SUPPLEMENTAL MATERIAL

Cleavage of E-cadherin and β-catenin by calpain affects Wnt signaling and spheroid formation in suspension cultures of human pluripotent stem cells

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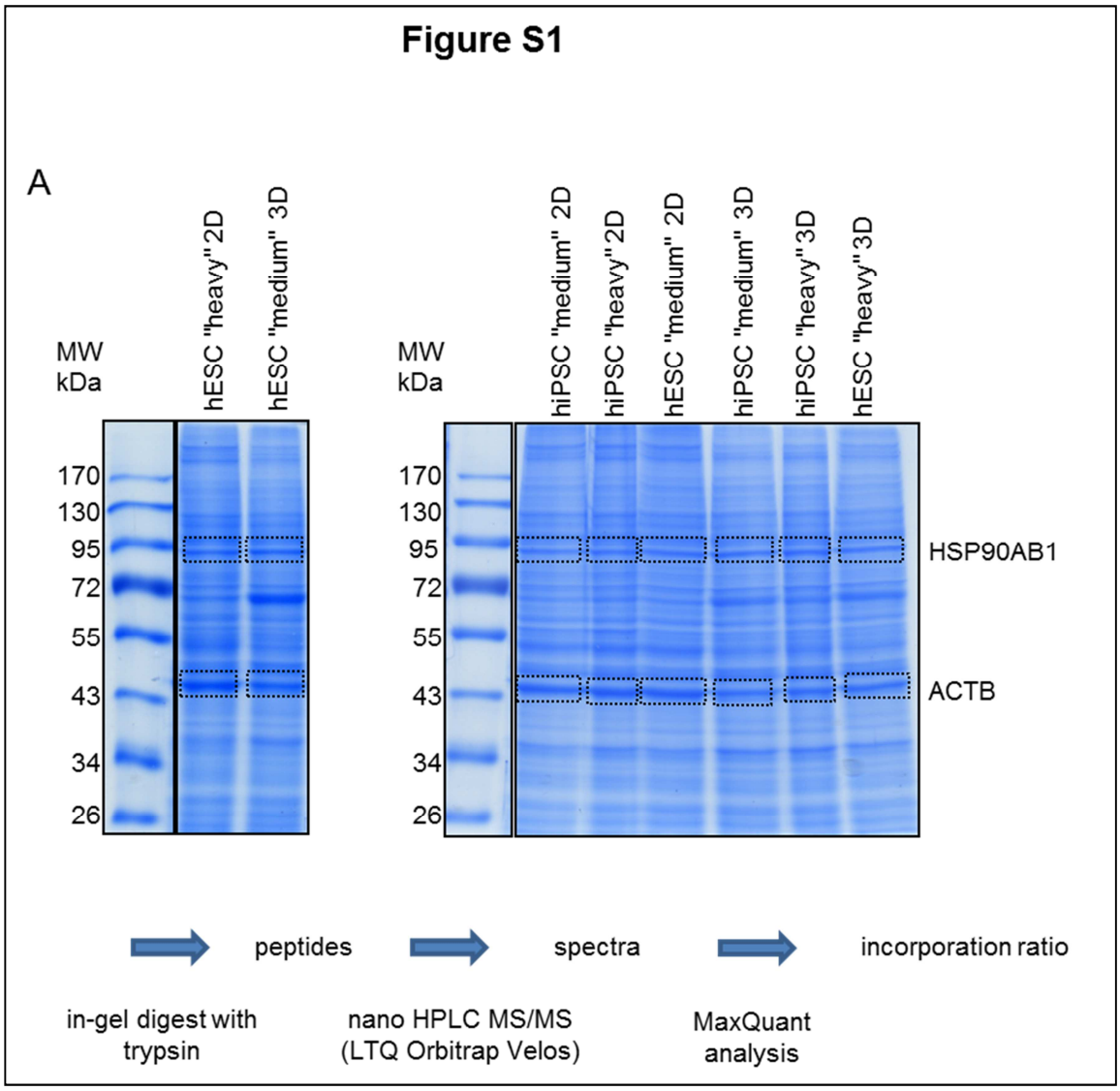
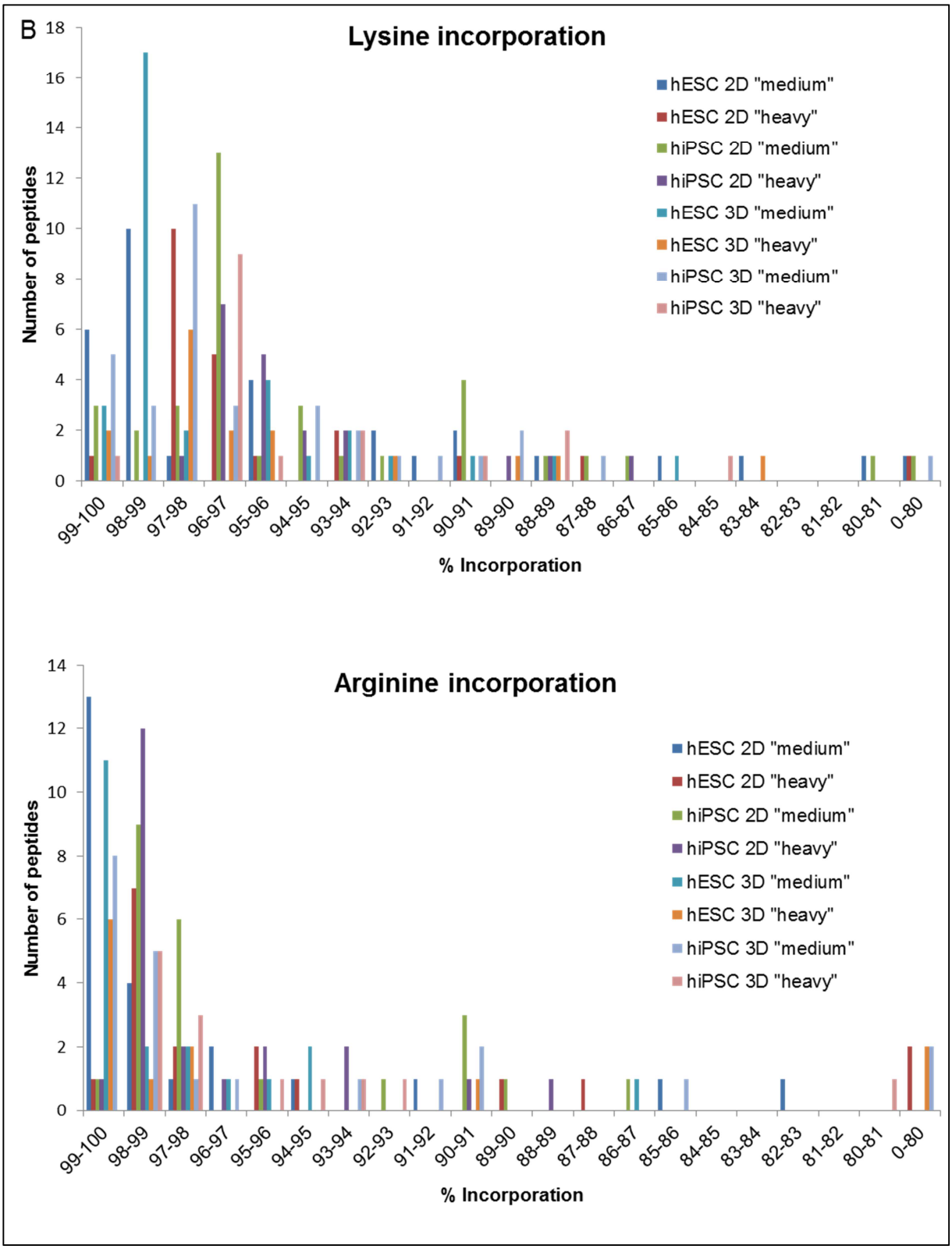
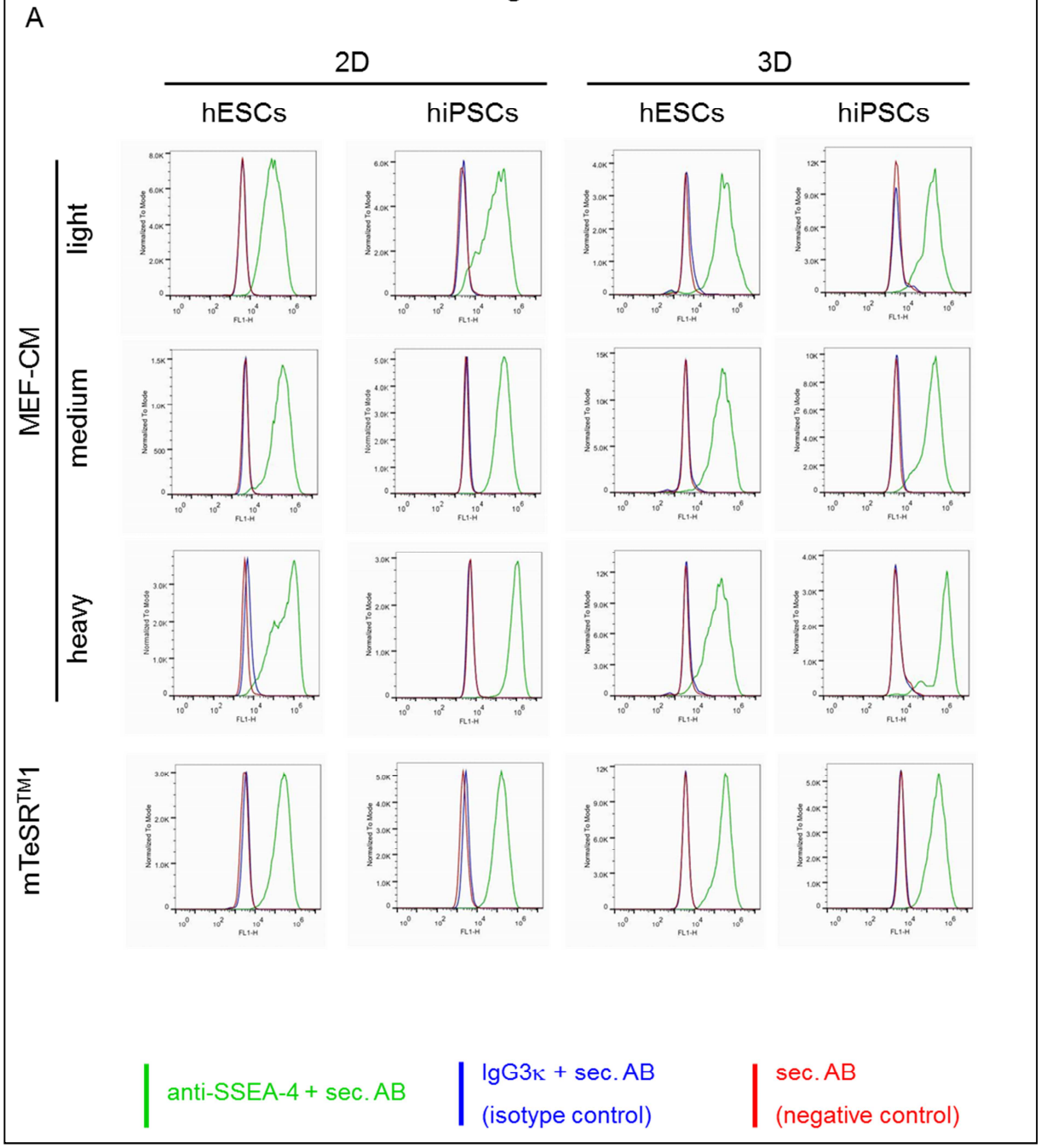
Supplementary Figures

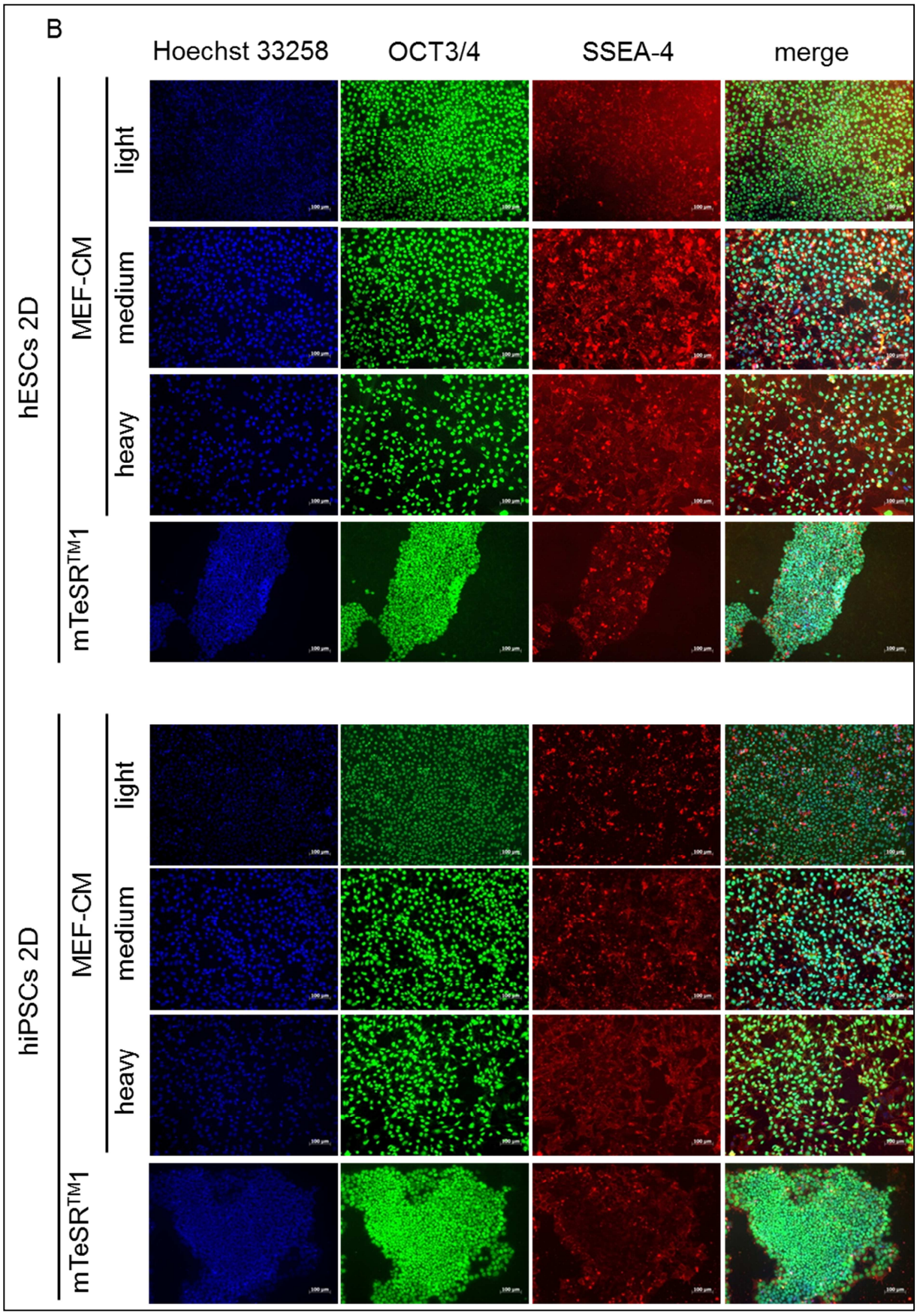


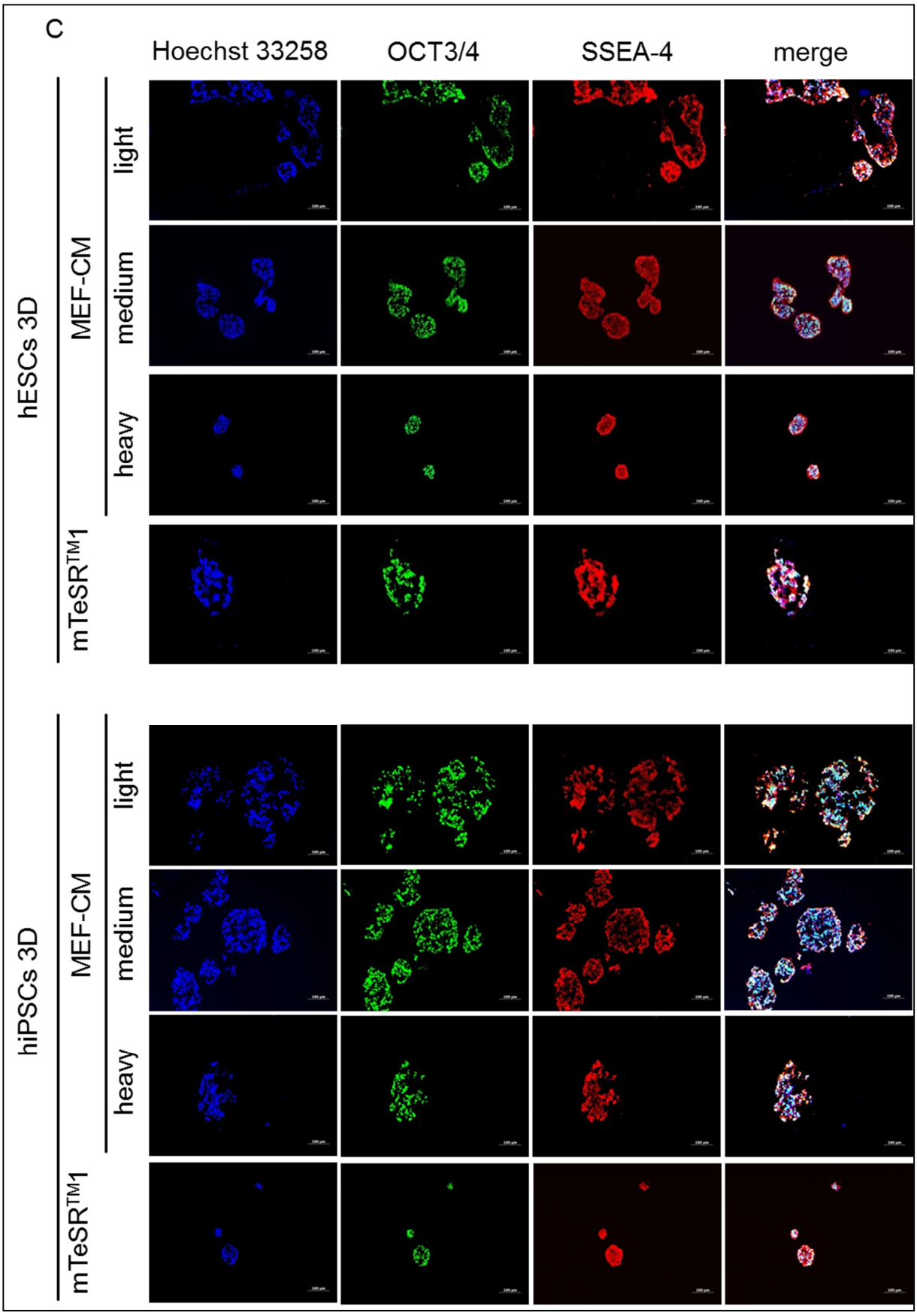

B
Lysine incorporation
Number of peptides
% Incorporation
hESC 2D "medium"
hESC 2D "heavy"
hiPSC 2D "medium"
hiPSC 2D "heavy"
hESC 3D "medium"
hESC 3D "heavy"
hiPSC 3D "medium"
hiPSC 3D "heavy"
0
2
4
6
8
10
12
14
16
18
99-100
98-99
97-98
96-97
95-96
94-95
93-94
92-93
91-92
90-91
89-90
88-89
87-88
86-87
85-86
84-85
83-84
82-83
81-82
80-81
0-80
Arginine incorporation
Number of peptides
% Incorporation
hESC 2D "medium"
hESC 2D "heavy"
hiPSC 2D "medium"
hiPSC 2D "heavy"
hESC 3D "medium"
hESC 3D "heavy"
hiPSC 3D "medium"
hiPSC 3D "heavy"
0
2
4
6
8
10
12
14
99-100
98-99
97-98
96-97
95-96
94-95
93-94
92-93
91-92
90-91
89-90
88-89
87-88
86-87
85-86
84-85
83-84
82-83
81-82
80-81
0-80

SUPPL. FIG. S1, related to Fig. 2. **Determination of SILAC incorporation efficiencies in hPSCs.** *A*, 10 µg of each “medium” or “heavy” SILAC labeled protein preparation of hESCs and hiPSCs grown under 2D or 3D conditions were separated by SDS-PAGE on 10% gels. Two distinct bands were cut out per lane, pooled and measured by LC-MS/MS. MaxQuant based analysis led to the identification of β-actin (ACTB) and heat-shock protein HSP 90-β (HSP90AB1). *B*, peptide lists were extracted for ACTB and HSP90AB1 from MaxQuant and incorporation efficiencies were calculated from the MaxQuant-derived ratio values for lysine and arginine terminated peptides, respectively. Incorporation efficiency = (1-1/(ratio+1)) x 100%. Peptides were assigned to categories representing the percentage of label incorporation.

Figure S2

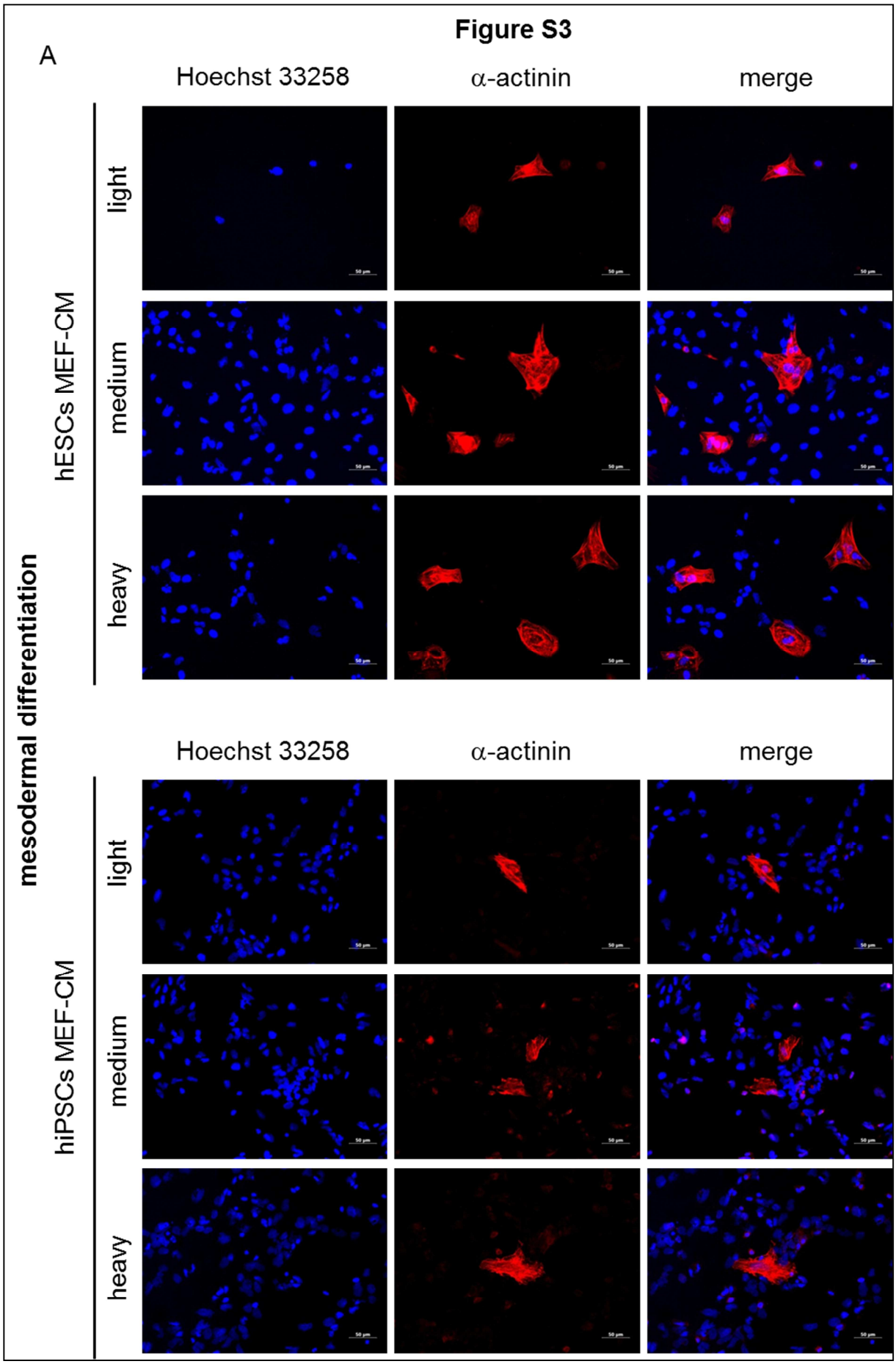

A
2D
3D
hESCs
hiPSCs
hESCs
hiPSCs
MEF-CM
light
medium
heavy
mTeSR™1
anti-SSEA-4 + sec. AB
IgG3κ + sec. AB
(isotype control)
sec. AB
(negative control)

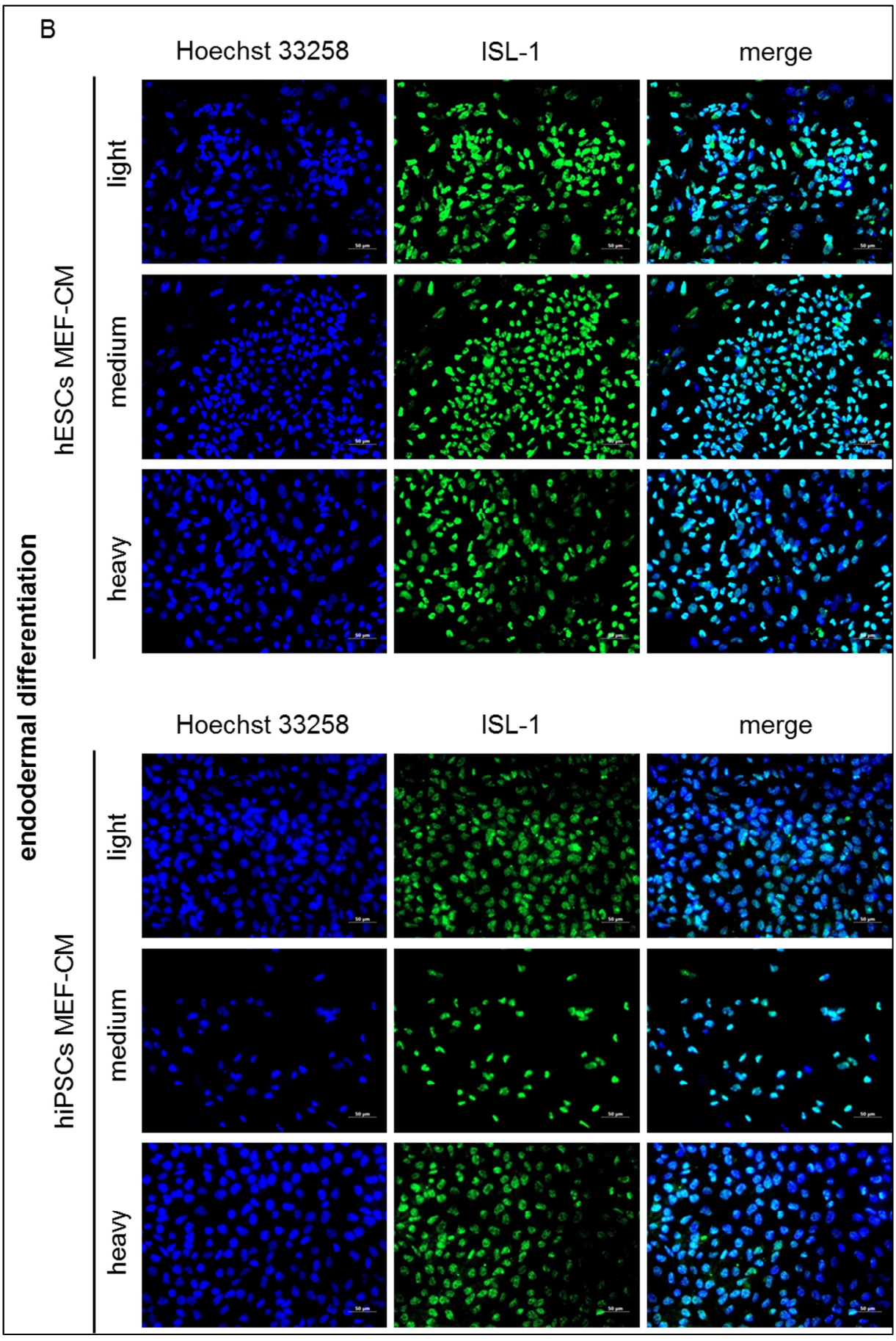

B
Hoechst 33258
OCT3/4
SSEA-4
merge
hESCs 2D
MEF-CM
light
medium
heavy
mTeSR™1
hiPSCs 2D
MEF-CM
light
medium
heavy
mTeSR™1

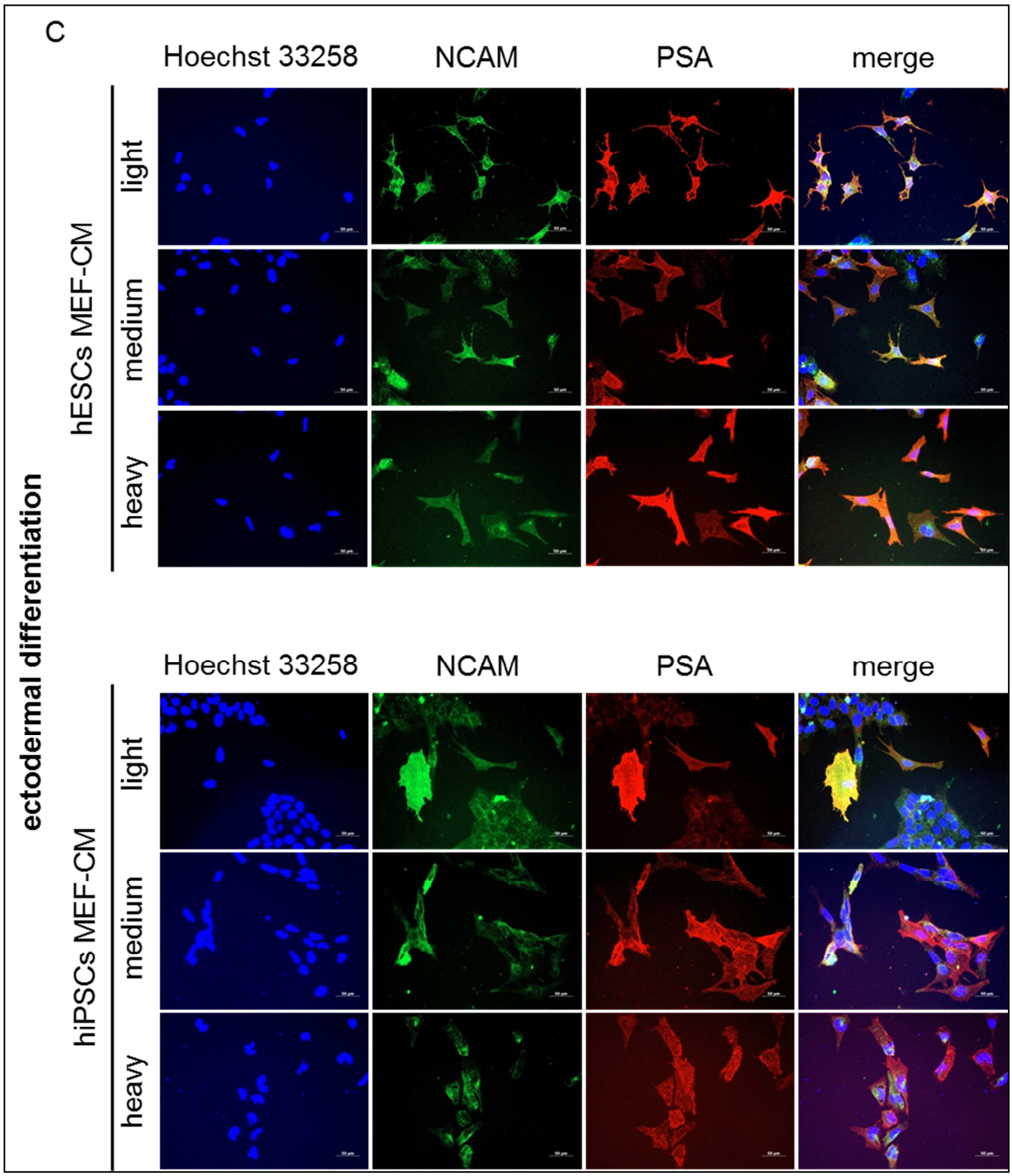

C
Hoechst 33258
OCT3/4
SSEA-4
merge
hESCs 3D
hiPSCs 3D
mTeSR™1
MEF-CM
heavy
medium
light

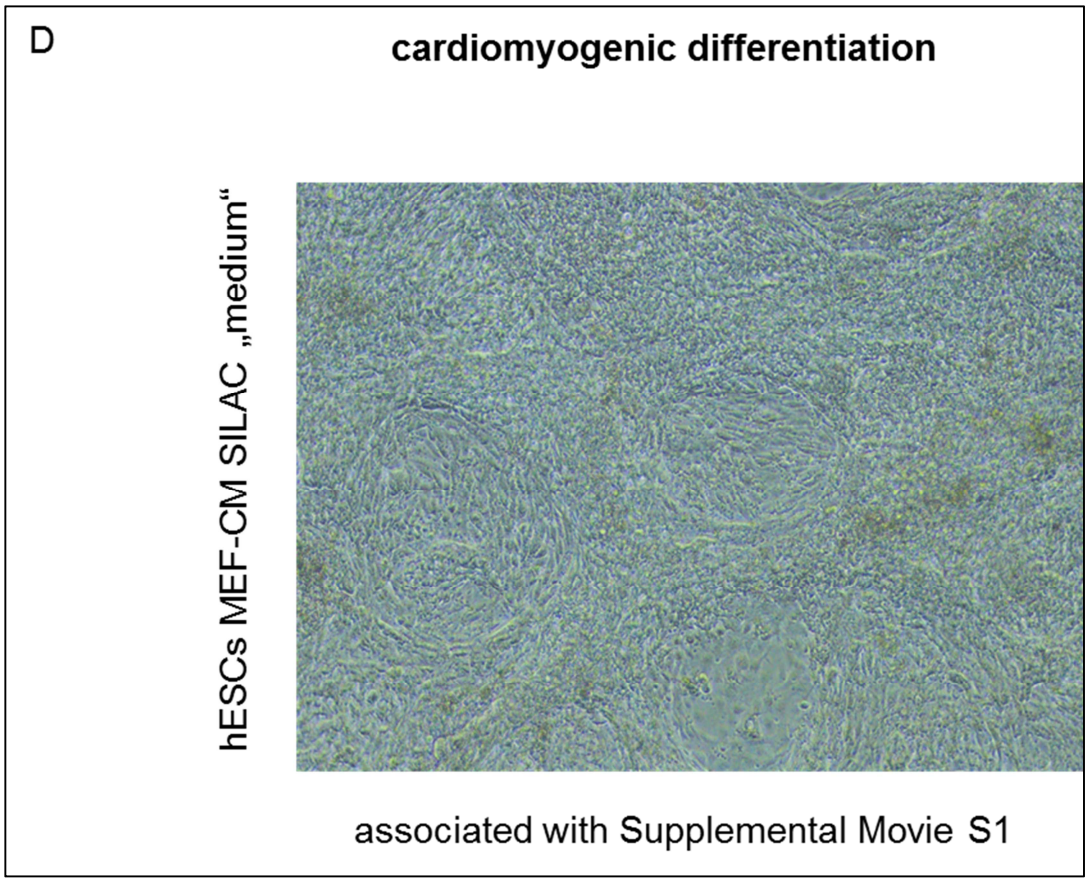
SUPPL. FIG S2 related to Fig. 2. **Analysis of pluripotency markers of hESCs and hiPSCs grown in 2D or 3D by flow cytometry and immunofluorescence microscopy.** *A*, analysis of SSEA-4 expression for hESCs and hiPSCs grown under 2D or 3D conditions by flow cytometry. The analysis was performed for each SILAC labeling condition (MEF-CM "light", "medium", and "heavy") and mTeSR^{TM}1 as a positive control. The green line represents the experimental analysis. The isotype control and the negative control are represented by the blue line and the red line, respectively. *B* and *C*, immunofluorescence microscopy for OCT3/4 and SSEA-4 expression in hESCs and hiPSCs grown under 2D ("*B"*) or 3D ("*C"*) conditions for each SILAC labeling condition (MEF-CM "light", "medium", and "heavy") and mTeSR^{TM}1 as a positive control. The scale bar represents 100 µm. hESCs and hiPSCs in SILAC MEF-CM including the Rho kinase inhibitor Y27632 (RI) grew as a monolayer under 2D conditions whereas hPSCs in mTeSR^{TM}1 without RI showed typical colony morphology.

Figure S3


A
Hoechst 33258
α-actinin
merge
light
medium
heavy
hESCs MEF-CM
mesodermal differentiation
Hoechst 33258
α-actinin
merge
light
medium
heavy
hiPSCs MEF-CM

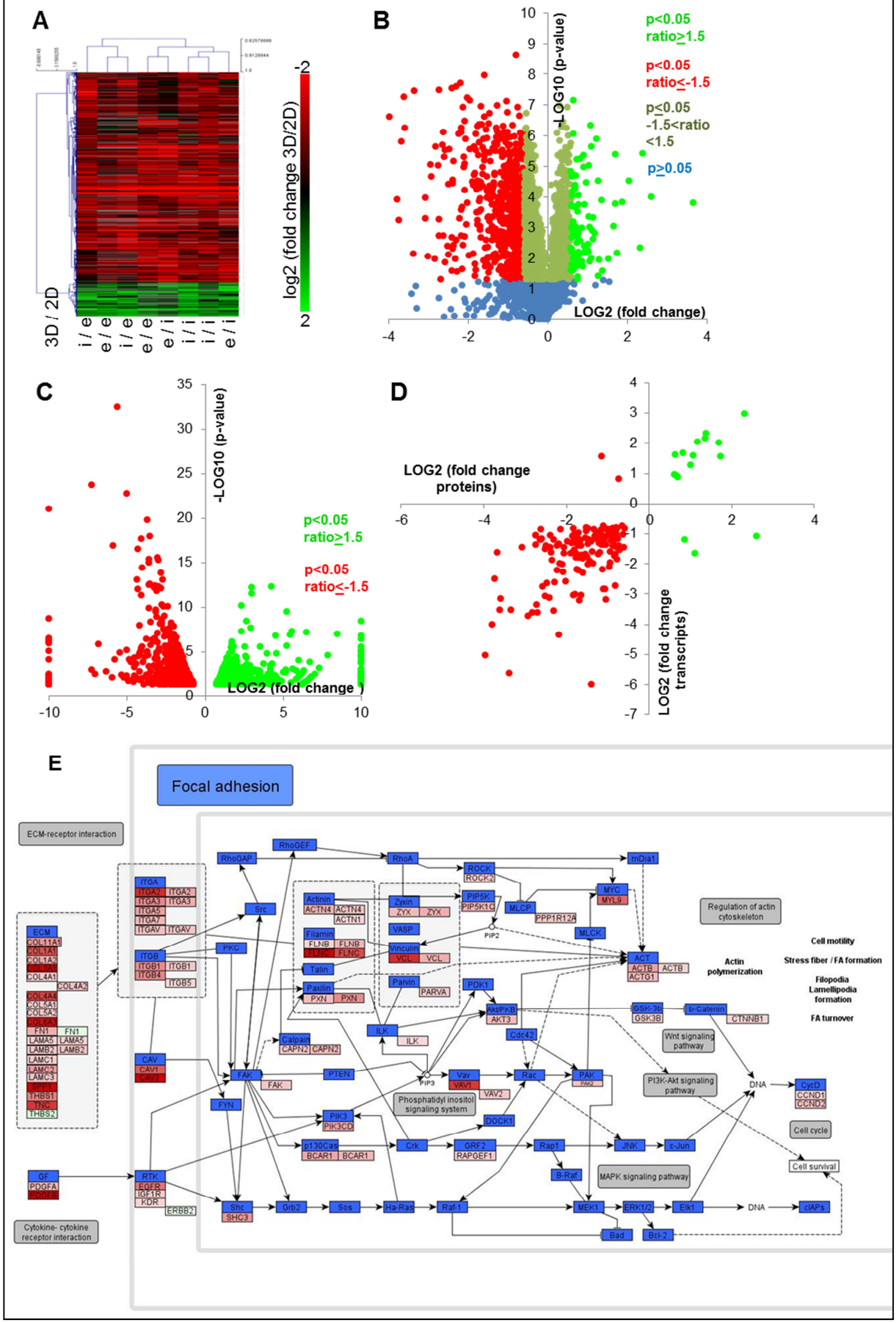

B
endodermal differentiation
hESCs MEF-CM
hiPSCs MEF-CM
Hoechst 33258
ISL-1
merge
light
medium
heavy


C
ectodermal differentiation
hESCs MEF-CM
hiPSCs MEF-CM
light
medium
heavy
Hoechst 33258
NCAM
PSA
merge



SUPPL. FIG S3 related to Fig. 2. **Differentiation of SILAC-labeled cells into derivatives of all three germ layers.** *A*, immunofluorescence microscopy for expression of the skeletal and cardiac muscle (mesoderm)-specific marker α-actinin after cardiomyogenic differentiation of SILAC-labeled hESCs and hiPSCs. The analysis was performed for each SILAC labeling condition (MEF-CM “light”, “medium”, and “heavy”). *B,* immunofluorescence microscopy for analysis of expression of the mesodermal/endodermal marker ISL-1 in hESCs and hiPSCs after initiation of endodermal differentiation. C, immunofluorescence microscopy for expression of the neuroectodermal markers neural cell adhesion molecule (NCAM) and polysialic acid (PSA). The scale bar represents 50 µm (A-C). D, light microscopy of SILAC labeled hESCs upon cardiomyogenic differentiation at 10-fold magnification. Supplemental Movie S1 (movieS1.mov) is associated with this figure showing autonomous contraction typical for cardiomyocytes. Beating cells do also develop from “light” and “heavy” labeled hESCs as well as from SILAC labeled hiPSCs upon cardiomyogenic differentiation (data not shown).

Figure S4


A
3D / 2D
i / e
e / e
i / e
e / e
e / i
i / i
i / i
e / i
log2 (fold change 3D/2D)
-2
2
B
-LOG10 (p-value)
LOG2 (fold change)
p<0.05
ratio≥1.5
p<0.05
ratio≤-1.5
p≤0.05
-1.5<ratio
<1.5
p≥0.05
C
-LOG10 (p-value)
LOG2 (fold change)
p<0.05
ratio≥1.5
p<0.05
ratio≤-1.5
D
LOG2 (fold change
proteins)
LOG2 (fold change
transcripts)
E
Focal adhesion
ECM-receptor interaction
Cytokine- cytokine
receptor interaction
RhoGEF
RhoGAP
RhoA
ROCK
ROCK2
mDia1
Src
Actinin
ACTN4
ACTN1
Zyxin
ZYX
PIP5K
PIP5K1C
MLCP
PPP1R12A
MYC
MYL9
Regulation of actin
cytoskeleton
VASP
Filamin
FLNB
FLNC
Vinculin
VCL
PIP2
MLCK
ACT
ACTB
ACTG1
Cell motility
Actin
polymerization
Stress fiber / FA formation
Filopodia
Lamellipodia
formation
FA turnover
Talin
Paxillin
PXN
Parvin
PARVA
PDK1
Akt/PKB
AKT3
GSK-3b
GSK3B
b-Catenin
CTNNB1
Wnt signaling
pathway
ILK
Calpain
CAPN2
Cdc42
PKC
CAV
CAV1
CAV2
FAK
PTEN
PIP3
Vav
VAV1
VAV2
Rac
PAK
PAK2
PI3K-Akt signaling
pathway
DNA
CycD
CCND1
CCND2
Cell cycle
FYN
Phosphatidyl inositol
signaling system
PIK3
PIK3CD
DOCK1
p130Cas
BCAR1
Crk
GRF2
RAPGEF1
Rap1
JNK
c-Jun
Cell survival
B-Raf
MAPK signaling pathway
GF
PDGFA
PDGFB
RTK
EGFR
IGF1R
KDR
ERBB2
Shc
SHC3
Grb2
Sos
Ha-Ras
Raf-1
MEK1
ERK1/2
Elk1
DNA
cIAPs
Bad
Bcl-2
ECM
COL11A1
COL1A1
COL1A2
COL3A1
COL4A1
COL4A2
COL4A4
COL5A1
COL5A2
COL6A3
FN1
LAMA5
LAMB2
LAMC1
LAMC2
LAMC3
SPP1
THBS1
TNC
THBS2
ITGA
ITGA2
ITGA3
ITGA5
ITGA7
ITGAV
ITGAV
ITGB
ITGB1
ITGB4
ITGB5

SUPPL. FIG S4 related to Fig. 3 and 4. **Bioinformatic analysis of proteomics and transcriptomics for hESCs and hiPSCs being taken together as hPSCs.** *A*, proteins significantly up- (green) or downregulated (red) comparing 3D vs. 2D culture with hESCs and hiPSCs being analyzed together. From the pooling of the differentially SILAC-labeled samples eight ratios of a 3D sample versus a 2D sample could be directly obtained from the MaxQuant analysis (four intra-cell-line comparisons [hESC 3D vs. hESC 2D or hiPSC 3D vs. hiPSC 2D] and four inter-cell-line comparisons [hESC 3D vs. hiPSC 2D or hiPSC 3D vs. hESC 2D] (Fig. 1)). The heatmap was generated with MeV V4.8.1 (http://www.tm4.org/mev/) from the eight 3D vs. 2D ratios for hESCs (e) and hiPSC (i) of 716 proteins that were significantly ($p<0.05$) regulated above the threshold of 1.5-fold and found in at least four comparisons. Significance and mean ratio were calculated from the eight different comparisons as indicated. Ratios were log2 transformed and hierarchical clustering was performed using "Pearson Uncentered" as "Distance Metric Selection". *B*, Volcano plot of 3521 different proteins identified by two or more peptides in at least four out of eight comparisons in a combined analysis of hESCs and hiPSCs. *C*, Volcano plot of 1298 different transcripts that were significantly ($p<0.05$) up- (504) or down- (794) regulated (>1.5-fold) in a combined analysis of hESCs and hiPSCs. *D*, comparison of ratios of the proteomic as well as the transcriptomic approach for proteins or transcripts that were identified significantly ($p<0.05$) above 1.5-fold by both approaches. *E*, representation of the KEGG pathway "focal adhesion" (blue boxes) including gene expression data (boxes directly below the blue boxes) and protein expression data (boxes shifted to the right below the blue boxes). Upregulation in 3D culture is indicated by greenish boxes, downregulation by reddish with the intensity of the green or red colour depending on the height of regulation. InCroMAP 1.4 software (http://www.ra.cs.uni-tuebingen.de/software/InCroMAP/) was used for importation of the KEGG pathway map as well as for importation and visualization of expression data. Final graphical layout was done manually with yEd Graph Editor Software Version 3.10.1 (http://www.yworks.com/de/products_yed_about.html).

Figure S5

A

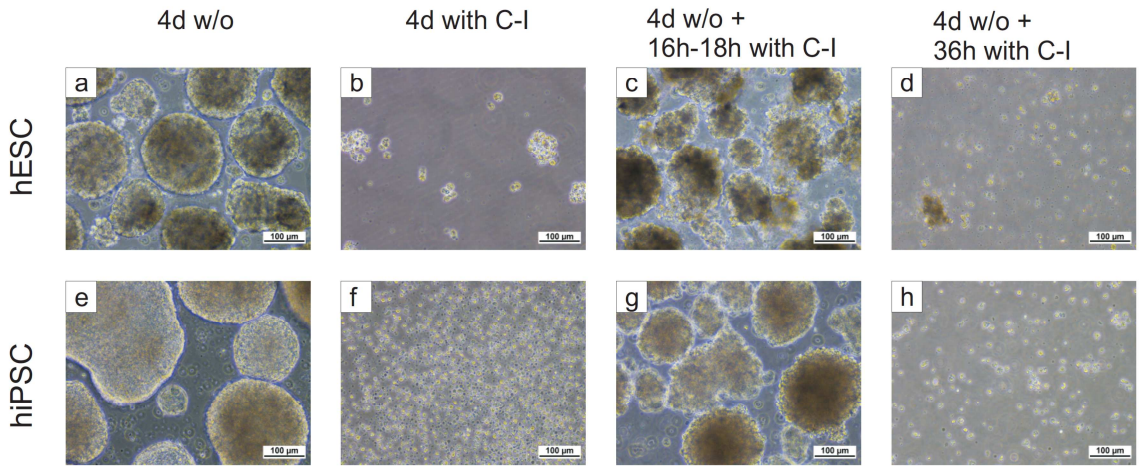

4d w/o
4d with C-I
4d w/o +
16h-18h with C-I
4d w/o +
36h with C-I
hESC
hiPSC
a
b
c
d
e
f
g
h
100 µm

Figure S5

B

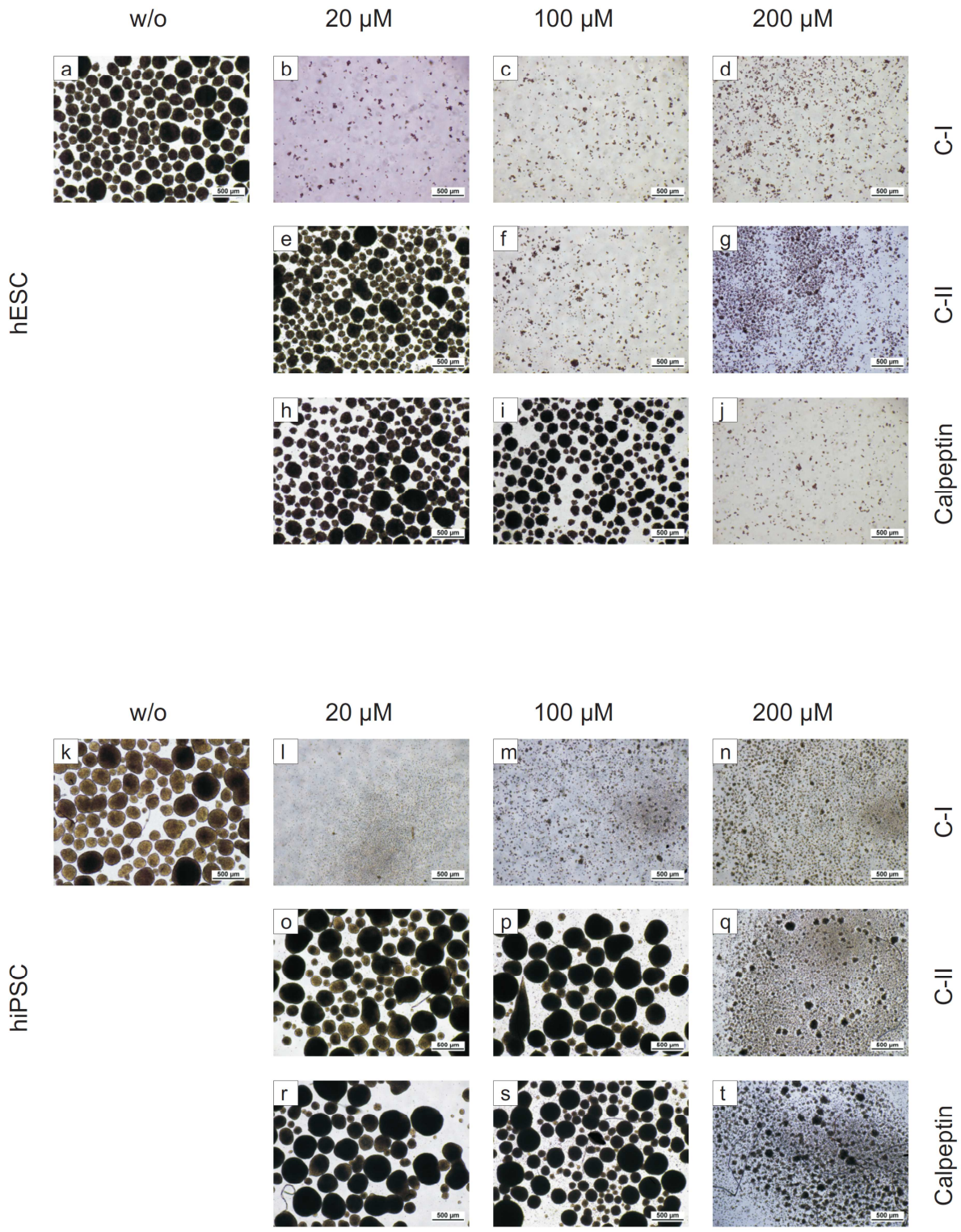
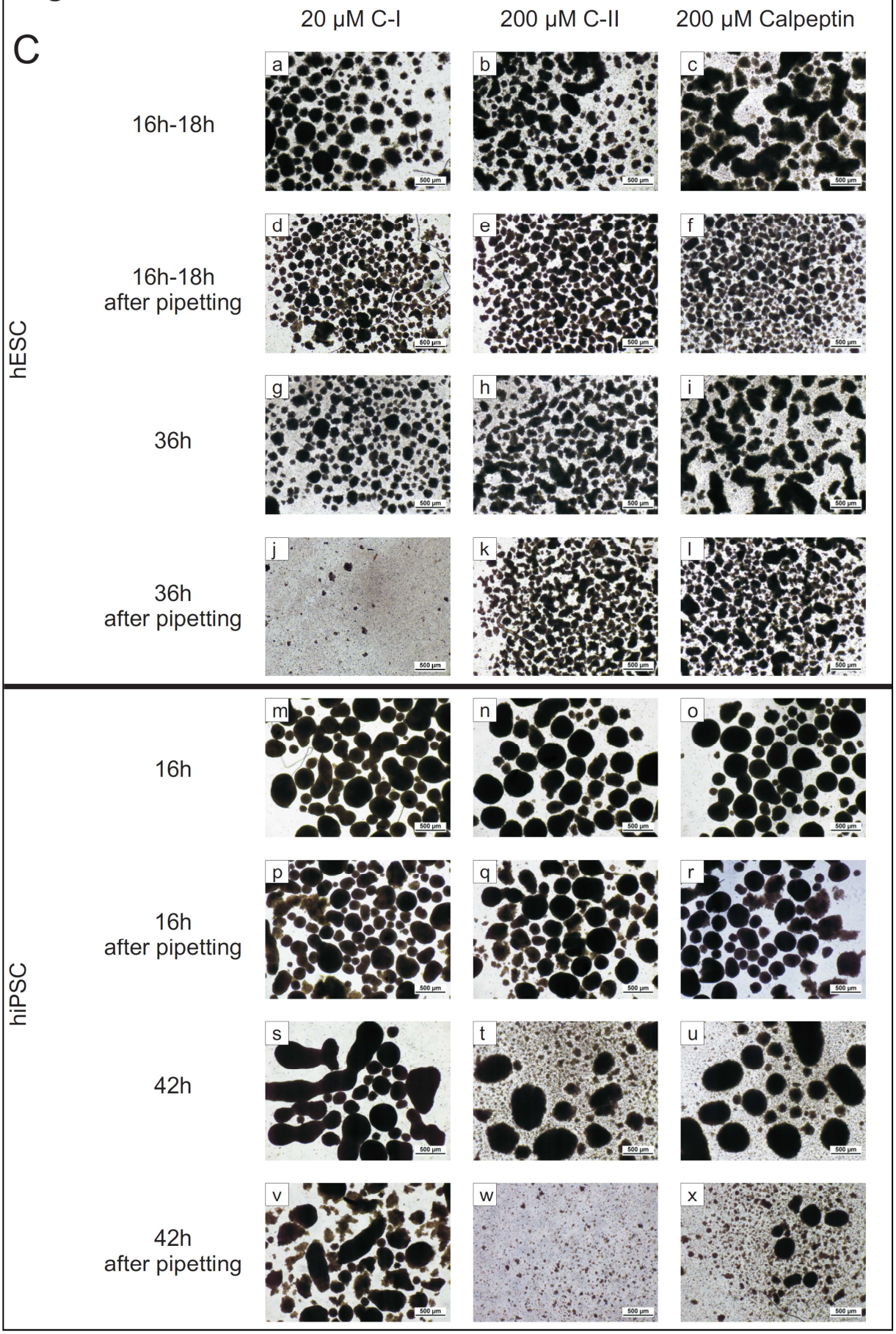

w/o
20 µM
100 µM
200 µM
hESC
C-I
C-II
Calpeptin
a
b
c
d
e
f
g
h
i
j
w/o
20 µM
100 µM
200 µM
hiPSC
C-I
C-II
Calpeptin
k
l
m
n
o
p
q
r
s
t

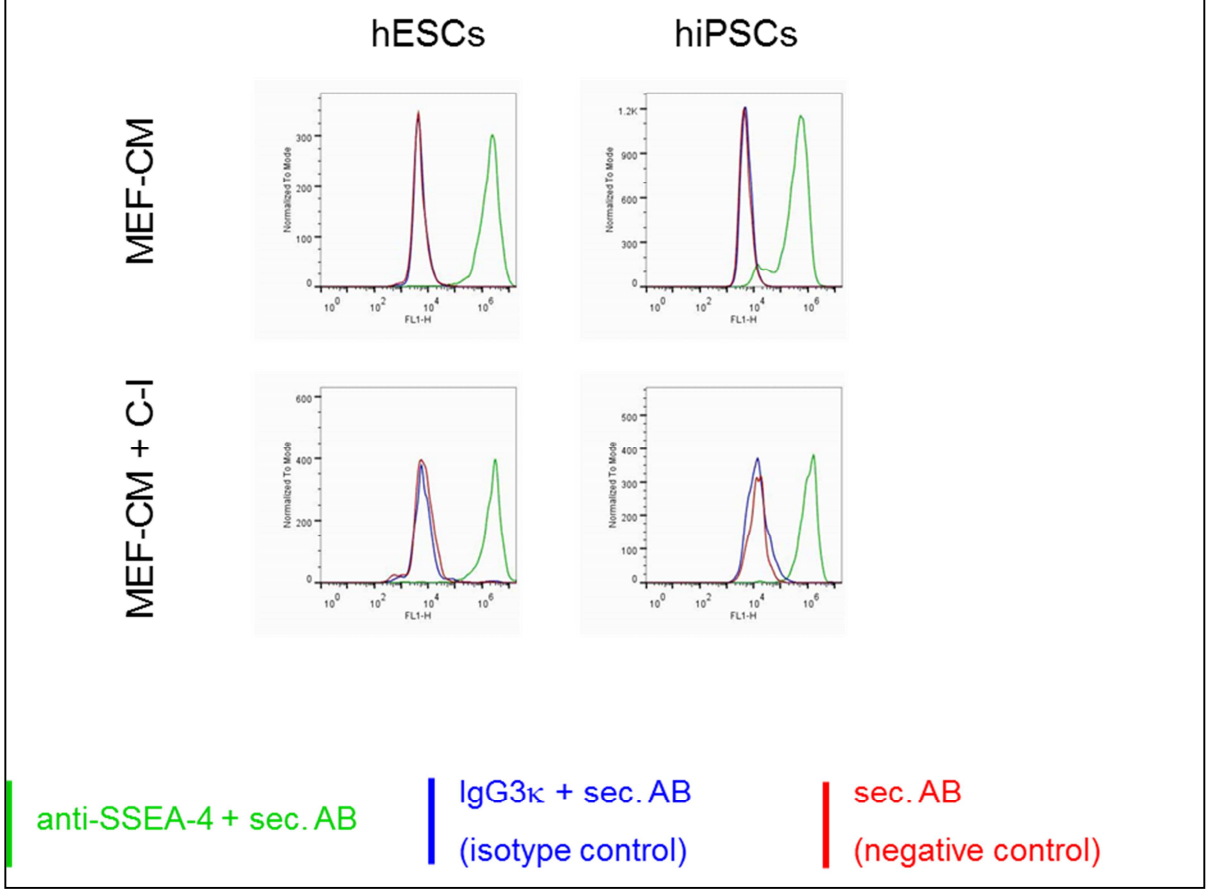
Figure S5

C


20 µM C-I
200 µM C-II
200 µM Calpeptin
hESC
16h-18h
16h-18h after pipetting
36h
36h after pipetting
hiPSC
16h
16h after pipetting
42h
42h after pipetting
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
500 µm

SUPPL. FIG S5, related to FIG. 7. **Effects of calpain inhibitors on spheroid genesis and on pre-formed spheroids.** *A*, this Figure is equivalent to Fig. 7, but pictures were taken at 10-fold magnification. *B*, addition of calpain inhibitors to the inoculum of suspension cultures inhibited spheroid formation. The suspension culture inoculum of hESCs or hiPSCs was supplemented with 20 µM, 100 µM or 200 µM of calpain inhibitor I (C-I), calpain inhibitor II (C-II) or calpeptin. For hESCs 20 µM C-I (b), 100 µM C-II (f) and 200 µM calpeptin (j) were sufficient to completely block spheroid formation. In case of hiPSCs spheroid formation was blocked with 20 µM C-I (l), 200 µM C-II (q) and 200 µM calpeptin (t). 100 µM C-II were sufficient to block spheroid formation of hESCs (f) but not of hiPSCs (p). Under standard culture conditions (w/o) spheroids developed as expected and spheroids of hiPSCs were slightly larger than those of hESCs. Solvents had no effect on spheroid formation (data not shown). Images were taken at 4-fold magnification. *C*, pre-formed spheroids could be disassembled by addition of calpain inhibitors to the culture media. 20 µM calpain inhibitor I (C-I), 200 µM calpain inhibitor II (C-II) or 200 µM calpeptin were added to 4d old pre-formed spheroids of hESCs and hiPSCs. Spheroid integrity was assessed after 16h to 18h and after 36h (hESCs) or 42h (hiPSCs). Images were taken at 4-fold magnification. To show loose cell-cell interactions of spheroids incubated with calpain inhibitors, they were pipetted up and down after the indicated times. Pipetting was not sufficient to disrupt spheroids grown in the absence of calpain inhibitors.

Figure S6


hESCs
hiPSCs
MEF-CM
MEF-CM + C-I
Normalized To Mode
FL1-H
anti-SSEA-4 + sec. AB
IgG3κ + sec. AB
(isotype control)
sec. AB
(negative control)

SUPPL. FIG S6 related to Fig. 7. **Analysis of pluripotency of hESCs and hiPSCs treated with calpain inhibitor I.** *A*, calpain inhibitor I was added to the inoculum of suspension cultures (MEF-CM + C-I). After 24 hours, control cells (MEF-CM) started formation of suspension culture spheroids whereas C-I treated cells remained as single cells. Flow cytometric analysis of hESCs and hiPSCs revealed that expression of SSEA-4 was unchanged in C-I treated cells compared to control. The green line represents the respective hPSC sample signal. The isotype control and the negative control are represented by the blue line and the red line, respectively.

SUPPL. TABLE S1, related to FIG. 1. **SILAC-based proteomic results.**

This table contains separate Excel sheets for proteins upregulated in 3D (i-iii) for hESCs (i), hiPSCs (ii) and hPSCs (iii, comprising a joint analysis of hESCs and hiPSCs) as well as for proteins downregulated in 3D (iv-vi) for hESCs (iv), hiPSCs (v) and hPSCs (vi). “Protein Names”, “Gene Names”, “Uniprot”, “Peptides”, “Sequence Coverage [%]”, “Mol. Weight [kDa]”, and “PEP” are direct output data of MaxQuant. “Gene Names” were searched at HGNC (HUGO Gene Nomenclature Committee, http://www.genenames.org/) in order to have a nomenclature comparable to transcriptomics data and are shown in row “HUGO Gene Names”. “Fold Change 2D/3D” represents the ratiometric mean of the respective protein amount under 2D conditions divided by the value under 3D culture conditions. This value was calculated from the three different biological repeats for hESCs and hiPSCs, each and for hPSCs, which is a joint analysis of hESCs and hiPSCs. Accordingly, the “Standard Deviation” and a “Student’s t-Test” were calculated.

SUPPL. TABLE S2, related to FIG. 1. **Deep-sequencing-based transcriptomic results.**

This table contains separate Excel sheets for transcripts upregulated in 3D (i-iii) for hESCs (i), hiPSCs (ii) and hPSCs (iii, comprising a joint analysis of hESCs and hiPSCs) as well as for transcripts downregulated in 3D (iv-vi) for hESCs (iv), hiPSCs (v) and hPSCs (vi). “id” depicts the nomenclature according to HGNC (HUGO Gene Nomenclature Committee, http://www.genenames.org/). “Base_Mean_2D” and “Base_Mean_3D” is a measure for the transcript amount in the sample. “Fold Change 3D/2D” is calculated as quotient of Base-Mean_3D vs. Base_Mean_2D and “Fold Change 2D/3D as quotient of Base-Mean_2D vs. Base_Mean_3D. Genes for which no transcript was detected either under 3D or 2D conditions are written in italics and the respective “Fold Change” was set to “Inf”. The three biological replicates for each, hESCs and hiPSCs, were pooled, and the pools of hESCs and hiPSCs were analysed individually. Results are shown for hESCs, hiPSCs and for hPSCs,

which is a joint analysis of hESCs and hiPSCs. The “p-Value” rather reflects the technical significance of the analysis than the biological.

SUPPL. TABLE S3, related to FIG. 4 and 5. **Primers used for qPCR analyses.**

Target gene name / full name	Primer name	Primer sequence (5' to 3')
Housekeeping genes		
ARPC1A / actin related protein 2/3 subunit 1A	ARPC1A_fw	GTGGAGCACGACTCATTTCT
	ARPC1A_rev	TGATCCTGCTGCCAGCAAAA
SDHB / succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	SDHB_fw	CTTCCGAAGATCATGCAGAG
	SDHB_rev	CATGTGTGGAAGAGGGTAGA
Pluripotency markers		
OCT3/4 / *POU5F1* / POU class 5 homeobox 1	OCT4_fw	AGAAGGAGAAGCUGGAGCAA
	OCT4_rev	CTTCCCAAATAGAACCCCCA
SOX2 / SRY (sex determining region Y)-box 2	SOX2_fw	GGACACTGGCTGAATCCT TCC
	SOX2_rev	CTCGCTGATTAGGCTCCAACC
LIN28 / lin-28 homolog A	LIN28_fw	ATGGAGAAAACCCGGTACGC
	LIN28_rev	TTTTGCGTGAGTGTGGATGG
NANOG / Nanog homeobox	NANOG_fw	TTGAGGAGCAGGCAGAGTGG
	NANOG_rev	TGCATTTGGACAGAGCATGG
PODXL / podocalyxin-like	PODXL_fw	ACAGCAGCATCAACTACCCA
	PODXL_rev	CTTCTCACTCTGTGTCTGTG
DNMT3B / DNA (cytosine-5-)-methyltransferase 3 beta	DNMT3B_fw	AAGTTTCTGCTGCTCACAGG
	DNMT3B_rev	ATCGAGTTCGACTTGGTGGT
Wnt target genes		
CCND1 / Cyclin D1	CCND1_fw	AATGTGTGCAGAAGGAGGTC
	CCND1_rev	AAGCGGTCCAGGTAGTTCAT
c-MYC / v-myc myelocytomatosis viral oncogene homolog	c-MYC_fw	CTTCTCTGAAAGGCTCTCCT
	c-MYC_rev	GTCGTAGTCGAGGTCATAGT
ETV4 / *PEA3* / ets variant 4	ETV4_fw	ATGAGAAACCTCTGCGACCA
	ETV4_rev	CAGCAAGGCCACCAGAAATT
SLUG / Snail homolog 2	SLUG_fw	ACTACAGCGAACTGGACACA
	SLUG_rev	GAAAGAGGAGAGAGGCCATT
CD44 / CD44 antigen, Epican	CD44 fw	TGGGTTCATAGAAGGGCATG
	CD44 rev	ATTGGGCAGGTCTGTGACT
FOSL1 / FOS-like antigen 1	FOSL1 fw	AACTGACCGACTTCCTGCA
	FOSL1 rev	CACTGGTACTGCCTGTGTC
L1CAM / L1 cell adhesion molecule	L1CAM fw 1	TATGGCCTTGTCTGGGATCT
	L1CAM rev 1	TAGGAGCTCTGGTTGTAGCT
PLAUR / plasminogen activator, urokinase receptor	PLAUR fw 1	GCATGCAGTGTAAGACCAAC
	PLAUR rev 1	TCTCTGAGTGGGTACAGCTT
Wnt antagonists		
CER1 / cerberus 1, cysteine knot superfamily, homolog	CER1_fw	GAAGTACATTGGGAGACCTG
	CER1_rev	ACAGTGAGAGCAGGAGGTAT
DKK1 / dickkopf 1 homolog	DKK1_fw	GTACCAGACCATTGACAACT
	DKK1_rev	TGCAGGCGAGACAGATTTG
DKK4 / dickkopf homolog 4	DKK4_fw	AACATCAGGAGCTCTGCTGA
	DKK4_rev	CACGACATGTAGCACAGAAC
FRZB / Frizzled-related protein B	FRZB_fw	GAAGATGAGGAACGTTCCAG
	FRZB_rev	GACTTCTGACTCTGAGTGGA
SFRP1 / Secreted frizzled-related protein 1	SFRP1_fw	TACTGGCCCGAGATGCTTAA
	SFRP1_rev	AAACTCGCTGGCACAGAGAT
WIF1 / WNT inhibitory factor 1	WIF1_fw	AAAGGTTACCAGGGAGACCT
	WIF1_rev	TGTATGAGGCTGGCTTCGTA
Adherens junction		
CDH1 / Cadherin-1 / E-cadherin	CDH1_fw	GGAGCCAGACACATTTATGG
	CDH1_rev	CTGTGTACGTGCTGTTCTTC
CTNNB1 / β-catenin	CTNNB1_fw	CTGCTAAATGACGAGGACCA
	CTNNB1_rev	GTATTCTGCATGGTACGTAC

SUPPL. TABLE S4, related to Fig. 4 and 5. **Primary antibodies used for western blots, flow cytometry and immunofluorescence.**

Target	Host species (isotype)	Supplier	Supplier #	Clone	Immunogen / Epitope *
Pluripotency markers					
OCT3/4 / Octamer-binding protein 3/4 / POU domain, class 5, transcription factor 1	mouse (IgG_{2b})	Santa Cruz Biotechnology	sc-5279	C-10	Raised against amino acids 1 - 134
SSEA-4 Stage-specific embryonic antigen-4	mouse ($IgG_{3\kappa}$)	BioLegend	330401	MC-813-70	Detects a glycolipid: NeuAcα2-3Galβ1-3GalNacβ1-R
E-Cadherin					
CDH1 / Cadherin-1 / E-Cadherin	rabbit	Cell Signaling Technologies	3195S	24E10	Raised against peptide around amino acid 780; the epitope is located C-terminal of amino acid 752, it contains at least amino acids 782 – 787 and ends C-terminal of amino acid 788 (personal communication with technical staff from Cell Signaling Technologies)
	mouse (IgG_{2a})	BD Transduction Laboratories	C20820	C-36	Raised against C-terminal recombinant E-cadherin (cytosolic part); epitope: amino acids 773 – 791 (1)
	goat	Santa Cruz Biotechnology	sc-1499	C-19	Raised against peptide within a C-terminal cytoplasmic domain; epitope: amino acids 863 – 882 (2)
β-Catenin					
CTNNB1 / β-catenin	rabbit	Cell Signaling Technologies	8480P	D10A8	Recognizes endogenous levels of total β-catenin protein; raised against peptide around amino acid 714
	rabbit	Millipore	ABE208		Epitope is corresponding to the consensus GSK3 phosphorylation site of β-catenin; detects total levels of β-catenin
	mouse ($IgG_{1\kappa}$)	Millipore	05-665	8E7	Epitope corresponds to amino acids 36 – 44 and is specific for the active form of β-catenin, dephosphorylated on Ser37 or Thr41
Other					
SFRP1 / Secreted frizzled-related protein 1	rabbit	Cell Signaling Technologies	3534S	D5A7	Detects endogenous levels of total SFRP1 protein
SPTAN1 / Spectrin alpha chain	mouse (IgG_1)	Millipore, Chemicon	MAB1622	AA6	Immunogen: Chicken red blood cell membranes purified by hypotonic lysis and mechanical enucleation
PSA (polysialic acid)	mouse			735D4	Immunogen: polysialic acid

	(IgG_{2a})				
NCAM	mouse (IgG_1)			123C3	Immunogen: all isoforms of human NCAM
Islet-1	rabbit	Abcam	ab20670		Raised against peptide conjugated to KLH from within residues 300 to the C-terminus of human Islet-1
α-actinin (sarcomeric)	mouse (IgG_1)	Sigma-Aldrich	A7811	EA-53	Raised against purified rabbit skeletal muscle α-actinin

* Numbers refer to amino acid positions of the respective human protein, if not stated otherwise; information was obtained from supplier's data sheets, if not stated otherwise

SUPPL. TABLE S5, related to Fig. 4 and 5. **Secondary antibodies used for western blots, flow cytometry and immunofluorescence.**

Target species (isotype)	**Host species**	**Supplier**	**Supplier #**	**Fluorochrome**	**Application**
goat IgG (H+L)	donkey	LiCor	926-32214	IRDye 800CW	Western blot (against CDH1 (C19))
anti-mouse IgG	goat	LiCor	926-32220	IRDye 680	Western blot
anti-mouse IgG	goat	LiCor	926-32210	IRDye 800CW	Western blot
anti-rabbit IgG	goat	LiCor	926-32221	IRDye 680	Western blot
anti-mouse IgG (H+L)	goat	Molecular Probes	A 11029	Alexa Fluor® 488	Flow cytometry (against SSEA-4)
anti-mouse IgG_{2a}	goat	Molecular Probes	A 21134	Alexa Fluor® 568	Immunofluorescence (against anti-PSA)
anti-mouse IgG_{2b}	goat	Molecular Probes	A 21141	Alexa Fluor® 488	Immunofluorescence (against anti-OCT3/4)
anti-mouse IgG_1	goat	Molecular Probes	A 21121	Alexa Fluor® 488	Immunofluorescence (against anti-NCAM)
anti-mouse IgG (H+L)	goat	Jackson Immuno Research Laboratories	115-165-003	Cy™3	Immunofluorescence (against anti-SSEA-4)
anti-rabbit IgG (H+L)	donkey	Molecular Probes	A21206	Alexa Fluor® 488	Immunofluorescence (against anti-Islet-1)

SUPPLEMENTAL RESULTS

Genes with increased expression in suspension culture spheroids encode nuclear factors — STRING database-based enrichment analyses performed with proteins and transcripts upregulated ($p<0.05$, >1.5-fold) under 3D culture conditions in hPSCs (combined analysis of hESCs and hiPSCs) revealed that 29 transcripts matching to the KEGG pathway "systemic lupus erythematosus" were found to be enriched with high significance ($p=6x10^{-13}$). All 29 matches were histone genes (Supplementary Table S2) and belong to the nucleosome. Gene ontology (GO)-enrichment analyses for "cellular component" and "biological process" confirmed the enrichment of transcripts upregulated in suspension culture to belong to the nucleosome or to be involved in chromatin assembly. With the exception of the core histone macro-H2A other histone proteins were not identified in the proteomic approach, because histones are generally smaller than 25 kDa, which was the lower size limit in the proteomic screen (Fig. 1*B*). Performing a search with the list of upregulated proteins for enrichment in "GO biological process" applying the STRING database, we identified categories like "telomere maintenance via semi-conservative replication", "telomere maintenance via recombination" or "chromosome organization" as the most significant hits. The nucleosome assembly protein 1-like 1 (NAP1L1) was found to be the most highly upregulated protein in the SILAC proteomic experiment. Other replication associated proteins including the endonuclease III-like protein 1 (NTH1), the core histone macro-H2A (H2AFY2), the DNA polymerase epsilon subunit A (POLE1) or the DNA polymerase delta subunit A were also among the highly upregulated proteins in 3D culture (Supplementary Table S1).

SUPPLEMENTAL DISCUSSION

Histone mRNAs are cell cycle-regulated and increase considerably when cells enter the S phase of the cell cycle where the chromosomal replication takes place. The histones are required in the S phase for packaging the newly synthesized DNA into chromatin. At the end of the S phase, before entering mitosis, histone expression drops to baseline levels (3). Pluripotent stem cells divide rapidly and have a considerably shortened G1 phase of the cell

cycle compared to somatic cells (4). Our finding of 3D induced upregulation of histone genes suggests that under 3D culture conditions more cells are in the S phase, which is indicative of higher proliferation rates and accelerated progression through the G1 phase. Calpains have been implicated in regulation of cell proliferation as calpain inhibitors have anti-mitogenic effects (5) and calpain activation shortens the G1 phase of transformed cells (6). Upregulation of the endogenous calpain inhibitor calpastatin as observed by us in 2D vs. 3D has been shown to decrease growth of CHO cells (7). We thus hypothesize that the observed activation of calpain might result in an increased proliferation of 3D cultured cells.

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