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# Polyacrylamide Hydrogels with Rigidity-Independent Surface Chemistry Show Limited Long-Term Maintenance of Pluripotency of Human Induced Pluripotent Stem Cells on Soft Substrates

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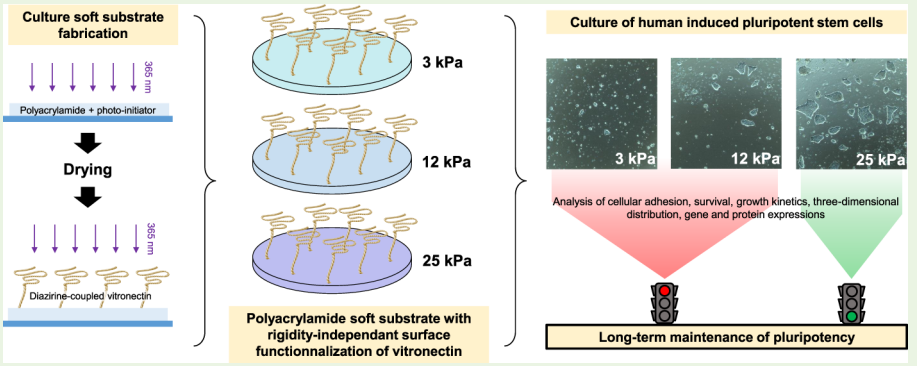
*Supporting Information*




**ABSTRACT:** In general, cells are cultured and adapted to the in vitro rigidities of plastic or glass ranging between 1 and 10 GPa, which is very far from physiological values that are mostly in the kilopascal range. Stem cells however show a high sensitivity to the rigidity of their culture environment, which impacts their differentiation program. Here, we address the impact of rigidity on the long-term maintenance of pluripotency in human induced pluripotent stem cells (hiPSCs) to determine whether soft substrates could provide a new standard for hiPSC expansion and maintenance. To do this, we set up a fabrication process of polyacrylamide-based culture supports with a rigidity-decoupled surface chemistry. Soft elastic substrates with uniform and reproducible physicochemical properties were designed. The maintenance of pluripotency of two hiPSCs lines on substrates with stiffnesses ranging from 3 to 25 kPa was studied with an identical chemical coating consisting of a truncated recombinant vitronectin with defined surface density. Based on the analysis of cellular adhesion, survival, growth kinetics, three-dimensional distribution, and gene and protein expressions, we demonstrate that below 25 kPa hiPSCs do not maintain pluripotency on long-term culture, while pluripotency and self-renewal capacities are maintained above 25 kPa. In contrast to previous studies, no drift toward a specific germ line lineage was revealed. On soft substrates, cell colonies started to grow in three-dimensional (3D), suggesting that softness allows cells to limit contact with the synthetic matrix and to build their own microenvironment. These observations drastically limit the benefit of using standardized soft substrates to expand hiPSCs, at least with the current culture conditions. The development of a robust technology for the design of soft substrates nevertheless opens up perspectives to fine-tune physicochemical properties of the culture environment in addition to or in replacement of soluble growth factors to finely direct cell fate.

**KEYWORDS:** *pluripotent stem cells, hydrogel, stiffness, surface chemistry, mechanobiology*

## INTRODUCTION

Over the last few decades, human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), have been at the heart of the most fascinating biomedical applications.[1−3] A key focus for scientists in the stem cell research field has always been to develop the most efficient and reliable culture methodology to maintain those cells in an undifferentiated and proliferative state. Chemical additives have been extensively tested, and current protocols to maintain hPSCs mostly involve the use of soluble factors and defined coating proteins. As important as these signaling molecules are, there

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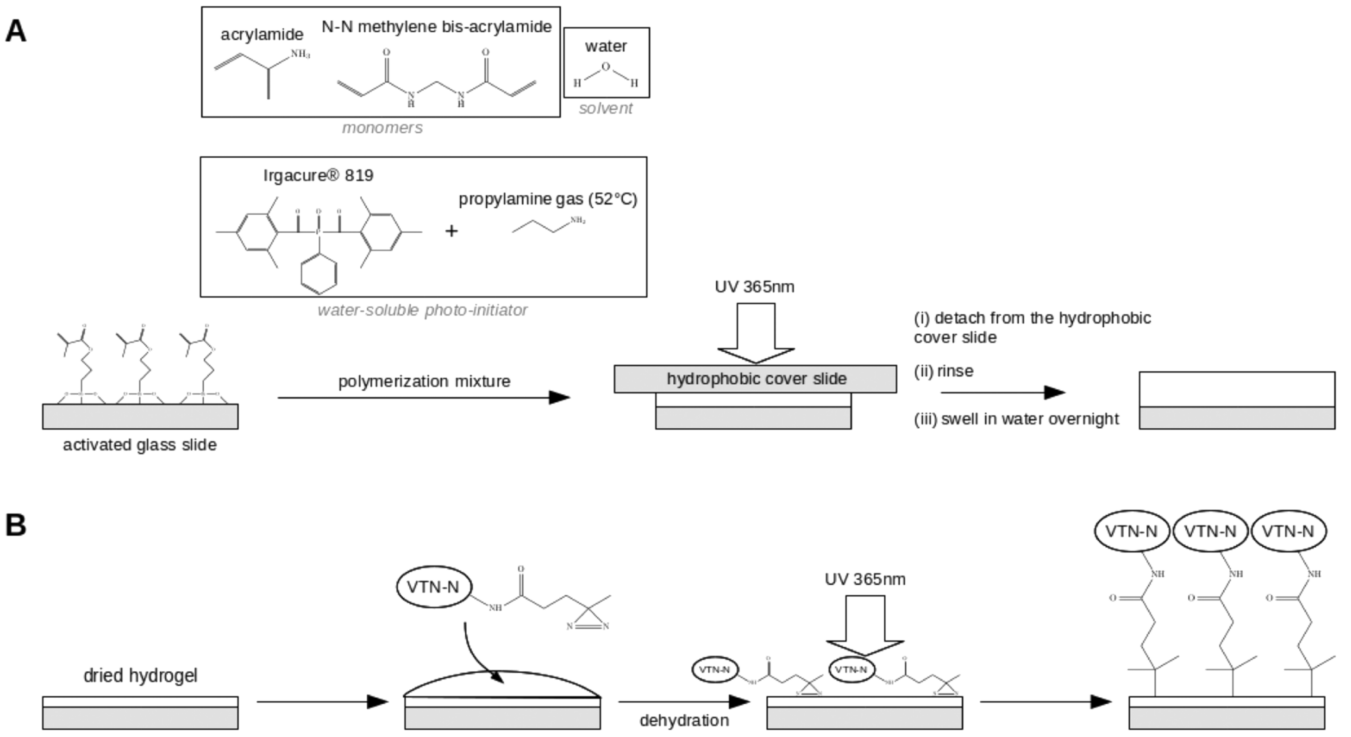


**Figure 1.** Schematic representation of the methods to obtain PSS for cell culture. (A) Fabrication of the polyacrylamide matrix from a UV-sensitive solution of monomers. (B) Covalent grafting of the surface chemistry in a rigidity-independent manner.

has been accumulating evidence suggesting that they are not the only factors influencing the in vitro maintenance and development of hPSCs. The physical properties of the local environment have also been identified as a promising parameter to control stem cell growth and differentiation.[4,5] For instance, the compliance of the extracellular matrix is also important since extracellular matrices with close-to-physiological mechanical properties have been shown to orient cell commitment to specific lineages.[6,7] For example, cardiac maturation was improved by using soft culture environments with a rigidity similar to that of cardiac tissues, of a few tens of kilopascal.[8,9]

While the use of extracellular matrices with close-to-physiological stiffness has proved beneficial for the differentiation of progenitor cells, there is no consensus on the influence of soft culture environments on hPSC. Several authors have reported stiffness-induced oriented differentiation of PSCs,[10−12] while others found that the differentiation of PSCs into mesenchymal stem cells was not sensitive to the stiffness of the culture environment.[13] Several studies have addressed the influence of the stiffness of the extracellular matrix on the maintenance of pluripotency. Scattered and sometimes contradictory results can be found in the literature. For instance, mouse embryonic stem cells (mESCs) were shown to maintain self-renewal and pluripotency on soft substrates around 0.6 kPa for up to 15 passages in the absence of the leukemia inhibitory factor (LIF), contrary to plastic where LIF was required.[14] In contrast to this, hESCs were shown to remain pluripotent on stiff supports and enter germ-layer differentiation on soft supports.[15−18] Together, these results suggest that mouse and human ESCs have distinct mechanosensitive responses, pointing the need to conduct investigations specifically on human and not mouse cells to evaluate the potential of induced PSCs for tissue engineering or regenerative medicine. However, reports on hiPSCs are less numerous and show scattered observations that are also not consistent. For instance, and contrary to,[15−18] very few studies found that the maintenance of pluripotency of hPSCs was not sensitive to rigidity.[13,19] Discrepancies may be due to hiPSCs lines derived from different cellular origins but also due to small but nonetheless significant differences in the physicochemical properties of the culture substrates (i.e., elastic versus viscoelastic, chemical composition, surface coating density, topography). To some extent, reports on hESC bear similar inconsistencies. While most observations on hESCs concluded that stiff environments are required throughout the process of amplification for maintenance of pluripotency, the reported stiffness values varied from 0.7 kPa[11] to several hundreds of megapascal.[16] Such discrepancies in cell responses compromise the potential of hiPSCs to ever achieve their full promises.

Development of "gold standard" culture methods for the maintenance of pluripotency has been extensively addressed in terms of media formula[20] and chemical culture environment for hiPSCs.[21] However, there is a lack of similar standardization in terms of physical and mechanical environments required for hPSCs. This is especially concerning specific applications where, for example, the maintenance of pluripotency in a soft environment needs to be finely controlled before a specific differentiation process is initiated. Several authors have reported stiffness-induced oriented differentiation of PSCs,[10−12] while others found that the differentiation of PSCs into mesenchymal stem cells was not sensitive to the stiffness of the culture environment.[13]

As mentioned previously, a comparison of studies that investigated the mechanosensitivity of stem cells has underlined the importance of considering all of the variations from the origin of the hiPSCs to the substrate properties at the


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macroscopic scale down to the molecular scale such as the precise rheology (elastic versus viscoelastic), the degradability, the surface chemistry, the coating density, and the topography.[22] In this context, this study aims to contribute to the design of robust elastic substrates, which could help decipher the potentialities of cultivating hPSCs on substrates with close-to-physiological elastic properties. For this purpose, we chose polyacrylamide as the mechanical basis for the substrate, as this material has a linear elastic behavior when submitted to cellular stresses, on the order of kPa (nN/$\mu m^2$).[23,24] We have developed a novel technology to make two-dimensional (2D) polyacrylamide soft culture environments with reproducible compliances and independent, decoupled surface chemistry, thus providing controlled mechanochemical properties. As described in detail below, achieving a robust decoupling between rigidity and surface chemistry is indeed critical for advances in biomaterials but is rarely robustly attained.[25] This study also used truncated human recombinant vitronectin (VTN-N) for surface coating instead of the more commonly used Matrigel coating, as it has long been recognized as a robust chemical environment for hPSCs culture.[26] These newly designed synthetic extracellular matrices were employed to investigate the mechanosensitivity of two hiPSCs lines. Differences in terms of cellular adhesion, survival, growth kinetics, three-dimensional distribution, maintenance of pluripotency, or derivation of hiPSCs lines were analyzed for up to 6 passages. In this context, we could evaluate the influence of soft compliances of the extracellular matrix on hiPSC lines during long-term culture in fabricated systems where the surface density of the adhesive coating protein is defined and rigidity-independent and where there is an absence of cell-scaled topography, a cue present in electrospun materials and known to impact hPSC fate.[27] Within these fully controlled and defined culture systems, we could unambiguously demonstrate that long-term culture on soft substrates was not able to maintain hiPSCs pluripotency, similarly to hESCs.

## MATERIALS AND METHODS

**Fabrication of Polyacrylamide Soft Substrate (PSS) for Cell Culture.** Different degrees of stiffness of polyacrylamide soft substrates (PSS) for cell culture, ranging from 3 to 25 kPa as attested by atomic force microscopy (AFM), were developed with patent protection (WO2013079231) (Figure 1). In brief, the monomer solution for polyacrylamide was prepared with a UV-sensitive initiator. First, 2 mg of Irgacure 819 (Ciba Specialty Chemicals, Basel, Switzerland) was dissolved into 10 $\mu$L of propylamine at 52 °C for 10 min. Then, 490 $\mu$L of deionized water, 250 $\mu$L of a 40% solution of acrylamide, and 250 $\mu$L of a 2% solution of *N*,*N*-methylene-bis-acrylamide (Bio-Rad, Marnes-la-Coquette, France) were added, leading to a 10−0.5% mixture of monomers. Coverslips of 20 mm diameter were chemically activated using Bind Silane (Thermo Fisher Scientific, Saint-Herblain, France) to allow stable, covalent bonding of the polyacrylamide hydrogels: the coverslips were first cleaned in a 0.1 M NaOH solution for 10 min and then rinsed in deionized water followed by an ethanol bath. The coverslips were dried using dry air. A solution containing 484 $\mu$L of acetic acid and 56 $\mu$L of BindSilane completed up to 15 mL with absolute ethanol was prepared. Next, 200 $\mu$L of this solution was pipetted to each coverslip and wiped off after a few seconds with a dust-free wiper (Figure 1A).

A droplet of 17 $\mu$L of the precursor solution was deposited on the activated coverslip. A hydrophobic glass slide equipped with 40 $\mu$m-thick wedges was placed onto the droplet to force the spreading of the precursor solution with a flat surface. The hydrophobic treatment was obtained by grafting a fluorinated silane onto it:[28] a clean glass slide was immersed into a 0.1% solution of Optool (Daikin Chemical, Düsseldorf, Germany) in perfluorohexane for 1 min. It was allowed to react for 1 h in water vapor at 65 °C. It was then rinsed for 10 min in perfluorohexane. The glass slide was fitted with 40 $\mu$m-thick wedges on its edges.

The PSS was reticulated by exposing the precursor solution sandwiched between the activated coverslip and the hydrophobic glass slide to UV-A (2 W/$cm^2$, Eleco UVP281). The duration of the illumination tuned the mechanical properties of the PSS. Illumination times were varied from 9 to 16 s to obtain rigidities from 3 to 25 kPa. The PSS was then immersed in water and carefully detached from the mask. It was rinsed three times in deionized water to remove unreacted compounds and stop the polymerization reaction. The PSS was then left to swell in deionized water overnight to complete the removal of unreacted compounds (Figure 1A).

**Characterization of the Rigidity of the PSS.** Young's moduli of the PSS were measured on Nanowizard II AFM (JPK Instruments, Berlin, Germany) in the force mapping mode. MLCT C tip cantilevers (Bruker, Palaiseau, France) with a nominal spring constant of 0.01 N/m were chosen for their ability to address Young's moduli between 0.1 and 50 kPa. Young's moduli were fitted to the indentation curves using a Hertz−Sneddon model. Three to five rigidity maps of 100 $\mu$m × 100 $\mu$m with 10 $\mu$m sampling were performed throughout each PSS.

**Rigidity-Decoupled Conjugation of the PSS with Adhesion Molecules.** Truncated recombinant human vitronectin (VTN-N, Gibco) was covalently cross-linked to the surface of the PSS using a photoactivatable heterobifunctional reagent sulfosuccinimidyl 6-(4,4′-azipentanamido)hexanoate (sulfo-LC-SDA, Pierce) cross-linker to obtain 0.5 $\mu$g/$cm^2$ VTN-N at the surface of each PSS (Figure 1B). To do so, the photosensitive cross-linker was first conjugated to VTN-N: 0.4 mg of sulfo-LC-SDA was dissolved in 1 mL of VTN-N and left to react for 30 min at room temperature, leading to an expected ratio of 86 molecules of sulfo-LC-SDA per VTN-N molecule. The solution was then dialyzed against PBS at 4 °C for 48 h through a 12−14 kDa membrane to remove the unreacted sulfo-LC-SDA molecules. The resulting diazirine-coupled VTN-N was then aliquoted and stored at −20 °C.

The concentration of the diazirine-coupled VTN-N was quantified by immunofluorescence. Uncoupled VTN-N with bulk concentration of 0.5 mg/mL as well as diazirine-coupled VTN-N with similar expected concentrations was evaporated onto glass slides and then rinsed with PBS. Both of the glass slides were stained for vitronectin: the glass slides were saturated with a solution of PBS containing 0.1% Tween and 2% BSA for 30 min at room temperature and incubated with a primary polyclonal antibody against vitronectin (Rabbit IgG, dilution 1:100, Thermo Fisher Scientific, Saint-Herblain, France) for 1 h at room temperature and then with an Alexa-conjugated secondary antibody A488 (Thermo Fisher Scientific, Saint-Herblain, France) (1 h, room temperature). Fluorescence intensities were compared using confocal microscopy (TCS SP5, Leica Microsystems, Nanterre, France): an image stack with 0.288 $\mu$m spacing was captured at a wavelength of 488 nm, thick enough to ensure the capture of the whole intensity signal. The mean of the intensity was calculated and compared between both conditions from images of 375 $\mu$m × 375 $\mu$m. The absolute concentration of the diazirine-coupled VTN-N had a value of 0.3 mg/mL.

The diazirine-coupled VTN-N was cross-linked to the surface of the PSS. The PSS were first dehydrated for at least an hour in a flow hood at room temperature until they became dry. Diazirine-coupled VTN-N was diluted in sterile, filtered, deionized water to reach a concentration of 5.25 $\mu$g/mL. Then, 300 $\mu$L of this solution was deposited on the PSS and left for approximately an hour, until the surface was dry, leading to the deposition of 0.5 $\mu$g/$cm^2$ diazirine-coupled VTN-N at the surface of the PSS. The diazirine-coupled VTN-N was then cross-linked to the PSS by 5 min of UV illumination (2 W/$cm^2$, Eleco UVP281) (Figure 1B). Dilution of the stock solution of diazirine-coupled VTN-N in water did not significantly affect the functionality of the VTN-N, as shown by the similar affinity


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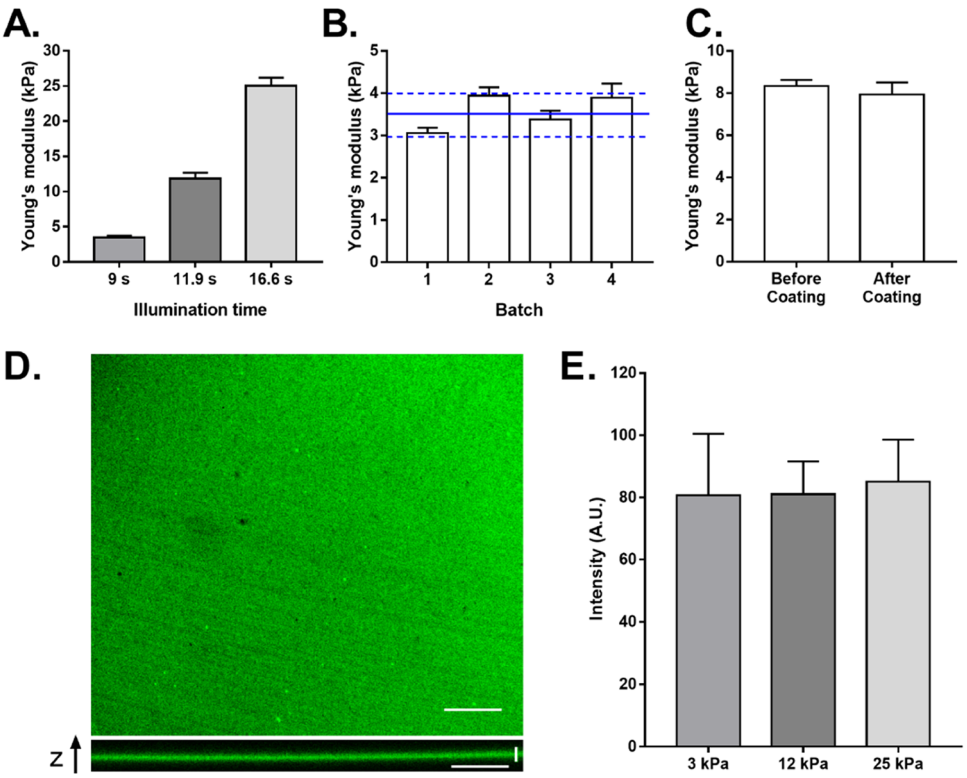


**Figure 2.** Physical characterization of the PSS. (A) Young's moduli of the samples used in this study ($n_{PSS}$ = 3−4, 300−500 measurement points per PSS, scattered on 7 cm$^2$). (B) The fabrication process limits the variability of the rigidity through the sample (dispersion ∼10% on 7 cm$^2$) and allows interbatch reproducibility with less than 15% variability (UV exposure time: 9 s; $n_{PSS}$ = 1 per batch; 300−500 measurement points scattered on 7 cm$^2$ for each batch). (C) UV cross-linking of the surface coating does not impact the rigidity of the matrix. All Young's modulus data obtained by atomic force microscopy (illumination time: 10.7 s; $n_{PSS}$ = 2 per condition; 400 measurement points scattered on 7 cm$^2$). (D) Surface coating with the diazirine-coupled VTN-N of the PSS. The VTN-N is covalently grafted to the surface and does not penetrate into the hydrogel. Horizontal bar is 50 $\mu$m. Vertical bar is 10 $\mu$m. (E) The surface density of VTN-N revealed by immunolabeling was independent of the rigidity of the hydrogel ($n_{PSS}$ = 2 per condition). Values are given as mean ± SD.

of the antivitronectin antibody compared to VTN-N-coated glass (data not shown).

The PSS was gently rinsed three times with PBS and immersed in PBS overnight at 4 °C for rehydration. One hour before cell seeding, the PBS was removed and replaced by a complete culture medium. The density of VTN-N at the surface of the PSS was characterized by immunostaining and confocal microscopy, following an identical staining protocol as described above.

**hiPSCs Culture.** Two hiPSCs lines (i90 and iKCL004) were used in this study. i90, derived from female human lung fibroblasts (IMR-90, ATCC, CCL-186), was generously provided by Dr. Xavier Nissan (I-Stem, Corbeil-Essonnes, France), and iKCL004, derived from human neonatal foreskin fibroblasts (BJ, ATCC, CRL-2522), was generously provided by Dr. Dusko Ilic (King's College London, Stem Cell Laboratory, London, U.K.).[29,30] Both hiPSCs lines were cultured under standard feeder-free conditions, with an mTeSR1 medium (Stem Cell Technologies, Grenoble, France) on plates coated with truncated recombinant vitronectin (VTN-N) (Life Technologies, Saint-Aubin, France) at 37 °C and 5% $CO_2$. The mTeSR1 medium was replaced daily. Once the cells reached 70−80% confluence, they were briefly treated with 0.5 mM ethylenediaminetetraacetic acid (EDTA) (Life Technologies, Saint-Aubin, France) and split at a 1:6 ratio into a new dish. After three passages to recover from thawing, cells were seeded either onto 0.5 $\mu$g/cm$^2$ vitronectin-coated plastic plates or onto PSS at an adapted split ratio of 1:4 and amplified throughout six passages. The values of the two cell lines were pooled to obtain the results presented in the figures.

**Cell Differentiation.** Embryoid bodies (EBs) were produced by the hanging drop method described by Lin and Chen, allowing the formation of single cell drops containing 2000 cells/20 $\mu$L.[31] After two days, incubated EBs were washed off into low attachment dishes. The medium was switched to E6 Medium (Life Technologies, Saint-Aubin, France) to allow spontaneous differentiation and changed every 2 days during 9 or more days. EBs were then spread into gelatin-coated wells following the manufacturer's instructions for 4 additional days, requiring a total of 15 days. At the end, single attached and spread EBs were suspended and lysed in TRIzol Reagent (Life Technologies, Saint-Aubin, France) and preserved at −80 °C to further measure gene expression.

Trilineage differentiation was carried out using the STEMdiff Trilineage Differentiation Kit (Stem Cell Technologies, Grenoble, France) following the manufacturer's protocol to differentiate hiPSCs into ectoderm, endoderm, and mesoderm. Briefly, cells were isolated with Accutase (Stem Cell Technologies, Grenoble, France) and plated at 200000 cells/cm$^2$ (ectoderm and endoderm) or 50 000 cells/cm$^2$ (mesoderm). The appropriate medium was changed daily, and cells were harvested on day 5 (endoderm and mesoderm) or day 7 (ectoderm) in TRIzol reagent for gene expression analysis.

Spontaneous differentiation was carried out using E6 Medium (Life Technologies, Saint-Aubin, France). Briefly, after passaging hiPSCs at 70−80% confluency with mTeSR1 medium, spontaneous differentiation was allowed by switching the cells to E6 Medium (Life Technologies, Saint-Aubin, France). Once confluency was reached, cells were recovered in TRIzol reagent (Life Technologies, Saint-Aubin, France) and stored at −80 °C prior to gene expression analysis.

**Immunostaining.** Cells were washed with PBS and fixed with cold methanol for 5 min. After two additional washing steps, cells were incubated with 5% bovine serum albumin (BSA) for 1 h, followed by a 90 min incubation at room temperature with antibodies against Nanog (mouse IgG, dilution 1:50, Santa Cruz Biotechnology, Heidelberg, Germany) and Ki67 (rabbit IgG antibody, dilution 1:100,


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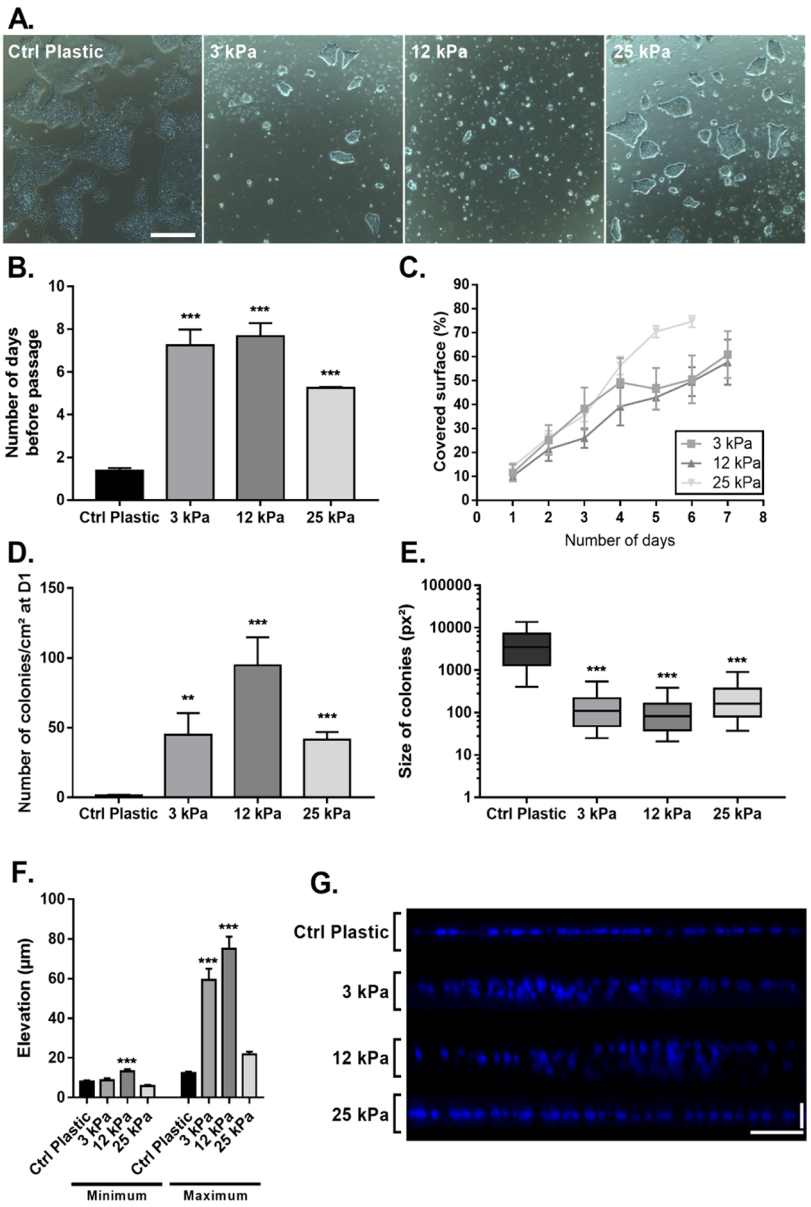


**Figure 3.** Impact of rigidity on the adhesion and growth of hiPSCs. (A) Adhesion of hiPSCs clumps after passage depends on the rigidity of the substrate (scale bar = 500 $\mu$m). (B) Incidence of the PSS rigidity on the growth of hiPSCs measured by the number of days required to be 70−80% confluent and ready for passage ($n$ = 4). (C) Growth kinetics of hiPSCs evaluated by cell culture surface coverage followed during six passages (day 0 corresponds to the day of passage). (D) Number of adherent colonies ($n$ = 6−12) and (E) distribution of size of hiPSCs' colonies 24 h after passage (at least 600 colonies were analyzed). (F) Quantification of minimum and maximum thicknesses of colonies of hiPSCs cultured for six passages on different PSS ($n$ = 8−10). (G) Orthogonal view of confocal z-stack images of the nuclei (Hoescht staining) of hiPSCs colonies cultured for six passages on different PSS demonstrating the superposition of nuclei when cells were cultured on 3 and 12 kPa PSS (horizontal scale bar = 50 $\mu$m; vertical scale bar = 30 $\mu$m). Statistical significance is indicated as $^{**}p$ <0.01; $^{***}p$ <0.001 compared with the plastic group. Nonsignificant differences are not indicated. Values are given as mean ± SEM.

Abcam, Paris, France). After three washes in phosphate-buffered saline (PBS), cells were incubated in the presence of secondary antibodies (Alexa 488 goat-antimouse and Alexa 568 goat-antirabbit, dilution 1:1000; Life Technologies, Saint-Aubin, France). After three additional washes in PBS, the samples were mounted with Mowiol containing 5 $\mu$g/mL Hoescht 33342 (Life Technologies, Saint-Aubin, France). Images were captured using a motorized confocal laser scanning microscope (TCS SP5, Leica Microsystems, Nanterre, France).

**Cytometry.** The analysis of pluripotency was performed after cell staining with the Human Pluripotent Stem Cell Transcription Factor Analysis kit (BD Biosciences, Le Pont de Claix, France) following the manufacturer's instructions. Then, cells were analyzed with MACSQuant analyzer 10 (Miltenyi Biotec, Paris, France) on the Imaging platform of Paris-Seine (IBPS, France).

**Gene Expression.** Total RNA was extracted from the cell pellets using TRIzol reagent (Life Technologies, Saint-Aubin, France). From 250 ng of extracted RNA, the first-strand cDNA was then synthesized using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Saint-Herblain, France) with the anchored-oligo(dT)18 primer and according to the manufacturer's instructions. Using the Light Cycler 480 system (Roche Diagnostics), the reaction was carried out in duplicate for each sample in a 6 $\mu$L reaction volume containing 3 $\mu$L of SYBR Green Master Mix, 500 nM of the forward and reverse primers each, and 3 $\mu$L of diluted (1:25) cDNA. The thermal profile for SYBR Green qPCR was 95 °C for 8 min, followed


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by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. To exclude PCR products amplified from genomic DNA, primers were designed, when possible, to span one exon−exon junction. The mean gene expression stability of two genes, SLC4A1AP (solute carrier family 4 anion exchanger member 1, adaptor protein) and NUBP1 (nucleotide binding protein 1), was used as the reference transcript. Data ($\Delta Ct$) were collected and analyzed using LightCycler 480 software release 1.5.0 (Roche Diagnostics). Lineage expression scores were calculated as follows: each gene expression $\Delta Ct$ value was weighted in relation to the highest value measured among the groups. Genes were then grouped into categories (a-pluripotency: *NANOG*, *OCT4*, *SOX2*; b-mesoderm: *T*, *MIXL1*, *NCAM*; c-endoderm: *CER1*, *FOXA2*, *HHEX*, or *DAF1*; d-ectoderm: *DLK1*, *LHX2*, *RAX*, or *PAX6*), and the weighted values for each category were averaged to obtain the scores. Specific lineage expression scores were calculated as the ratio between the lineage (mesoderm, ectoderm, or endoderm) expression score and the pluripotency expression score.

**Statistics.** The dispersion of the physical characteristics of the PSS was evaluated as follows. Within a batch that consisted of a new solution of polyacrylamide precursors, the rigidity of $n_{PSS}$ gels was characterized by AFM. Maps of 100 measurement points were performed as described above. The mean value and the standard deviation of the rigidity were calculated from the concatenation of all of the measurement points of all of the batches (Figure 2A,C) or within a batch (Figure 2B). Concerning cell analysis, conditions were statistically compared using GraphPad Prism 7.00 (GraphPad Software Inc.). Normality was checked using the Shapiro−Wilk normality test, and equal variance was checked with the test of Bartlett. If normality of distributions and/or equal variance was not assumed, the nonparametric Kruskal−Wallis test was used instead of analysis of variance (ANOVA). If a significant difference was found, then multiple comparison tests were performed to compare the different groups analyzed (Dunn's or Bonferroni's multiple comparison test following Kruskal−Wallis or ANOVA, respectively). The level of significance was set to 0.05. Values are given as means ± standard error of the mean (SEM).

## RESULTS

**Physical Characteristics of Polyacrylamide Soft Substrate (PSS).** The main goal of this study was to determine the effect that the degree of stiffness of the underlying substrate has on hiPSCs culture behavior. To this end, we fabricated PSS of different rigidities and functionalized with truncated recombinant vitronectin (VTN-N) using a novel technology as described in Figure 1. PSS with three different degrees of stiffness (3, 12, and 25 kPa) were obtained by varying the illumination time of a photosensitive solution of polyacrylamide precursors.[32] The Young's modulus of the substrate was then assessed by atomic force microscopy (Figure 2A) and demonstrated a high reproducibility among the different batches fabricated with a low standard deviation, in the range of 15% (Figure 2B). We also demonstrated that the functionalization procedure of PSS using the diazirine-coupled VTN-N did not affect the Young's modulus (Figure 2C). Moreover, we assessed the uniformity of the VTN-N functionalization by immunofluorescence and demonstrated that this procedure allows a homogeneous coating of VTN-N all over the surface of the PSS (Figure 2D). More importantly, we observed that the functionalization procedure of PSS as described in Figure 1B allowed a reproducible coating of VTN-N with a surface density that is independent of the degree of stiffness (Figure 2E). As shown in Supporting Information Figure S1, this result is a true improvement in the fabrication process of PSS, as surface coating of polyacrylamide hydrogels is generally coupled to the stiffness of the PSS in the absence of the newly introduced dehydration step before protein cross-linking (Figure 1B).[25] Moreover, this dehydration step permitted the concentration of the whole protein content of the solution of diazirine-coupled VTN-N onto the surface of the PSS, independent of the stiffness of the PSS. As shown on the confocal cross section in Figure 2D, proteins did not diffuse into the PSS, thus allowing a reliable quantification of the surface coating density (Figure 2E). Altogether, we conclude that the fabrication process leads to VTN-N-coated PSS with less than 15% variability in their degree of stiffness and a rigidity-independent surface density of VTN-N of 0.5 $\mu g/cm^2$.

**Rigidity of Culture Substrate Induces a Change in the Behavior of hiPSCs.** To effectively determine the effect that different rigidities have on hiPSCs culture, two hiPSCs lines were cultured for six passages on the three different fabricated PSS with rigidities close to 3, 12, and 25 kPa and functionalized with 0.5 $\mu g/cm^2$ VTN-N. In parallel, tissue culture polystyrene plates coated with 0.5 $\mu g/cm^2$ VTN-N (plastic) were used as controls with a stiffness evaluated around 3 GPa. All were compared and analyzed for their ability to support the different cell functions, such as adhesion, survival, proliferation, and maintenance of pluripotency.

Differences between PSS on the first day after passage were observed (Figure 3A). On the 3 and 12 kPa PSS, colonies of hiPSCs were poorly attached, leaving few colonies to grow, whereas on 25 kPa PSS, colonies of hiPSCs attached very well to the culture surface and presented similar morphologies compared with colonies of hiPSCs on plastic. These observations demonstrated that the behavior of hiPSC regarding adhesion seemed to be different on lower stiffness PSS when compared with what was observed for 25 kPa and the control.

Partly due to adhesion differences described previously, growth kinetics also displayed significant variations between conditions (Figure 3B,C). As demonstrated in Figure 3B, the mean number of days needed for passage was 1 day for plastic, 5 days for 25 kPa, and over 7 days for 3 and 12 kPa. The number of days needed to reach 70−80% confluency during the six passages remained strongly constant as indicated by the low dispersion of data from the mean. In the cases of 3 and 12 kPa PSS, longer times, associated with large variations and significant differences compared with plastic, demonstrated their unpredictability in terms of growth kinetics. Then, the percentage of surface covered by the cells every day of each passage was measured (Figure 3C). For the 3 first days, the cell spreading was similar for the different rigidities tested. However, from the fourth day, cell spreading seemed to be accelerated for hiPSCs cultured on 25 kPa PSS compared with the PSS of 3 and 12 kPa, where the spreading of cells took more time. Therefore, differential growth kinetics were evidenced according to different rigidities of the culture substrate. The observed differences concerning growth kinetics could be partially due to a lower number of adherent colonies and/or to their smaller size on softer rigidities. Following this, the number and the size of hiPSCs colonies were evaluated 24 h after plating (Figure 3D,E). At 24 h, the number of colonies in the control condition was very low, since in this condition most of the colonies were touching at least one neighboring colony. As shown in Figure 3D, 50−100 colonies per $cm^2$ were observed for 3, 12, and 25 kPa PSS conditions and showed significant difference compared with plastic. Concerning the size of colonies, hiPSCs colonies were found to be significantly larger when cultured on rigid plastic dishes coated with


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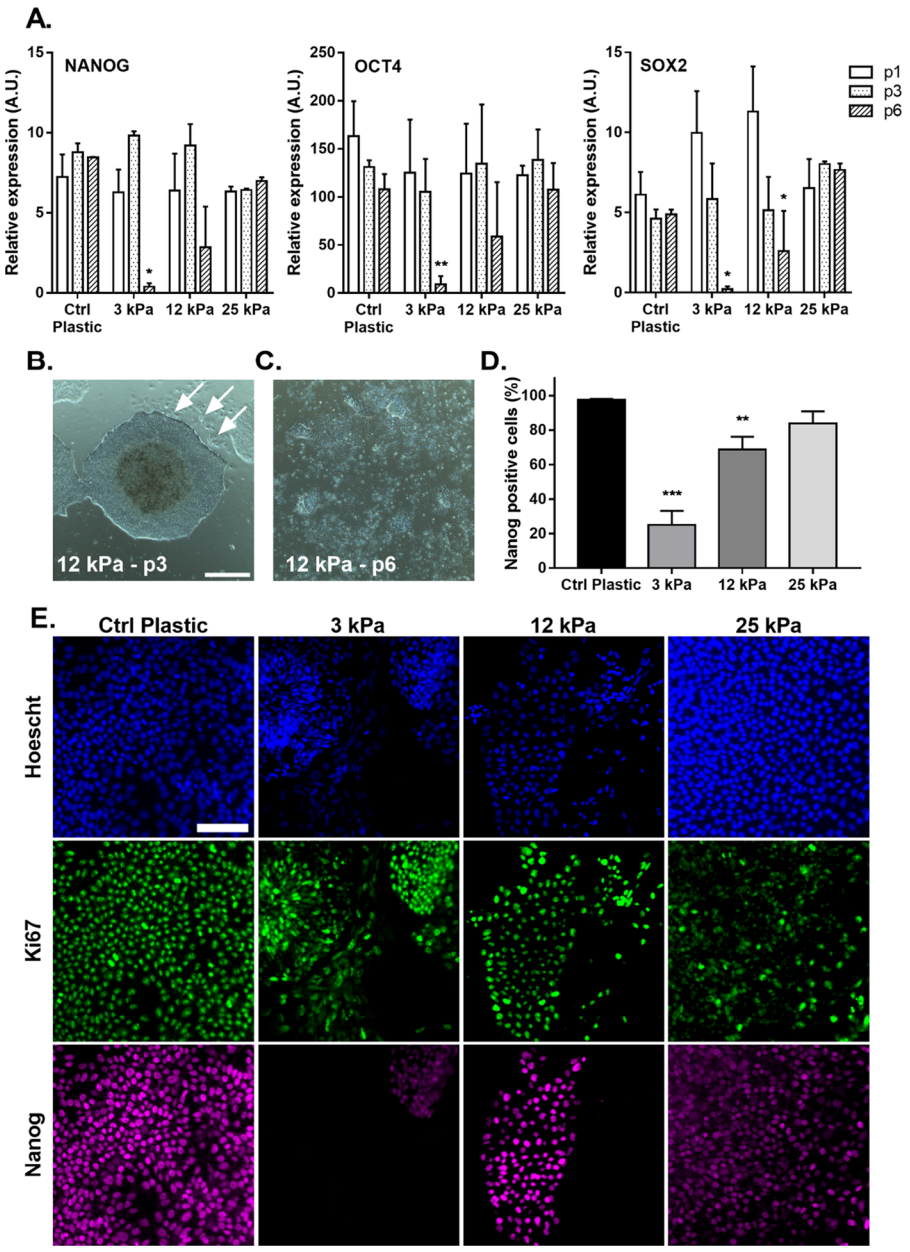


**Figure 4.** Impact of rigidity on maintenance of pluripotency of hiPSC. (A) Relative expression of pluripotency gene markers throughout passage 1 (p1), passage 3 (p3), and passage 6 (p6) on the different PSS ($n = 2$). (B, C) Phase contrast images of cellular derivation between passages 3 and 6 on 12 kPa PSS. (D) Quantification of Nanog positive cells after immunostaining of hiPSCs cultured for six passages on different PSS. (E) Representative images of hiPSCs cultured for six passages and stained for nuclei (Hoescht, blue), Ki67 (green), and Nanog (pink). Scale bar is 100 $\mu$m. Statistical significance is indicated as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared with the p1 group (A) or the plastic group (D). Nonsignificant differences are not indicated. Values are given as mean ± SEM.

Matrigel compared with the different PSS (Figure 3E). Because all of the PSS tested had a similar impact on either the number or the size of hiPSCs colonies, the rigidity of the culture surface appeared to be the main parameter explaining the differences in cell spreading and proliferation rates between 25 kPa PSS and 3 or 12 kPa PSS.

We also observed, during the course of the experiment, the formation of dense cell structures developing at the center of hiPSCs colonies. More specifically, hiPSCs, rather than growing outward by spreading, seemed to grow on top of each other, resulting in the appearance of highly dense three-dimensional (3D) areas visible to the naked eye (Supporting Information Figure S2). Significantly, these dark areas only appeared on PSS and never on coated polystyrene well-plates. In accordance with this observation, the quantification of the thickness of colonies via confocal microscopy (Figure 3F,G) exposed a maximum of 60−75 $\mu$m for colonies cultured on 3 and 12 kPa PSS, significantly different from the plastic (13 $\mu$m of maximum thickness). Colonies cultured on 25 kPa did not show any significant differences compared with the control. These results clearly demonstrated a significant increase in the thickening of colonies cultured on 3 and 12 kPa PSS.

**Softer Rigidities Induce a Loss of Pluripotency of hiPSC.** Nanog, Oct3/4, and Sox2 are transcriptional factors involved in the maintenance of pluripotency and stemness.[33] Expressions of these pluripotency markers were followed by


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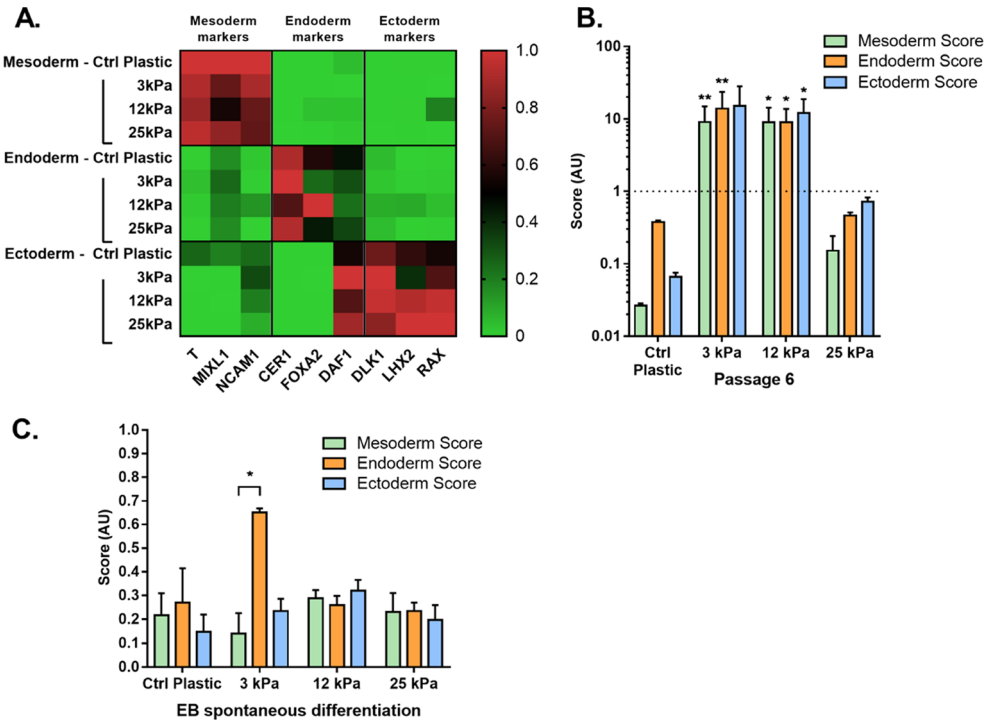


**Figure 5.** Impact of rigidity on commitment and lineage specification of hiPSC. (A) Heatmap of gene expression of three mesoderm, endoderm, and ectoderm markers after induction of trilineage differentiation following 1 passage on different PSS ($n$ = 3). (B) Specific lineage expression scores of hiPSCs after six passages on PSS ($n$ = 4). Dotted line represents the specific pluripotency score for each group. (C) Lineage expression scores of spontaneous differentiated embryoid bodies obtained from hiPSCs after passages on PSS ($n$ = 4). Statistical significance is indicated as *$p$ < 0.05; **$p$ < 0.01 compared with the respective lineage score on plastic (B) or with the two other scores of the group (C). Nonsignificant differences are not indicated. Values are given as mean ± SEM.

qPCR over six passages, precisely at the first, third, and sixth passages for each different PSS (Figure 4A). Our results demonstrate that the expression of NANOG was significantly downregulated on 3 kPa PSS at the sixth passage compared with the first and third ones. Similar observations were performed at passage 6 for the expressions of OCT4 and SOX2 that were downregulated on both 3 and 12 kPa PSS but not for hiPSCs cultured on 25 kPa PSS and plastic. Maintenance of pluripotency for hiPSCs cultured on 25 kPa PSS was also confirmed by flow cytometry throughout three passages for the same markers, Nanog, Oct3/4, and Sox2 (Supporting Information Figure S3). These results clearly indicated that a loss of expression of pluripotency markers occurred between the third and the sixth passage when hiPSCs were cultured on 3 and 12 kPa PSS but not on plastic or on 25 kPa PSS.

The morphology of hiPSCs' colonies cultured on 3 and 12 kPa PSS was also monitored (Figure 4B,C). We observed the first cells with differentiated morphologies from the third passage (see arrows shown in Figure 4B) even if the vast majority of colonies had undifferentiated morphologies. After the sixth passage, few colonies had undifferentiated morphologies and many cells demonstrated morphological signs of derivation (Figure 4C).

In comparison, when hiPSCs were cultured on 25 kPa PSS or plastic, no morphological signs of pluripotency loss were detected (data not shown). To better characterize passage 6, immunostaining was conducted for Nanog as a pluripotency marker and Ki67 as a proliferation marker because it is strictly expressed during active phases of the cell cycle and is only detected in the nuclei when a mitosis occurs.[34] We showed that quantification of Nanog positive cells clearly emphasized the maintenance of pluripotency on 25 kPa PSS and plastic control, while the number of Nanog positive cells decreased sharply on 3 and 12 kPa PSS (Figure 4D,E). Consistently, hiPSCs cultured on plastic and 25 kPa PSS were strongly positive for Nanog and Ki67 labeling (Figure 4E). In contrast, 3 and 12 kPa PSS showed a loss of signal for Nanog although it remained mostly positive for the Ki67 proliferative marker, demonstrating a loss of pluripotency even if cells kept their proliferative capacities. Relative to 3D structures reported earlier (Supporting Information Figure S2), immunolabeling further testified the hypothesis of a cellular growth in 3D colonies and provided evidence that these structures remained pluripotent (data not shown).

**Soft Rigidities Do Not Induce a Specific Lineage Commitment of hiPSCs.** Because we observed a loss of pluripotency of hiPSCs cultured for six passages on 3 and 12 kPa PSS, we first characterized if a specific lineage commitment can occur from the first passage on PSS. To verify this hypothesis, after one passage on PSS, single hiPSCs were cultured with specific lineage inducing media (mesoderm, endoderm, and ectoderm). Gene expressions of different germ-layer markers were measured (Figure 5A). We have shown that hiPSCs cultured for only one passage on PSS maintained their ability to generate mesoderm, endoderm, and ectoderm similar to the control. We then examined whether hiPSCs were committed to a specific lineage after six passages with mTeSR1 on 3 and 12 kPa PSS. Gene expressions of embryonic germ-layer markers were measured by semiquantitative PCR and allowed to obtain a lineage score based on *T*, *MIXL*1, and *NCAM*1 for mesoderm, *CER*1, *FOXA*2, and *DAF*1 for endoderm, and *DLK*1, *LHX*2, and *RAX* for ectoderm. These


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ACS Biomater. Sci. Eng. XXXX, XXX, XXX−XXX

scores were normalized to the pluripotency score measured with the expressions of *NANOG*, *OCT4*, and *SOX2* to obtain specific lineage expression scores (Figure 5B). We observed that after six passages, mesoderm, endoderm, and ectoderm scores were significantly enhanced for hiPSCs cultured on 3 and 12 kPa PSS. In addition, these scores were also higher than the pluripotency score (dotted line), indicating again a loss of pluripotency markers compared with the markers of the three germ layers. Regarding 25 kPa PSS, cells behaved similar to the plastic condition. We could also note that the three different calculated scores were not significantly different from one another in either 3 or 12 kPa, suggesting that differentiated cells were not primed toward a preferential lineage. To confirm this last observation, we analyzed EBs cultured for 13 days without factors of pluripotency (E6 medium, spontaneous differentiation) obtained from cells cultured on PSS for six passages. Previously described lineage scores were calculated for the spontaneously differentiated EBs, and results demonstrated no significant differences between them for each PSS condition, except for the endodermal score in the case of 3 kPa PSS (Figure 5C). This last result indicated that hiPSCs cultured on 3 kPa PSS seemed to be slightly primed toward an endoderm lineage even if this rigidity did not induce an exclusive differentiation toward this lineage. Together, the results demonstrated that the pluripotency loss from cells cultured on the two softer PSS for six passages, namely, 3 and 12 kPa, presented no preferential lineage commitment and subsequently were able to generate all three embryonic germ layers on EBs.

## DISCUSSION

The main aim of our work was to investigate whether mechanical cues could provide a more efficient and physiological approach for expanding and differentiating hiPSCs. In this context, we used polyacrylamide as a soft substrate and VTN-N as chemical coating with the specific requirement of implementing a robust reproducible fabrication technology capable of providing standardized culture conditions. This technology, developed with patent protection (WO2013079231), allowed us to prepare 2D soft culture environments with reproducible rigidities and decoupled surface chemistry offering controlled mechano-chemical properties. The choice of polyacrylamide was made because it responds only elastically to cellular stresses. In the field, materials with viscoelastic rheology have also been investigated but without paying attention to the impact of viscosity. As a matter of fact, viscosity was shown to influence stem cell responses. For instance, several authors have shown that viscoelastic plasma desorption mass spectrometry (PDMS)[35] or fibrin[13] made human mesenchymal stem cell differentiation insensitive to the mechanical properties of the extracellular matrix molecules, in contrast to elastic polyacrylamide.[35] Moreover, hPSCs responses to the physical microenvironment depend to a large extent on the surface chemistry, activating different mechano-transduction pathways. Historically, PSCs were cultured on feeder layers of mouse embryonic fibroblasts. This raised several issues, among them the complexity of coculture and the difficulty of removing contaminating animal-derived fibroblasts. Subsequently, different extracellular matrix proteins or RGD peptides were used as attachment molecules to avoid feeder cells and to use chemically defined culture environments.[11,16,17,21,22] Finally, the consensus of several studies was to use recombinant vitronectin (VTN-N) as the best alternative to Matrigel or other animal-derived extracellular matrix molecules, to sustain the self-renewal and pluripotency of different hESCs lines on plastic.[22,36]

In this study, we have investigated the behavior of two hiPSCs lines on PSS characterized by different rigidities, specifically 3, 12, and 25 kPa, and VTN-N surface coating with surface density of 0.5 $\mu g/cm^2$. The choice of these rigidities is based on the elasticity values of undifferentiated pluripotent stem cells in the literature, which are around 5 kPa when measured with AFM or optical tweezers.[5,37,38] Following this knowledge, in this study, we explore two rigidities around 5, 3, and 12 kPa, and one a more rigid surface but still within the physiological range of tissues,[39] 25 kPa. Our data clearly demonstrated that substrate stiffness had a strong influence on hiPSCs cell behavior, guidance, proliferation, maintenance of pluripotency, as well as responses to differentiation. These conclusions could be achieved as cells were cultured on soft supports with controlled, well-defined, and stiffness-independent surface coating. Consequently, the contribution of the elasticity of the extracellular matrix could be identified in cell responses with no bias arising from variations in the surface chemistry, a pitfall often encountered with hydrogels (see Supporting Information Figure S1). We observed that, over time, low rigidities, between 3 and 12 kPa, did not allow total maintenance of hiPSCs pluripotency during long-term culture. hiPSCs sensitivity to rigidity became significant after 3 passages, which corresponded to 15−24 days of culture depending on the rigidity. On the contrary, we noticed that a substrate with a stiffness of 25 kPa was compatible with maintenance of hiPSCs pluripotency markers. Our study, conducted with two hiPSCs lines, led to results that are in agreement with past studies on hESCs, which showed an increase in the maintenance of pluripotency in stiff environments compared with soft ones.[18,22]

More precisely, similar to hESC, we observed on VTN-N-coated PSS that attachment and proliferation rates were higher than on stiffer substrates. We also observed that the rigid substrates and not the softer ones appeared to better support pluripotency for hiPSC, similar to reports on hESCs. Another study performed on polyacrylamide substrates coated with Matrigel solely investigated short-term culture.[11] After 3 days, they observed that the size of the colonies is influenced by the stiffness of the PSS but not the number of colonies or the cell density and the pluripotency. After 9 days, softer substrates around 0.1 and 0.7 kPa promoted neuronal and early ectodermal differentiation in contrast to 75 kPa. In slightly longer-term cultures of 12 days, Maldonado et al.[40] reported a spontaneous differentiation toward the ectodermal lineage on soft electrospun materials coated with Collagen 1 at a Young's modulus of 19 kPa. Compared with these studies, we did not observe any significant spontaneous differentiation during the first three passages for all conditions, which corresponds to 15−21 days, and is thus a much longer culture period than reported in these previous studies.[11,40] Spontaneous differentiation was characterized after the sixth passage, but we could not confirm the derivation toward ectodermal lineage as reported previously.[40] Actually, we observed spontaneous differentiation toward the three germ lines, endoderm, mesoderm, and ectoderm in the case of the softer PSS, of 3 and 12 kPa, as shown in Figure 5. Different from Maldonado et al.,[40] pluripotency was maintained on the PSS with 25 kPa (see Figure 4 and Supporting Information Figure S3). This observation was however consistent with that of Musah et


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ACS Biomater. Sci. Eng. XXXX, XXX, XXX−XXX

al.[17] who showed that PSS with stiffness above 10 kPa were the only ones effective not only for expanding hESCs but also for propagating no more than five hiPSCs lines. Discrepancy with Maldonado et al.[40] may come from the culture support. First, the surface coating differs between both studies (collagen 1 in ref 40, vitronectin-derived GAG in ref 17, and VTN-N in our study). Second, nonuniform elastic electrospun fibers, which are known to be soft at the cell scale but very stiff at the subcellular scales, are used in Maldonado et al.[40] It should be noted that cytoskeletal tension must adapt to this heterogeneous elasticity. As cytoskeletal tension regulates morphogenetic signals, this may consequently impact stem cell spontaneous differentiation.[41] Finally, similar to previous studies,[11,40] we observed that the cellular colonies were larger on the stiffer PSS and took a rounded, 3D shape on the softer PSS. Values of stiffness differ between reports, but as shown in Maldonado et al.[40] where two surface treatments are tested, this effect is strongly related to the surface coating.

In vivo, pluripotent stem cells are only present in the inner cell mass of the developing embryo. At this state, the stiffness of this biological structure was found to be about 200 Pa.[42] Because pluripotency is a transient state, we can hypothesize that the biophysical environment of the developing embryo is not favorable to the maintenance of pluripotency. From our study, we can also conclude that the use of stiffer elastic culture environments, close to adult in vivo rigidities in the kPa range (between 3 and 12 kPa), is not prone to maintaining hiPSCs pluripotency in long-term cultures. However, this conclusion is to be balanced by the fact that the hiPSCs lines currently used are adapted to nonphysiological environments (1−10 GPa). Accordingly, medium, coating proteins, and other currently used culture methodologies are adapted to these extremely rigid environments. In summary, taking this hypothesis into account, it would be interesting to reprogram somatic cells directly on substrates respecting physiological elastic moduli. Perhaps hiPSCs or cells derived from these hiPSCs lines could provide a better model to mimic the bona fide human body. Other perspectives also arise from this study since it provides some clues to the development of more appropriate biomaterials for hPSCs culture and for their subsequent applications. For instance, conventional protocols to direct differentiation of stem cells are mainly use soluble factors. However, these practices are not fully efficient, yielding a heterogeneous population with immature phenotypes. Thus, in addition to biochemical factors, the properties of the surrounding microenvironment could be modulated to more finely direct cell fate. As demonstrated, substrate stiffness acted as a primary determinant in influencing cell behavior, guiding proliferation and differentiation responses in hiPSCs. The understanding of hPSCs mechanobiology will guide the future design of more biomimetic in vitro environments and biomaterials.

## CONCLUSIONS

In summary, we have developed and tested new culture supports to explore the mechanosensitivity of hiPSCs. As a proof of concept, our observations were consistent with previous works and confirmed that soft mechanical environments prevent the maintenance of hiPSCs pluripotency, as has been reported for hESCs. Our observations have provided additional support that currently generated hiPSCs have a mechanosensitivity that is very similar to hESCs lines, thus extending the similarities between hESCs and hiPSCs in terms of their morphology, growth behavior, surface marker expression, pluripotency maintenance, in vivo teratoma formation capacity, or global gene expression profiles[43,44] to their mechanosensitivity. In addition, the newly designed PSS, with uniform elastic properties and stiffness-independent surface chemistry, provides quantitatively consistent observations on cell proliferation, colony morphology, and the maintenance of pluripotency, thus reinforcing its ability to become "standard" culture support. As an interesting result obtained from the two cell lines, low rigidities, below 25 kPa, induced spontaneous differentiation toward the three germline lineages, with no predominance of the ectoderm lineage as previously suspected on shorter-term cultures.[40] Comparison with the state-of-the-art however showed that the values of stiffness that allow the maintenance of pluripotency, above 12 kPa here, are definitely linked and dependent on the surface coating (i.e., chemical composition, surface coating density). Thus, our work highlights the importance of being able to develop culture substrates with defined but also decoupled mechanical properties and surface chemistry.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.9b01189.

Absence of dehydration in the functionalization process leads to stiffness-dependent surface density of the VTN-N (Figure S1); three-dimensional structures of hiPSCs cultured on PSS (Figure S2); maintenance of pluripotency for hiPSCs cultured on 25 kPa PSS (Figure S3) (PDF)

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‖S.P. and P.J. contributed equally.


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### Notes

The authors declare the following competing financial interest(s): C.M. is founder and CEO of the spin-off Cell&Soft which sells pre-coated soft hydrogels for cell culture as used in this work. A.N. is co-founder, shareholder and scientific advisor of Cell&Soft.


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