH 6.8). Cells were then fixed with 4 % PFA (Paraformaldehyde) for 10 minutes, briefly rinsed with 0.1 M Tris-HCl followed by 1× PBS wash. This was followed by permeabilization with 0.5 % Triton for 10-15 minutes. Overnight incubation in 20% glycerol at 4 °C, and then 5 - 6 freeze-thaw cycles in liquid nitrogen followed. After this, cells were washed with 1× PBS a few times, before and after treatment with 0.01 % HCl for 5-10 minutes, followed by digestion with 0.002 % porcine pepsin (Sigma Aldrich, USA) in 0.01N HCl at 37 °C for 4 minutes. Cells were then fixed with 1 % PFA for 4 minutes, briefly rinsed in 1× PBS before being treated with RNAse (from Promega, USA, 200 µg/ml made in 2× SSC-0.3M sodium chloride and 30mM trisodium citrate) at 37°C for 15-20 minutes. The cells were then washed with 2× SSC and equilibrated in 50 % Formamide / 2× SSC [(pH 7.4) overnight at 4 °C. Hybridization was set up the following day. Chromosome paints (Chrombios, Germany) tagged with different

fluorophores were thawed to room temperature, and mixed with hybridization buffer provided by the supplier. Cells were denatured in 50 % Formamide / 2× SSC at 85 °C for 2-3 minutes and then incubated with the fluorescently labeled mouse chromosome FISH probe mix; the slides were then sealed with a Sigmacote (Sigma-Aldrich) coated hydrophobic coverslip and rubber cement to incubate overnight in a moist chamber at 37 °C with shaking. Chromosomes were pair-wisely painted and 3 representative pairs were selected for analysis: Chr11-Chr15, Chr5-Chr9 and Chr3-Chr6. At the end of the incubation period, slides were washed thrice each in 50 % Formamide / 2× SSC at 45 °C and 0.1× SSC at 60 °C. Finally, the nuclei were stained with Hoechst 33342 (Sigma-Aldrich) for 10 minutes and then mounted with Prolong Gold anti-fade mounting medium (Thermo Fisher Scientific), sealed with a coverslip, and imaged.

Endoderm Differentiation: The ten-day old spheroids (developed from NIH3T3 cells) were collected from the RE micropattern by mild trypsinization (30 seconds — 1 minute). The collected spheroids were plated onto 100 mm gelatinized tissue culture dishes in 1□10 dilutions and incubated at 37°C with 5% CO_2 with 15% FBS, 0.1 mM non-essential amino acids, 1 mM MTG, 2 mM of L-glutamine, 10^{-7} M of all-trans-retinoic acid (Sigma) and 25 ng/ml of human basic fibroblast growth factor (bFGF) (Stem Cell Technologies), and high glucose DMEM for 3 days. The cells generated in previous step were then maintained at 37°C with 5% CO_2 with 15% FBS containing high glucose DMEM supplemented with 0.1 mM non-essential amino acids, 1 mM MTG, 2 mM of L-glutamine in the presence of 25 ng/ml of bFGF for up to 14 days (2). At this time, the cells were trypsinized, collected, seeded onto petri dishes, and incubated for 6 days in suspension at 37°C with 5% CO_2 with DMEM/F12 (1:1) basal media (GIBCO) supplemented with 1x N2 supplement-A, bFGF, 1x B27 supplement (GIBCO) to induce formation of cell clusters. The cell clusters from previous step were cultured for 6 days at 37°C with 5% CO_2 in the presence of 2×10^{-3} M dibutyryl cAMP in DMEM/F12 (1:1) basal media (GIBCO) supplemented supplemented with 1x N2 supplement and 10 mM nicotinamide.

Dopaminergic neuronal differentiation: As illustrated in SI Appendix, Fig. S19, the isolated ten day old spheroids were cultured on Pluronic-F127 treated bacterial culture plate in prepared ESC media for 4 days at 37 °C and 5% CO2. On day 12, media was changed by allowing the spheroids suspension to stand for approximately 5 minutes in a 15 mL tube. After settling down of spheroids, old media was replaced with fresh media and this was followed by gentle pipetting and the transfer of spheroids back into the original plates. On day 14, spheroids were collected as mentioned above and transferred on 6 well tissue culture plate and cultured for 2 hours in ESC media. On day 15, the ESC media was replaced with ITS/Fibronectin media and cultured in it for another 6-8 days at 37 °C and 5% CO2 with media replacement every alternative day. ITS/Fibronectin media was prepared with DMEM/F-12, glucose, L-glutamine, NaHCO3, ITS supplement and fibronectin according to manufacturer's protocol. After 6-8 days, cells were typsinized and seeded on Poly-L-Lysine/Fibronectin-coated coverslips and cultured in N-2 MAX/FGF basic/FGF-8b/ Shh-N/Ascorbic Acid Media. In this condition, cells were cultured for 4-6 days with media replacement every other day. Cells from this stage were cultured in N-2 MAX/Ascorbic Acid Media (without growth factors) for 10-15 days with every alternative day media replacement. After 10-15 days, cells were immuno-stained for

dopaminergic neurons by staining with Tyrosine Hydroxylase and Neuron-specific β-III Tubulin antibodies. For control differentiation assay, these spheroids were cultured under similar conditions but in absence of dopaminergic neuronal differentiation factors

Mammosphere culture condition: For MCF-7 mammosphere culture, cells were suspended at 1×10^5 cells/mL and seeded into ultra-low attachment surface (0.2% Pluronic F127 treated surface). In serum free DMEM/F12 (1:1) supplemented with 10 ng/mL basic fibroblast growth factor (b-FGF, R&D Biosystem), 20 ng/mL epidermal growth factor (EGF, R&D Biosystem), 5 µg/mL ITS (insulin + transferrin + selenium, GIBCO), with B27 (GIBCO) (3). Every two days 2mL of fresh media was replaced. Cells grown in these conditions becomes non-adherent spherical clusters of cells (named ‘‘mammospheres’’) were collected on day 10 s by gentle centrifugation.

Image Analysis:

Nuclear area fluctuation and dynamics: ImageJ was used to calculate the projected nuclear area fluctuation (PNAF) of H2B-EGFP nucleus and generate time-lapse merged nuclear periphery data. PNAFs were combined from multiple cells and time points for each condition to obtain a normal distribution. SD (σ) of such distribution indicates the amplitude (in percentage) of area fluctuations and was performed in ORIGIN. The nuclear circularity for fibroblasts seeded onto micropattern were calculated according to the formula, circularity = $4\pi A/P^2$, where A is nucleus projection area and P is nucleus perimeter. The corresponding projected area and perimeter were calculated in ImageJ. In order to estimate nuclear orientation for fibroblasts seeded onto micropattern, the angle between major axis and cell basal surface plane was calculated using custom written code in MATLAB. Confocal images of the nucleus stained with DAPI were used for this purpose.

The mean square displacement (MSD) is calculated for the centroid of each nucleus by finding the average displacement between two time points with a time interval $\tau = n\Delta t$, where n takes integer values (1 to N), as follows:

$$< r^2(\tau) >= \frac{1}{N-n}\sum_{k=1}^{N-n}[r\big((k-1)\Delta t + n\Delta t\big) - r\big((k-1)\Delta t\big)]^2$$

Here r is the position vector of the particle at each time point and N is the total number of measured points.

Chromosome Intermingling: A semi-automated algorithm written in MATLAB (Mathworks, USA) was used to analyze 3D chromosome FISH images, where both nuclei and chromosomes were manually selected according to the signal distribution on the Z-maximum projected images. Maximum projected masks for nuclei and chromosomes were then generated. This was used to multiply the entire Z-stack of the nucleus and chromosome to remove the background noise, which would undermine the segmentation accuracy. For 3D segmentation, the mean and standard deviation of intensity was computed throughout the entire Z-stack. The criteria for

setting a pixel as 1 or 0 is based on the mean ± (standard deviation × a), where a is a value to adjust the criteria. Pixels above mean ± (standard deviation × a), were set as 1, and those below were set as 0. The segmentation procedure was monitored by merging the outline of the segmented object with the original object. This resulted in best 3D masking for both nucleus and chromosomes. To compute the intermingling degree, the intermingling volume between two chromosomes was first estimated. To estimate the intermingling volume, the segmented images of the two chromosomes were multiplied, and only the overlapping region resulted in pixels with value of 1. The number of pixels with value 1 represents intermingling volume. The intermingling degree was then defined as the intermingling volume normalized to the volume of the two chromosomes and their homologues (V_1, $V_{1'}$, $V_{1''}$, V_2, $V_{2'}$, $V_{2''}$),

$$Intermingling\ degree = \frac{V_{1\&2}}{V_1 + V_{1'} + V_{1''} + V_2 + V_{2'} + V_{2''}}$$

SUPPLEMENTARY FIGURES

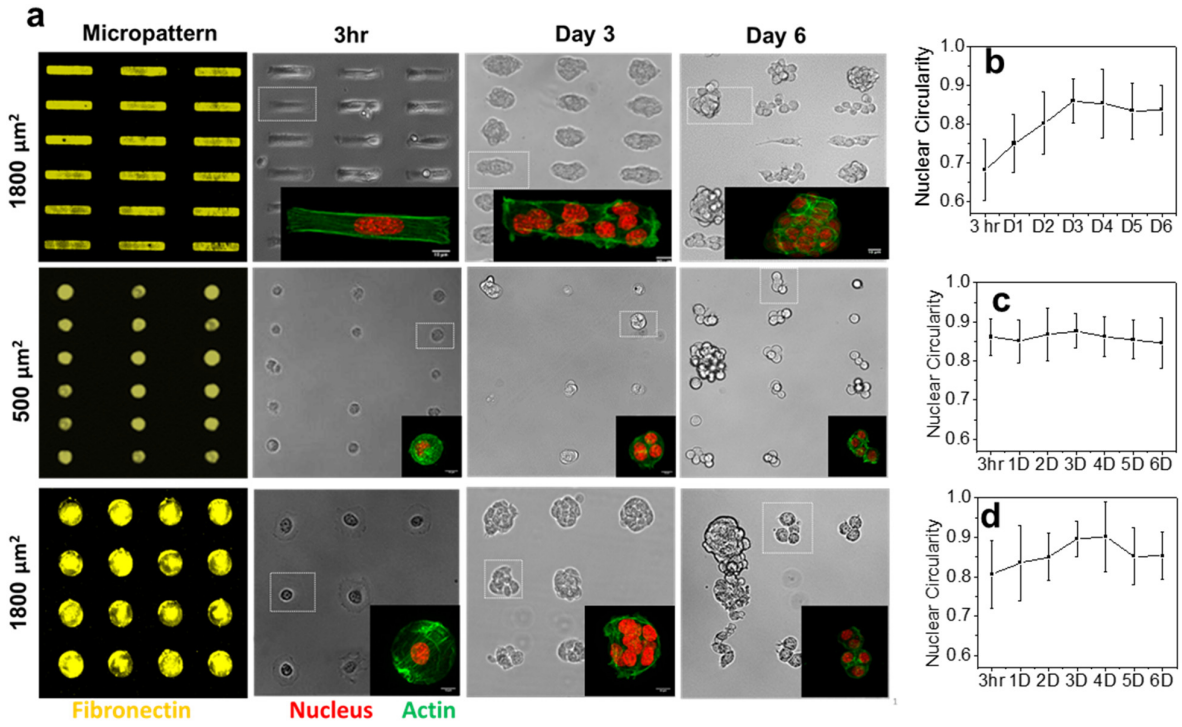

Figure S1. Laterally confined growth of cell on different micropatterns. (a) The left column represents fibronectin RE, SC, BC micropatterns. The phase contrast images of NIH 3T3 mouse fibroblasts cells cultured on micropatterns upto six days. Inset shows a fluorescent image of cells highlighted by a white dotted box on the micropattern stained with nucleus (red) and actin (green). (b-d) Nuclear circularity plots represent the temporal evolution of nuclear morphology for six days on different micropatterns. Error bars represent SD.

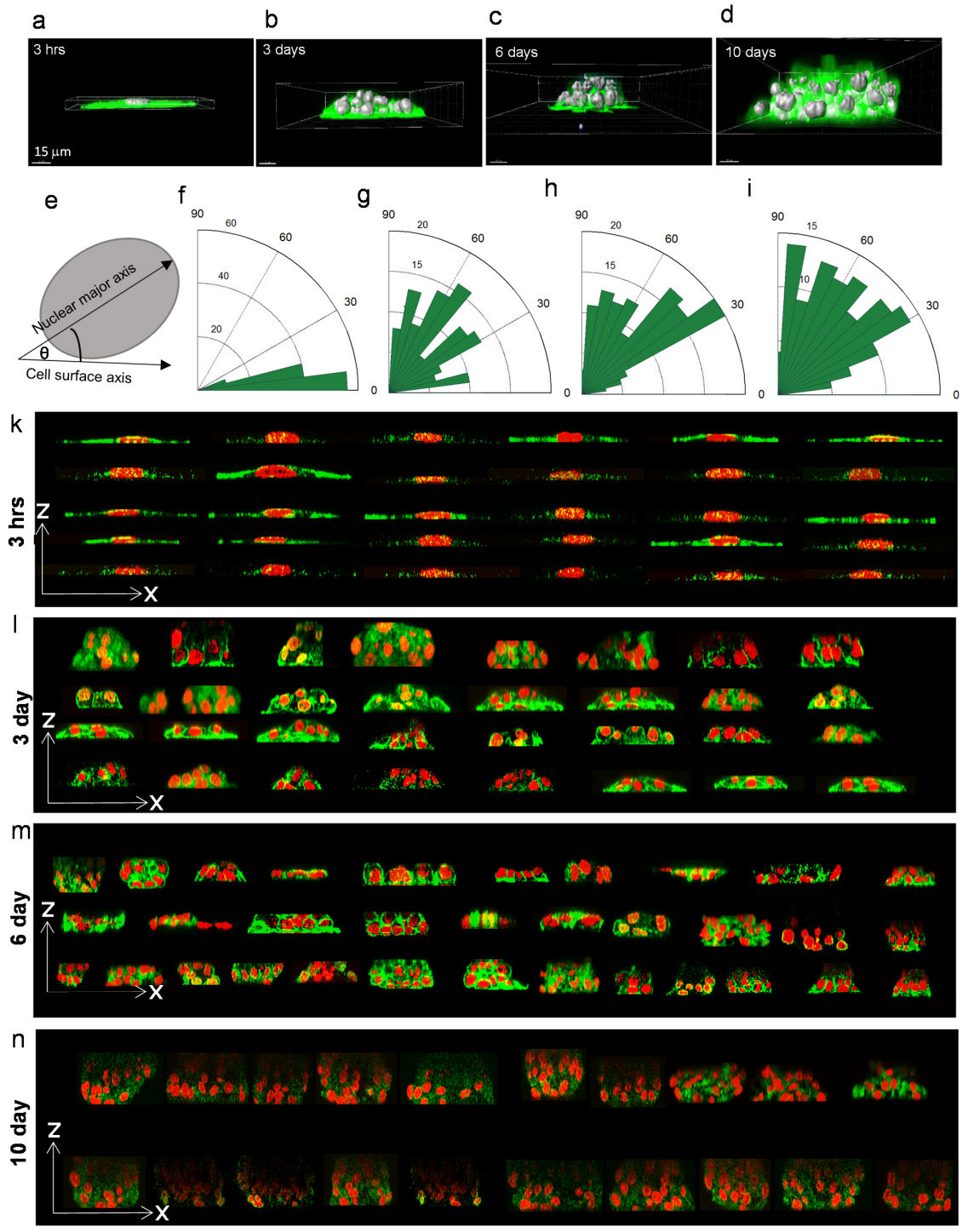

Figure S2. Changes to nuclear orientation. (a-d) 3D view of the nuclear orientation of the cells cultured on RE upto 10 days. (e-i) Distribution of the angle between major axis and cell basal surface plane in cell cultured on RE at 3hr (n = 45), day 3 (n = 65), day 6 (n = 71), and 10days (n=75). (k-n) Collage of cross sectional images of cells represents the change of nuclear orientation. Actin (green) and Nucleus (red).

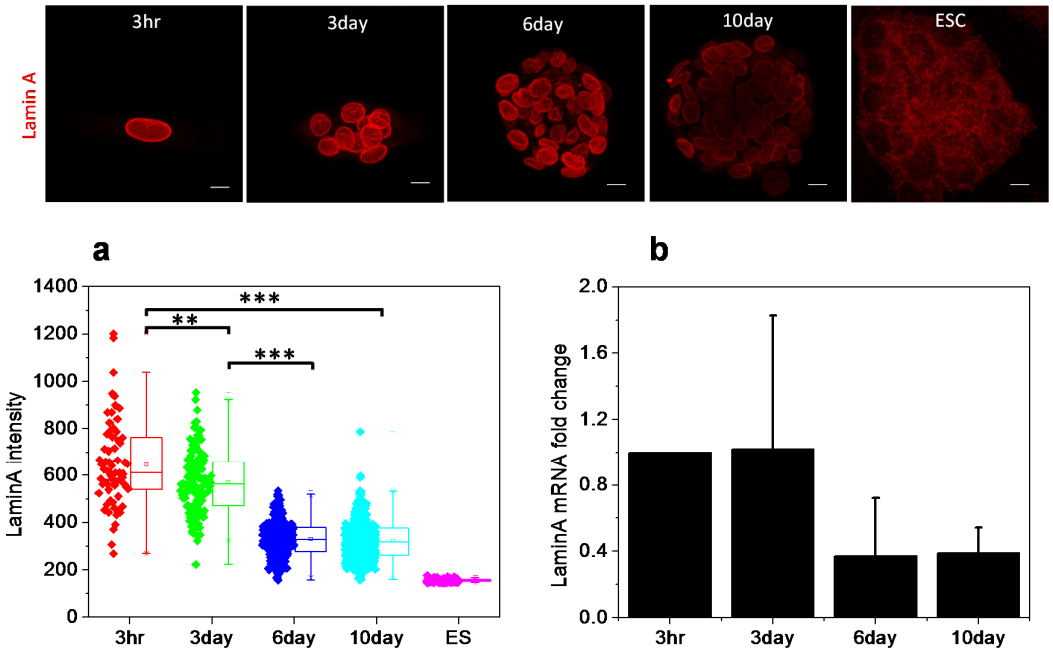

Figure S3. Changes in Lamin A expression levels during nuclear reprogramming. (a) Representative images of Lamin A levels during the lateral confined growth of cells on RE and ESC as a control. (b) Intensity plot of Lamin A. Error bars represent standard deviation. (c) Lamin A mRNA levels obtained by qRT-PCR for cells with prolonged geometry driven cellular confinement normalized with respect to NIH3T3 grown on RE for 3hr cells (n = 3 samples). Error bars represent SD. ***P < 0.001.

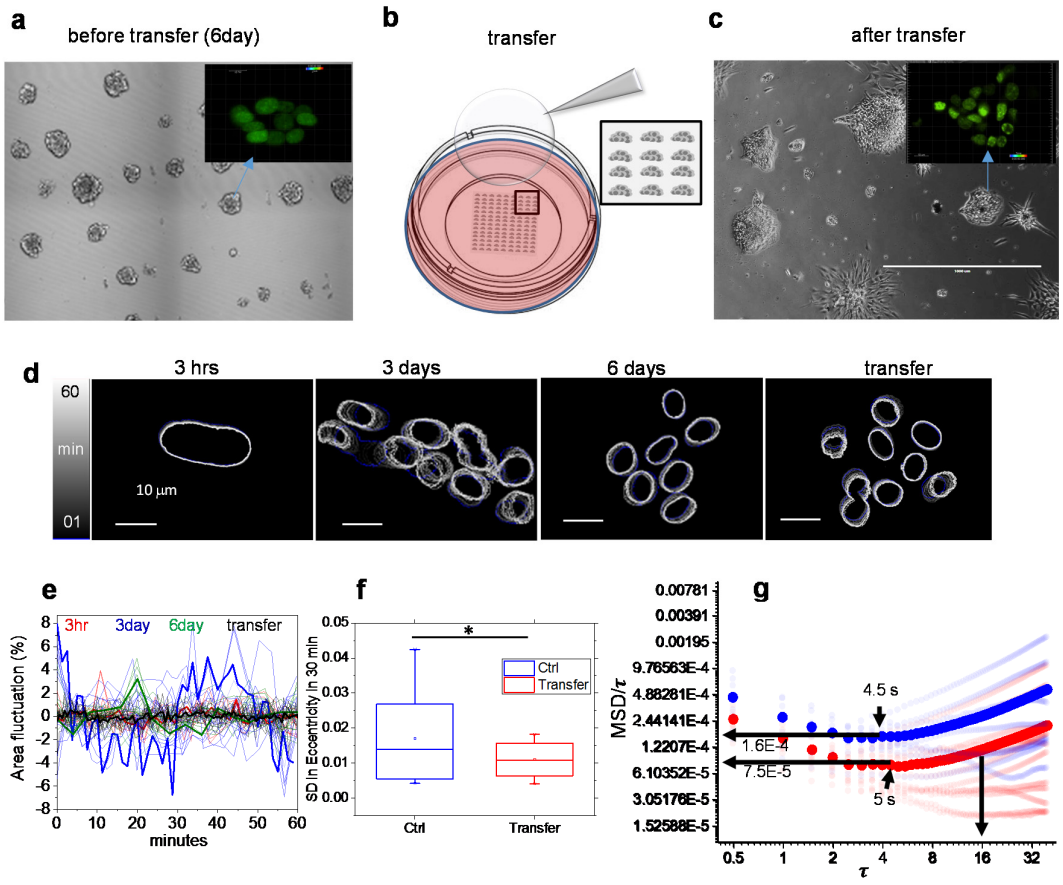

Figure S4. Nuclear dynamics. (a) Cells in six day old spheroid, (b and c) formation of colony upon transfer on unpatterned surface. (d) Time-lapse merged image of the nuclear periphery of cells expressing H2B-EGFP grown on RE at 3hr, 3 days, 6 days and after transfer (scale bar 10 μm). (e) Plot of the projected nuclear area fluctuations vs. time for multiple cells on RE at 3hr (n=21), 3 days (n=43), 6 days (n=52) and after transfer (n=31). (f) Standard deviation in nuclear eccentricity in 30 minutes after transfer (red) and in control untransformed cells (blue). (g) MSD/t plot represents the nuclear dynamics after transfer (red) and control untransformed cells (blue).

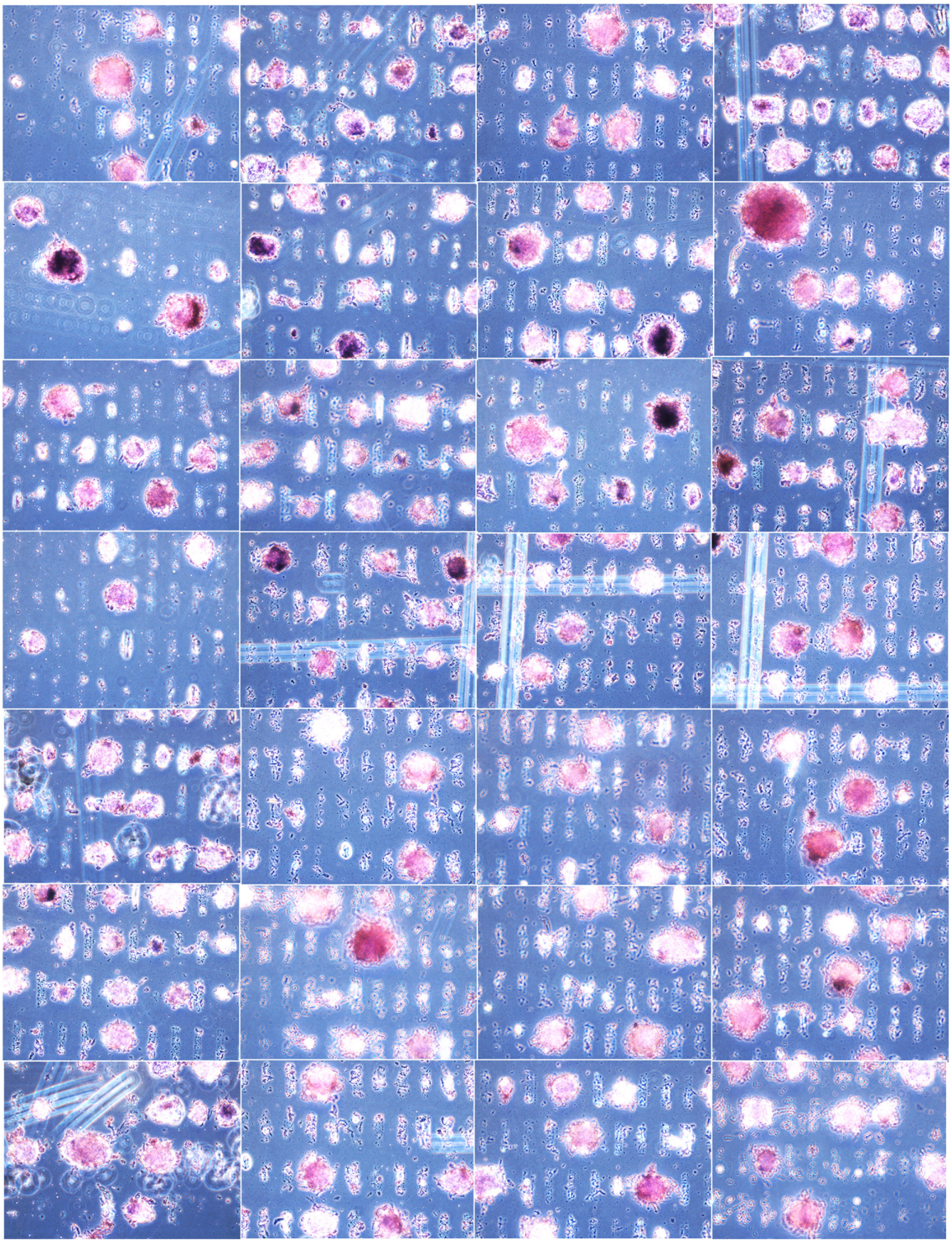

Figure S5. Montage of alkaline phosphatase staining of 10 day spheroids. Differential alkaline phosphatase activity (pink colour) demonstrate the heterogeneous nature of these spheroids.

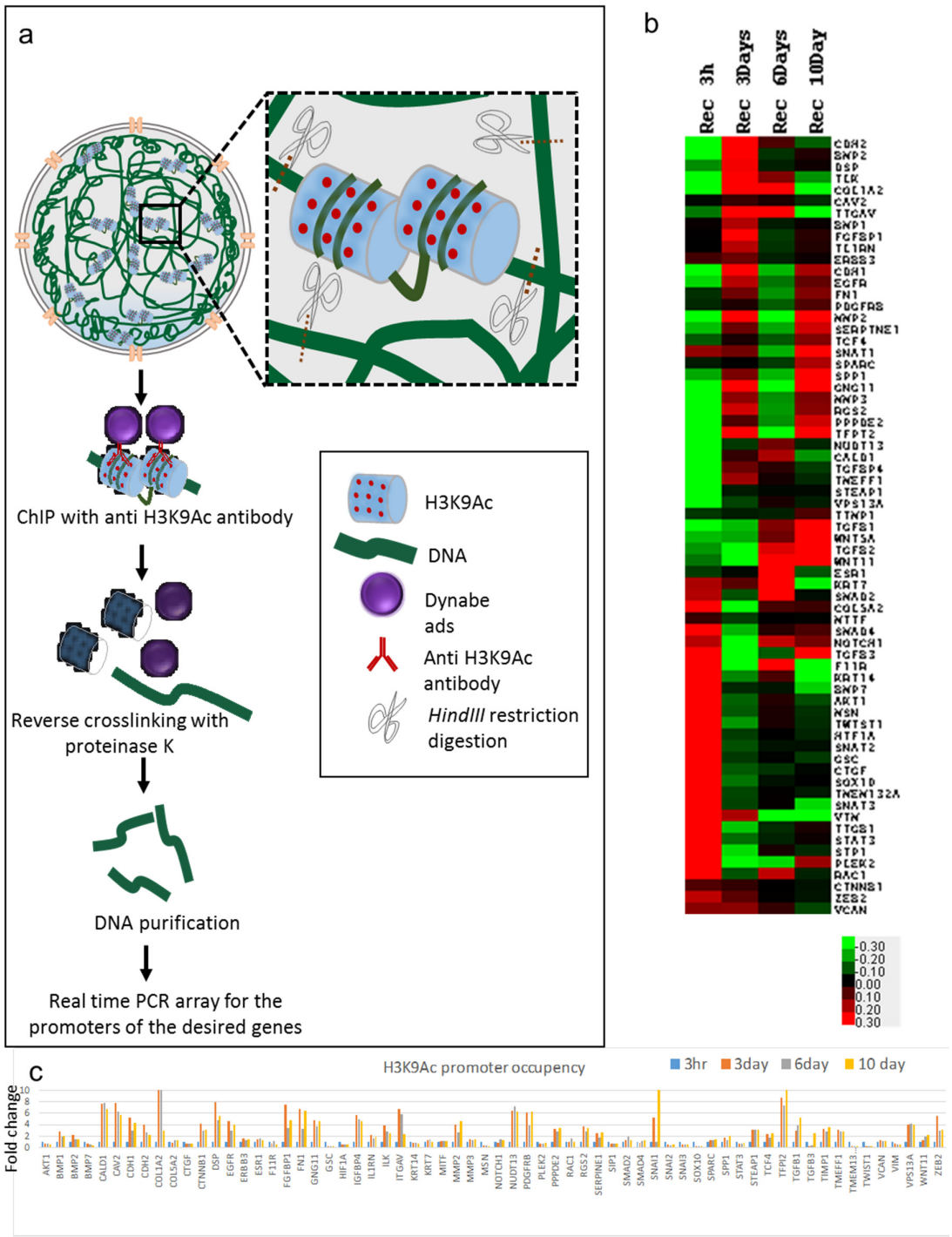

Figure S6. Epithelial to mesenchymal transition (EMT) EpiTect ChIP-qPCR assay. (a) Brief overview of the assay. DNA is digested by HindIII within the intact nucleus. For EpiTect ChIP analysis, digested chromatin fragments are pulled down with magnetic beads coated with an antibody recognizing the H3K9Ac. Chromosomal contacts associated with H3K9Ac are reverse cross-linked, and the DNA is purified and amplified, before performing qRCR array for the promoter of H3K9Ac occupancy of the genes related EMT. (b-c) heat map representing differential histone acetylation at respective gene promoters and (c) bar plot represents relative fold enrichment of H3K9Ac at the promoter regions of all genes responsible for EMT process.

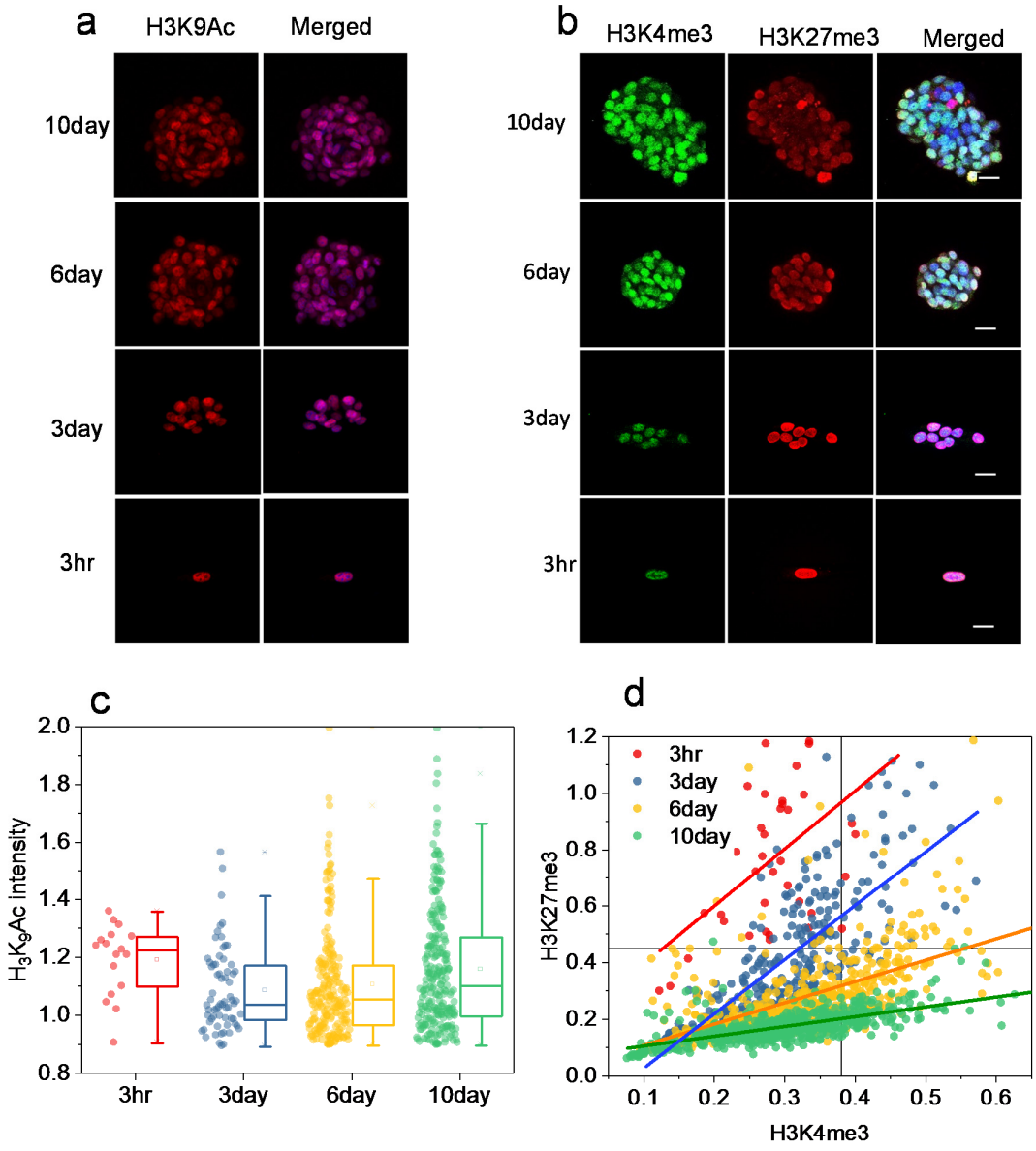

Figure S7. (a) Representative confocal micrographs of cells cultured under geometry driven induced cellular confinement for 10 days. Cells were immuno-stained for H3K9Ac (red); and (c) corresponding intensity plot; (b) Cells were immuno-stained for H3K4me3 and Cells were immuno-stained for H3K27me3 and (d) corresponding intensity plot.

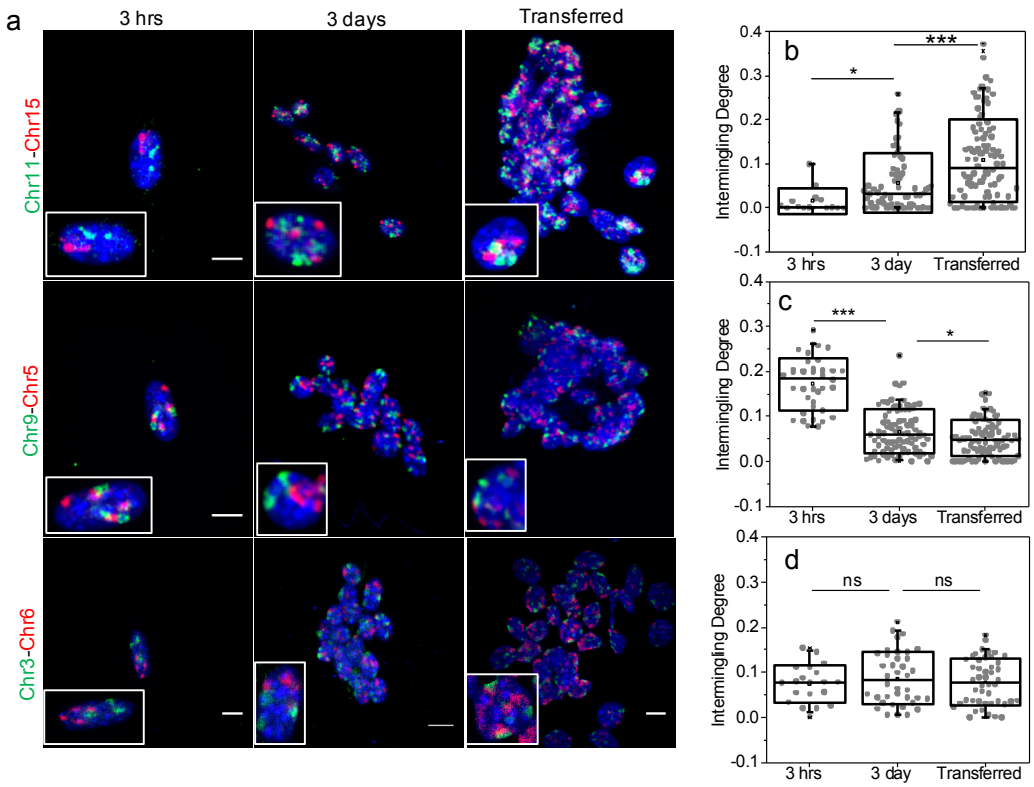

Figure S8. Changes in chromosome intermingling degree. (a) Representative images showing intermingling in different chromosome pairs (Ch11-Ch15, Ch9-Ch5 and Ch3-Ch6) in 3 hr, 3 days and transfer conditions. (Scale bar, 5 um). (b-d) Quantification of the intermingling degree of the respective chromosome pairs. ***P < 0.001.

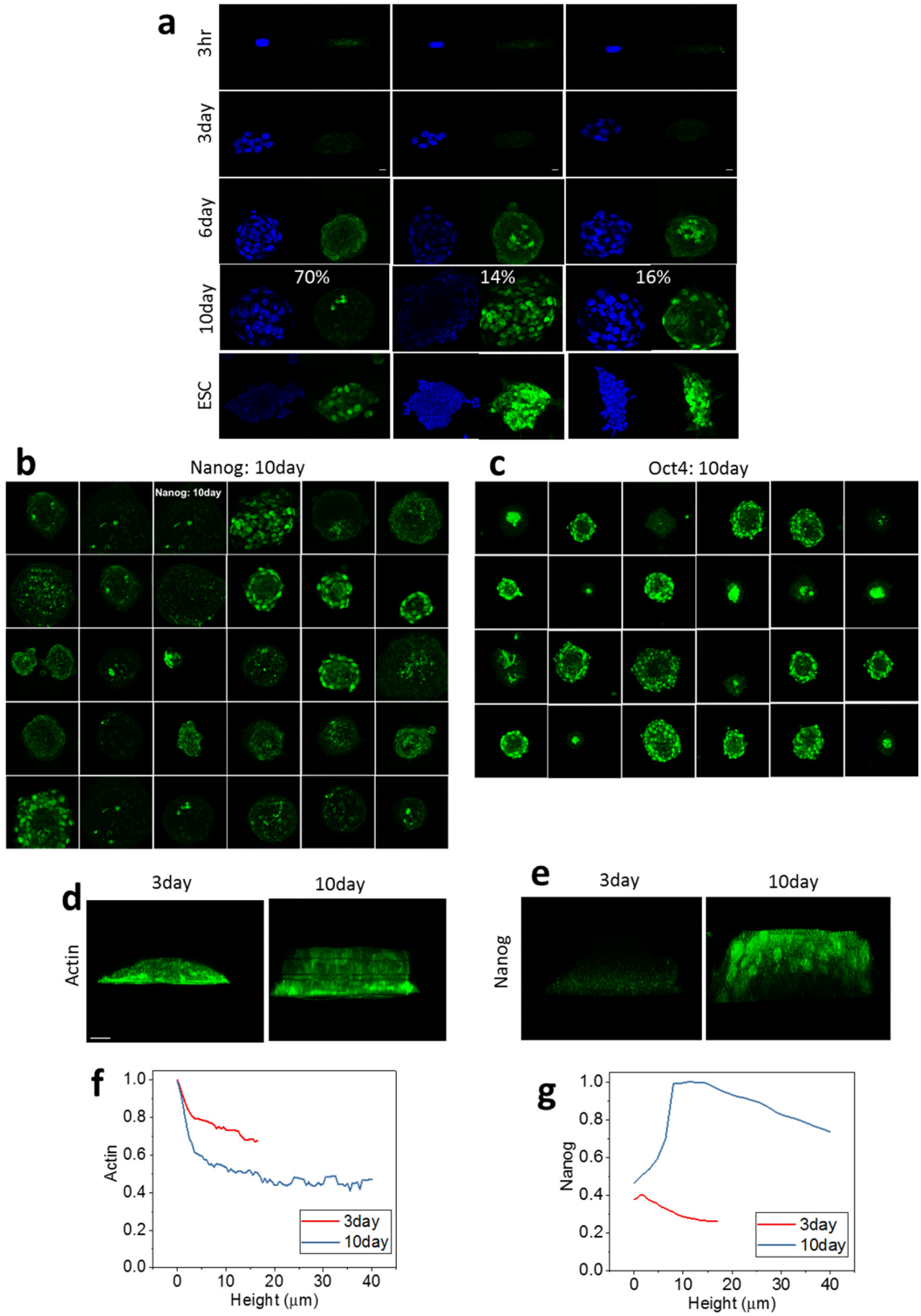

Figure S9. Heterogonous expression of Nanog and Oct4 in spheroids. (a) Representative Images of Nanog immunostaining of cells grown on RE at different time points. (b) Collage of Nanog immunostaining of 10 day spheroids. (c) Collage of micrograph of Oct4 immunostaining of 10 day spheroids; (d) YZ actin (stained with phalloidin) projection of 3 day and 10 day spheroid obtained by laterally confined growth of 3T3 cell on RE fibronectin

micropattern. (e) YZ projection of 3 day and 10 day spheroid immunostained with nanog antibody; (f-g) Corresponding actin and nanog intensity profile of 3 day and 10 day spheroid with respect to height.

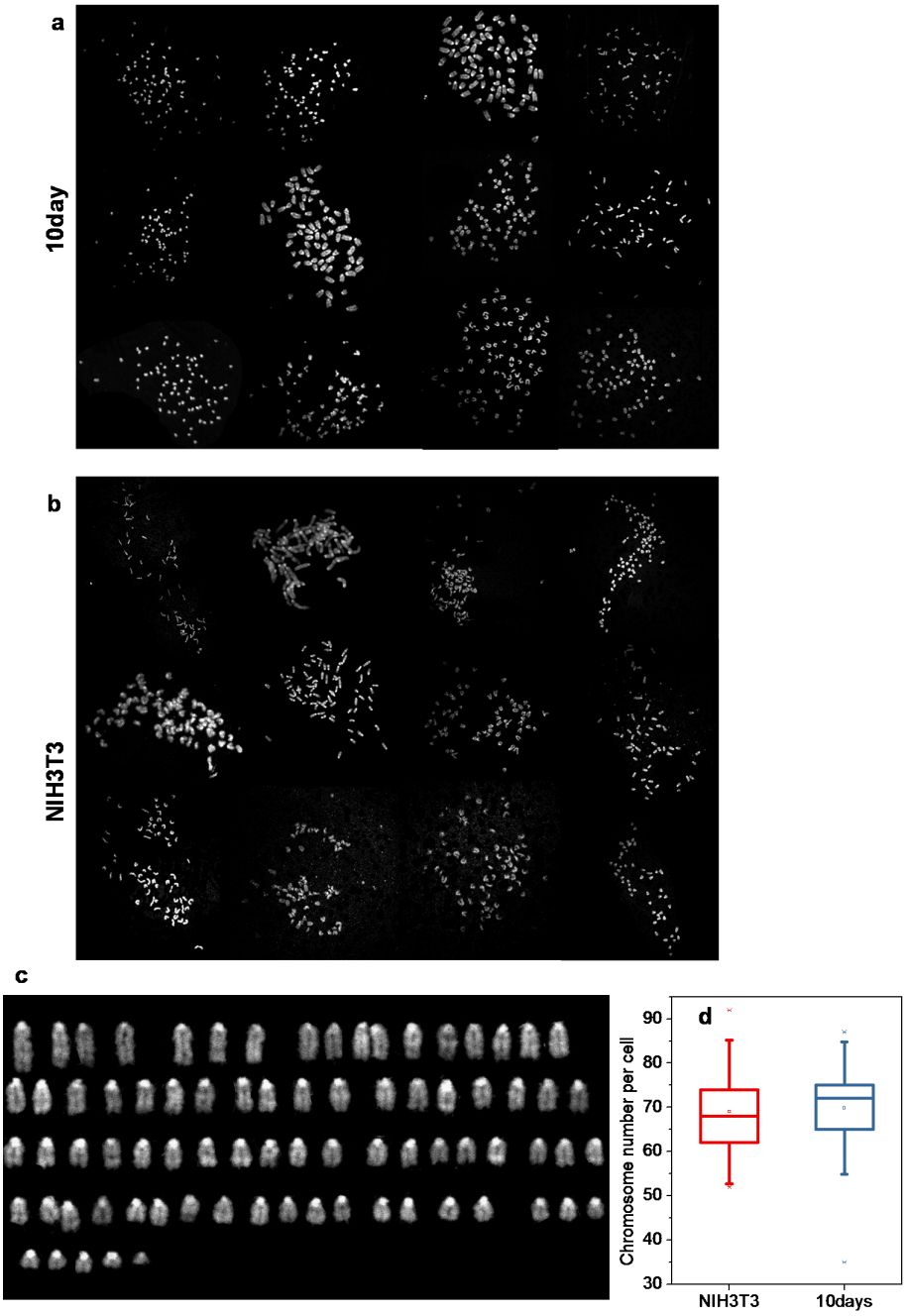

Figure S10. Metaphase chromosome spread. (a-b) Representative collage of metaphase chromosomes of ten day-old spheroids and control NIH3T3 cells stained with DAPI. (c) Typical metaphase spreads in ten day condition. Chromosomes are arranged using length as a parameter. (d) Plot represents the distribution of chromosome number in control NIH3T3 cell and transformed cells (10days).

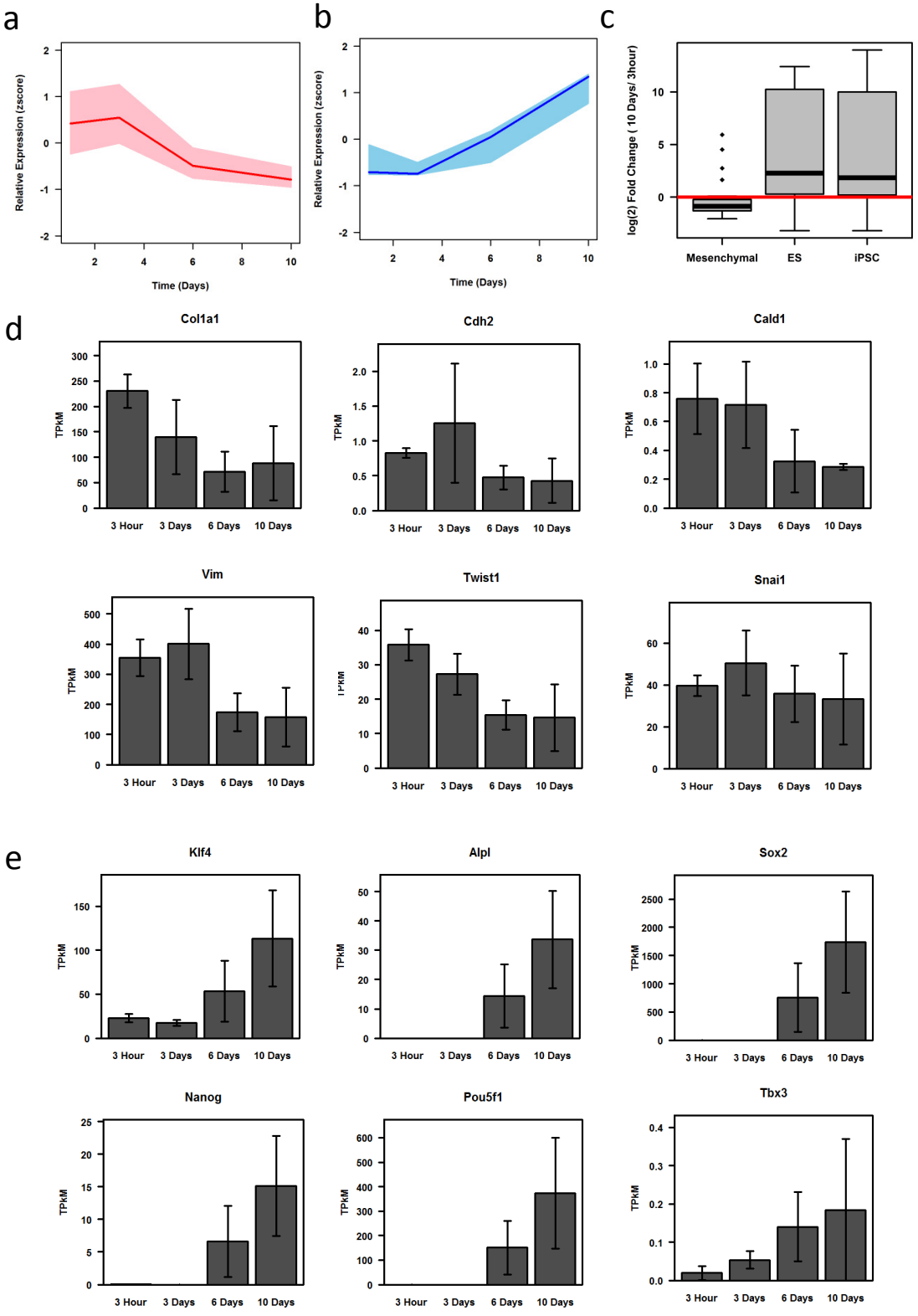

Figure S11. Changes in the transcription profile of candidate genes during nuclear

reprogramming. A lineplot illustrating the temporal changes in the expression of the Mesenchymal genes (a) and Stem Cell genes (b). The bold line represents the median expression and the upper and lower bounding curves represent 75th and 25th Percentile expression respectively. (c) A boxplot depicting the Log(base2) Fold change in the Mesenchymal, Embryonic Stem Cell (ES) and induced Pluripotent Stem cell (iPSC) genes between Day10 and 3hours samples. Bar plots depicting the changes in expression of representative Mesenchymal (d) and Stem Cell (e) genes. Note: The error bars represent 95% CI.

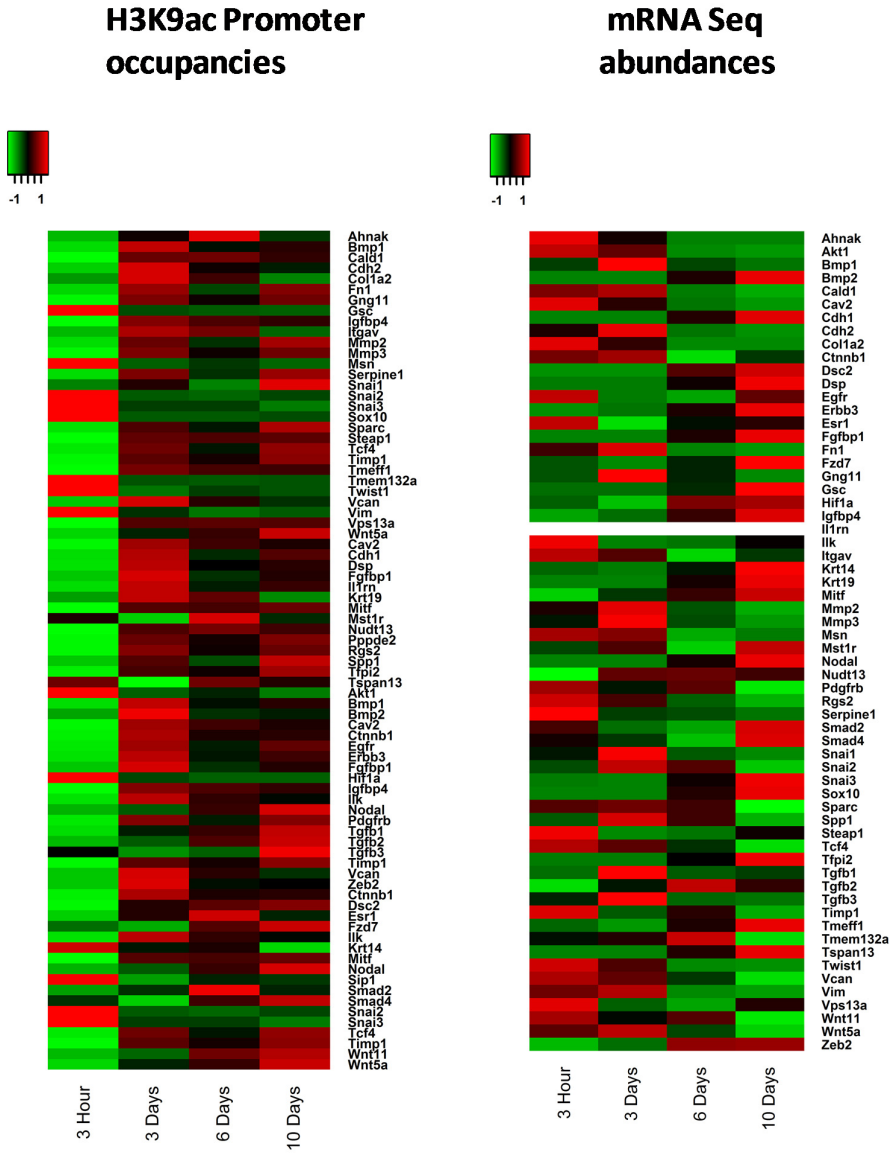

Figure S12. Correlation between H3K9Ac occupancy results obtained from EpiTect assay and mRNA abundance results obtained from RNAseq analysis.

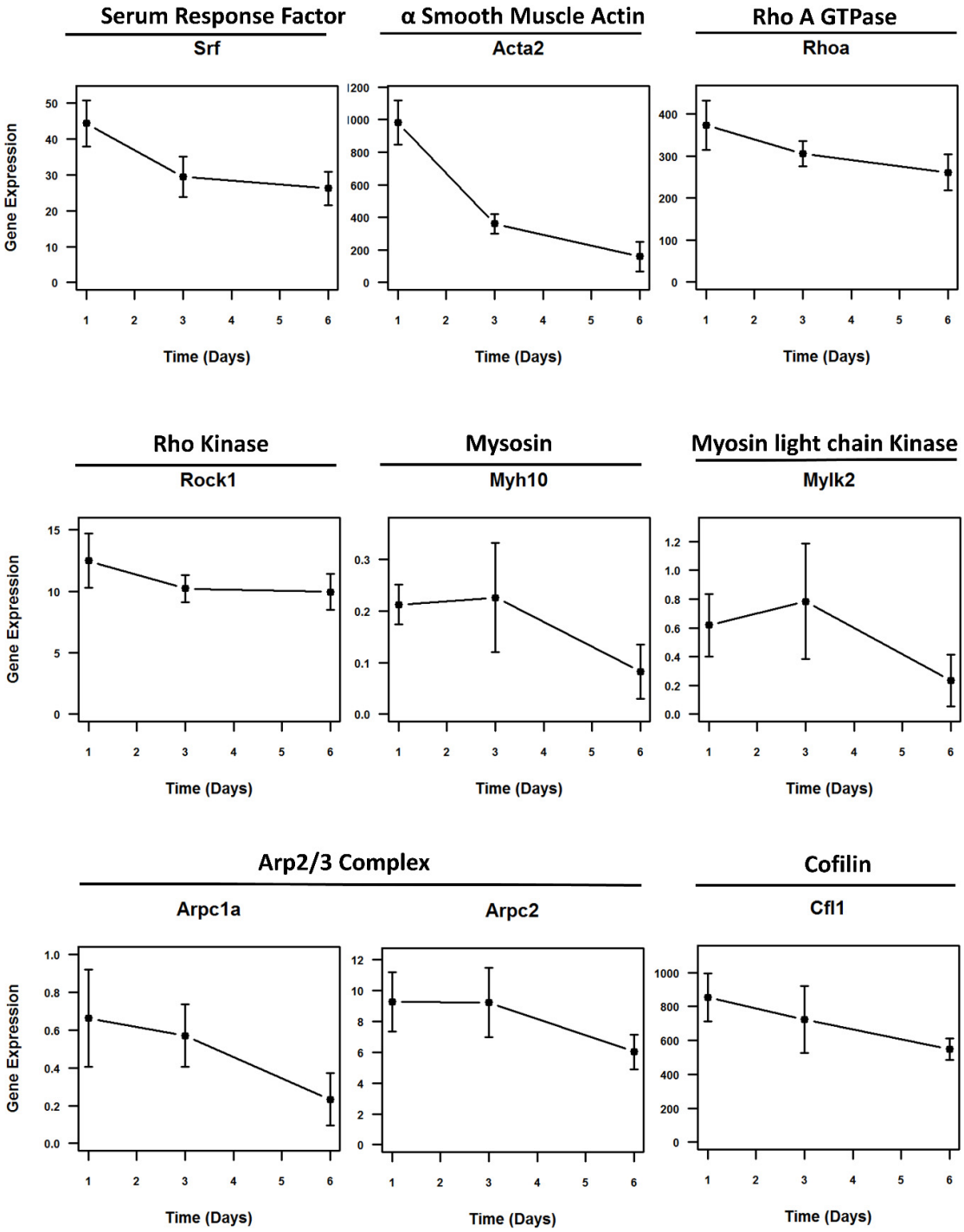

Figure S13. Expression of genes involved in Actin filament assembly and contractility is reduced by 6 Days. Each line plot depicts the normalized gene expression of respective genes over 6 Days. Note: The error bars represent 95% CI.

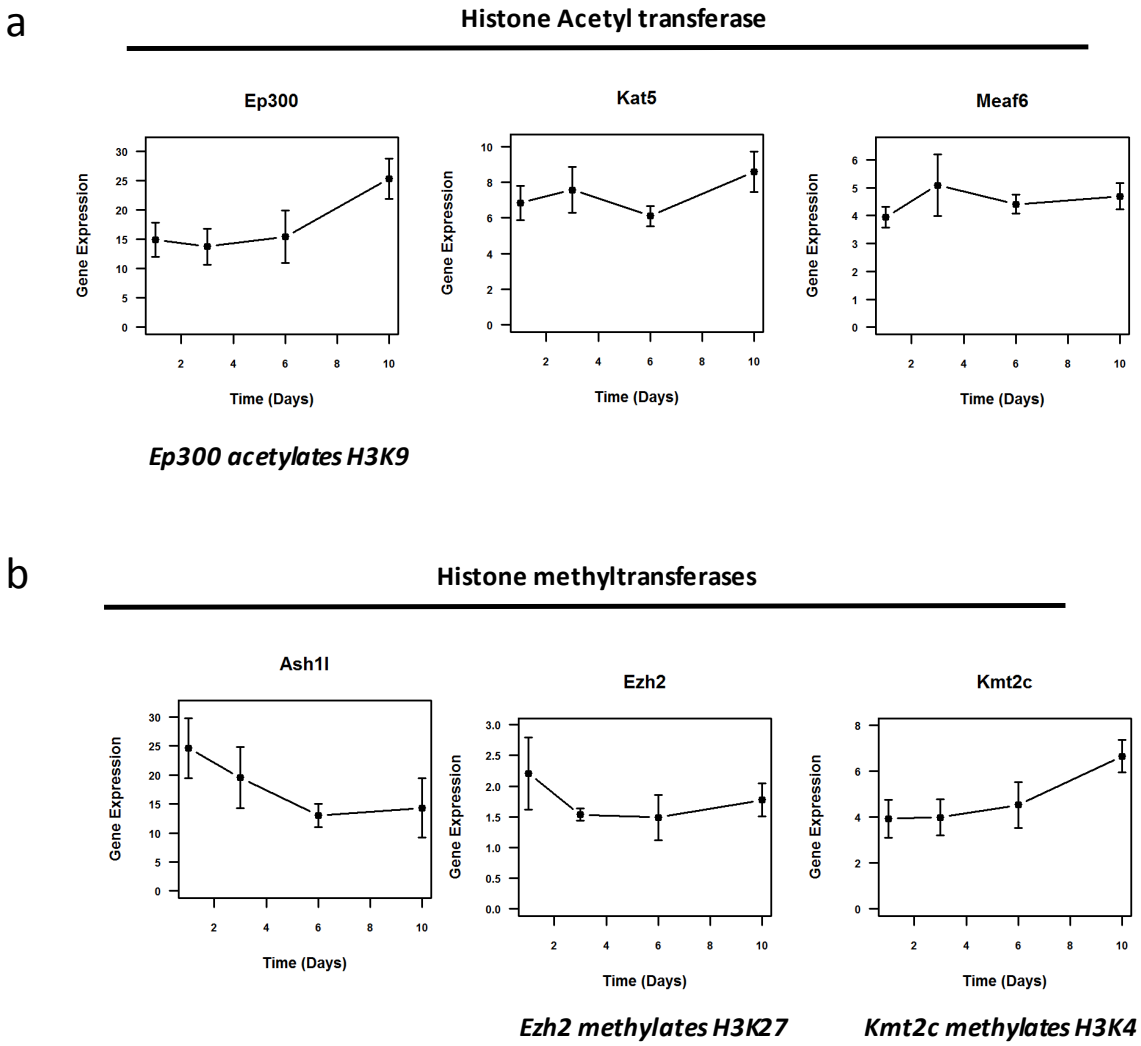

Figure S14. Expression of genes involved in regulating chromatin epigenetics. (a) FIllustrates Histone Acetyl Transferases (HATs), (b) illustrates Histone Methyl Transferases (HMTs). Each line plot depicts the normalized gene expression of respective genes over 10 Days. Note: The error bars represent 95% CI.

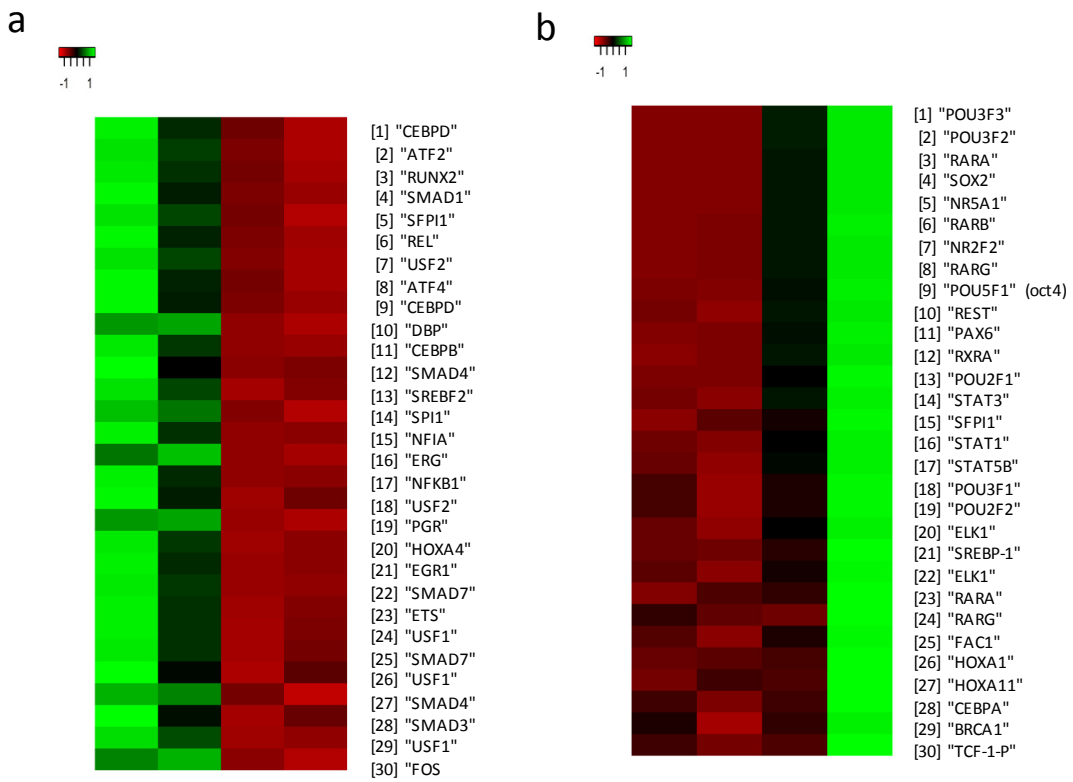

Figure S15. Heat map illustrating the top 30 Transcription Factors whose target genes are transcriptionally activated (a) or repressed (b) from Day 1 to Day 10.

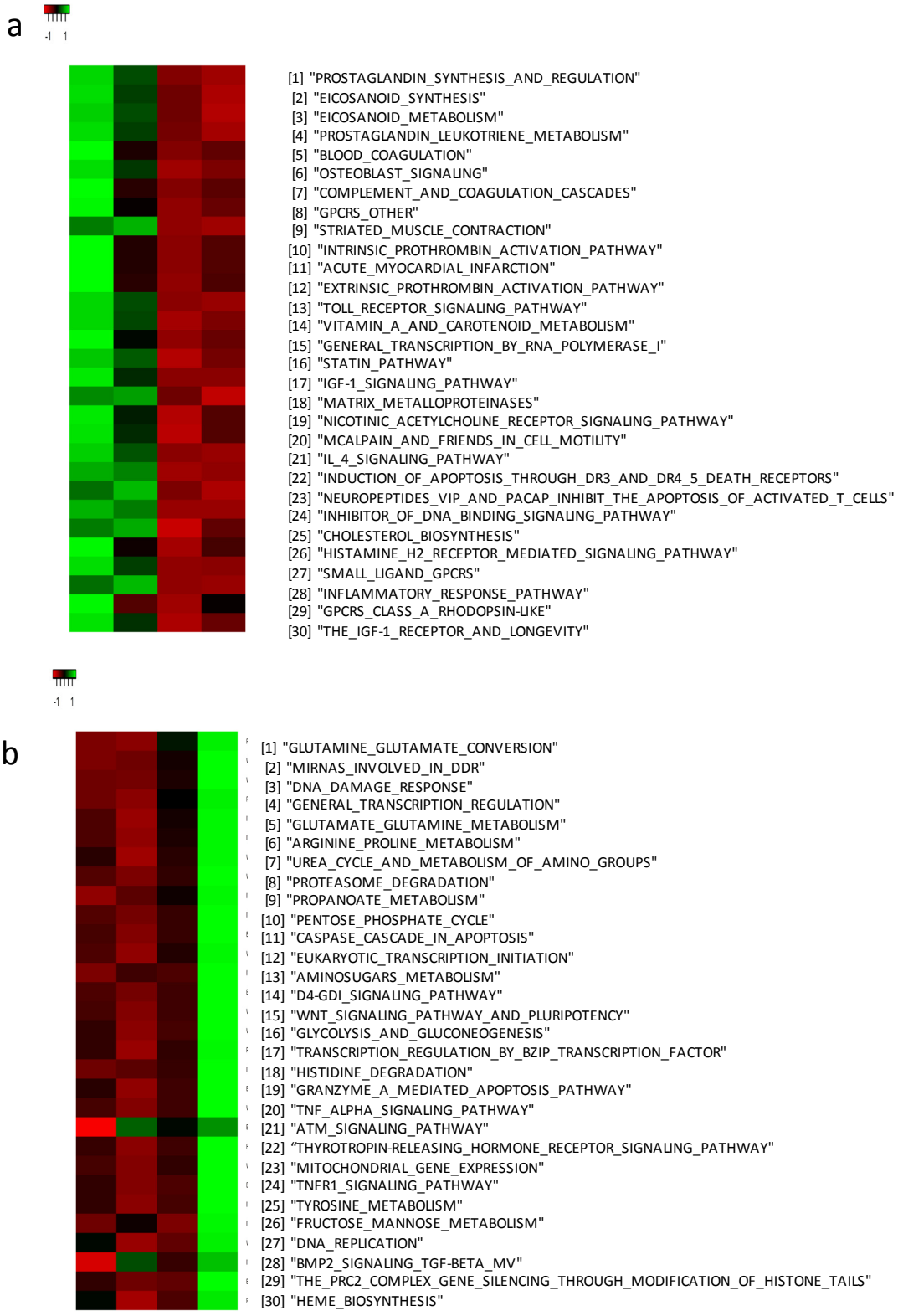

Figure S16. Heat map illustrating the top 30 Transcription Factors whose target genes are transcriptionally activated (a) or repressed (b) from Day 1 to Day 10.

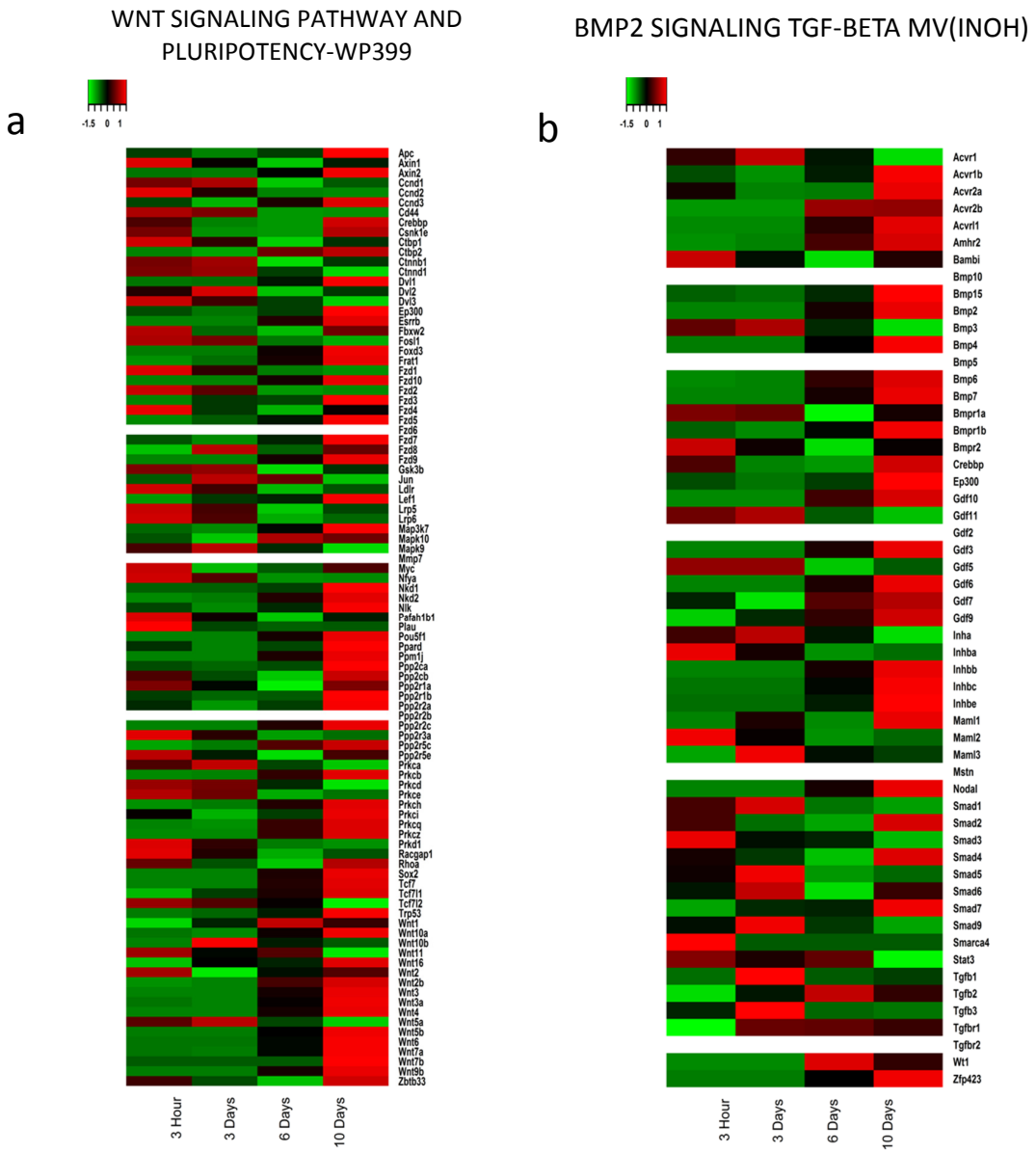

Figure S17. Heat map illustrating the expression of gene participating in (a) WNT SIGNALING PATHWAY AND PLURIPOTENCY pathway (WikiPathwayWP399) and (b) BMP2 TGFbeta SMAD pathways (INOH).

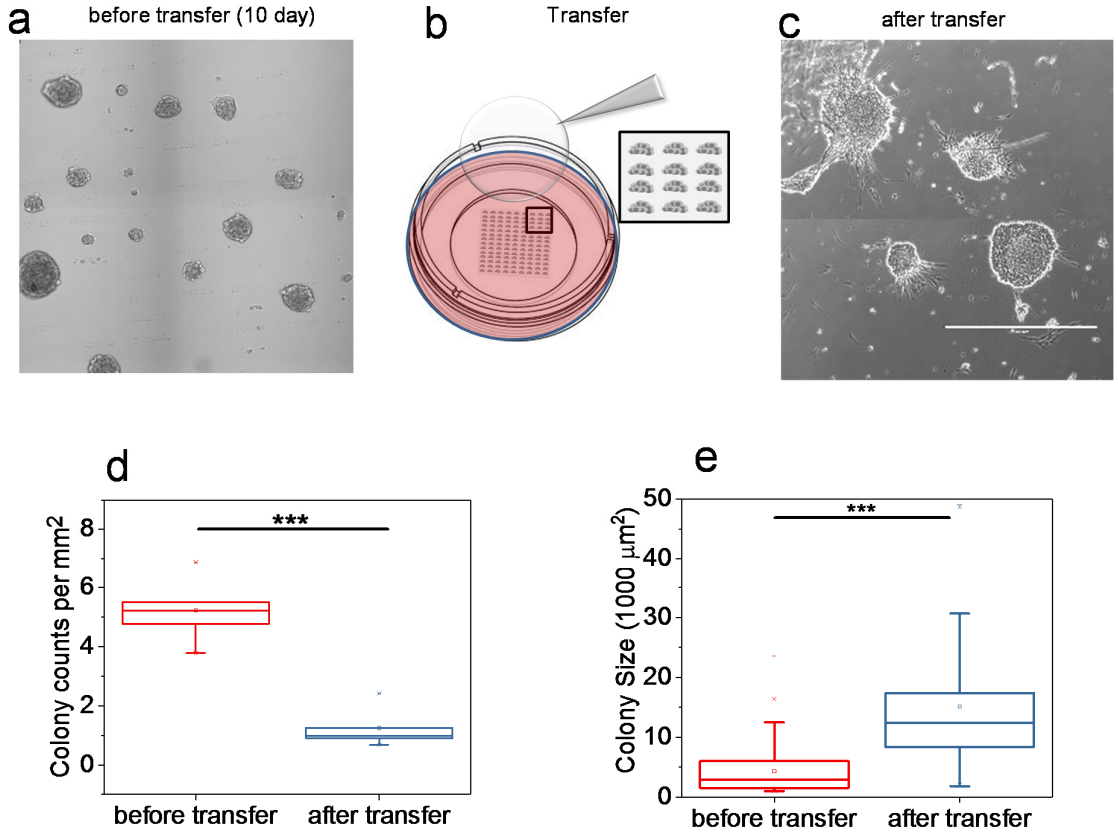

Figure S18. Transfer efficiency. Ten day-old spheroids (a) maintain the ES like colony formation upon transfer onto unpatterned glass surface (c). (b) Represents the transfer protocol on to unpatterned glass surface. (d) Plots represents number of colonies per mm^2 and (e) colony size before and after transfer. ***P < 0.001.

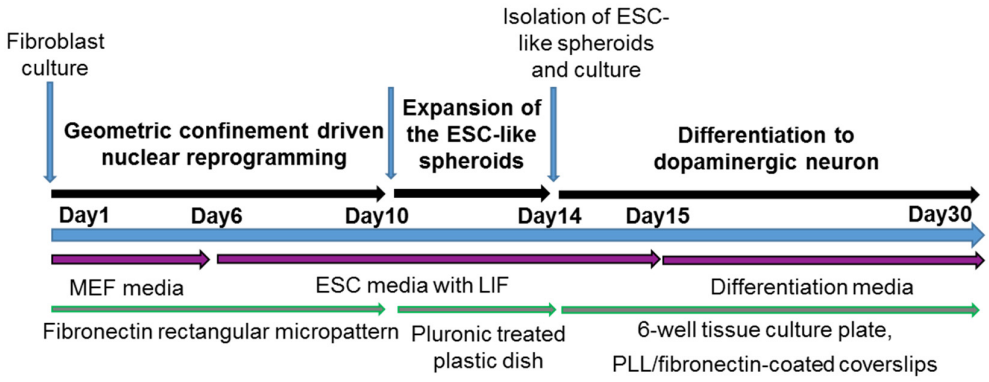

Figure S19. Schematic protocol of dopaminergic neuronal differentiation of the 10 day old spheroids.

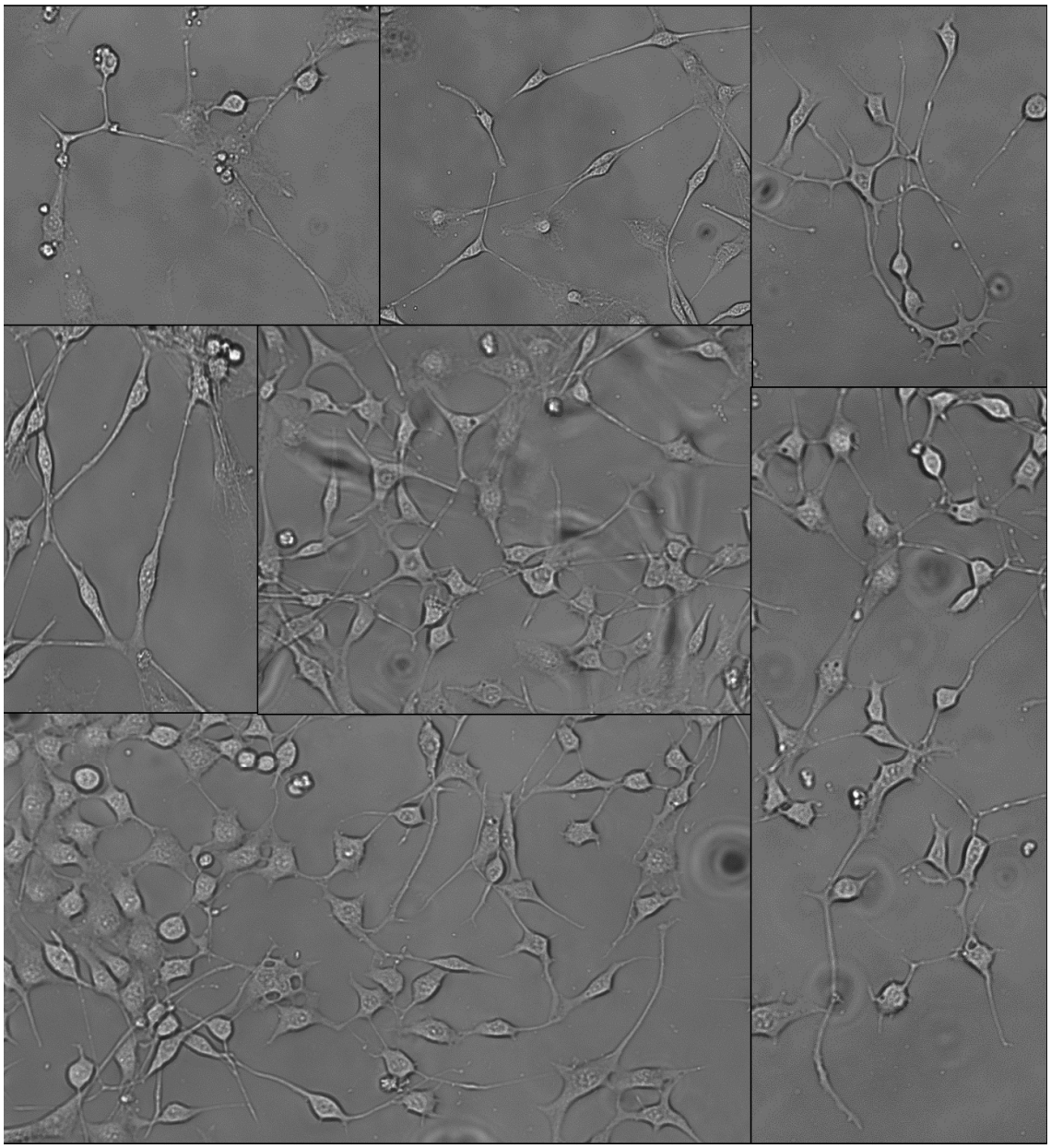

Figure S20. Collage of the bright field micrograph of dopaminergic neuron generated through neuronal differentiation of the 10 day spheroids.

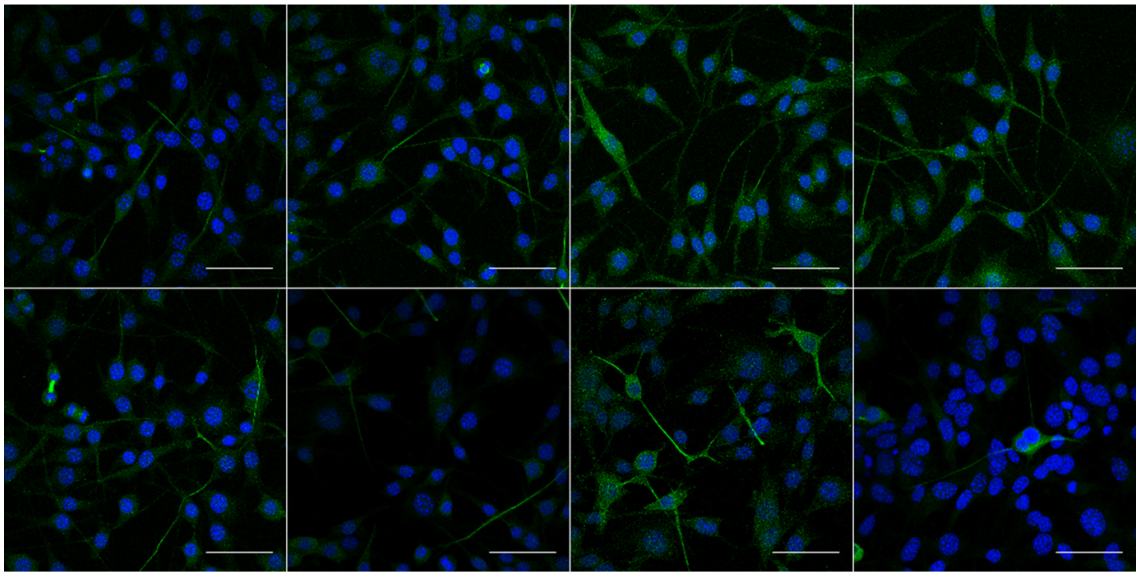

Figure S21. Collage of the micrograph of dopaminergic neuron generated through neuronal differentiation of the 10 day spheroids and immunostained with β III-tubulin antibodies.

SUPPLEMENTARY TABLE

Table S1. Primers used for qRT-PCR

Genes	Forward	Reverse
Mouse gene Primers		
Pcna	TGCTCTGAGGTACCTGAACT	TGCTTCCTCATCTTCAATCT
Gapdh	GACCAGGTTGTCTCCTGCGACTT	CCATGAGGTCCACCACCCTGTT
Dsp	CAGAGTGTTCCCAGTTCAAGG	TTGATCTGACCGTAGCACTTG
Snai2	TGTCTGCAAGATCTGTGGC	CTGTCTGCAAAAGCCCTATTG
Twist1	TGTCCGCGTCCCACTAG	ACTGTCCATTTTCTCCTTCTCTG
Nanog	TGAATTTGGAAGCCACTAGGG	CCCAGATGTTGCGTAAGTCTC
Lin28	GAAGAGATCCACAGCCCTG	CCAAAGAATAACCCTGACTCCTG
LaminA	GTACAACCTGCGCTCACGCACCGT	CACTGCGGAAGCTTCGAGTGACT
Acta2	GCTGACAGGATGCAGAAGGAG A	GCTGACAGGATGCAGAAGGAGA
Actb	CTAAGGCCAACCGTCAAAG	ACCAGAGGCATACAGGGACA
Snai2	TGTCTGCAAGATCTGTGGC	CTGTCTGCAAAAGCCCTATTG
Sox17	GCTAGGCAAGTCTTGGAAGG	CTTGTAGTTGGGGTGGTCCT
Foxa2	TGGCTGCAGACACTTCCTA	AAGCTCTCCCAAAGTCTCCA
Nestin	GCTGGAGGCTGAGAACTC	GAAAGGCTGTCACAGGAG
Human gene primers		
ALDH1A3	AAAAAGAGCGAATAGCACCG	GCATAGAGGGCGTTGTAGCA
OCT4	CGAAAGAGAAAGCGAACCAG	AACCACACTCGGACCACATC
CD24	GCTCCTACCCACGCAGATT	GGTGGTGGCATTAGTTGGAT
CD44	GAAGAAGGTGTGGGCAGAAGA	ACCATTTCCTGAGACTTGCTG
SNAi1	TCGGAAGCCTAACTACAGCGA	AGATGAGCATTGGCAGCGAG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

SUPPLEMENTARY REFERENCES:

1. Hooper M, Hardy K, Handyside A, Hunter S, Monk M (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326:292–295.
2. Kim TP, et al. (2010) Differentiation of Mouse Embryonic Stem Cells into Endoderm without Embryoid Body Formation. *PloS one* 5(11): e14146.
3. Wang R, et al. (2014) Comparison of mammosphere formation from breast cancer cell lines and primary breast tumors. *J Thorasic Dis.* 6(6): 829-837.