Spatial cell fate manipulation of human pluripotent stem cells by controlling the microenvironment using photocurable hydrogel

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Abstract

Human pluripotent stem cells (hPSCs) sense the surrounding chemical, and physical microenvironment and adjust their behavior accordingly. However, current *in vitro* research is mostly conducted in plastic culture wares which provides an oversimplified microenvironment compared to that *in vivo*. Increasing evidence has highlighted the importance of the physical microenvironment in hPSCs differentiation and maintenance. Here, hPSCs were cultured on homogeneous substrates with specific stiffness. Biocompatible material that allows spatial control of the chemical and mechanical environment for hPSC culture has not yet been developed. Culture substrates that allow flexible manipulation of spatial physical properties can assist the recapitulation of *in vivo* environment, and assist tissue engineering.

We developed a photocurable polyethylene glycol–polyvinyl alcohol (PVA-PEG) hydrogel which allows spatial control of the surface stiffness and structure at a micrometer level. The developed hydrogel can be functionalized using different extracellular matrix (ECM) proteins. Laminin 511 functionalized PVA-PEG gel supports hPSCs growth and differentiation. Furthermore, by spatially controlling the stiffness of the patterned gel, we could selectively differentiate cells, generating complex patterned structures.

Keywords: Human pluripotent stem cells, microenvironment, differentiation, biocompatible.

Introduction

hPSCs are intensively used in regenerative medicine and developmental biology research. Many types of cells and tissues have been generated from hPSCs, providing a powerful tool for *in vitro* research. However, the current culture system is distinct from the *in vivo* microenvironment, limiting the generation of complex structures *in vitro*. Pluripotent stem cells (PSC) can be cultured for differentiation in either two- or three-dimensions (2D or 3D). 2D culture provides a simple, reproducible, and robust culture condition, and many cell differentiation protocols have been developed in 2D. In contrast, in 3D culture, cells form aggregates that float in the culture medium or are embedded in an ECM (Gattazzo et al., 2014; Simunovic and Brivanlou, 2017). In both culture systems, stem cells are differentiated by adjusting the composition of the culture medium. 2D culture systems are subject to mechanical constraints on tissue shape because cells are cultured on a rigid culture dish, whereas in 3D culture systems, induced tissues are not mechanically constrained and can freely change their shape. During development, stem cells form organs under weak mechanical constraints through chemical and mechanical signals mediated by tissue-tissue interactions (Simunovic and Brivanlou, 2017). Therefore, to induce functional tissues with appropriate shapes *in vitro*, an ideal environment allows tissue deformation with weak mechanical constraints.

Increasing evidence suggests the importance of physical cues on cell behavior and fate (Candiello et al., 2013; Chen et al., 2020; Keung et al., 2012; Maldonado et al., 2017; Muduli et al., 2017; Musah et al., 2014; Przybyla et al., 2016a; Rosowski et al., 2015). Soft substrates and high mechanical tension enhance hPSC mesodermal differentiation (Muncie et al., 2020b; Przybyla et al., 2016a). Simultaneously, substrate geometry and structures are also critical to complex tissue structure generation (Karzbrun et al., 2021; Warmflash et al.,

2014). During development, neighboring cells communicate with each other, sensing the surrounding chemical and mechanical cues, and differentiate accordingly. Eventually, cells gain differentiated cell fate and cooperate with one another to form organs with complex structures. Therefore, it is important to spatially control the microenvironment to generate complex cellular structures. To this end, we aimed to develop a culture substrate with adjustable mechanical properties.

Hydrogels have been used intensively in biomedical research as cell culture substrates due to their mechanical and chemical similarity with the ECM(Tibbitt and Anseth, 2009). Furthermore, hydrogels can be functionalized to have different properties, such as photocurability. Photocurable hydrogels based on hyaluronic acid and gelatin have been developed(Chen et al., 2019; Tibbitt and Anseth, 2009). By controlling the light source, gels with different shapes and structures can be generated. However, these gels were used for hPSCs encapsulation, but not for attachment due to poor adhesivity on the synthetic surface of hPSCs (Tibbitt and Anseth, 2009; Virdi and Pethe, 2021) In contrast, some hydrogels allow good hPSC attachment, such as polyacrylamide or polydimethylsiloxane (PDMS), that have been used intensively in studies regarding substrate stiffness (Chen et al., 2019; Chen et al., 2020; Maldonado et al., 2017; Millar-Haskell et al., 2019; Perez-Puyana et al., 2020; Tibbitt and Anseth, 2009). However, they lack flexibility in terms of gel structure and mechanical property control. Driven by this discussion, we decide to develop a photocurable hydrogel with ideal hPSCs attachment.

hPSCs attach to the substrate via integrin-ECM protein interactions. Therefore, ECM protein engraftment on the hydrogel is necessary. Previously, ECM protein functionalized PVA could be used for hPSCs culture (Muduli et al., 2017). Polyvinyl alcohol (PVA), a type of polymer with a simple structure, can form a stable and biocompatible hydrogel upon cross-linking. We used PVA as the backbone and further functionalized PVA to have photocurability and ECM proteins to facilitate hPSCs attachment.

Here, we demonstrated a composite photocurable PVA and PEG hydrogel with adjustable stiffness and shape. Our hydrogel supports hPSCs growth and differentiation, and the differentiation tendency of hPSCs was compared with cells cultured on plastic dishes. Most importantly, we demonstrated that, by controlling the local substrate's physical property, stem cell fate could be spatially manipulated at a micrometer level.

Results

1. Synthesis of photocurable PVA-methacrylate hydrogel

To develop a photocurable hydrogel with protein binding ability, we used a denatured Polyvinyl alcohol (PVA-COOH) which contains randomly integrated carboxyl groups as starting material. The carboxyl group in the PVA backbone can be activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide(NHS/EDC) and further bind with the N end of proteins to allow cell attachment (Figure 1b). To obtain photocurability, a methacrylate functional group was introduced into the PVA backbone to obtain photocurable PVA-MA. Lithium phenyl-2,4,6-trimethyl-benzoyl phosphonate (LAP) (0.5%) was used as the photoinitiator (Figure 1a). To evaluate the protein binding ability of PVA-MA, activated PVA-MA, and non-protein binding hydrogel PEG were incubated with a fluorescent protein. After incubation, fluorescence intensity was determined to quantify the hydrogel protein binding ability. Compare with non-protein binding PEG, activated gel showed a

significantly(4 times) higher fluorescent signal (Figure 1b, c) indicating that the protein was sufficiently bound to our hydrogel.

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Increasing evidence suggests the importance of the diameter of colony and substrate geometry in generating complex tissue *in vitro* (Karzbrun et al., 2021; Warmflash et al., 2014). However, current micropatterned cultures are typically constructed on glass or plastic cultureware, and their physical property *in vitro* differs from that under *in vivo* conditions. Taking advantage of the flexibility of photoinitiated crosslinkability of our hydrogel, we attempted to develop a patterned culture method using our hydrogel. As seen in Figure 2c, we generated a spacer with a round hole in the center using PDMS. The spacer was then put on the glass-bottom dish. Hydrogel prefabrication solution was added into the hole and then covered by a coverslip. We then used a direct-exposure photolithography system to initiate patterned UV light to initiate gelation. Using this method, we could generate various-shaped gels at a micrometer level (Figure 1c, d). However, we found that PVA-MA gel alone has substantially low elasticity or a narrowed elasticity range. To improve the elasticity range, we added 4-arm PEG to form a composite hydrogel. By using 4-arm PEG with different molecular weights in different ratios, we could obtain hydrogel with stiffness ranging from ~5 kPa to ~500 kPa. Additionally, we could control the stiffness of PVA-PEG composite hydrogel by giving a different dosage of UV irradiation (Figure 2e). In this study, 3% PVA and 15% 5K 4-arm PEGweres are used, since it gives us a suitable range of stiffness. To this end, we have developed a photocurable hydrogel substrate with an adjustable stiffness, ranging from soft (~20 kPa) to hard (~100 kPa).

2. PVA-PEG gels support hPSC survival and outgrowth while maintaining pluripotency

Next, we tested if our gel supports hPSC growth. We generated a PVA-PEG gel by using sufficient UV to generate a ~100 kPa (hard) gel. The gel was then activated and grafted with laminin 511 as ECM proteins for hPSCs culture. To analyze cell proliferation, we generated a nucleus reporter and the H2B-mCherry hPSCs line to allow cell tracking and cell cycle analysis. H2B-mCherry was seeded on culture dishes or hydrogels, and live imaging was performed to track cell proliferation and nuclear division. In Figure 3, cells cultured on the gel showed a similar proliferation rate compared with those cultured on glass-bottom dishes. Immunofluorescence (IF) suggests that hPSCs cultured on our hydrogel maintained their pluripotency within one passage, indicated by the expression of pluripotent markers (SOX2, OCT4, NANOG) of Day 6 cultures on the gel; suggesting that pluripotency and proliferation of hESCs can be maintained on hydrogels as well as on rigid culture dishes.

3. hPSCs can be differentiated into all germ layers on PVA-PEG gel

Since our hydrogel could be used for hPSCs culture, we next evaluated how our gel would affect stem differentiation. Cells passaged on the PS dish were collected and seeded on Hard PVA-PEG gel and subsequently differentiated towards neural ectoderm (NE),

definite endoderm (DE), paraxial mesoderm (PM), and lateral plate mesoderm (LPM) following a previously reported protocol(D'Amour et al., 2005; Loh et al., 2016; Surmacz et al., 2012).

For NE, hPSCs were differentiated using the previously reported dual SMAD inhibition method(Chambers et al., 2009). After 6 days of differentiation, cells on both dish and gel cultures largely expressed the NE markers, PAX6 and SOX1, suggesting successful NE differentiation. qPCR suggests cells differentiated on the dish cultures have similar or slightly higher expression of NE genes which is consistent with the previous report when they compared NE differentiation on relatively harder hydrogel (75 kPa and 300 kPa) and culture dish (2.28–3.28 GPa) (Keung et al., 2012; Maldonado et al., 2017).

For DE, cells were first differentiated to the anterior primitive streak (APS) for 24 h and then towards DE (D'Amour et al., 2005; Evseenko et al., 2010). As Figure 3b shows, cells on both culture dishes and gels were successfully differentiated, supported by the NE marker SOX17 expression in most cells. Interestingly, qPCR revealed a notably enhanced expression of APS and DE-related genes. Consistent with a previous study, DE differentiation was enhanced on hydrogels (3 kPa and 165 kPa) and culture dishes (2.28–3.28 GPa). Our developed hydrogel model promotes DE differentiation likely by enhancing APS and DE-related gene expression on a softer substrate.

For Mesodermal development, the PM and LPM fates separate at the early stages of differentiation, therefore we tested both. For PM, hESCs were differentiated towards APS fo,r 24 h, and then APS was directed towards PM for 24 h (Loh et al., 2016). Although soft substrate was shown to enhance mesendoderm fate (Chen et al., 2020; Przybyla et al., 2016a), IF and qPCR reflected similar expression of LPM markers. It has been previously reported that in the range of 3 kPa to 1GPa, the expression of APS and mesendoderm genes (*EOMES* and *Brachyury*) and DE genes (*FOXA2* and *SOX17*) is inversely proportional to the substrate stiffness, but a soft substrate seems to inhibit pan primitive streak gene, *CDX2*, and mesodermal genes, *FOXC2* and *NKX2*.5(Chen et al., 2020). It was also shown that substrate stiffness affects the cell fate decision developmental stage specifically. It is not always enhanced or decreased but rather dynamic (Maldonado et al., 2017). Given the fact that we only investigated limited markers and time points, PM-related genes remained at similar expression levels and can be explained by different observed markers and the relatively hard stiffness of the gel used in this experiment (~100 kPa).

For LPM hESCs, cells were differentiated toward mid primitive streak (MPS) for 24 h and then towards LPM for 24 h following the previously reported protocol (Loh et al., 2016). IF suggested that differentiation both on the gel and culture dish was successful. Cells differentiated on gels expressed a higher level of *HAND1* and *ISL1* but expressed a lower level of *NKX2.5*. The *NKX2.5* downregulation was expected since it matches the previous report (Chen et al., 2020).

To summarize, we demonstrated that our hydrogel can also be used for the differentiation of hPSCs.

4. Soft hydrogel upregulates NODAL signaling while maintaining pluripotency

Next, we evaluated how different stiffness of hydrogel impact hPSC gene expression. Day 4 hPSC cultures cultured on the hard gel (~100 kPa), soft gel (~20 kPa), and glass-bottom dish were collected and subjected to RNA-seq. In total, 34032 transcripts were assembled.

Within these transcripts, 1288 genes were upregulated on the softer gel compared to the hard gel, and pluripotent genes such as *SOX2, OCT3/4,* and *NANOG* were not notably different among each sample. In contrast, NODAL signaling-related genes such as *ANXA1, CER1, FOXA2, LEFTY1, LEFTY2, SMAD7, SRPINE1*, and *TIMP4* were upregulated in softer gel compared to hard gels and culture dishes. Gene Ontology (GO) analysis indicated that soft hydrogels enrich genes related to axis specification, bilateral symmetry determination, symmetry specification, endoderm differentiation, gastrulation, pattern specification, and anterior/posterior pattern specification (Figure 4d). During gastrulation in the embryo, NODAL signaling was induced posteriorly by Wnt3a which is induced by BMP4. Posteriorly localized NODAL and WNT signaling together initiate the primitive streak to be formed and generate mesendoderm. Softer hydrogel likely enhances posteriorization via upregulated NODAL signaling and further induced APS genes such as *FOXA2*, consistent with the previous reports (Chen et al., 2020; Muncie et al., 2020c).

To summarize, hPSCs cultured on softer hydrogel showed unregulated NODAL signaling while maintaining pluripotency.

5. Manipulating cell fate of hPSCs on the patterned gel by controlling substrate stiffness

Since our gel has a spatially adjustable stiffness and the softer hydrogel was shown to enhance the NODAL signaling, we next wondered if it is possible to spatially manipulate stem cell fate by generating locally soft hydrogel.

During gastrulation, the mesoderm is differentiated posteriorly in the embryo by NODAL and WNT signaling which are induced by BMP4. Since soft hydrogel promotes NODAL signaling, we hypothesized that it is possible to generate locally soft patterned hydrogel and selectively differentiate cells on the soft part towards the mesoderm. We first generated soft (20 kPa) and hard (80 kPa) patterns and differentiated hPSCs on gels towards the mesoderm by administering 10 ng/mL BMP4 and bFGF(Evseenko et al., 2010) in differentiating medium. After 24 h of differentiation, cells on the soft gel were differentiated to a mesendoderm fate which was indicated by the expression of mesodermal and primitive streak marker T. Most cells on hard gels do not express T, except a few cells on the edge (Figure 5a), probably due to weaker cell-cell junction and higher tension (Muncie et al., 2020c; Przybyla et al., 2016a). These results indicate that it is possible to manipulate stem cell fate by adjusting the stiffness of the hydrogel. Since the stiffness of the gel is easily controlled by UV dosage, we next generated a hydrogel with locally different stiffness using a patterned irradiating system. As Figure 5b shows, hPSCs were seeded on a patterned hydrogel which was half hard and half soft. The colony was then differentiated towards the mesoderm for 24 h. Cells in hard areas remained pluripotent, which was indicated by the *SOX2* positive and T negative assays, while cells in the softer areas gained mesodermal fate, indicated by their T expression and absence of *SOX2* expression (Figure 6b). Regardless, cells were differentiated in the same dish. We next calculated T^+ cells per area based on the IF image, as shown in Figure 5c, and cells on a softer gel pattern had a significantly higher (~200 times) rate of T^+ cells. Figure 5d shows the T^+ cells per area on soft/hard areas on the same patterned gel.

Despite the significant difference in T^+ cell number after 24 h of differentiation, we found that if the differentiation was extended to in h, cells on both the hard and soft areas became T^+. We next tested if it is possible to maintain the effect of selective differentiation

by administering a pulse of morphogen stimulation. hPSCs were seeded on hydrogel patterns and cultured in the pluripotency maintenance medium. BMP4 and bFGF (10 ng/mL) were directly added into the maintenance medium for 24 h and then withdrawn by medium change. The patterned culture was cultured in a maintenance medium for another 48 h. As Figure 5e shows, cells on the soft gel are largely T positive, while only a few T-positive cells on hard gels. On partially soft gels, T-positive cells are mostly restricted in soft areas and lose *SOX2* expression. In contrast, cells cultured on hard gels mostly retained the *SOX2* expression. These data suggest that we could selectively differentiate cells by spatially controlling the hydrogel stiffness.

Discussion

We developed a photocurable hydrogel for hPSCs culture and by controlling the UV light source, we could control the shape of the gel pattern and the local stiffness. Laminin511 E8 fragment functionalized hydrogel supports hPSCs growth and maintained pluripotency. hPSCs can be differentiated towards all three germ layers on our developed hydrogel. We also demonstrated when cultured on the softer gel, NODAL signaling is upregulated compared to hard gels and PS dishes. Most importantly, by generating locally soft gels, we could selectively differentiate hPSCs towards the mesoderm and manipulate stem cell fate spatially.

To overcome the limitation of traditional 2D/3D cultures, many soft substrates have been developed for cell cultures, such as various hydrogels, electro-spun fibers, and PDMS. However, due to the poor adhesivity of hPSCs on the synthetic surface (Tibbitt and Anseth, 2009; Virdi and Pethe, 2021), only limited materials can be used for attachment culture. PA has been used for hPSCs attachment culture; however, the pluripotency cannot be maintained. Here, Laminin 511 E8 was covalently bonded to our hydrogel and provided a strong attachment for hPSCs to enable an ideal culture of hPSCs while maintaining pluripotency.

Soft substrates such as PVA, electro-spun fibers, PA gel, and PDMS have previously been used for hPSCs attachment culture (Chen et al., 2020; Hsu et al., 2018; Keung et al., 2012; Kumbar et al., 2008; Muduli et al., 2017; Muncie et al., 2020c; Musah et al., 2014; Pagliari et al., 2021; Przybyla et al., 2016a; Virdi and Pethe, 2021). However, these methods usually coat the whole surface of the culture dish, and the local physical property cannot be adjusted. Recent research suggests the importance of the stiffness and geometry constraint in differentiating complex structures (Chen et al., 2020; Karzbrun et al., 2021; Keung et al., 2012; Martyn et al., 2018; Muncie et al., 2020a; Musah et al., 2014; Przybyla et al., 2016b; Rosowski et al., 2015; Virdi and Pethe, 2021). Our hydrogel provided a simple and flexible platform for generating a geometry- and local stiffness-defined culture substrate.

Currently, most of the differentiation protocol was developed by adjusting the culture medium composition. However, organs are an organized structure of different lineages. Differentiation of different lineages in a homogenous culture system by only adjusting medium composition is challenging. Here, we selectively differentiate hPSCs towards the mesoderm by adjusting local stiffness; this provided a new tool for generating complex structures and contributing to regenerative medicine and tissue engineering.

The physical microenvironment plays a critical role in stem cell fate determination. We noticed that when differentiating hESCs on different stiffness substrates, the expression changes of marker genes do not appear linear with stiffness (Maldonado et al., 2017; Musah

et al., 2014). Furthermore, even for a similar stiffness range, studies using different materials, such as electrospun nanofibers, have different results from those using hydrogels (Chen et al., 2020; Maldonado et al., 2017; Przybyla et al., 2016a), indicating the culture substrate might not only affect cells by mechanotransduction but also other factors such as the substrate dimensionality and surface chemistry. Although these factors are beyond this study's scope, the mechanism of how our hydrogel, with other substrates, affects stem cell behavior is an important aspect to explore. Most studies discussing substrate stiffness used hydrogels (Chen et al., 2020; Keung et al., 2012; Maldonado et al., 2017; Muduli et al., 2017; Muncie et al., 2020c; Pagliari et al., 2021; Przybyla et al., 2016a; Virdi and Pethe, 2021). Although hydrogel stiffness can be easily controlled, this is usually accomplished by adjusting the cross-linking concentration of the hydrogel. However, these adjustments will not only affect the stiffness but also other properties. Hydrogels are polymer networks containing water molecules within these networks. Changing the number of cross-links also changes the mesh size and surface chemistry, thus affecting the diffusion of the soluble factors at the basal side of the cell. It is also important to include these aspects when discussing substrate stiffness. Simultaneously, hydrogel substrates also provide different dimensionality compared to culture dishes, since it allows the soluble factors to contact cells from the basal side. These could potentially play an important role in stem cell differentiation.

Our experiment has shown that hPSCs when maintained on a soft substrate, showed up-regulated NODAL signaling which is downstream of Activin. Activin is a soluble component added to maintain pluripotency in many hPSCs culture media (Beattie et al., 2005). Additionally, cells on softer substrates show enhanced differentiation efficiency by BMP4. BMP4 and activin both belong to the transforming growth factor beta family (TGF beta). The TGF beta family receptor's subcellular localization is precisely regulated, and in epithelium cells, TGF beta receptors localize basal-laterally, causing the apical side to be relatively insensitive to stimulation (Derynck and Budi, 2019). Softer hydrogels have a large mesh size and might also affect cells by enhancing the TGF beta signaling since it allows more activin and BMP4 to contact the basal side of cells, thus promoting TGF beta downstream genes and promoting mesendoderm fate.

In summary, we have developed a novel method for the fabrication of hPSCs culture substrate. Taking advantage of the flexibility of gel geometry and stiffness, we could precisely control the geometry and spatial stiffness. By adjusting the stiffness locally, we could selectively differentiate hPSCs toward the mesoderm, providing a novel method to generate more complex tissue structures *in vitro*. Further investigation is required regarding the molecular mechanism of hydrogel substrate stiffness-mediated differentiation of hPSCs.

Methods

Synthesis and preparation of hydrogel prefabrication solution

PVA (AP-17, Japan Vinyl Acetate & Poval Co., Ltd. Mn= 80,000–90,000) was reconstituted into a 5% PVA solution by slowly adding PVA into 50–60℃ H_2O while stirring. The temperature was then increased to 90℃ while stirring for 60 min for PVA to completely dissolve. The solution was subsequently cooled to 50 ℃ while stirring. Sulfuric acid (400 µL) was added to the PVA solution dropwise while the temperature was maintained at

approximately 50℃. Methacrylic anhydride (1.5 mL) (Catalog #276685, Sigma-Aldrich, St. Louis, United States) was added to the mixture dropwise while stirring until the mixture became cloudy. More sulfuric acid was added dropwise until the solution became clear. The temperature was maintained at approximately 50℃. The reaction proceeded with stirring at 50℃ overnight. The reaction product was transferred into a dialysis tube (Standard RC Tubing 6–8 kD Catalog #**15370752**, Spectrum Laboratories, Inc., Göteborg, Sweden) and dialyzed at 50℃ for 7–10 times. Obtained PVA-MA solution was then lyophilized to obtain PVA-MA powder. Prefabrication solution was prepared by reconstituting PEG, PVA-MA, and LAP in PBS to a final concentration of 10%, 5%, and 0.5% respectively. The solution was then adequate and stored in dark at -20℃. Prefabrication solution was hardened whether by using a patterned UV irradiation device or desktop UV stove in all experiments.

Activation of hydrogel and protein binding

The carboxyl groups of the prepared hydrogels were activated by incubating the hydrogel with 10 mg/ml each of NHS/EDC in MES solution at 37℃ for 2 h. The solution was replaced with Laminin 511 E8 fragment (Imatrix511Catalog #T304, TAKARA BIO, Kusatsu, Japan) solution (30 µL in 1 mL of PBS) incubated at 37℃ overnight. The next day, the hydrogel was washed three times with PBS and used for cell culture following standard protocol.

Cell culture

The human embryonic stem cell line, KhES-1, was used in this study. All cells used in this research are below passage number 50. KhES-1 was cultured at feeder-free conditions using StemFit® AK02N (Catalog #RCAK02N, TAKARA BIO, Kusatsu, Japan) with daily medium change. Cells were routinely passaged every week. On passage day, cells were washed in PBS twice and digested using 50% CTS™ TrypLE™ Select Enzyme (Thermo fisher, Catalog #A1285901) at 37°C for 5 min. CTS™ TrypLE™ Select Enzyme was then replaced with 2 mL PBS. Cells were collected by gently pipetting and centrifuging at 600g for 5 min. ROCK Inhibitor Y-27632 (Catalog #72302, STEMCELL Technologies, Vancouver, Canada) (10 M) was added during passage to improve survival and removed the next day. Laminin 511 E8 fragment (Takara, Imatrix511 Catalog #T304, TAKARA BIO, Kusatsu, Japan) (2.5 µg/mL) was added into the medium during passage as the cell culture matrix.

Generation of H2B-mCherry cell line

PiggyBac donor vector containing H2B fusion mCherry-IRES-puromycin resistant gene under a CAG promoter, PB-CAG-H2B-mCherry-IPuroR is a kind gift from Dr.Ohgushi. pCMV-hyPBase is a kind gift from Dr. Yusa(Yusa et al., 2011). PB-CAG-H2B-mCherry-IPuroR and pCMV-hyPBase were co-transfected into KhES-1 using Fugene 6 (Promega, E269A) transfection reagent following the manufacturer's instruction. Three days after transfection, 1 µg/mL of puromycin was added to the culture medium to eradicate none integrated cells. Single colonies with a strong H2B-mCherry fluorescence signal were picked and expanded.

Cell cycle analysis

To compare the proliferation rate of cells cultured on gel and glass, half of the bottom of one 35 mm Glass dish was coated with PVA-PEG hydrogel and hardened to ~100 kPa following the previously described protocol. H2B-mCherry cells (1.5×10^5) were seeded into

each dish and cultured following the standard protocol. Time-lapse was performed using Olympus LCV100 confocal live imaging system with an interval of 15 min/frame for 72 h. Cells (~100) inside similar size colonies on gel or glass from 3 dishes were manually tracked for cell cycle analysis. The time between each nuclei division was measured according to 15 min/frame, the cell cycle of each cell was tracked at least three times and the mean value was recorded.

Cell culture on hydrogel

Glass bottom dishes (Catalog #3961-035, AGC TECHNO, Shizuoka, Japan) was coated using Bindsilane (Catalog # 10600047, GE Healthcare, Illinois, United States) following the manufacturer's instruction to improve hydrogel binding. Dishes were then coated with MPC polymer (Catalog # Lipidure-CM5206, NOR Corporation, Tokyo, Japan) to avoid attachment of cells on the none gel part of the dish following manufacturer instructions. The prefabrication solution was thawed and briefly vortexed. A prefabrication solution (20 µL) was added to one dish. Cover glass coated with Sigmacote® (Catalog # SL2-25ML, Sigma-Aldrich, St. Louis, United States) was gently put on the gel while avoiding generating bubbles to create a flat gel surface. The gel was then hardened using Spot Type UV Curing Systems (Catalog #ANUJ3500-NC, Panasonic, Osaka, Japan). All gel was hardened same distance from the light source. For proliferation assay and differentiation of hESCs, the gel was hardened with 100% output power UV for 2 min. For RNA-seq, the Hard gel was hardened using 100% output power UV for 2 min and the soft gel was hardened using 50% output power UV for 10 s. After gelation, the cover glass was gently removed, and then hydrogel was activated and functionalized using the Laminin511 E8 fragment. Cells were seeded following standard protocol. The culture medium was half replaced the next day of passage and were all replaced 2 days after passage to gradually remove Y-27632 to avoid detachment of cells. Cells were then maintained following a standard medium change schedule.

Stiffness Measurement of Hydrogels Using Atomic Force Microscope (AFM)

All stiffness in this research was represented by the elastic moduli. All hydrogels' young's modulus was measured using AFM (NanoWizardIIR, BRUKER, Billerica, United States). Briefly, 100 µm diameter beads were attached to the tipless cantilever (NANOSENSORS™, TL-CONT-20) and used for subsequent measurement. Measurements were performed in the Force Spectroscopy mode in water, and Young's modulus was calculated after removing noise in NanoWizard software(V7, BRUKER, Billerica, United States).

Micropatterned culture on gel

In patterning culture, cells should be seeded at high density since they are cultured on top of the hydrogel patterns. Therefore, the cell seeding area was limited using a PDMS spacer. First, PDMS sheets were prepared by mixing the main agent: hardener = 9:1 for 15 min, and then pour onto a flat surface and cured by heating at 80°C for 15–20 min. The sheet was made as thin as possible for subsequent patterning culture. A hole of 6 mm diameter was made in the center of the formed PDMS sheet using a hole punch. PDMS spacer was attached to Bindsilane coated 35 mm glass-bottom dish and 20 µL of PVA-PEG prefabrication solution was added into the hole. Then Sigmacote® coated coverslip was gently put on top of the gel solution. The patterned gel was then cured by irradiating

patterned UV using Desktop Maskless Lithography System (DDB-701-DL, NEOARK CORPORATION, Hachioji, Japan). Gel with different stiffness was generated by giving spatially different UV exposure. Then coverslip was removed and hydrogel was activated and functionalized with Laminin511 E8 fragment. hESCs (KhES-1) (1×10^6) were collected and resuspended in 200 µL of AK02N medium with 10 µM Y27632.The suspension (20 L) was added on top of the gel pattern and incubated at 37 °C, 5% CO_2 for 30 min to allow cells to attach. The gel was washed with PBS once to remove exceeded cells and the PDMS spacer was removed. AK02N medium (1 mL) with 10uM Y27632 was added to the dish. Half of the medium was replaced with AK02N the next day. Then Cells were cultured following standard protocol.

Measuring protein binding ability of PVA-MA

PVA-MA hydrogel was prepared and activated as described previously. The hydrogel was then incubated with a fluorescent 2nd antibody (Catalog # A-11003, ThermoFisher, Massachusetts, United States) following the protein binding protocol described previously. Photos were taken using a fluorescence inverted microscope (SP8 LIGHTNING Confocal Microscope, Leica, Wetzlar, Germany) and the fluorescence intensity was quantified using Image J2(V 2.9.0, National Institutes of Health, Maryland, Unites states).

Immunofluorescence

The culture medium was removed from the sample. Samples were then fixed by incubating in 2 mL of 4% PFA in PBS on ice for 20 min. the sample was washed in PBS, three times for 5 min each. PBS was replaced with 0.3% Triton-X100/PBS and samples were incubated for 5 min at 25℃. Samples were then blocked with Blocking One (Catalog #03953-66, NACALAI TESQUE, Kyoto, Japan) for 1 h at room temperature, then incubated with primary antibody diluted in blocking one at 4°C overnight. primary antibody was Removed then samples were washed with 0.05% Tween 20/PBS for 5 min, and repeated three times. Then samples were incubated with secondary antibodies and DAPI diluted in the blocking solution, 1/500 for 1 h at room temperature. Secondary antibodies were removed and samples were washed in 0.05% Tween 20/PBS for 5 min, thrice. The samples were then observed using a microscope.

RNA extraction and quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Catalog #74106, QIAGEN, Hilden, Germany) following the manufacturer's instructions. cDNA was reverse transcripted from 500ng RNA using PrimeScript™ II 1st strand cDNA Synthesis Kit (Catalog # 6210A TAKARA BIO, Kusatsu, Japan) following the manufacturer's instructions. qPCR was performed using Power™ SYBR™ Green Master Mix (Catalog #4368577, Applied Biosystems™, Massachusetts, United States). The expression level of mRNAs was calculated and normalized based on indicated housekeeping gene (*HPRT1*, *PBGD*).

RNA-seq

Gel-coated 35 mm glass-bottom dishes were prepared as previously described, Gel was hardened by 50% for 10 s (soft gel), or 100% for 2 min (hard gel) using a UV desktop oven. KhES-1 was seeded on the soft gel, hard gel, or uncoated glass-bottom dish and cultured for 4 days or 7 days before they were collected as previously described. Total RNA was

extracted using RNeasy Mini Kit (Cat#74106, QIAGEN, HILDEN, GERMANY,) following the manufacturer's instruction. Two samples for each group, 12 samples in total were collected. Libraries were prepared from 100 ng of total RNA using the following kits, NEBNext Poly(A) mRNA Magnetic Isolation Module (Catalog # E7490, NEW ENGLAND BIOLABS, MASSACHUSETTS, UNITED STATES), NEBNext Ultra II Directional RNA Library Prep Kit (Catalog # E7760, NEW ENGLAND BIOLABS,MASSACHUSETTS, UNITED STATES), NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) (Catalog # E6440, NEW ENGLAND BIOLABS,MASSACHUSETTS, UNITED STATES) following the manufacturer's instructions. Sequencing was performed with the paired-end mode (more than 50 bp at both ends) to have at least 15 clusters per sample using the NextSeq550 NGS system. The generated raw RNA-seq files were quality trimmed with Trimomatic-0.39 and removed rRNA with bowtie2. This highly qualified sequence reads were mapped and annotated with STAR-2.7.6a against the GRCh38, and then the count data which was estimated with featureCounts was subjected to differential expressed gene analysis using the bioconductor package DESeq2_1.30.1. The DEGs were defined as genes that were expressed with a false discovery rate < 0.01. Metascape database platform was used to enrich and analyze the GO (Gene ontology) annotation.

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Author contributions

Z.W., A.N., and M.E. designed the research; Z.W. and A.N. performed the research; Z.W., A.N., F.W., and Y.O. analyzed the data. Z.W. and M.E wrote the paper.

Data availability statement

The data and materials supporting this research are available from the authors on reasonable request.

Additional information

The authors declare no competing interests.

Figure legends

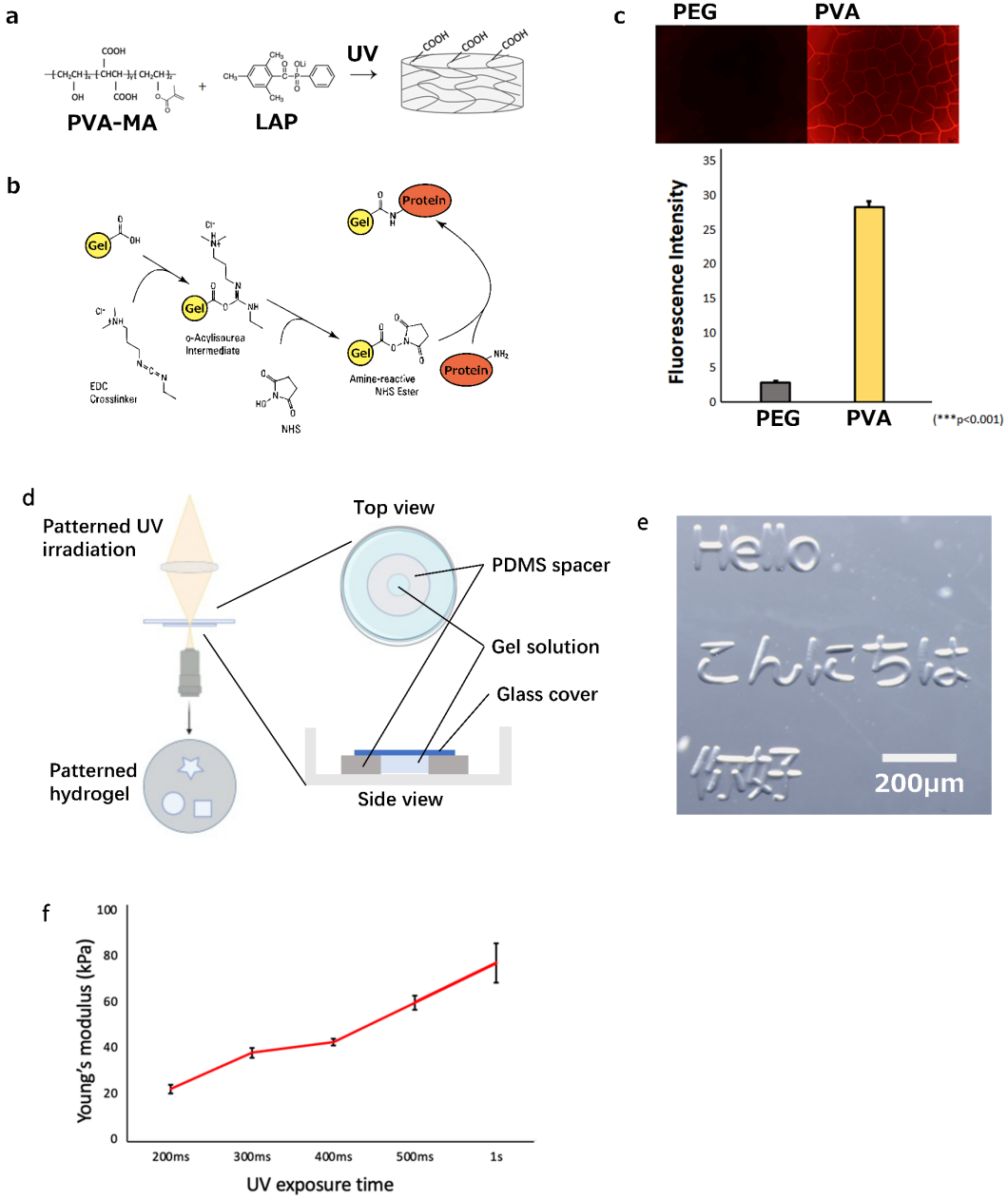
Figure 1. Schematic illustration of photocurable PVA-PEG hydrogel (**a**) PVA-MA forms COOH-containing hydrogel upon ultraviolet (UV) irradiation. (**b**) COOH group of the hydrogel can be activated to bond with protein using NHS/EDC. (**c**) Protein binding ability of PVA gel. (**d**) Schematic illustration of generating patterned hydrogel. (**e**) Patterned hydrogel. Scale bar, 200 μm. (**f**) Characteristics of PVA-PEG hydrogels.

Figure 2. Human pluripotent stem cells (hPSCs) proliferate on hydrogel while maintaining pluripotency. (**a**) Live imaging of hPSCs H2B-mCherry cultured on gel and dish for 3 days. (**b**) Cell cycle analysis of hPSCs cultured on a gel and dish, each dots represent one cell. (**c**) Immunofluoresence (IF) of Day 6 hPSCs cultured on gels.

Figure 3. Immunofluorescence (IF) and quantitative polymerase chanin reaction (qPCR) of lineage makers for each germ layer.

Figure 4. RNA-seq of human pluripotent stem cells (hPSCs) cultured on hard and soft hydrogel, and polystyrene(PS) dish. (**a**) Cluster dendrogram of hPSCs cultured on soft and hard gel, and PS dish. (**b**) Enhanced volcano plot of differently expressed genes between hPSCs cultured on the soft (right) and hard (left) gel, NS, not significant. Y-axes represent possibility, and X-axes represent fold change. (**c**) Heat map of Nodal family expression. (**d**) Top six enriched gene groups by gene ontology (GO) analysis.

Figure 5. Manipulating stem cell fate by adjusting local stiffness of the hydrogel. (**a**) Differentiation timetable for mesoderm differentiation. (**b**) Immunofluorescence (IF) of 24 h mesoderm differentiation on soft and hard gels. (**c**) IF of 24 h mesoderm differentiation on gel with locally different stiffness. (**d**) Quantification of T+ number/area on soft and hard gels (n=5). (**e**) Quantification of T+ number/area on each gel with locally different stiffness, dots connected by the same line represent one gel pattern (n=6). (**f**) Differentiation timetable for pulse differentiation. (**g**) IF of pulse differentiation on soft and hard gels. (**h**) IF of pulse differentiation on gel with locally different stiffness. Scale bar, 100 µm.


a
PVA-MA
LAP
UV
COOH
b
Gel
Protein
o-Acylisourea Intermediate
EDC Crosslinker
NHS
Amine-reactive NHS Ester
c
PEG
PVA
Fluorescence Intensity
(***p<0.001)
d
Patterned UV irradiation
Top view
PDMS spacer
Gel solution
Glass cover
Patterned hydrogel
Side view
e
Hello
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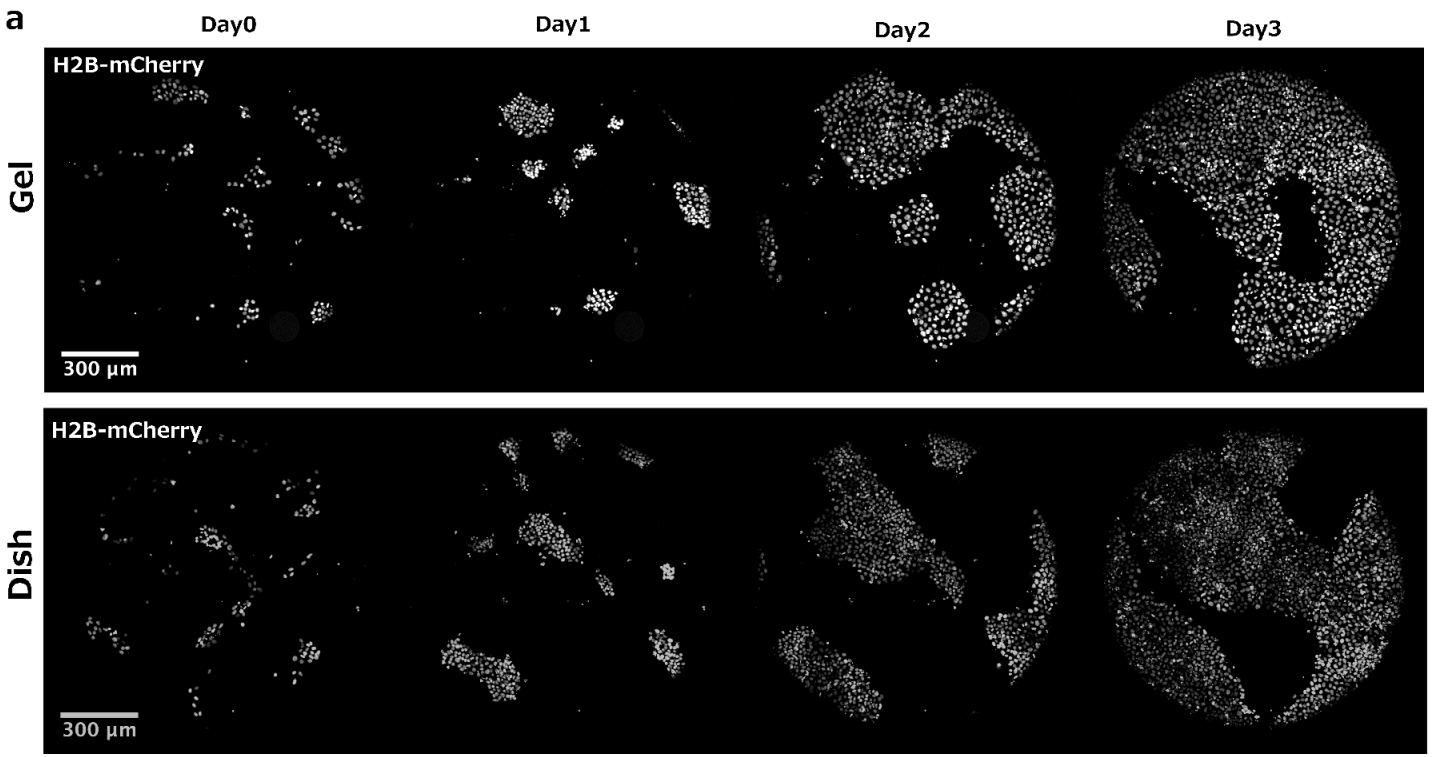
你好
200μm
f
Young's modulus (kPa)
200ms
300ms
400ms
500ms
1s
UV exposure time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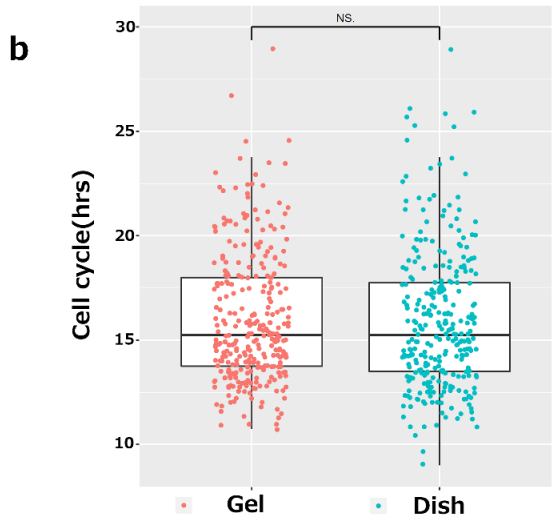
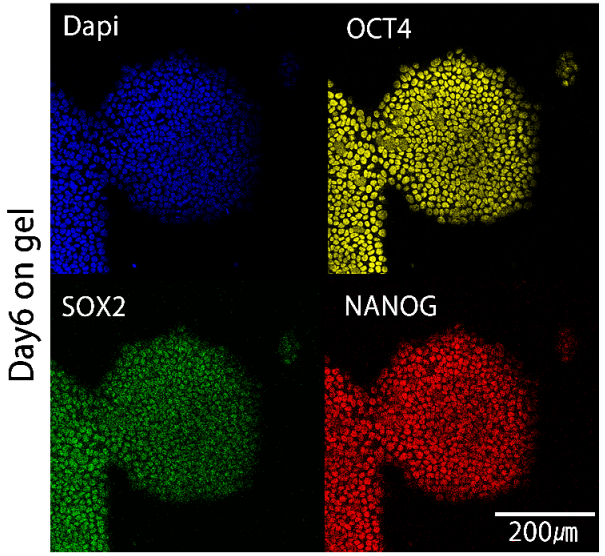
Day0
Day1
Day2
Day3
Gel
H2B-mCherry
300 µm
Dish
H2B-mCherry
300 µm


b
NS.
Cell cycle(hrs)
30
25
20
15
10
Gel
Dish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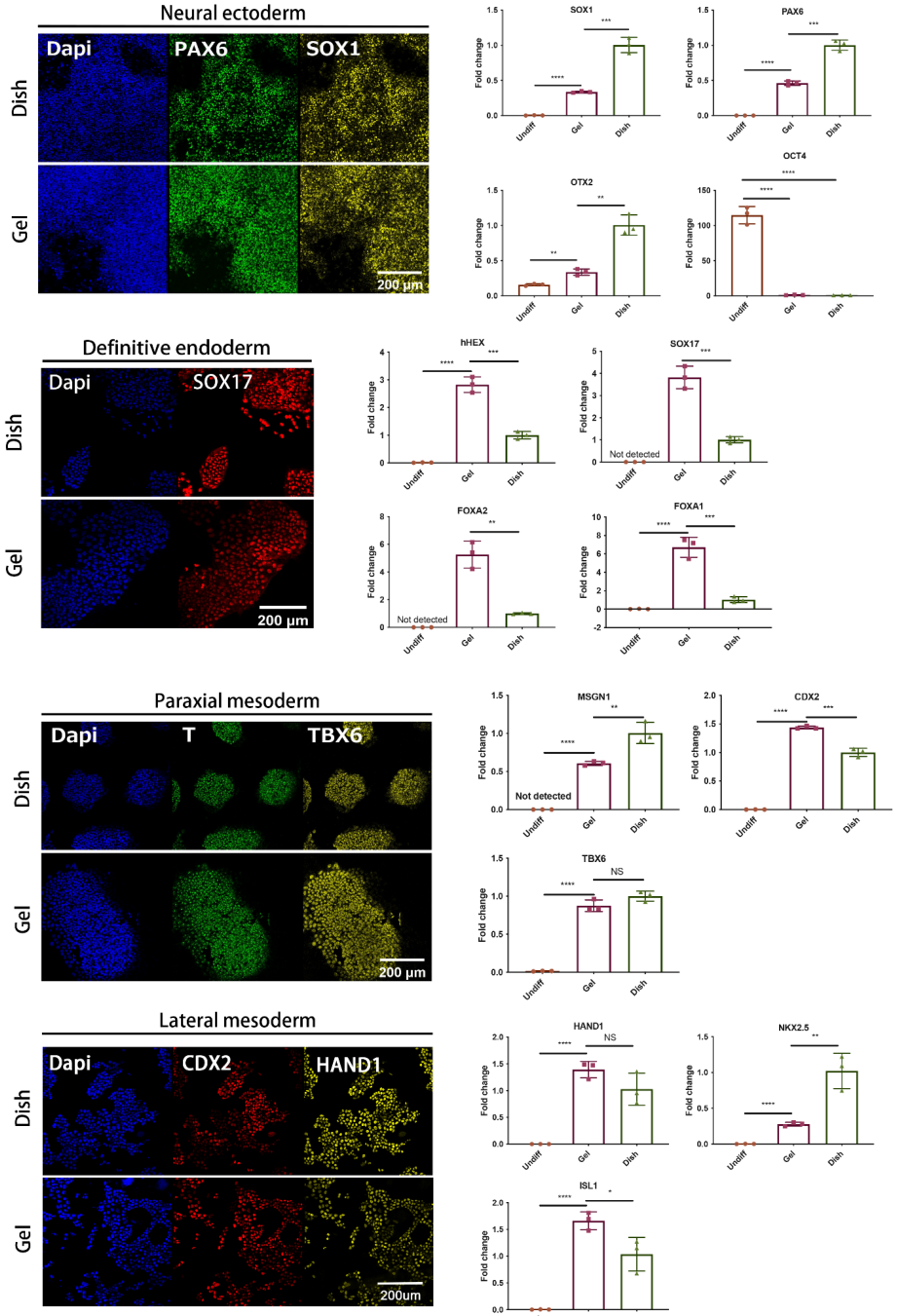
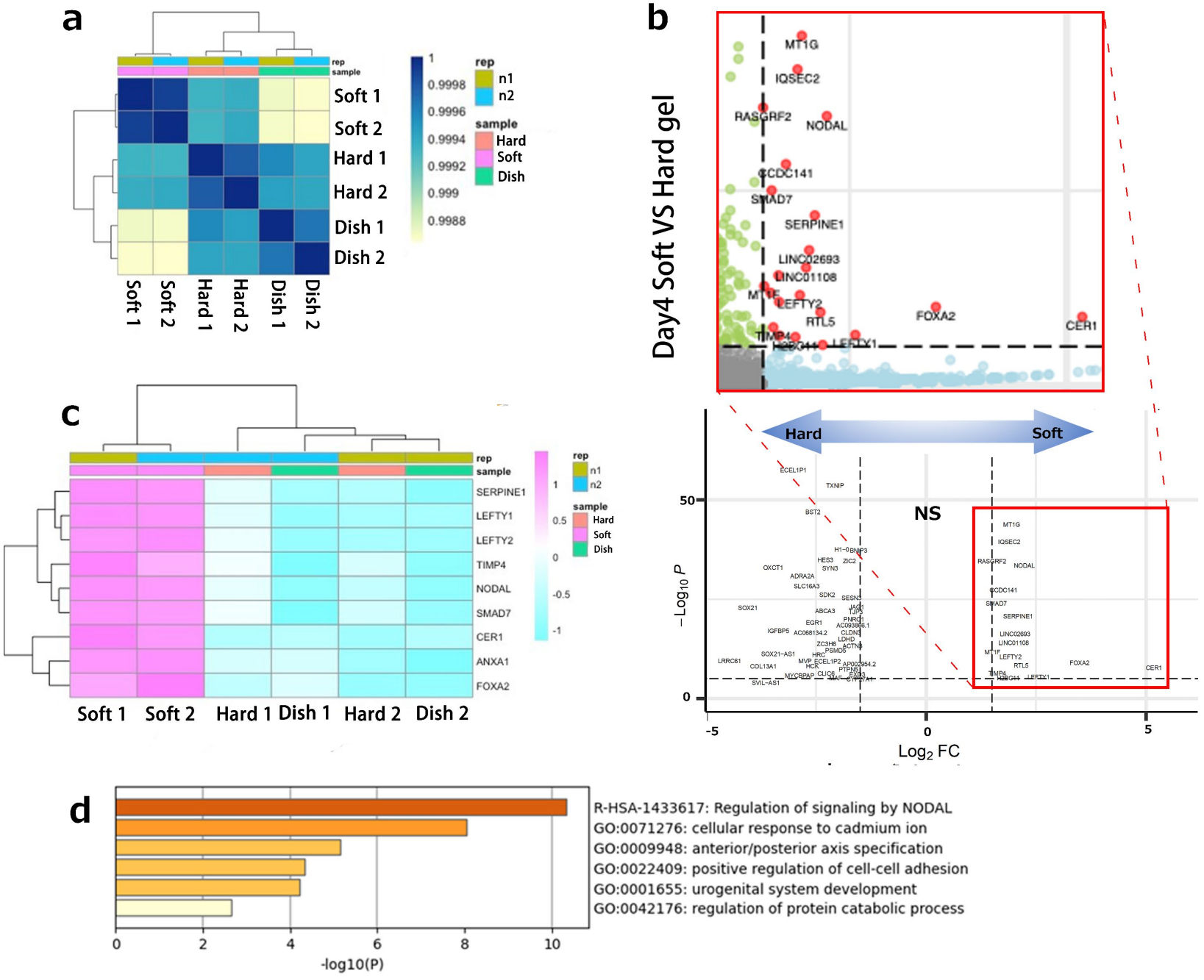
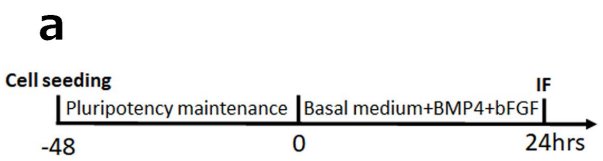
Day6 on gel
Dapi
OCT4
SOX2
NANOG
200µm


Neural ectoderm
Dapi
PAX6
SOX1
Dish
Gel
200 µm
SOX1
PAX6
OTX2
OCT4
Fold change
Undiff
Gel
Dish
Definitive endoderm
Dapi
SOX17
hHEX
SOX17
FOXA2
FOXA1
Not detected
Paraxial mesoderm
Dapi
T
TBX6
MSGN1
CDX2
TBX6
NS
Lateral mesoderm
Dapi
CDX2
HAND1
HAND1
NKX2.5
ISL1
200um


a
rep
sample
Soft 1
Soft 2
Hard 1
Hard 2
Dish 1
Dish 2
1
0.9998
0.9996
0.9994
0.9992
0.999
0.9988
rep
n1
n2
sample
Hard
Soft
Dish
b
Day4 Soft VS Hard gel
MT1G
IQSEC2
RASGRF2
NODAL
CCDC141
SMAD7
SERPINE1
LINC02693
LINC01108
LEFTY2
RTL5
FOXA2
CER1
Hard
Soft
NS
−Log10 P
Log2 FC
c
rep
sample
SERPINE1
LEFTY1
LEFTY2
TIMP4
NODAL
SMAD7
CER1
ANXA1
FOXA2
Soft 1
Soft 2
Hard 1
Dish 1
Hard 2
Dish 2
d
R-HSA-1433617: Regulation of signaling by NODAL
GO:0071276: cellular response to cadmium ion
GO:0009948: anterior/posterior axis specification
GO:0022409: positive regulation of cell-cell adhesion
GO:0001655: urogenital system development
GO:0042176: regulation of protein catabolic process
-log10(P)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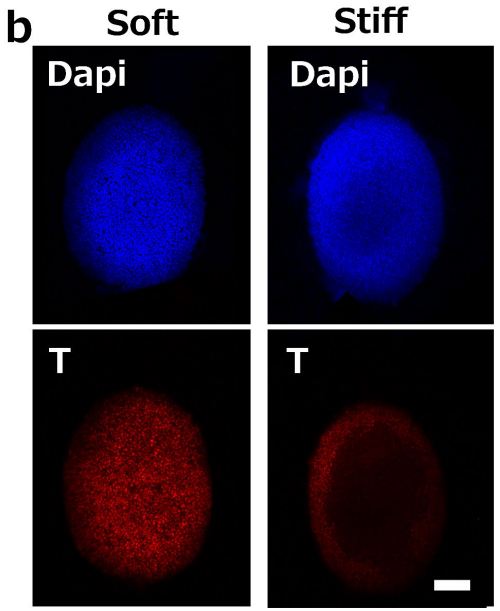
Cell seeding
Pluripotency maintenance
Basal medium+BMP4+bFGF
IF
-48
0
24hrs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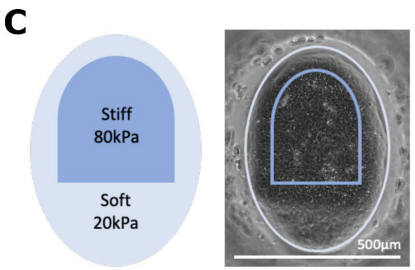
Soft
Stiff
Dapi
Dapi
T
T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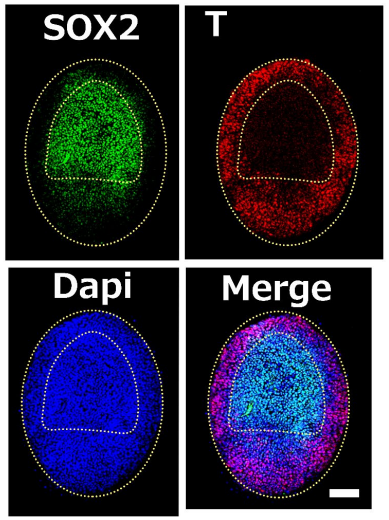
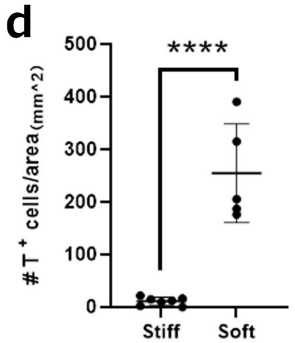
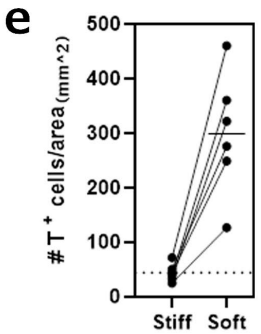
Soft
20kPa
500μm


SOX2
T
Dapi
Merge


d
****
T + cells/area (mm^2)
500
400
300
200
100
0
Stiff
Soft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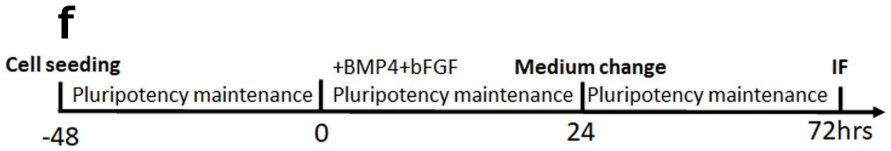
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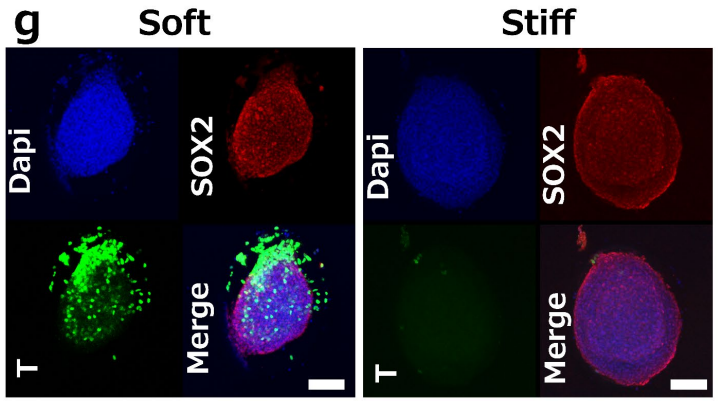
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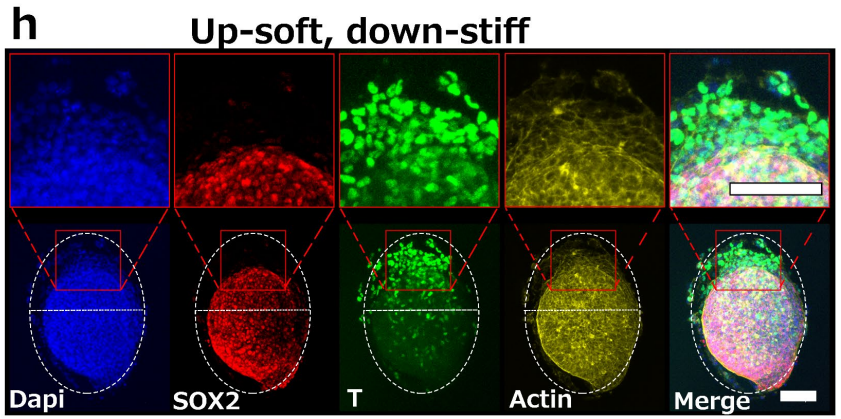
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Actin
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