# Supporting Information

## Glycosaminoglycan-Binding Hydrogels Enable Mechanical Control of Human Pluripotent Stem Cell Self-Renewal

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### Supplementary Methods

**Preparation of "reactive" and siliconized coverslips.** For attachment of hydrogels to a surface, "reactive" and siliconized coverslips were prepared. Functionalization of both "reactive" and siliconized coverslips started by rinsing 22 mm sq. No. 1 cover glass (Corning Inc.) with ethanol, and allowed to air dry. Coverslips were then prepared by following established protocol.[1] Briefly, "reactive" coverslips were etched by treatment with 0.1 M NaOH for 3 min, and then aminosilanized by exposure to 97% 3-aminopropyltriethoxysilane (3-APTES) (Sigma) for 5 min. The coverslips were washed several times with distilled water, and dried by aspiration. A solution of 0.5% glutaraldehyde (Fluka BioChemika) was added to the aminosilanized coverslips, and the mixture was allowed to react for 30 min. The "reactive" coverslips were washed several times with water and set aside to air dry. Another set of coverslips were siliconized by reacting with 10% Surfasil™ siliconizing fluid (Thermo Scientific) in chloroform for a minimum of 15 min at room temperature. Siliconized coverslips were first washed with chloroform, then several times with water, and then allowed to air dry before use.

**Measurement of hydrogel elasticities by atomic force microscopy.** All elasticity measurements were obtained with a Multi-Mode IV Atomic Force Microscope (AFM) equipped with a Nanoscope IV Controller and a fluid cell (Digital Instruments). Hydrogels were probed with a borosilicate sphere-tipped cantilever with a radius of 2.5 μm and nominal spring constant of 0.06 N/m (Novascan Technologies Inc.). Force-indentation profiles were collected on fully hydrated hydrogels and fit up to 1 μm tip deflections by using the Hertz sphere-on-flat model where the force ($F$) is defined as:

$$F = \frac{4}{3}\frac{E}{(1-v^2)}\sqrt{R}\delta^{3/2}$$

Where $E$ is the elasticity, $v$ is the Poisson ratio (which is typically 0.5 for soft materials including hydrogels), $R$ is the radius of the AFM probe, and $\partial$ is the indentation.[2]

**Preparation of hydrogels for cell culture.** Hydrogels were washed several times with PBS for ~10 min per wash, and then sterilized with 70% ethanol for ~5 min. After several PBS washes, the hydrogels were transferred to non-tissue culture treated 6-well plates (Becton Dickinson Labware) under sterile conditions. Hydrogels were washed twice with sterile PBS and several times with DMEM/F12 (Gibco) supplemented with penicillin-streptomycin (Gibco), and then stored in DMEM/F12 at 4 $^{o}$C until needed. Prior to cell plating, hydrogels were allowed to equilibrate at room temperature (~30 min). Hydrogels were washed three times with DMEM/F12 before plating cells.

**Peptide functionalization of polyacrylamide hydrogels.** Hydrogels were synthesized as described above and functionalized with a fluorescein-labeled GRGDSC

peptide. The peptide used possessed an aminocaproic acid (Acp) linker (FITC-Acp-GRGDSC). Hydrogels were dialyzed in PBS with frequent buffer exchange to remove unreacted FITC-Acp-GRGDSC. For hydrogels of varying elasticities, FITC-conjugated GKKQRFRHRNRKGC peptide (FITC-Acp- GKKQRFRHRNRKGC) was used. Fluorescence microscopy images were acquired at constant exposure time for all samples with a Hamamatsu digital camera mounted on Olympus IX81 inverted microscope. Microscopy images were analyzed with Adobe Photoshop CS3. For plate reading, hydrogels were transferred to Greiner™ clear bottom 96-well plates (Sigma-Aldrich) and fluorescence intensities were measured at 485 nm excitation and 535 nm emission wavelengths with a Tecan Infinite M1000 plate reader.

**Cell adhesion and viability assays.** For serum-free adhesion on hydrogels, EC cells were detached with 0.05% trypsin-EDTA (Gibco) by incubating for approximately 2 min. Cells were washed three times in serum-free medium (RPMI-1640) by centrifugation at 1200 rpm for 5 min. Supernatant was removed and cells were resuspended in the serum-free medium and plated on the hydrogels at a density of $12x10^4$ cells/mL. After 1 hour incubation, nonadhering cells were removed by rinsing with RPMI-1640. Bright field images were captured with Olympus IX81 microscope mounted with Hamamatsu digital camera. For prolonged culture, cells were incubated with regular NCCIT medium, which was replenished daily. To quantify the amount of metabolically active cells on the hydrogels, cells were lysed with RIPA buffer (Thermo Scientific) after 1 hour serum-free culture. Cell lysates were mixed with CellTiterGlo™ luminescent cell viability reagent (Promega). The mixture was allowed to establish

luminescence at room temperature for 5 min, and luminescence was recorded with 20/20$^{n}$ luminometer (Turner Biosystems).

**Proliferation of hES cells on hydrogel scaffolds.** Hydrogels were synthesized onto 18 mm circular coverslips (Fisher Scientific) by following the above mentioned protocols. The hydrogels were placed into non-tissue culture treated 12-well-plates for cell culture and subsequent analysis. Human ES and iPS cells at ~70% confluency were detached from Matrigel by treatment with Hank's-based enzyme-free cell dissociation buffer (Sigma) for about 10 min at 37 $^{o}$C. Cells were resuspended in mTeSR1 medium (Stem Cell Technologies) and centrifuged twice in mTeSR1 medium at 1200 rpm for 5 min. Cells were then resuspended in mTeSR1 medium supplemented with 5 μM ROCK inhibitor Y-27632 (Calbiochem)[3] and plated on hydrogels at 15x10$^{4}$ cell/mL. Cells were incubated at 37 $^{o}$C with 5% $CO_2$, and the medium was refreshed daily. The amount of viable cells on the hydrogels at each time point was examined with the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies Inc.) by following manufacture protocol. Briefly, cells were treated with 500 μL of defined medium to which 50 μL of CCK-8 reagent was added. Cells were then incubated at 37 $^{o}$C for 2 hr. For plate reading, 100 μL of the incubated mixture was transferred to 96-well plate and the absorbance at 450 nm was measured with Tecan Infinite M1000 plate reader.

**Flow cytometry analysis**. Human ES cells were dissociated by treatment with 0.05% trypsin-EDTA. The dissociation process was stopped by the addition of 10% FBS in DMEM (Gibco). For alkaline phosphatase staining, cells were resuspended in wash buffer (2% BSA in PBS) and incubated with alkaline phosphatase conjugated to

allophycocyanin (R&D Systems) for 30 min at 4 °C. Cells were washed with PBS by centrifugation at 1000 rpm for 10 min, and fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. Cells were then washed twice with PBS. For Oct-4 staining, cells were fixed with 2% paraformaldehyde in PBS, and permeabilized with permeabilize buffer (0.1% saponin and 0.1% BSA in PBS). Cells were incubated with Oct-4 antibody conjugated to phycoerythrin (BD Biosciences) overnight at 4 °C. The cells were washed twice with permeabilize buffer. Data were acquired with FACSCalibur and analyzed using FlowJo software. The percentage of positive cells was determined by comparing experimental cells to spontaneously differentiated hES cells.

**Embryoid body formation and characterization.** For embryoid body formation, hydrogels were directly synthesized into non-tissue culture treated 6-well plates (2 mL of hydrogel solution per well) in the absence of acrylic-NHS ester and without peptide functionalization. Hydrogels were prepared for plating as described above. Human ES cells were detached from the hydrogels by treatment with dispase (2 mg/mL) for ~7 min. Proteolysis was stopped by the addition of EB medium consisting IMDM (Gibco), 15% FBS, 1% MEM non-essential amino acids (Gibco), and 200 μL/100 mL of 2-mercaptoethanol. Cells were centrifuged twice in EB medium (1000 rpm for 1 min each). Supernatant was removed and cells were resuspended in EB medium. Cells were plated on peptide-free hydrogels at approximately 50 colonies per well. The medium was refreshed every other day. After 14 days of growth, gene expression was analysed by following the protocols below. For immunostaining analysis, EBs were dissociated by exposure to 0.05% trypsin-EDTA (approximately 15 min), and washed twice with EB medium by centrifugation at 1200 rpm for 5 min. EB-derived cells were then cultured

on Matrigel-coated plates for 5 days with of EB medium, and immunostained for markers of all three embryonic germ layers.

**Karyotype analysis**. Human ES cells were harvested by following standard protocols and submitted to the WiCell Research Institute for standard G-banded cytogenetic testing. Detailed protocols can be obtained at www.wicell.org.

**Supplementary Figure Legends**

**Figure S1.** Comparison of peptide level incorporation in polyacrylamide hydrogels. (*A*)Fluorescence intensity of polyacrylamide hydrogels generated from the indicated ratio of amino maleimide to glucamine and subsequently functionalized with a fluorescein-conjugated peptide (FITC-Acp-GRGDSC). Error bars represent the standard deviation for the mean ($n$ = 3). Dashed line denotes background fluorescence. (*B*)Measurement of hydrogel thickness. Cross-sectional view of a fluorescein-Acp-GRGDSC functionalized hydrogel imaged via fluorescence microscopy. Hydrogel thickness was aproximately 150 μm. Scale bar: 250 μm.

**Figure S2.** Quantitation of cell adhesion and viability on hydrogels. Embryonal carcinoma cells were cultured for 1 h under serum-free conditions on hydrogels generated using the indicated ratios of aminomaleimide to glucamine and then functionalized with CGRGDS. The number of metabolically active cells that attached to the hydrogels was quantified by using the CellTiter Glo™ luminescent cell viability assay. Error bars denote standard deviation of the mean ($n$ = 2).

**Figure S3**. Proliferation of of hES cells on hydrogels functionalized with integrin and GAG-binding peptides. Human ES cells (H9) were cultured on polyacrylamide hydrogels presenting CRGDS or CGKKQRFRHRNRKG with the defined medium mTeSR. Cell proliferation was examined by using the CCK-8 assay. The absorbance at 450 nm is directly proportional to the amount of viable cells present in the experimental sample. The control indicates background absorbance from culture medium that was

incubated with the CCK-8 reagent. Error bars represent the standard deviation for the mean ($n$ = 3).

**Figure S4**. Characterization of hydrogels of varying elasticity and their ability to support hES cell proliferation. (*A*)AFM force-indentation plot for hydrogel elasticity measurements. Representative force-indentation plot for hydrogels of different elasticities are shown. The *E* values denote the elastic modulus derived from fitting. (*B*) Comparison of peptide substitution levels in hydrogels of different elasticities. Fluorescence intensity of polyacrylamide hydrogels of varying elasticities functionalized with FITC-conjugated GKKQRFRHRNRKGC peptide (FITC-Acp-GKKQRFRHRNRKGC). All hydrogels were generated using a 1:1 ratio of amino maleimide to glucamine. No significant differences were observed in the level of peptide substitution as the elasticity of the hydrogels was varied. Error bars represent the standard deviation for the mean ($n$ = 8). (*C*)Proliferation of hES cells on polyacrylamide hydrogels of varying elasticities. Human ES cells (H9) were cultured on polyacrylamide hydrogels with elasticities of approximately 0.7, 3, or 10 kPa. Each hydrogel was functionalized with CGKKQRFRHRNRKG peptide (as in Figure S6). Cells were cultured with defined medium, and cell proliferation was examined by using the CCK-8 assay. The absorbance at 450 nm is proportional to the amount of viable cells present in the experimental sample. The control indicates background absorbance from culture medium that was incubated with the CCK-8 reagent. Error bars represent the standard deviation for the mean ($n$ = 3).

**Figure S5.** Adhesion and growth of several hES cell lines on hydrogels of varying elasticities. Bright field images of the H1, H7, H14, and SA02 cell lines cultured for 7 days on polyacrylamide hydrogels of different elasticities. All hydrogels were functionalized with the CGKKQRFRHRNRKG peptide as described in Figs. S7 and S8. Scale bars: 100 μm.

**Figure S6**. Elasticity-dependent hES cell adhesion and growth on polyacrylamide hydrogels functionalized with Matrigel. Bright field images of H9 cell line cultured for 7 days on the indicated hydrogels. Scale bars: 500 μm.

**Figure S7.** Expression and subcellular localization of YAP/TAZ in hES cells (H9) cultured for 24 h on polyacrylamide hydrogels of varying stiffness. (*A*) Bright field images of the cells prior to fixation and immunostaining. (*B*) Confocal images of the cells immunostained for YAP/TAZ (green), and counterstained with DAPI (blue). (C) Quantification of YAP/TAZ colocalization with DAPI in hES cells cultured on 0.7 kPa (circles) or 10 kPa (diamond shape) hydrogel. Black lines indicate average Pearson's Coefficient where 0 stands for no correlation and 1 stands for complete positive correlation, $n$ = 14. All hydrogels were functionalized with the CGKKQRFRHRNRKG peptide as described in Figs. S4-S5. . Scale bars: (*A*) 100 μm, (*B*) 10 μm.

**Figure S8.** Pluripotency marker expression in multiple hPS cell lines cultured on 10 kPa hydrogels. Microscopy images of hES (H1, H7, H14 and SA02) and iPS (IMR-90-1, iPS-BM1i, iPS-CBT4, iPS Vector Free and iPS-Foreskin-1) cells cultured on 10 kPa hydrogels for 7 days under chemically defined conditions. All hydrogels were generated

using a 1:1 ratio of maleimide to glucamine and then functionalized with the CGKKQRFRHRNRKG peptide. Cells were immunostained for pluripotency markers Oct-4 (green) and SSEA-4 (red), and counterstained with DAPI (blue). Scale bars: 100 μm

**Figure S9.** Long-term cultured hES cells on 10 kPa hydrogel functionalized with the peptide CGKKQRFRHRNRKG. Human ES cells (H9) were cultured for 38 days on hydrogels with defined medium. Hydrogels were generated using a 1:1 ratio of amino maleimide to glucamine and then functionalized with the CGKKQRFRHRNRKG peptide. (*A*) Microscopy images of cells immunostained for markers of pluripotency Oct-4 (green) and SSEA-4 (red), and counterstained with DAPI (blue). Scale bar: 100 μm. (*B*) Flow cytometry analysis of cells for the expression of pluripotency markers Oct-4 and alkaline phosphatase. (*C*)Karyogram of hES cells cultured long-term on 10 kPa hydrogels. H9 cells were cultured for 31 days on 10 kPa hydrogel functionalized with CGKKQRFRHRNRKG peptide and cytogenetically tested for chromosomal stability. Cells retained a normal karyotype after prolonged culture on the hydrogels. (*D*) Embryoid bodies developed from long-term hES cell cultures. Bright field image of long-term cultured hES cells (H9) that were transferred to peptide-free 10 kPa hydrogel for suspension culture, which results in EB development. Scale bar: 500 μm

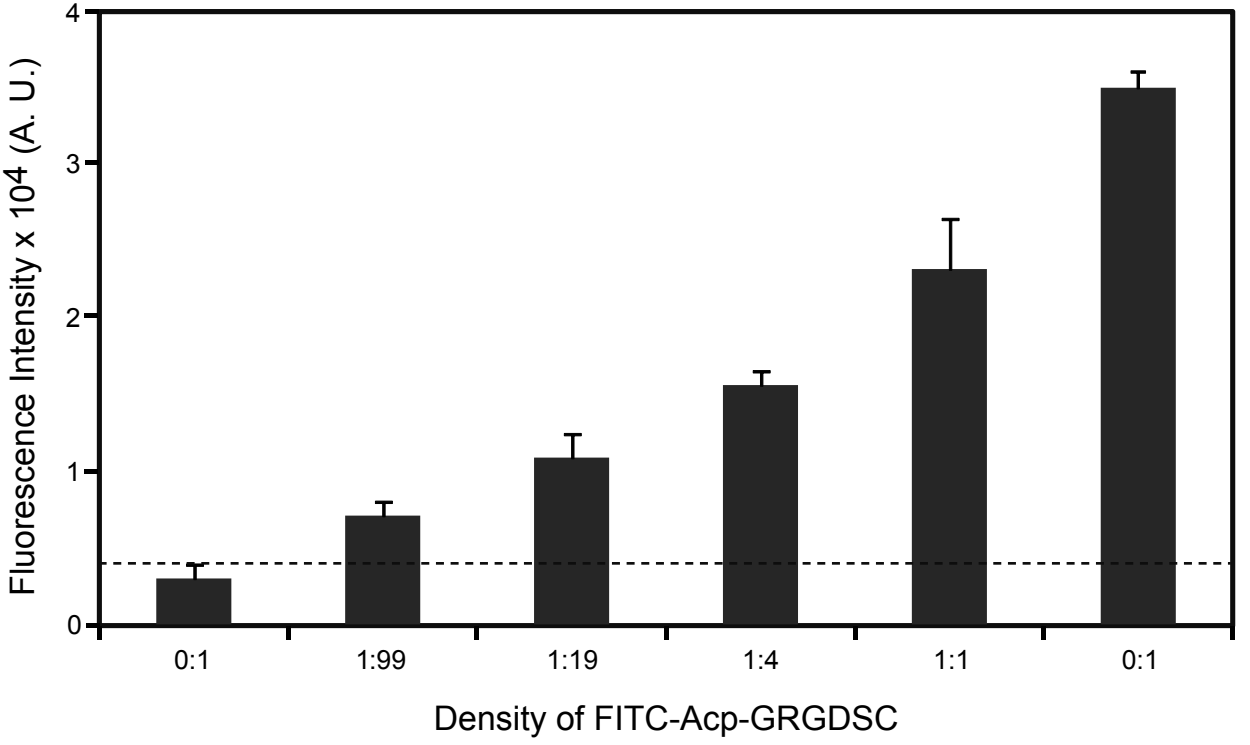
**Table S1**. Quantitative gene expression profile of hES cells cultured long-term (60 days) on the 10 kPa hydrogel. (*A*) Gene expression levels of hES cells (H9) cultured for 60 days on polyacrylamide hydrogels in the presence of defined medium. Hydrogels were functionalized with the CGKKQRFRHRNRKG peptide at 50 percent density. The

level of gene expression is relative to cells cultured on Matrigel-coated plates. (*B*) Gene expression levels of EBs generated from the long-term (60 days) cultured hES cells. The level of gene expression is relative to undifferentiated cells cultured on the peptide-bearing hydrogels. For both tables, genes that were up-regulated by more than four-fold are shown in red and those down-regulated by more than four-fold are shown in blue.

## Supplemental References


1. Klein, E. A.; Yung, Y.; Castagnino, P.; Kothapalli, D.; Assoian, R. K. Cell Adhesion, Cellular Tension, and Cell Cycle Control. In *Integrins*; Elsevier Academic Press Inc: San Diego, 2007; pp 155-175.
2. Radmacher, M.; Fritz, M.; Hansma, P. K. Imaging Soft Samples with the Atomic Force Microscope - Gelatin in Water and Propanol. *Biophys. J.* **1995**, 69, 264-270.
3. Watanabe, K.; Ueno, M.; Kamiya, D.; Nishiyama, A.; Matsumura, M.; Wataya, T.; Takahashi, J. B.; Nishikawa, S.; Muguruma, K.; Sasai, Y. A Rock Inhibitor Permits Survival of Dissociated Human Embryonic Stem Cells. *Nat. Biotechnol.* **2007**, 25, 681-686.

A


Fluorescence Intensity x 10^4 (A. U.)
4
3
2
1
0
0:1
1:99
1:19
1:4
1:1
0:1
Density of FITC-Acp-GRGDSC

B

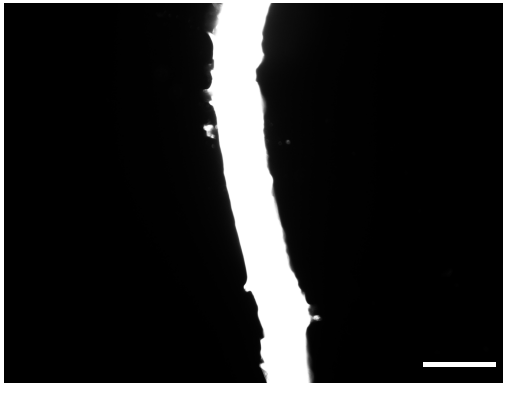


Figure S1

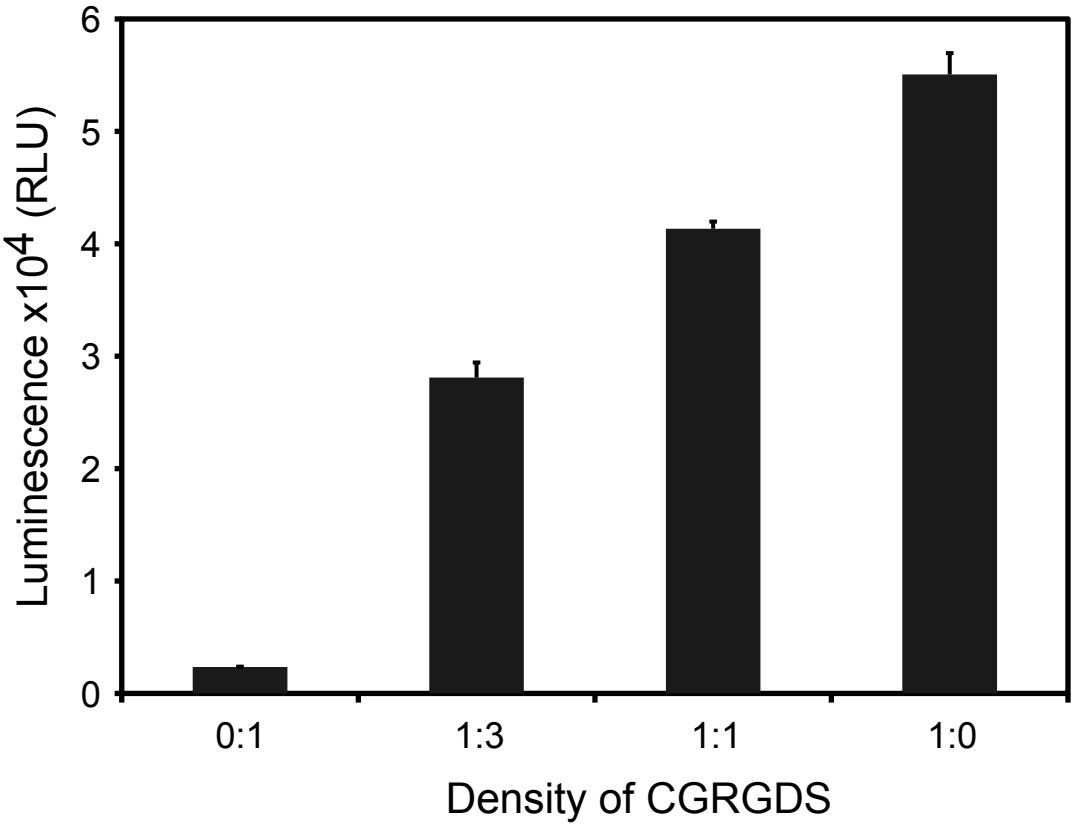



Figure S2

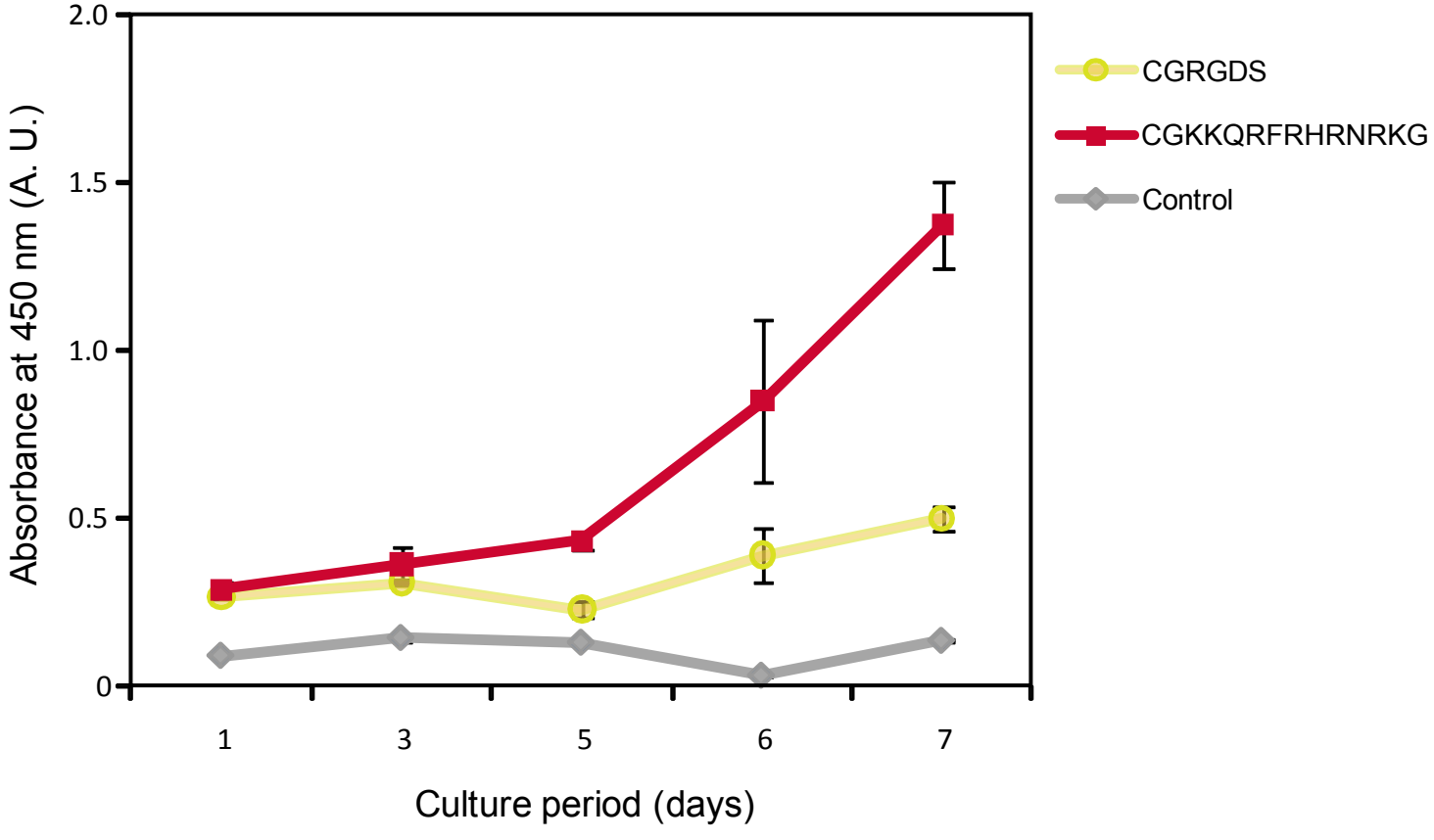
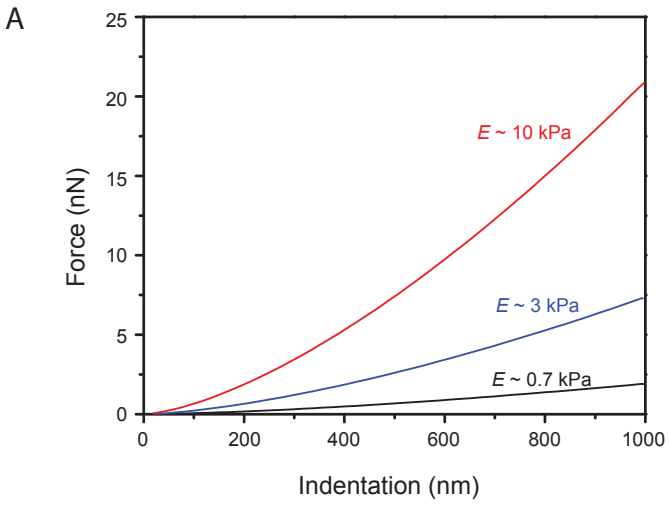
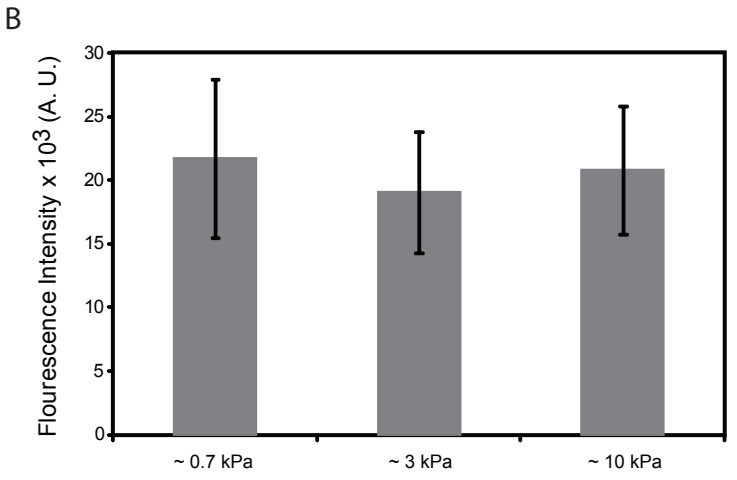

2.0
1.5
1.0
0.5
0
Absorbance at 450 nm (A. U.)
1
3
5
6
7
Culture period (days)
CGRGDS
CGKKQRFRHRNRKG
Control

Figure S3


A
Force (nN)
Indentation (nm)
E ~ 10 kPa
E ~ 3 kPa
E ~ 0.7 kPa
0
5
10
15
20
25
0
200
400
600
800
1000


B
Flourescence Intensity x 10^3 (A. U.)
~ 0.7 kPa
~ 3 kPa
~ 10 kPa
0
5
10
15
20
25
30

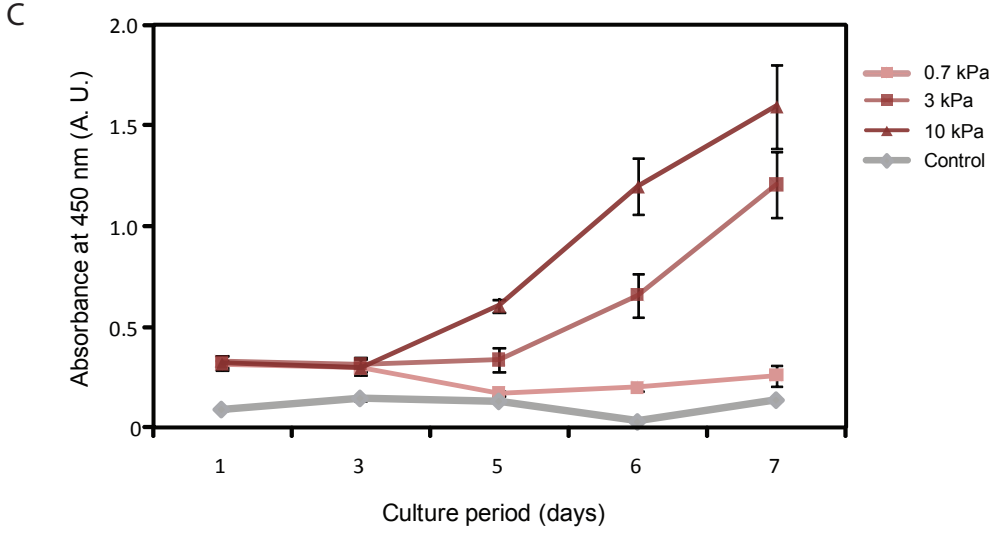

C
Absorbance at 450 nm (A. U.)
Culture period (days)
0.7 kPa
3 kPa
10 kPa
Control
0
0.5
1.0
1.5
2.0
1
3
5
6
7

Figure S4

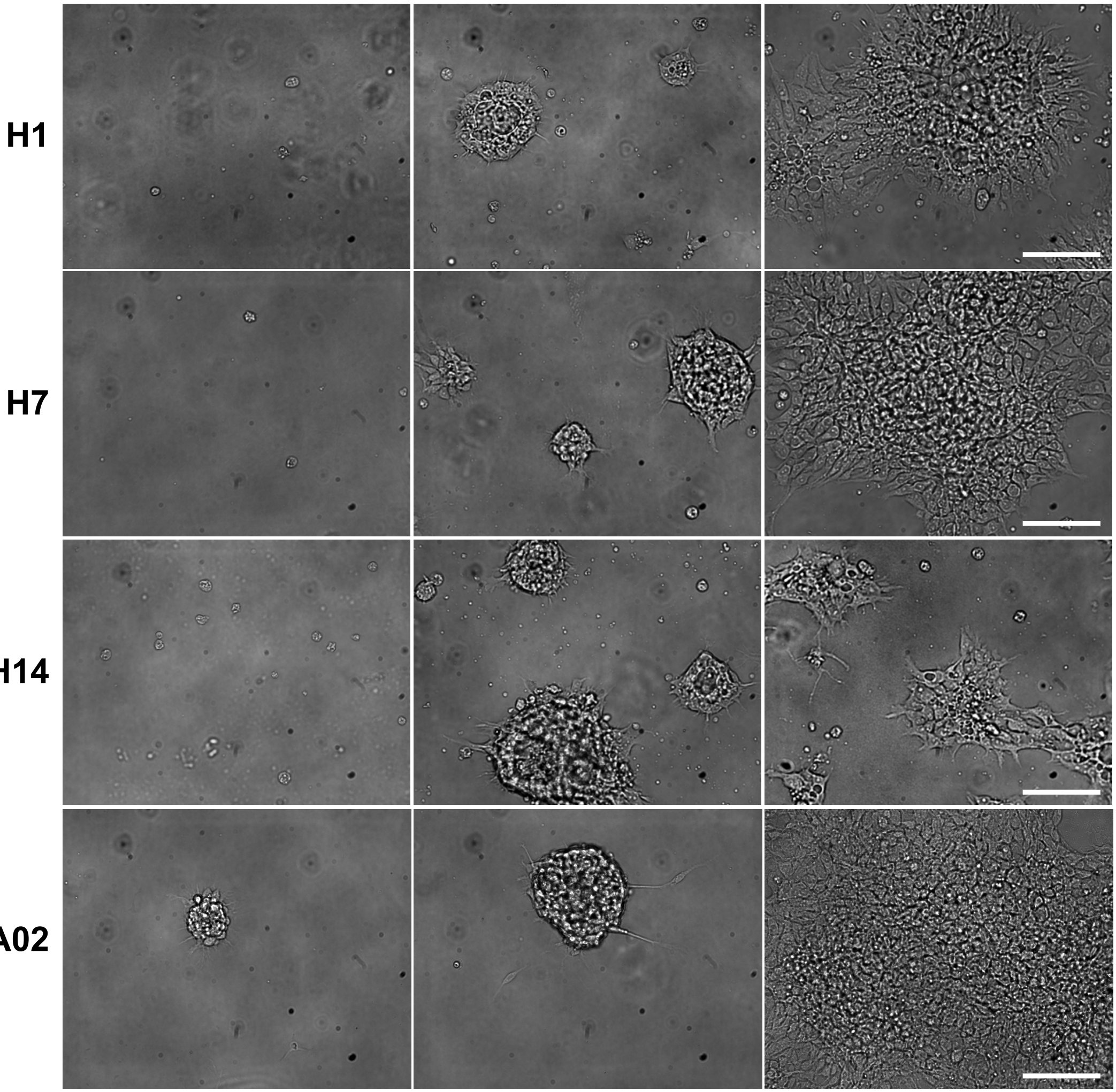



Figure S5

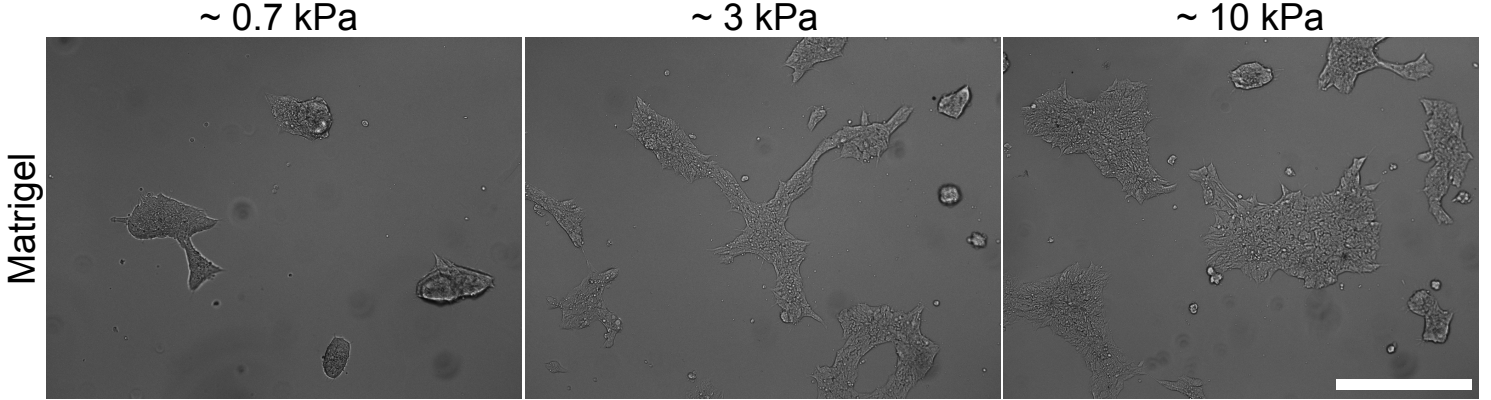



Figure S6

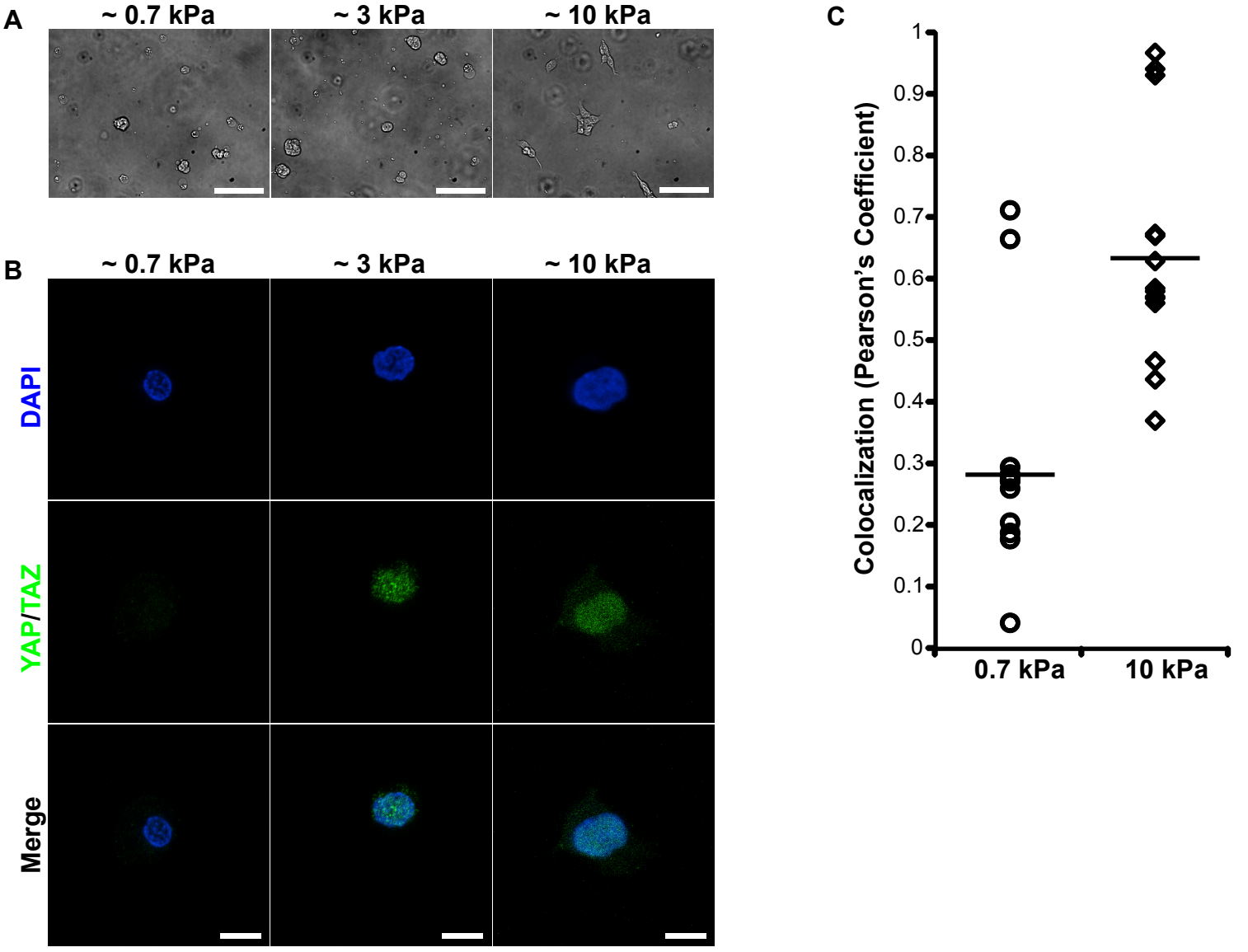

A
~ 0.7 kPa
~ 3 kPa
~ 10 kPa
B
~ 0.7 kPa
~ 3 kPa
~ 10 kPa
DAPI
YAP/TAZ
Merge
C
Colocalization (Pearson's Coefficient)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.7 kPa
10 kPa

Figure S7

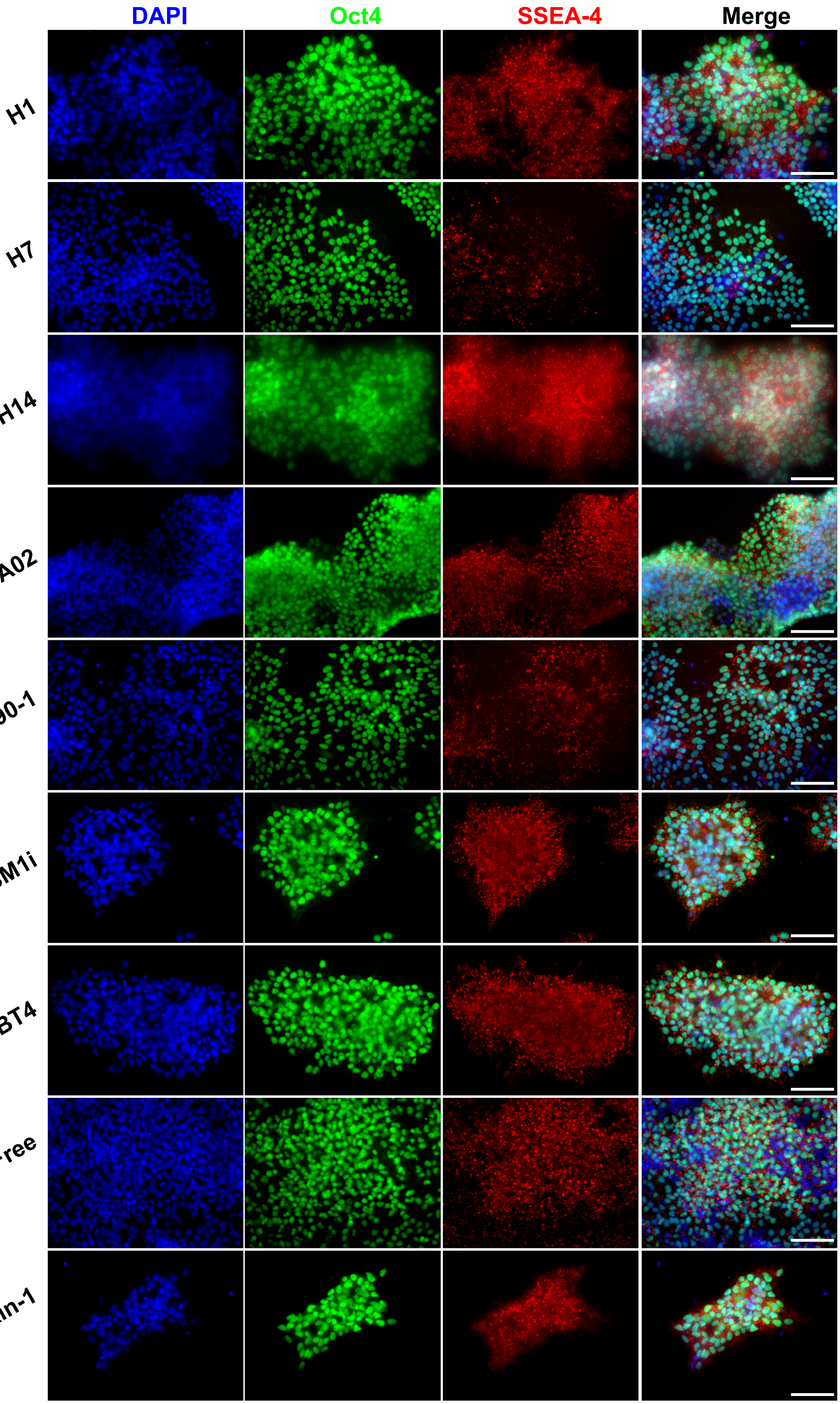
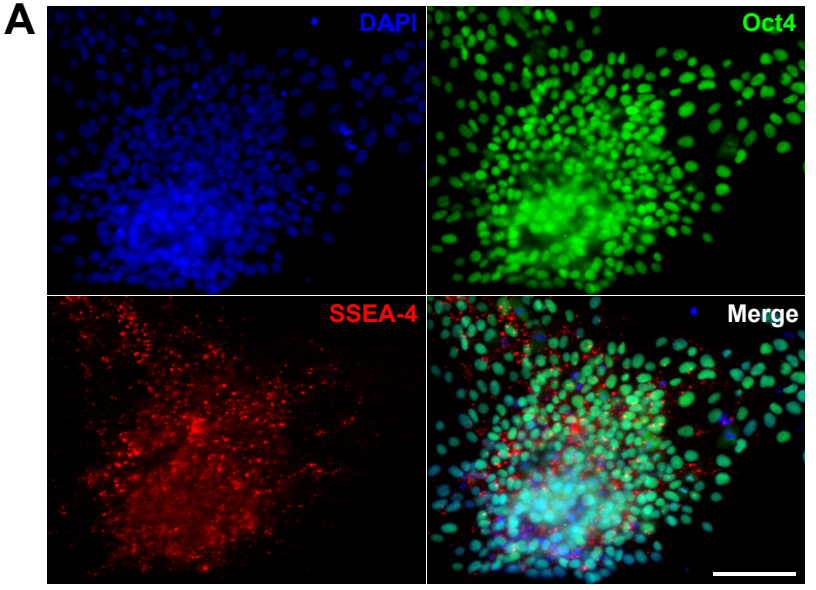
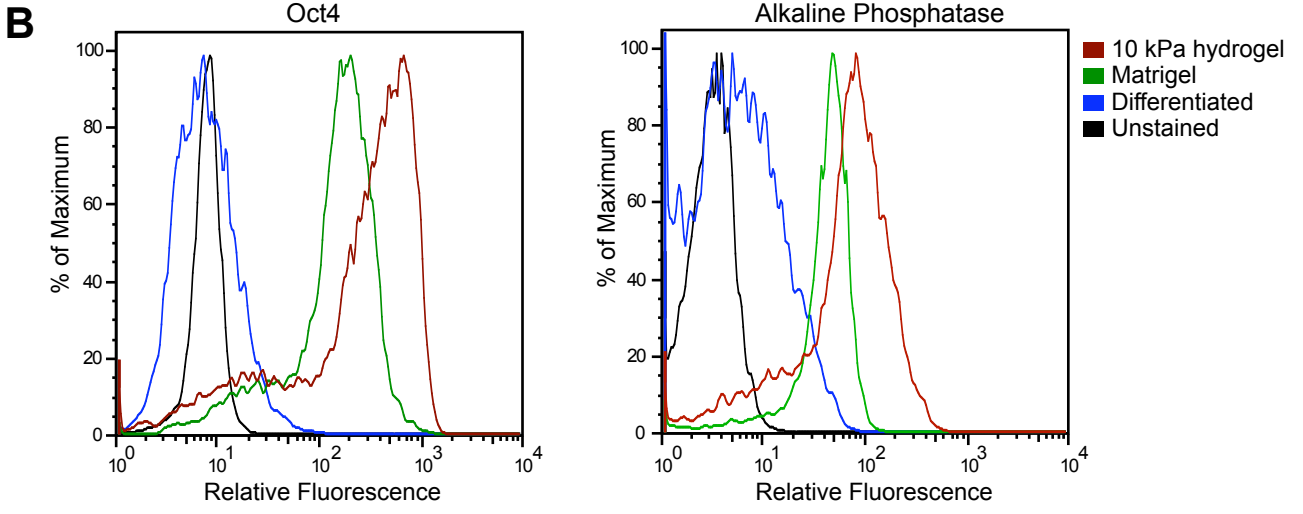
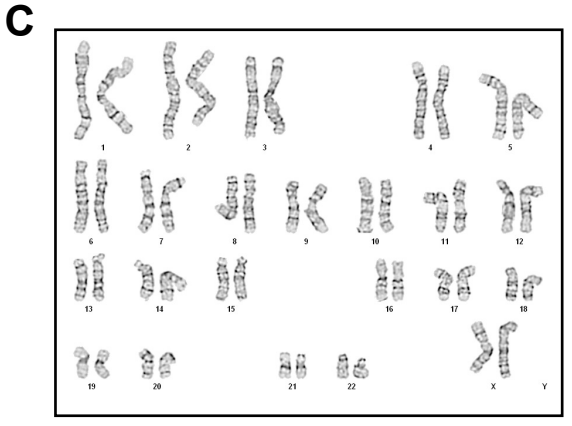
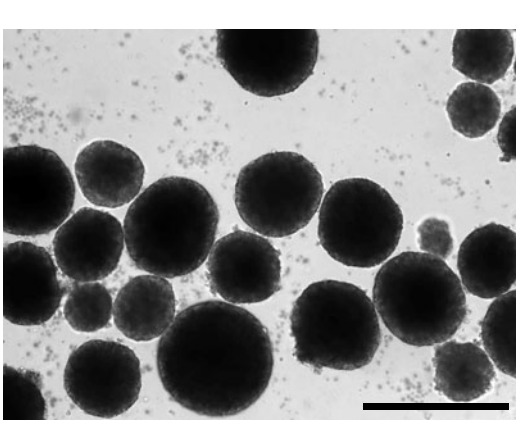

DAPI
Oct4
SSEA-4
Merge
H1
H7
H14
SA02
IMR-90-1
iPS-BM1i
iPS-CBT4
iPS Vector Free
iPS-Foreskin-1

Figure S8


A
DAPI
Oct4
SSEA-4
Merge


B
Oct4
Alkaline Phosphatase
% of Maximum
Relative Fluorescence
10 kPa hydrogel
Matrigel
Differentiated
Unstained


C
46, XX


D

**A**

| | Gene symbol | Fold change |
|---|---|---|
| **Pluripotency** | GBX2 | 1.434 |
| | SOX2 | 1.366 |
| | PODXL | 1.2311 |
| | REST | 1.0943 |
| | BXDC2 | 1.0792 |
| | IGF2BP2 | 1.0425 |
| | LIN28 | 1.021 |
| | DIAPH2 | 1 |
| | SEMA3A | 0.9931 |
| | DNMT3B | 0.9862 |
| | POU5F1 | 0.9266 |
| | NR6A1 | 0.9202 |
| | NR5A2 | 0.9138 |
| | NANOG | 0.9075 |
| | UTF1 | 0.8827 |
| | TERT | 0.8827 |
| | ZFP42 | 0.8351 |
| | CD9 | 0.8293 |
| | IFITM2 | 0.8123 |
| | FOXD3 | 0.5704 |
| | TFCP2L1 | 0.4633 |
| **Extra-Embryonic** | EOMES | 7.1107 |
| | CDX2 | 5.8563 |
| | SERPINA1 | 5.6569 |
| | PTF1A | 2.4967 |
| | LAMB1 | 1.2658 |
| | LAMA1 | 1.0425 |
| | KRT1 | 0.9526 |
| | FN1 | 0.9202 |
| | LAMC1 | 0.9138 |
| | GATA4 | 0.8236 |
| | GCM1 | 0.7579 |
| | AFP | 0.7474 |
| **Ectoderm** | NEUROD1 | 4.5002 |
| | PAX6 | 2.395 |
| | NES | 0.9526 |
| | OLIG2 | 0.8645 |
| **Mesoderm** | T | 1.6021 |
| | RUNX2 | 1.4044 |
| | WT1 | 1.0425 |
| | DES | 0.9862 |
| | MYOD1 | 0.8236 |
| | HBZ | 0.7684 |
| | COL1A1 | 0.7579 |
| | HBB | 0.6552 |
| | MYF5 | 0.5987 |
| **Definitive Endoderm** | SST | 0.9013 |
| | SOX17 | 0.8066 |
| | PAX4 | 0.7684 |
| | IAPP | 0.6113 |
| | TAT | 0.4061 |
| | FOXA2 | 0.2117 |

**B**

| | Gene symbol | Fold change |
|---|---|---|
| **Pluripotency** | GATA6 | 266.8712 |
| | TERT | 2.0279 |
| | NANOG | 1.9725 |
| | SEMA3A | 1.6702 |
| | REST | 1.2834 |
| | DIAPH2 | 1.257 |
| | BXDC2 | 0.8645 |
| | IFITM2 | 0.8526 |
| | IGF2BP2 | 0.7474 |
| | SOX2 | 0.6071 |
| | TFCP2L1 | 0.5105 |
| | NR5A2 | 0.4147 |
| | FOXD3 | 0.1948 |
| | CD9 | 0.1241 |
| | GBX2 | 0.0698 |
| | DNMT3B | 0.0629 |
| | PODXL | 0.0625 |
| | LIN28 | 0.0504 |
| | NR6A1 | 0.0497 |
| | UTF1 | 0.0138 |
| | POU5F1 | 0.0025 |
| | ZFP42 | 0.0009 |
| **Extra-Embryonic** | CDX2 | 15.455 |
| | FN1 | 10.6295 |
| | GATA4 | 6.5432 |
| | KRT1 | 1.9453 |
| | LAMC1 | 1.879 |
| | EOMES | 1.6472 |
| | GCM1 | 1.6245 |
| | LAMB1 | 0.9526 |
| | PTF1A | 0.8586 |
| | LAMA1 | 0.7792 |
| | SERPINA1 | 0.5322 |
| | AFP | 0.507 |
| **Ectoderm** | PAX6 | 72.5046 |
| | NEUROD1 | 13.9288 |
| | NES | 1.9053 |
| | OLIG2 | 0.6926 |
| **Mesoderm** | RUNX2 | 7.6741 |
| | HBB | 5.7358 |
| | WT1 | 4.0278 |
| | COL1A1 | 2.9079 |
| | MYOD1 | 2.4284 |
| | DES | 1.9319 |
| | HBZ | 1.434 |
| | MYF5 | 0.9659 |
| | T | 0.1224 |
| **Definitive Endoderm** | SST | 91.1392 |
| | GCG | 16.7955 |
| | PAX4 | 2.2815 |
| | SOX17 | 1.7532 |
| | IAPP | 1.0353 |
| | FOXA2 | 0.4698 |

Table S1