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

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Summary:

Human pluripotent stem cells(hPSCs) sense the surrounding chemical, and physical microenvironment and adjust their behaviors accordingly. However, current *in vitro* research is mostly conducted in plastic culture wares which provides an oversimplified microenvironment compared with *in vivo*. Recently, increasing evidence has shown that the physical microenvironment plays a critical role in hPSCs differentiation and maintenance. However, current studies were conducted by a bulk culture of hPSCs on substrates with different stiffness. The development of biocompatible materials that allow spatial control of the chemical and mechanical environment for human PSC culture has not yet been achieved. Culture substrates allowing flexible manipulation of physical properties spatially would help us better recapitulate the *in vivo* environment, and benefit tissue engineering.

In this study, we developed a photocurable hydrogel (PVA-PEG gel) which allows us to spatially control the stiffness and the structure at the micrometer level. The generated hydrogel can be functionalized using different 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 on the same patterned gel, generating complex patterned structures.

Introduction:

hPSCs have been used intensively 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 largely different from the in vivo microenvironment, limiting the ability to generate complex structures in vitro. PSC differentiation culture can be largely divided into 2D and 3D. 2D culture provides a simple, reproducible, and robust culture condition and many cell differentiation protocols have been developed in 2D. On the other hand, in 3D culture, cells form aggregates floating in the culture medium or embedded in an extracellular matrix (ECM)[1,2]. 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[1]. Therefore, to induce functional tissues with appropriate shapes in vitro, the ideal environment allows tissue deformation with weak mechanical constraints.

Increasing evidence has been suggesting the importance of physical cues on cell behavior and cell fate decisions[3-10]. Soft substrate and high mechanical tension were shown to enhance mesodermal differentiation of hESCs [10,11]. At the same time, substrate geometry and structures were also shown critical to generating complex tissue structures[12,13]. During development, neighboring cells communicate with each other, sensing the surrounding chemical and mechanical cues, and differentiate accordingly, eventually, they gain differentiated cell fate and corporate with each other to form organs with complex structures. Therefore, it is important to spatially controllable microenvironment to generate complex cellular structures. To fulfill these requirements, we aim 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 extracellular matrix[14]. Furthermore, hydrogel could be functionalized to have different properties, such as photocurability. Photocurable hydrogels based on hyaluronic acid and gelatin have been developed. By controlling the light source, gel with different shapes and structures could be generated. However, these gels were used by encapsulating hPSCs but not attachment culture due to their poor adhesivity on the synthetic surface of hPSCs[14,15]. On the other hand, some hydrogel allows good attachment of hPSCs such as polyacrylamide or PDMS. They have been used intensively in studies about substrate stiffness[3,6,14,16-18]. However, they lack flexibility in terms of controlling gel structure and mechanical properties. Taken together we decide to develop a photocurable hydrogel with ideal hPSCs attachment.

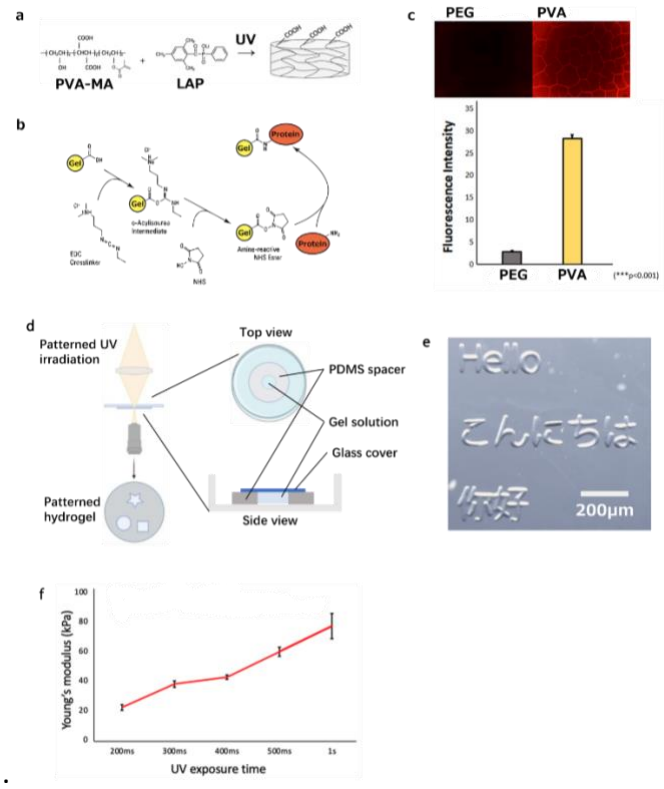
hPSCs attach to the substrate through integrin-ECM protein interaction. Therefore, the engraftment of ECM protein on the hydrogel is necessary. Previously, it was shown that ECM protein functionalized PVA could be used for hPSCs culture[8]. Polyvinyl alcohol (PVA) is a type of polymer with a simple structure and can form a hydrogel upon cross-linking. It's stable and biocompatible. Taken together, we decided to use PVA as the backbone and further functionalize PVA to have photocurability and ECM protein to acquire hPSCs attachment ability.

In this study, we demonstrated a composite photocurable hydrogel of PVA and PEG 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 the micrometer level.

Result:

1. Synthesis of photocurable PVA- Methacrylate hydrogel

To develop a photocurable hydrogel with protein binding ability, we used a denatured Poly (vinyl alcohol) (PVA-COOH) which has randomly integrated carboxyl groups as starting material. The carboxyl group in the PVA backbone can be activated by NHS/EDC and further bind with the N end of proteins to allow cell attachment (Figure 1, b). To obtain photocurability, Methacrylate functional group was introduced into the PVA backbone to obtain photocurable PVA-MA. 0.5%Lithium phenyl-2,4,6-trimethyl-benzoyl phosphonate (LAP) was used as the photoinitiator (Figure 1, a). To evaluate the protein binding ability of PVA-MA, activated PVA-MA and none protein binding hydrogel polyethylene glycol (PEG), was incubated with a fluorescent protein. After incubation with a fluorescent protein, fluorescence intensity was measured to quantify the hydrogel protein binding ability. Compare with none protein binding PEG, activated gel shows a significantly higher fluorescent signal (figure1, b, c) indicating protein was sufficiently bound to our hydrogel

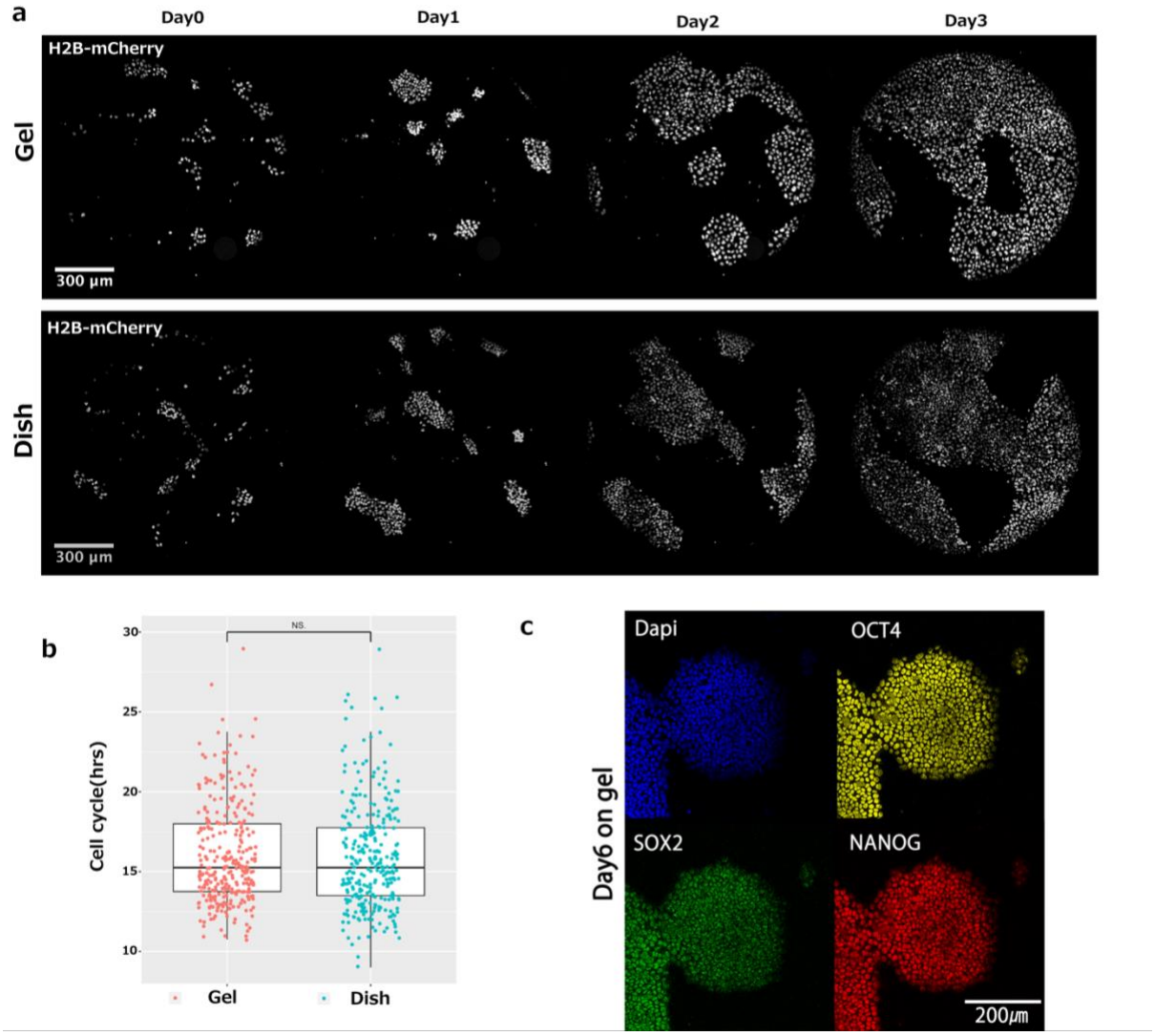


Increasing evidence is suggesting the importance of the diameter of colony and substrate geometry in generating complex tissue in vitro[12,13]. However current micropatterned cultures are performed on glass or plastic cultureware, their physical property largely different from in vivo. Taking advantage of the flexibility of photoinitiated crosslinkability of our hydrogel, we tried to develop a patterned culture method using our hydrogel. As figure 2, c shows, 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 gel at the micrometer level (figure1 c, d). However, we found that PVA-MA gel alone has very low elasticity and narrowed elasticity range. to increase the range of elasticity, we added 4-arm PEG to form a composite hydrogel. Using 4 Arm PEG with different molecular weights in different ratios we could obtain hydrogel with stiffness ranging from ~5kPa to ~500kPa. We could also control the stiffness of PVA-PEG composite hydrogel by giving a different dosage of UV irradiation (figure 2, e). In the following research, 3%PVA, 15% 5K 4-arm PEG was used, since it gives us a suitable range of stiffness. To this end, we have a photocurable hydrogel substrate with an adjustable stiffness, ranging from soft(~20Kpa) to hard(~100kPa).

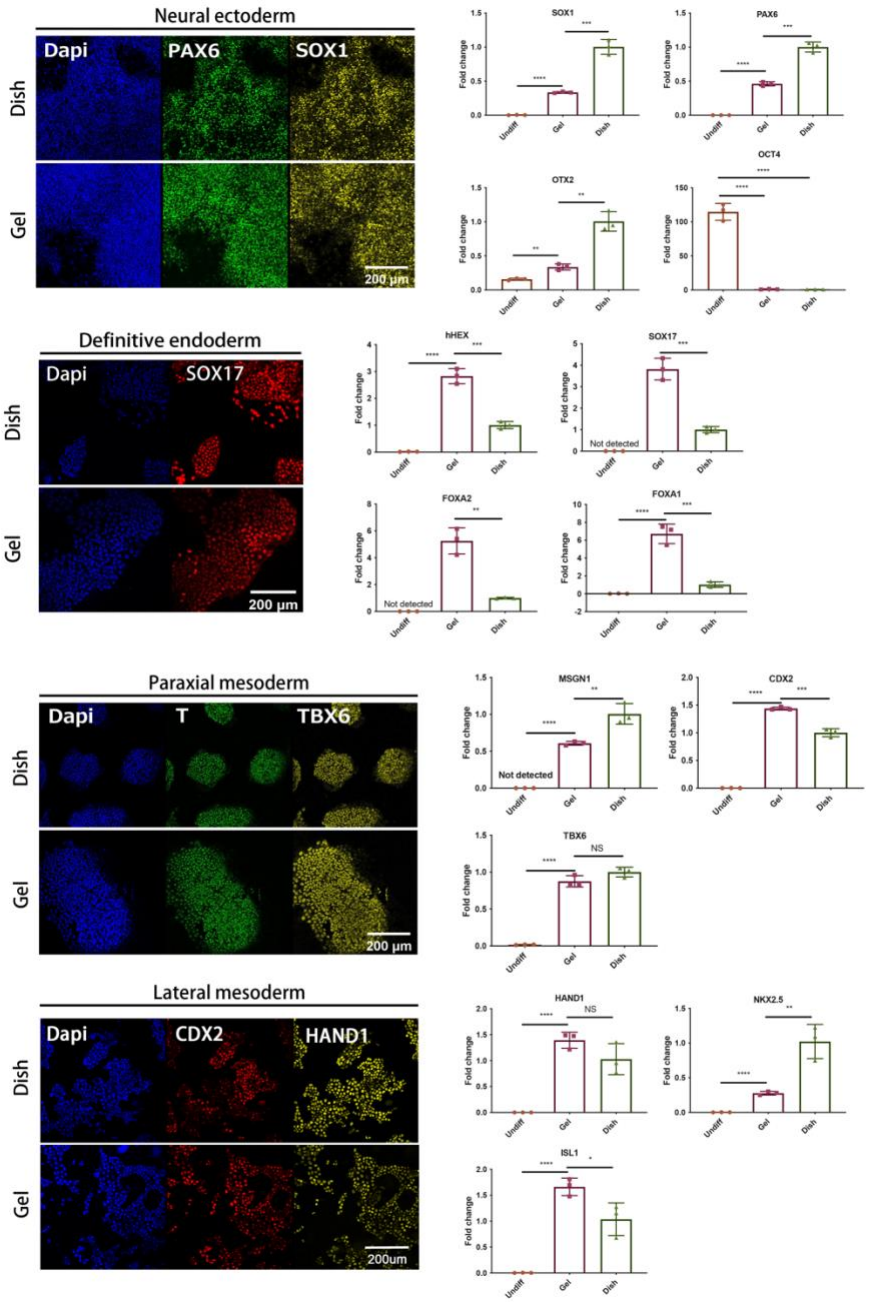
2. PVA-PEG gels support human pluripotent stem cell survival and outgrowth of hPSC while maintaining pluripotency

We next tested if our gel supports the growth of human pluripotent stem cells(hPSCs). We generated gel by giving sufficient UV to generate ~100kPa 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, the H2B-mCherry hPSCs line to allow cell

tracking and cell cycle analysis. H2B-mCherry was seeded on culture dishes or hydrogel, and live imaging was performed to track cell proliferation and nuclear division. As figure 3 shows, cells cultured on gel showed a similar proliferation rate compared with those cultured on glass-bottom dishes. Immunofluorescence (IF) suggests hPSCs cultured on our hydrogel have maintained pluripotency within 1 passage which was indicated by the expression of pluripotent markers of day6 culture on the gel. Suggesting pluripotency and proliferation of hESCs can be maintained on hydrogel as well as on rigid culture dishes.



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



Since our hydrogel can be used for hPSCs culture we next 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 previously reported protocol.

For NE, hESCs were differentiated using the previously reported dual SMAD inhibition method[19]. After 6 days of differentiation, cells on both dish and gel largely expressed NE marker PAX6 and SOX1. Suggesting successful differentiation of NE. qPCR suggests cells differentiated on dish 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 (75kPa and 300kPa) and culture dish(~GPa)[6,7].

For DE, cells were differentiated toward anterior primitive streak (APS) first for 24hrs and then towards DE[20,21]. As Figure 3, b shows, Cells on both dish and gel were successfully

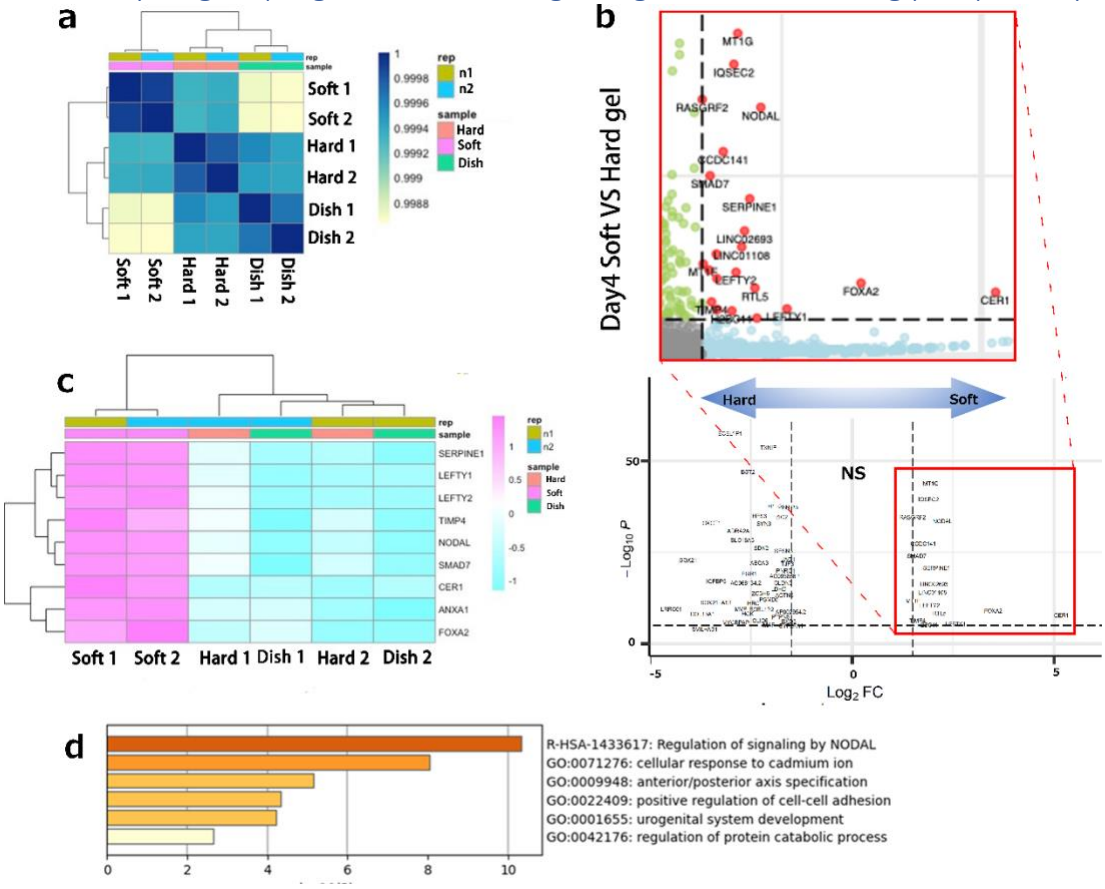
differentiated, supported by the expression of NE marker SOX17 in most of the cells. Interestingly, qPCR shows a notably enhanced expression of APS and DE-related genes. Consist with a previous study showing DE differentiation is enhanced on hydrogel (3kPa and 165kPa) and dish(~GPa). Our hydrogel promotes DE differentiation probably by enhancing APS and DE-related gene expression on a softer substrate.

For Mesodermal development, since PM and LPM fate separate at the early stage of differentiation, therefor we tested both. For PM, hESCs were differentiated towards APS for 24hrs and then APS was directed towards PM for 24hrs[22]. Although soft substrate was shown to enhance mesendoderm fate[3,10], IF and qPCR showed similar expression of LPM markers in our case. Previously it was reported that in the range of 3kPa to ~GPa, the expression of APS and mesendoderm genes EOMES and Brachyury and DE genes FOXA2 and SOX17 is inversely proportional to the substrate stiffness, but soft substrate seems to inhibit pan primitive streak gene CDX2 and mesodermal gene FOXC2 and NKX2.5[3]. It was also shown that substrate stiffness affects cell fate decision developmental stage specifically. It is not always enhanced or decreased but rather dynamic[6]. Given the fact that we only investigated limited markers and limited time points, PM-related genes remain at similar expression levels and can be explained by different observed markers and the relatively hard stiffness of the gel used in this experiment(~100kPa).

For LPM hESCs were differentiated toward mid primitive streak (MPS) for 24hrs and then towards LPM for 24hrs following previously reported protocol[22]. IF suggesting differentiation both on the gel and on the dish was successful. Cells on gel expressed a higher level of HAND1 and ISL1 but expressed a lower level of NKX2.5. The downregulating of NKX2.5 was expected since it matches the previous report[3].

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

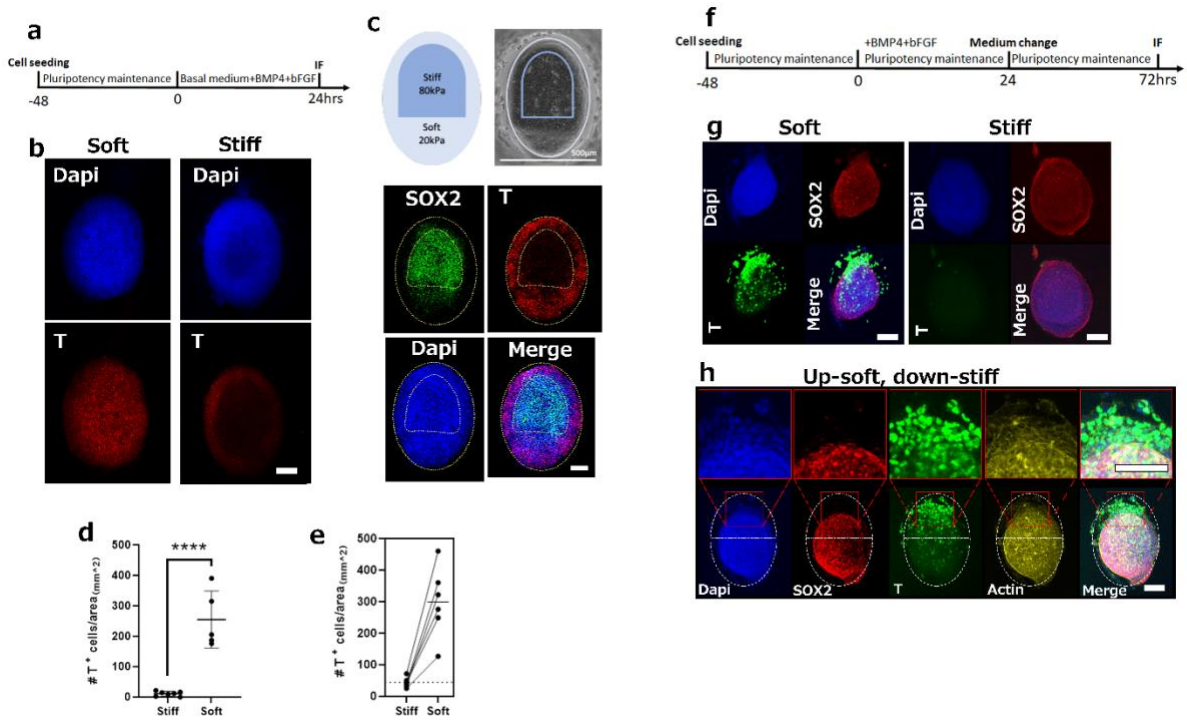
4. Soft hydrogel upregulates NODAL signaling while maintaining pluripotency.



Next, we asked how different stiffness of hydrogel impact hPSC gene expression. Day4 culture of hPSCs on the hard gel(~100kPa), soft gel(~20kPa), and glass-bottom dish was collected and performed RNA-seq respectively. In total 34032 transcripts were assembled. Within these transcripts, 1288 genes were shown to upregulated on softer gel compared with hard gel, pluripotent genes such as SOX2, OCT3/4, and NANOG were not significantly different among each sample. On the other hand, NODAL signaling-related genes such as ANXA1, CER1, FOXA2, LEFTY1, LEFTY2, SMAD7, SRPINE1, and TIMP4 were upregulated in softer gel compared with hard gel and dish. GO analysis shows that softer hydrogel enriches genes from axis specification, determination of bilateral symmetry, specification of symmetry, Endoderm differentiation, gastrulation, pattern specification process, and anterior/posterior pattern specification (fig 4, d). 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 probably enhances posteriorization through upregulated NODAL signaling and further induced APS genes such as FOXA2, consistent with the previous reports[3,23].

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 softer hydrogel was shown to enhance the NODAL signaling, we next asked if it is possible to spatially manipulate stem cell fate, by generating locally soft hydrogel.

During gastrulation, 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 it is possible to generate locally soft patterned hydrogel and selectively differentiate cells on the soft part toward mesoderm. We first generated soft(20kPa) and hard(80kPa) patterns and differentiated hESCs on gel towards mesoderm by giving 10ng/ml BMP4 and bFGF[21] in differentiating medium. After 24hrs of differentiation, cells on the soft gel were differentiated to a mesendoderm fate which is indicated by the expression of mesodermal and primitive streak marker T, while most of the cells on hard gel do not express T, except few cells on the edge (fighure5, a), probably due to weaker cell-cell junction and higher tension[10,23]. These results indicate it is possible to manipulate stem cell fate by adjusting the stiffness of our hydrogel. Since the stiffness of our gel is easily controlled by UV dosage, we next generated hydrogel with locally different stiffness using a patterned irradiating system. As Fig 5 b shows, hPSCs were seeded on a patterned hydrogel which is half hard and half soft. This colony was then differentiated toward mesoderm for 24hrs. Cells in hard areas remained pluripotency, which is indicated by the SOX2 positive and T negative. While cells in the softer area gain mesodermal fate indicated by their expression of T and absence of SOX2 expression (Figure6, b). regardless they were differentiated inside the same dish. We next calculated T^+ cells per area based on the IF image, as shown in fig5 c, cells on a softer gel pattern have a significantly higher rate of T^+ cells. Fig5 d shows the T^+ cells per area on soft/hard areas on the same patterned gel.

Despite the significant difference in T^+ cell number after 24hrs of differentiation, we found that if the differentiation was extended to 48hrs, cells on both hard and soft areas will

become T^+. We next tested if it's possible to maintain the effect of selective differentiation by giving a pulse of morphogen stimulation. hPSCs were seeded on hydrogel patterns and cultured in the pluripotency maintenance medium. 10ng/ml BMP4 and bFGF were directly added into the maintenance medium for 24hrs and then withdrawn by medium change. The patterned culture was cultured in a maintenance medium for another 48hrs. As fig5, e shows, cells on the soft gel are largely T positive while only a few on hard gels. On partially soft gel, T-positive cells are mostly restricted in soft areas and lost the expression of SOX2. In contrast cells on hard gel mostly remained SOX2 expression. These data suggest we could selectively differentiate cells by spatially controlling the hydrogel stiffness.

Discussion:

In this study, we developed a photocurable hydrogel for hPSCs culture and by controlling the UV light source, we can control the shape of the gel pattern and the local stiffness of the gel. Laminin511 E8 fragment functionalized hydrogel supports hPSCs growth and maintained pluripotency. hPSCs can be differentiated towards all 3-germ layers on our hydrogel. We also demonstrated that on a softer gel, Nodal signaling is upregulated compared with hard gel and PS dish. Most importantly by generating locally soft gel we could selectively differentiate hPSCs towards mesoderm, and manipulate stem cell fate spatially.

To overcome the limitation of traditional 2D/3D culture, many soft substrates have been developed for cell cultures, such as various hydrogels, electro-spun fibers, and Polydimethylsiloxane (PDMS). However, due to the poor adhesivity of hPSCs on synthetic surface[14,15], only limited materials can be used for attachment culture. Polyacrylamide (PA) has been used for hPSCs attachment culture. however, pluripotency cannot be maintained. In this study, Laminin 511 E8 was covalently bonded on our hydrogel, providing a strong attachment for hPSCs enable an ideal culture of hPSCs while maintaining pluripotency.

Soft substrates such as PVA, Electro-spun fibers, Polyacrylamide (PA) gel, and PDMS have been used for hPSCs attachment culture[3,7-10,15,23-26]. However, these methods usually coate the whole surface of the cultural dish, the local physical property cannot be adjusted. Recent research has been suggesting the importance of the stiffness and geometry constraint in differentiating complex structure[3,5,7,9,13,15,27-29]. Our hydrogel provided a simple and flexible platform for generating 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 lineage. Differentiation of different lineage in a homogenous culture system by only adjusting medium composition is challenging. In this study we selectively differentiate hPSCs towards mesoderm by adjusting local stiffness, providing 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 change of marker genes, doesn't appear linear with stiffness[6,9]. Furthermore, even for a similar stiffness range, studies using different materials such as electrospun nanofiber have different results from those using hydrogel[3,6,10], indicating the culture substrate might not only affect cells by mechanotransduction but also other factors such as the substrate dimensionality and surface chemistry. Although beyond this paper's scope, the mechanism of how our hydrogel together with other substrates affects stem cell behavior is an important question to ask. We noticed that most studies discussing substrate stiffness used

hydrogel[3,6-8,10,15,23,25]. Although hydrogel stiffness can be easily controlled, this is usually accomplished by adjusting cross-linking in hydrogel or by adjusting the concentration of hydrogel. However, these adjustments will not only affect the stiffness but also the other properties. Hydrogels are polymer networks containing water molecules within 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. At the same time, hydrogel substrate also provides different dimensionality compared with culture dish 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 when maintained on a soft substrate, hPSCs showed up-regulated NODAL signaling which is downstream of Activin. Activin is one of the soluble contents added to maintain pluripotency in many hPSCs culture mediums[30]. And cells on softer substrate show enhanced differentiation efficiency by BMP4. BMP4 and Activin both belong to the transforming growth factor beta family (TGF beta). 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 [31]. Softer hydrogel has a bigger 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 a hPSCs culture substrate. Taking advantage of the flexibility, we could precisely control the geometry and the stiffness spatially. By adjusting the stiffness locally, we could selectively differentiate hPSCs toward mesoderm. Providing a novel method to generate more complex tissue structures in vitro. Further investigation should be done 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 5w% 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 .400ul of sulfuric acid was added to the PVA solution dropwise while the temperature was maintained at around 50℃. 1.5 ml of methacrylic anhydride (Sigma-Aldrich) was added to the mixture dropwise while stirring, then the mixture became cloudy. More sulfuric acid was added dropwise until the solution becomes clear. The temperature was maintained at around 50℃. The reaction proceeded while stirring at 50℃ overnight. The reaction product was transferred into a dialysis tube (Standard RC Tubing 6-8kD, Spectrum Laboratories, Inc.) 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 10w%, 5w%, and 0.5w% 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 10mg/ml each of NHS/EDC in MES solution at 37℃ for 2h. The solution was replaced with Laminin 511 E8 fragment (Takara, Catalog #T304) solution (30 µL in 1mL 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

Human embryonic stem cell line KhES-1 was used in this research. All cells used in this research are below passage number 50. KhES-1 was cultured at feeder-free conditions using StemFit® AK02N (Takara, Catalog #RCAK02N) with daily medium change. Cells were routinely passaged every week. On passage day, cells were washed in PBS 2 times and digested using 50% CTS™ TrypLE™ Select Enzyme (Thermo fisher, Catalog #A1285901) at 37°C for 5min. CTS™ TrypLE™ Select Enzyme was then replaced with 2ml PBS. Cells were collected by gently pipetting and centrifuging at 1000rpm 5min. 10uM ROCK Inhibitor Y-27632(STEMCELL Technologies, Catalog #72302) was added during passage to improve survival and removed the next day. 2.5ug/ml of Laminin 511 E8 fragment (Takara, Catalog #T304) 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[32]. 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. 3 days after transfection, 1µg/ml of puromycin was added into 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 35mm Glass dish was coated with PVA-PEG hydrogel and hardened to ~100kPa following the previously described protocol. 1.5*10^5 H2B-mCherry cells 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 15min/frame for 72hrs. ~100 cells 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 15min/frame, the cell cycle of each cell was tracked at least 3 times and the mean value was recorded.

Cell culture on hydrogel

Glass bottom dishes (Iwaki, Catalog #3961-035) was coated using Bindsilane (GE Healthcare, Catalog # 10600047) following the manufacturer's instruction to improve

hydrogel binding. Dishes were then coated with MPC polymer (NOR corporation, LIPIDURE®) to avoid attachment of cells on the none gel part of the dished following manufacturer instructions. The prefabrication solution was thawed and briefly vortexed. 20 µL prefabrication solution was added to one dish. 22mm round cover glass coated with Sigmacote® (Sigma-Aldrich, Catalog # SL2-25ML) 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 (Panasonic, Catalog #ANUJ3500-NC). 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 2min. For RNA-seq, the Hard gel was hardened using 100% output power UV for 2min and the soft gel was hardened using 50% output power UV for 10sec. After gelation, the Cover glass was gently removed, and then hydrogel was activated and functionalized using Laminin511 E8 fragment. Cells were seeded following standard protocol. The culture medium was half replaced the next day of passage and were all replaced 2days 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 (BRUKER, NanoWizardIIR). 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 V7 software.

Micropatterned culture on gel

In patterning culture, cells need to 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 35mm glass-bottom dish and 20ul 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 (NEOARK CORPORATION, DDB-701-DL). Gel with different stiffness was generated by given spatially different UV exposure. Then coverslip was removed and hydrogel was activated and functionalized with Laminin511 E8 fragment. $1*10^6$ hESCs (KhES-1) were collected and resuspended in 200µL of AK02N medium with 10uM Y27632. 20ul of suspension was added on top of the gel pattern and incubated at 37 °C, 5% CO_2 for 30min to allow cells to attach. The gel was washed with PBS once to remove exceeded cells and the PDMS spacer was removed. 1ml AK02N medium 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 (ThermoFisher, Cat # A-11003) following the protein binding protocol described previously. Photos were taken using a fluorescence inverted microscope (Leica, SP8 LIGHTNING Confocal Microscope) and the fluorescence intensity was quantified using Image J.

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, 3 times for 5min each. PBS was replaced with 0.3% Triton-X100/PBS and samples were incubated 5min at room temperature. Samples were then blocked with Blocking One (NACALAI TESQUE, 03953-66) for 1h 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 3 times. Then samples were incubated with secondary antibodies and Dapi diluted in blocking one, 1/500 for 1h at room temperature. Secondary antibodies were removed and samples were washed in 0.05% Tween 20/PBS for 5 min, 3 times. The samples were then observed using a microscope.

RNA extraction and quantitative PCR

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

RNA-seq

Gel-coated 35mm glass-bottom dishes were prepared as previously described, Gel was hardened by 50% 10sec (soft gel) or 100% 2min (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 (QIAGEN, Cat#74106) following the manufacturer's instruction. 2 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 (NEB, Cat# E7490), NEBNext Ultra II Directional RNA Library Prep Kit (NEB, Cat# E7760), NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) (NEB, Cat# E6440) 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 NovaSeq6000 NGS system. GRCh38 p13 (ver103) was used for transcriptome annotations. Normalization was performed by TMM (Trimmed mean of M values), Pearson correlation, and Ward's method. p-value was adjusted < 0.01, LFC > 0 (up): 1313, 3.9%. LFC < 0 (down): 1062, 3.1%, outliers: 0, 0%, low counts: 19597, 58% (mean count < 19).

Figure legends

Figure 1: Schematic illustration of photocurable PVA-PEG hydrogel

(a) PVA-MA forms COOH-containing hydrogel upon 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. The scale bar is 200µm.
(f) Characteristics of PVA-PEG hydrogels.

Figure 2: 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 gel and dish, each dots represent one cell.
(c) IF of Day6 hPSCs cultured on gels.

Figure 3: Immunofluorescence and qPCR of lineage makers for each germ layer.

Figure 4: RNA-seq of hPSCs cultured on hard, soft hydrogel, and PS dish

(a) Cluster Dendrogram of hPSCs cultured on soft, hard gel and PS dish.
(b) Enhanced volcano plot of differently expressed gene between hPSCs cultured on the soft(right) and hard(left) gel, NS stands for not significant. Y-axes represent possibility, and X-axes represent fold change.
(c) Heat map of Nodal family expression.
(d) Top 6 enriched gene groups by GO analysis

Figure 5: Manipulating stem cell fate by adjusting local stiffness of the hydrogel

(a) Differentiation timetable for mesoderm differentiation
(b) IF of 24hrs mesoderm differentiation on soft and hard gels.
(c) IF of 24hrs 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 100µm.

Supplementary

List of oligos

	Forward	Reverse	Ref
Oct4	CCTGAAGCAGAAGAGGATCACC	AAAGCGGCAGATGGTCGTTTGG	*Origene, HP206340*
PAX6	CTGAGGAATCAGAGAAGACAGGC	ATGGAGCCAGATGTGAAGGAGG	*Origene, HP225517*
OTX2	GGAAGCACTGTTTGCCAAGACC	CTGTTGTTGGCGGCACTTAGCT	*Origene, HP214209*
SOX1	GAGTGGAAGGTCATGTCCGAGG	CCTTCTTGAGCAGCGTCTTGGT	*Origene, HP209017*
PBGD	GGAGCCATGTCTGGTAACGG	CCACGCGAATCACTCTCATCT	*Kyle M. Loh et al., 2014*
FOXA1	AAGGCATACGAACAGGCACTG	TACACACCTTGGTAGTACGCC	*Ang et al., 1993; Sasaki and Hogan, 1993*
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA	*Ang et al., 1993; Sasaki and Hogan, 1993*
Hhex	CACCCGACGCCCTTTTACAT	GAAGGCTGGATGGATCGGC	*Thomas et al., 1998*
SOX17	CGCACGGAATTTGAACAGTA	GGATCAGGGACCTGTCACAC	*Kanai-Azuma et al., 2002*
TBX6	AAGTACCAACCCCGCATACA	TAGGCTGTCACGGAGATGAA	*Kyle M. Loh et al., 2016*
MSGN1	CGGAATTACCTGCCACCTGT	GGTCTGTGAGTTCCCCGATG	*Kyle M. Loh et al., 2016*
CDX2	GGGCTCTCTGAGAGGCAGGT	CCTTTGCTCTGCGGTTCTG	*Kyle M. Loh et al., 2016*
HAND1	GTGCGTCCTTTAATCCTCTTC	GTGAGAGCAAGCGGAAAAG	*Kyle M. Loh et al., 2016*
ISL1	AGATTATATCAGGTTGTACGGGATCA	ACACAGCGGAAACACTCGAT	*Kyle M. Loh et al., 2016*

NKX2.5	CAAGTGTGCGTCTGCCTTT	CAGCTCTTTCTTTTCGGCTCTA	*Kyle M. Loh et al., 2016*

List of antibodies

Target	Manufacturer	Host animal	Dilution
T	R&D, AF2085	Goat	500
SOX2	Abcam, ab79351	Mouse	100
TBX6	R&D, AF4744	Goat	500
T	Abcam, ab20680	Rabbit	100
SOX17	R&D, AF1924	Goat	40
PAX6	R&D, AF8150	Mouse	1000
SOX1	Cell signaling, #4194	Rabbit	500
CDX2	Abcam, ab76541	Rabbit	500
HAND1	R&D, AF3168	Goat	200
NANOG	Abcam, #4893	Goat	500
OCT3/4	Abcam, ab181557	Rabbit	500
Alexa Fluor™ 546 Phalloidin	Invitrogen, A22283		500
Alexa Fluor™ 647 Phalloidin	Invitrogen, A22287		500

Ethics declarations

Competing interests

The authors declare no competing interests.

Contributions

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

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