# Human cell dedifferentiation in mesenchymal condensates through controlled autophagy


## AUTHORS

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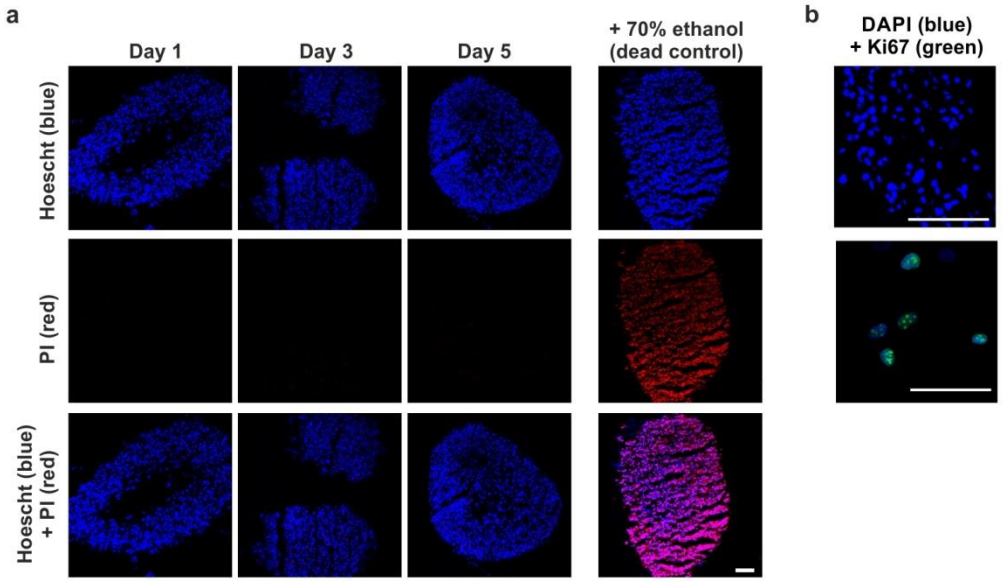
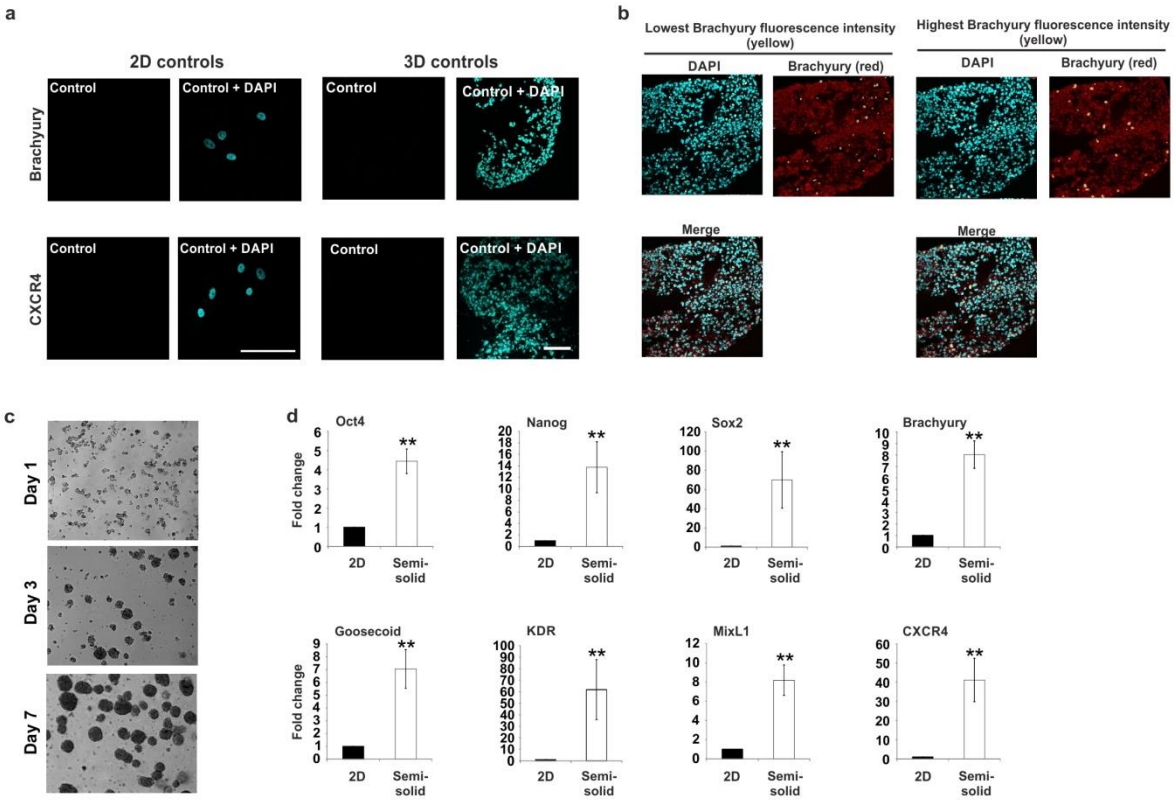
## SUPPLEMENTARY FIGURES

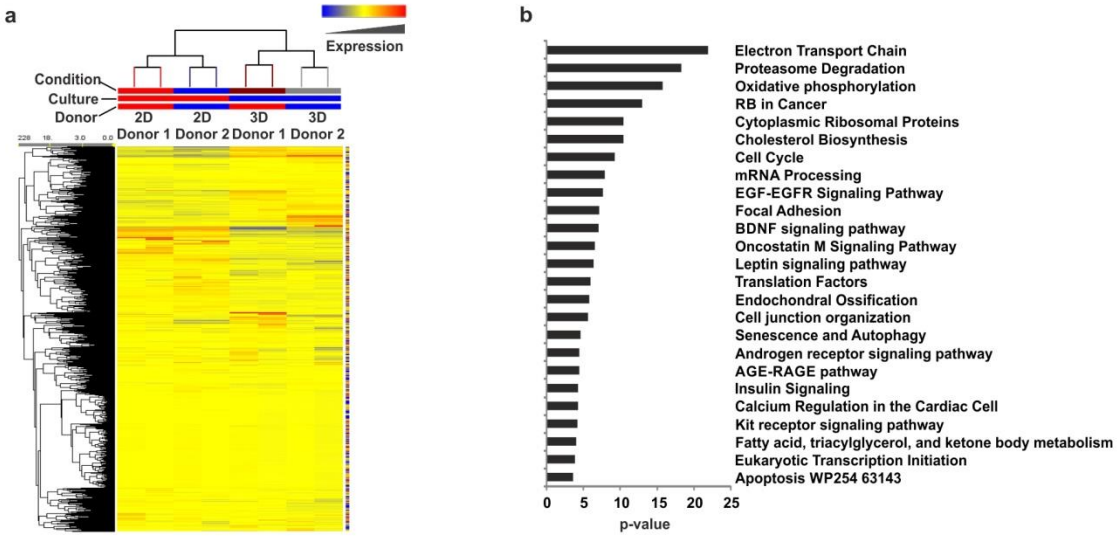
### Supplementary Figure S1.



## Supplementary Figure S2.



**Supplementary Figure S3.**



## SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Figure S1. Cell viability and proliferation in 3D MSCs**

(a) Staining of 3D MSCs with PI and Hoechst 33342. Representative images of Day 1, Day 3 and Day 5 3D MSCs and killed control 3D MSCs + 70% ethanol are shown (scale bar = 100µm).

(b) Immunofluorescent detection of Ki67 in 3D MSCs (2D MSCs are shown as a positive control), scale bar = 50µm

**Supplementary Figure S2. Analysis of dedifferentiation status of 3D MSCs**

(a) Brachyury and CXCR4 isotype controls in 2D MSCs and 3D MSCs (scale bar = 100µm). Nuclei identified by DAPI staining (blue fluorescence).

(b) Image analysis of Brachyury-immunopositive nuclei in 3D MSCs, showing lowest- (yellow, left panel) and highest- (yellow, right panel) expressing cells, distributed evenly throughout the spheroid. DAPI nuclear staining shown as blue fluorescence.

(c) Cell growth assays following disaggregation of 3D MSCs and re-suspension in semi-solid media (scale bar = 100µm).

(d) QPCR analysis of pluripotency and mesendodermal marker expression following disaggregation of 3D MSCs and re-suspension in semi-solid media. Data represent two separate experimental donors and are shown as mean values ± SEM, ** = $p<0.01$.

**Supplementary Figure S3. Transcriptomic analyses of differentially expressed pathways in 3D MSCs versus 2D MSCs**

(a) Clustering of 2D MSCs and 3D MSCs from 2 different donors according to culture condition (2D or 3D) and donor.

(b) The top 25 significantly differentially expressed pathways in 3D MSCs versus 2D MSCs from gene pathway analysis.

**SUPPLEMENTARY TABLES**

| Gene name | Fold change | Gene name | Fold change |
|---|---|---|---|
| ND5 | -6.1126 | ATP5S | -1.57138 |
| ND4L | -5.05674 | AQDQ | -1.56911 |
| ND3 | -4.04979 | ATP5G3 | -1.5637 |
| ND4 | -3.03716 | ATP5L | -1.54443 |
| ATP5G1 | -2.84382 | ATP5G2 | -1.53316 |
| NDUFS8 | -2.20634 | ATP5O | -1.53188 |
| B18 | -2.03715 | NDUFA11 | -1.52613 |
| NDUFB2 | -2.01686 | B14 | -1.52465 |
| ATP5J2 | -1.98975 | CI-SGDH | -1.50213 |
| ATP6 | -1.9447 | NDUFA2 | -1.49991 |
| NDUFA4 | -1.93893 | ATP5J | -1.438 |
| B14.5a | -1.93 | NDUFS5 | -1.39763 |
| ATP5F1 | -1.89167 | ATP5E | -1.37441 |
| ATP5D | -1.8098 | ATP6AP1 | -1.37354 |
| NDUFS6 | -1.79843 | CI-51kD | -1.36005 |
| ATP5H | -1.77936 | CI-75Kd | -1.34605 |
| KFYI | -1.76233 | ATP6AP2 | -1.34393 |
| ATP5A1 | -1.75855 | B22 | -1.33539 |
| ATP5I | -1.75112 | B14.5b | -1.2961 |
| B9 | -1.66852 | NDUFS3 | -1.26757 |
| GZMB | -1.65155 | NDUFV2 | -1.25547 |
| B17 | -1.63827 | NDUFS7 | -1.2454 |
| CI-SGDH | -1.62896 | CI-42KD | -1.1436 |
| ASHI | -1.60262 | ND2 | -1.091 |
| NDUFV3 | -1.59722 | B13 | -1.086 |
| NDUFA8 | -1.5948 | B15 | 1.046901 |
| ATP5B | -1.58734 | ND1 | 1.089706 |
| NDUFB10 | -1.57776 | NDUFS2 | 1.108659 |
| NDUFA9 | -1.575 | NUOMS | 3.654102 |
| FASN2A | -1.57454 | ND6 | 4.23435 |

**Supplementary Table S1.** Fold change expression of genes assigned to the mitochondrial oxidative phosphorylation pathway (using Genespring v12.1) in 3D MSCs vs 2D MSCs.

| Gene name | Fold change | Gene name | Fold change |
|---|---|---|---|
| ND4L | -5.05674 | NDUFA8 | -1.5948 |
| SLC25A4 | -3.34134 | ATP5B | -1.58734 |
| ATP5G1 | -2.84382 | NDUFB10 | -1.57776 |
| COX7A1 | -2.61804 | NDUFA9 | -1.575 |
| ATPIF1 | -2.48431 | NDUFAB1 | -1.57454 |
| NDUFS8 | -2.20634 | ATP5S | -1.57138 |
| SLC25A5 | -2.09295 | NDUFS4 | -1.56911 |
| COX8A | -2.07 | ATP5G3 | -1.5637 |
| NDUFB7 | -2.03715 | ATP5L | -1.54443 |
| NDUFB2 | -2.01686 | COX6B1 | -1.53532 |
| ATP5J2 | -1.98975 | ATP5G2 | -1.53316 |
| NDUFA4 | -1.93893 | ATP5O | -1.53188 |
| NDUFA7 | -1.93 | COX7B | -1.52843 |
| COX5A | -1.89533 | NDUFA6 | -1.52465 |
| ATP5F1 | -1.89167 | COX7C | -1.51127 |
| UCRC | -1.84434 | NDUFB5 | -1.50213 |
| DAP13 | -1.84087 | NDUFA2 | -1.49991 |
| ATP5D | -1.8098 | COX5B | -1.4669 |
| UQCRFS1 | -1.80153 | ATP5J | -1.438 |
| NDUFS6 | -1.79843 | SURF1 | -1.41699 |
| ATP5H | -1.77936 | NDUFA1 | -1.40509 |
| NDUFB3 | -1.77505 | NDUFS5 | -1.39763 |
| NDUFC1 | -1.76233 | SCO1 | -1.3874 |
| ATP5A1 | -1.75855 | ATP5E | -1.37441 |
| COX6A1 | -1.75063 | NDUFS1 | -1.34605 |
| QP-C | -1.70969 | NDUFB9 | -1.33539 |
| ATP5C1 | -1.69572 | UQCRB | -1.3215 |
| SDHB | -1.68605 | NDUFC2 | -1.2961 |
| NDUFA3 | -1.66852 | NDUFS3 | -1.26757 |
| NDUFB6 | -1.63827 | NDUFS7 | -1.2454 |
| NDUFB1 | -1.62896 | UQCRC1 | -1.23871 |
| SDHC | -1.6136 | NDUFA10 | -1.1436 |
| UQCRH | -1.61309 | COX11 | 1.054393 |
| NDUFB8 | -1.60262 | | |

**Supplementary Table S2.** Fold change expression of genes assigned to the electron transport chain pathway (using Genespring v12.1) in 3D MSCs vs 2D MSCs.

## SUPPLEMENTARY METHODS

### Assay of dead cell numbers in 3D MSCs

3D spheroids initiated from 60,000 MSCs were collected at days 1, 3, 5. A sample at each time point was incubated with 70% ethanol for 45 minutes, then rinsed in PBS and incubated with 1µg/ml PI for 4 hours. These samples served as dead controls. For the test samples at each time point, samples were rinsed in PBS and incubated with 1µg/ml PI for 4 hours. Samples were then isolated, snap frozen and sectioned. After sectioning, samples were stained with Hoechst 33342. Imaging was performed using the LSM510 confocal imaging system, all samples were imaged using identical imaging conditions.

### Quantitative polymerase chain reaction (QPCR)

| **Gene** | **Primer sequences - forward and reverse (5'- 3')** | **GenBank accession number** |
|---|---|---|
| GAPDH | TGCACCACCAACTGCTTAGC<br>GGCATGGACTGTGGTCATGAG | NM_002046 |
| Oct4 | CCCACACTGCAGCAGATCAG<br>CACACTCGGACCACATCCTTCT | NM_002701 |
| Nanog | CCTCCATGGATCTGCTTATTCAG<br>TGCGACACTATTCTCTGCAGAAG | NM_024865 |
| Sox2 | GAGAACCCCAAGATGCACAAC<br>CGCTTAGCCTCGTCGATGA | NM_003106 |
| TFEB | CCAGAAGCGAGAGCTCACAGAT<br>TGTGATTGTCTTTCTTCTGCCG | NM_007162 |
| Brachyury (T) | GGGTCCACAGCGCATGAT<br>TGATAAGCAGTCACCGCTATGAA | NM_003181 |
| Goosecoid | GATGCTGCCCTACATGAACGT<br>GACAGTGCAGCTGGTTGAGAAG | NM_173849 |
| KDR | TGATGCCAGCAAATGGGAAT<br>CCACGCGCCAAGAGGCTTA | NM_002253 |
| MIXL1 | AAGCCCCAGCTGCCTGTT<br>CCCTCCAACCCCGTTTG | NM_031944 |
| CXCR4 | CGCCTGTTGGCTGCCTTA<br>ACCCTTGCTTGATGATTTCCA | NM_003467 |
| CXCL12 | CCGTCAGCCTGAGCTACAGAT<br>GACGTTGGCTCTGGCAACA | NM_199168.3 |

Table showing primer sequences used for quantitative polymerase chain reaction (QPCR).

**Semi-solid expansion assay**

For semi-solid expansion studies, MSCs were cultured as 60,000 cell spheroids for 5 days and disaggregated to small cell clumps as described above. Cell suspensions in DMEM, high glucose, were then mixed with semi-solid media (either medium 1: DMEM high glucose supplemented with 5% FBS and 1% methyl cellulose (final concentrations), or medium 2: ESC medium supplemented with 1% methyl cellulose (final concentration)). MSCs were cultured at 37°C, 5% CO2 for 7 days. For RNA extraction, MSC aggregates were isolated by washing with PBS and then pelleted by centrifugation, before RNA extraction was performed using Trizol following manufacturer's instructions (Gibco). cDNA synthesis and QPCR was performed as described above.

**Global gene expression analysis**

For global gene expression analyses, duplicate RNA samples from two donors, maintained for 5 days in 2D or 3D culture were harvested as previously described. RNA quality was assessed using the Agilent 2100 Bioanalyzer with RNA 6000 nano chips and 150ng high quality RNA per sample (with RNA integrity numbers of 9.8 or greater), spiked with control RNA species from the Agilent One color spike-in kit, were Cy3-labelled using the Agilent One Color Low Input Quick Amp labeling kit. 600ng Cy3-labelled RNA per sample was then hybridized onto an Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarray and washed as per the manufacturer's guidelines. Arrays were scanned using an Agilent DNA Microarray Scanner (G2565CA) with SureScan High-Resolution Technology. Data were analyzed using Genespring version 12.1 software (Agilent technologies), and all quality control checks met the standards required. This software was then used to draw clustered heatmaps of global expression data, and perform relevant statistical analyses. A paired sample t-test was employed, using the Benjamini - Hochberg

algorithm to control for the effects of multiple testing. This procedure allowed for the adjustment of the p-values and the lowering of the false discovery rate whilst keeping the sensitivity for true positive results. Genes with a minimum two-fold difference in expression level and an adjusted p-value of $<0.05$ were considered differentially expressed. Subsequent pathway analysis was performed on lists of genes differentially expressed in response to change in culture conditions, using the pathway analysis tool within Genespring v12.1 software.

**Ethical Approvals**

The *in vivo* study was performed independently by Reinnervate Ltd (Co. Durham, UK). Mouse ESCs (E14) included as positive controls were cultured and prepared by Reinnervate Ltd. All work was carried out in accordance with ethical guidelines under the Home Office project licence of Reinnervate Ltd. Human MSCs were obtained following full informed consent under approval of the York Local Research Ethical Committee.