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Short Report

Substrate elasticity does not impact DNA methylation changes during differentiation of pluripotent stem cells

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Background aims: Substrate elasticity may direct cell-fate decisions of stem cells. However, it is largely unclear how matrix stiffness affects the differentiation of induced pluripotent stem cells (iPSCs) and whether this is also reflected by epigenetic modifications.
Methods: We cultured iPSCs on tissue culture plastic (TCP) and polydimethylsiloxane (PDMS) with different Young's modulus (0.2 kPa, 16 kPa or 64 kPa) to investigate the sequel on growth and differentiation toward endoderm, mesoderm and ectoderm.
Results: Immunofluorescence and gene expression of canonical differentiation markers were hardly affected by the substrates. Notably, when we analyzed DNA methylation profiles of undifferentiated iPSCs or after three-lineage differentiation, we did not see any significant differences on the three different PDMS elasticities. Only when we compared DNA methylation profiles on PDMS-substrates versus TCP we did observe epigenetic differences, particularly on mesodermal differentiation.
Conclusions: Stiffness of PDMS substrates did not affect directed differentiation of iPSCs, whereas the moderate epigenetic differences on TCP might also be attributed to other chemical parameters.
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Introduction

The application of induced pluripotent stem cells (iPSCs) requires efficient protocols for differentiation into specific cell types [1]. However, directed differentiation protocols usually remain incomplete, and cells do not recapitulate the fully differentiated phenotype [2]. In this study, we aimed to investigate whether substrate elasticity can enhance early cell fate decisions of iPSCs toward endoderm, mesoderm and ectoderm—which should conversely also be reflected by corresponding epigenetic changes.

Physical parameters—such as surface chemistry, micro-topography or substrate elasticity—can affect the growth and differentiation of stem cells *in vitro* [3,4]. It is often anticipated that stem cells can sense the mechanical properties of their environment and differentiate accordingly. For example, it has been suggested that very soft tissues like the brain, with a Young's modulus of less than 1 kPa, direct toward neurogenic lineage; 8–17 kPa might support myogenic; and 25–40 kPa might rather correspond to cross-linked collagen of bones and hence enhance osteogenic differentiation [5]. The effects of substrate elasticity have been extensively investigated for adult stem cells, such as mesenchymal stromal cells [5,6], whereas the effects on pluripotent stem cells have been less studied. For the latter, it has been suggested that substrate elasticity seems to alter the expression of germ layer markers during directed differentiation [7,8] and affect differentiation of iPSC-derived embryoid bodies [9]. In contrast, there are studies that did not observe any effect of elasticity on directed germ layer specification of iPSCs [10,11]. Moreover, descriptions of unequivocal effects of substrate elasticity on differentiation of pluripotent cells are hampered by different substrate chemistries, topography or two-dimensional versus three-dimensional approaches [7,9,12,13]. Thus, it is still under debate whether matrix elasticity really is a central parameter to direct differentiation of pluripotent cells—and whether potential phenotypic modifications also would be reflected in the epigenetic makeup, which ultimately defines cellular differentiation [11,14].

DNA methylation (DNAm) is an epigenetic modification that changes in a highly concerted and reproducible manner during cellular differentiation [15]. In fact, the DNAm pattern is very cell type specific [16,17], and it can be used to track early differentiation events of iPSCs [18,19]. We have therefore systematically

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investigated direct differentiation of iPSCs toward specific germ layers on polydimethylsiloxane (PDMS) with different Young's modulus (0.2 kPa, 16 kPa or 64 kPa), along with tissue culture plastic (TCP) as a control material. If the elasticity of PDMS directs cell fate decisions, we would anticipate finding reproducible changes in the cell-type specific DNAm patterns.

Methods

Cell culture and differentiation of iPSCs

Three human iPSC lines were used in this study with hPSCreg accessions (UKAi009-A, UKAi010-A and UKAi011-A). They were generated from bone marrow−derived mesenchymal stromal cells from three different donors (iPSC-102-2, 104-12 and 106-3), reprogrammed using episomal plasmids expressing *OCT4, L-Myc, SOX2, KLF4* and *Lin28A* and clonal iPSC lines were then established by picking of individual colonies [11]. The study was approved by the local ethics committee, and all samples were taken after written consent (permit number: EK206/09). Cells were cultured regularly on TCP coated with vitronectin (0.5 μg/cm^2) in StemMACS iPS-Brew (Miltenyi Biotec, Bergisch Gladbach, Germany) and passaged regularly using intermittent ethylenediaminetetraacetic acid treatment.

To investigate the effect of substrate elasticity, iPSCs were dissociated with ACCUTASE (STEMCELL Technologies, Vancouver, Canada) and seeded (0.5−3 $\times$ 10^5 cells/cm^2) on either PDMS with different Young's modulus (0.2 kPa, 16 kPa or 64 kPa; Advanced Biomatrix, Carlsbad, CA, USA) or TCP (approximately 1 GPa; TPP, Trasadingen, Switzerland) with Matrigel coating. Differentiation toward endoderm and mesoderm was carried out for 5 days and ectoderm for 7 days using the STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies). Alternatively, endoderm differentiation was performed according to the protocol by Wang *et al.* [20] on vitronectin-coated substrates. The endoderm differentiation medium consisted of RPMI-1640 base medium (Gibco, Carlsbad, CA, USA) supplemented with 1% non-essential amino acids, L-glutamine and penicillin/streptomycin (all from Gibco) with the addition of 1% B27 supplements (Gibco), CHIR99021 (6.45 μmol/L; Tocris Biosciences, Bristol, UK) and Activin A (100 ng/mL; Proteintech, Rosemont, IL, USA) for the first day, and only Activin A (100 ng/mL) for the following 3 days.

Immunostaining and image analysis

Immunofluorescence was analyzed after differentiation toward endoderm and mesoderm for 5 days and ectoderm for 7 days, as indicated previously. For non-differentiation conditions, the cells also were harvested after 5 days. Cells were fixed using 4% paraformaldehyde for 10 minutes, treated with 0.1% Triton X-1000 (Carl Roth GmbH, Karlsruhe, Germany) and 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline for 20 minutes and then incubated overnight at 4°C with primary antibodies against OCT4 (clone C-10), PAX6 (clone AD2.35; both from Santa Cruz Biotechnology, Dallas, TX, USA), Brachyury (R&D Systems, Minneapolis, MN, USA) or GATA6 (clone D61E4; Cell Signaling Technology, Danvers, MA, USA). Secondary antibody staining was carried out at room temperature for 1 hour using donkey anti-goat (Alexa Fluor 488), goat anti-rabbit (Alexa Fluor 594) and goat anti-mouse (Alexa Fluor 594; all from Invitrogen, Carlsbad, CA, USA). Samples were counterstained with 4′,6-diamidino-2-phenylindole for 15 minutes. Imaging was performed using the Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany) or EVOS FL Auto (Life Technologies, Carlsbad, CA, USA). Quantification of fluorescence images was carried out using cellpose python package (v2.2) [21]. Fluorescence markers and DAPI images were segmented using the pre-trained "nuclei" model with default parameters and the number of cells was counted from the generated segmentation mask using scikit-image [22].

Semi-quantitative reverse-transcription polymerase chain reaction (qPCR)

Total RNA was harvested after differentiation toward endoderm and mesoderm for 5 days and ectoderm for 7 days, as indicated previously. For non-differentiation conditions, the cells were also harvested after 5 days. RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), quantified with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). qPCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems) and primers for germ layer differentiation and pluripotency marker genes (supplementary Table 1) using StepOnePlus machine (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase was used for normalization.

DNAm analysis

To investigate early epigenetic effects of the substrates on cell-fate decisions, genomic DNA was harvested after differentiation towards endoderm and mesoderm for 5 days and ectoderm for 7 days, as indicated previously. For non-differentiation conditions, the cells were also harvested after 5 days. DNA was isolated using the NucleoSpin Tissue Kit (Macherey-Nagel) and quantified on a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). A total of 1.2 μg of DNA was bisulfite converted and analyzed using the EPIC BeadChip microarray (Illumina, San Diego, CA, USA) by Life & Brain company (Bonn, Germany). The data were analyzed with the minfi R package [23] and normalized using ssNoob [24]. CpG sites on XY chromosomes, non-CG probes and single-nucleotide polymorphism−associated CpGs were removed. In addition, the detection *P* value of the remaining CpGs was evaluated using SeSAMe package, and CpG sites with detection $P > 0.05$ were removed [25]. Limma R package was used for identification of differentially methylated CpGs. Significant CpG sites were defined as showing at least 10% difference in mean beta values and a Benjamini-Hochberg−adjusted *P* value smaller to 0.05. Over-representation analysis of the significant CpGs was carried out using the MissMethyl package employing the gene ontology database [26]. Estimation of germ layer specification was carried with GermLayerTracker (alternatively called PluripotencyScreen), which is based on DNAm at 12 CpGs [19]. As SeSAMe masking is stringent and resulted in removal of several CpGs associated with the GermLayerTracker, we carried out the deconvolution using the more permissive detection *P* values from the minfi package instead.

Statistical analysis

All experiments were performed with all three iPSC lines. For immunofluorescence, we analyzed 5−10 representative images per cell line and condition. Statistical analysis was carried out using the *t*-test from the statannotations python package for quantification of immunofluorescence imaging or the moderated *t*-test from the limma R package for differential methylation analysis. Analysis of variance (ANOVA) test was carried out to test between-group differences in qPCR results. *P* was adjusted for multiple testing using the Benjamini-Hochberg procedure when appropriate, and $P < 0.05$ was considered significant.

Data availability

DNAm profiles can be accessed from the Gene Expression Omnibus under accession number: GSE253190. DNAm profiles for iPSCs cultured and differentiated on TCP are accessible under GSE214400 [27].

Results

Substrate elasticity hardly affects differentiation of iPSCs

Human iPSCs were cultured on soft (0.2 kPa), medium (16 kPa) or stiffer PDMS substrates (64 kPa) and for comparison on conventional TCP (about 1 GPa). Immunophenotypic analysis revealed similar expression of OCT4 (Figure 1A) and absence or very low expression of the germ layer−specific markers GATA6 (endoderm), Brachyury (mesoderm) and PAX6 (ectoderm, supplementary Figure 1A). When we induced differentiation toward endoderm, mesoderm and ectoderm, we observed the clear presence of GATA6, Brachyury and PAX6 protein, respectively, and there were again no clear differences on the different substrates (Figure 1A). Furthermore, we carried out multi-group comparison using ANOVA and pair-wise *t*-test of gene expression of pluripotency markers *OCT4* and *NANOG*; mesoderm markers *TBXT* (Brachyury) and *MIXL1;* endoderm markers *GATA6* and *SOX17* and ectoderm markers *PAX6* and *LHX5.* Most genes showed no significant differences in ANOVA and pair-wise comparisons on the different substrates (Figure 1B, supplementary Figure 1B). Only *GATA6* showed a significant difference between the groups using ANOVA ($P < 0.05$), and a moderate up-regulation of the endodermal differentiation markers *GATA6* and *SOX17* was observed on 0.2 kPa substrates (*t*-test; n = 6; $P < 0.05$) as compared with differentiation on PDMS substrates of greater stiffness and TCP (Figure 1B). However, this could not be validated in immunofluorescence imaging, even after quantification of GATA6-positive cells by image segmentation using a neural network model (three biological replicas; n > 20 per substrate; *t*-test; supplementary Figure 1C). We alternatively tested a different differentiation regimen toward endoderm on TCP and 64 kPa PDMS, and there was no significant difference in immunofluorescence of GATA6, nor did the coating with either vitronectin or Matrigel impact on the differentiation (n > 10; supplementary Figure 2A, B). The up-regulation of gene expression of *GATA6, SOX17* or *NANOG* was also the same on PDMS and TCP (n = 6; supplementary Figure 2C). Taken together, they retained their stemness and revealed very similar differentiation on the different substrates, which is in line with previous observations [10].

DNAm changes during differentiation are not affected by elasticity

Subsequently, we investigated how the DNAm profiles were affected by culture and differentiation of iPSCs on the different substrates. A multidimensional scaling plot for the top 10 000 variable CpG sites revealed that samples mainly separated according to their state of differentiation (Figure 2A). Initially, we focused on these germ layer−associated epigenetic modifications across all different substrates and observed significant DNAm changes at 16 024 CpGs during endodermal differentiation, in 63 729 CpGs during mesodermal differentiation and in 22 059 CpGs during ectodermal differentiation (delta mean DNAm >10%, Adj. $P < 0.05$; Figure 2B). These epigenetic modifications were enriched in Gene Ontology categories that may be relevant for the corresponding differentiation process (supplementary Figure 3). Furthermore, GermLayerTracker analysis [19] could clearly classify all cell preparations as undifferentiated, endodermal, mesodermal and ectodermal differentiation, but there was no clear effect from the substrates (Figure 2C).

To further investigate whether substrate elasticity had a significant effect on DNAm profiles, we performed pairwise comparison between the three replicates of each culture condition. There were

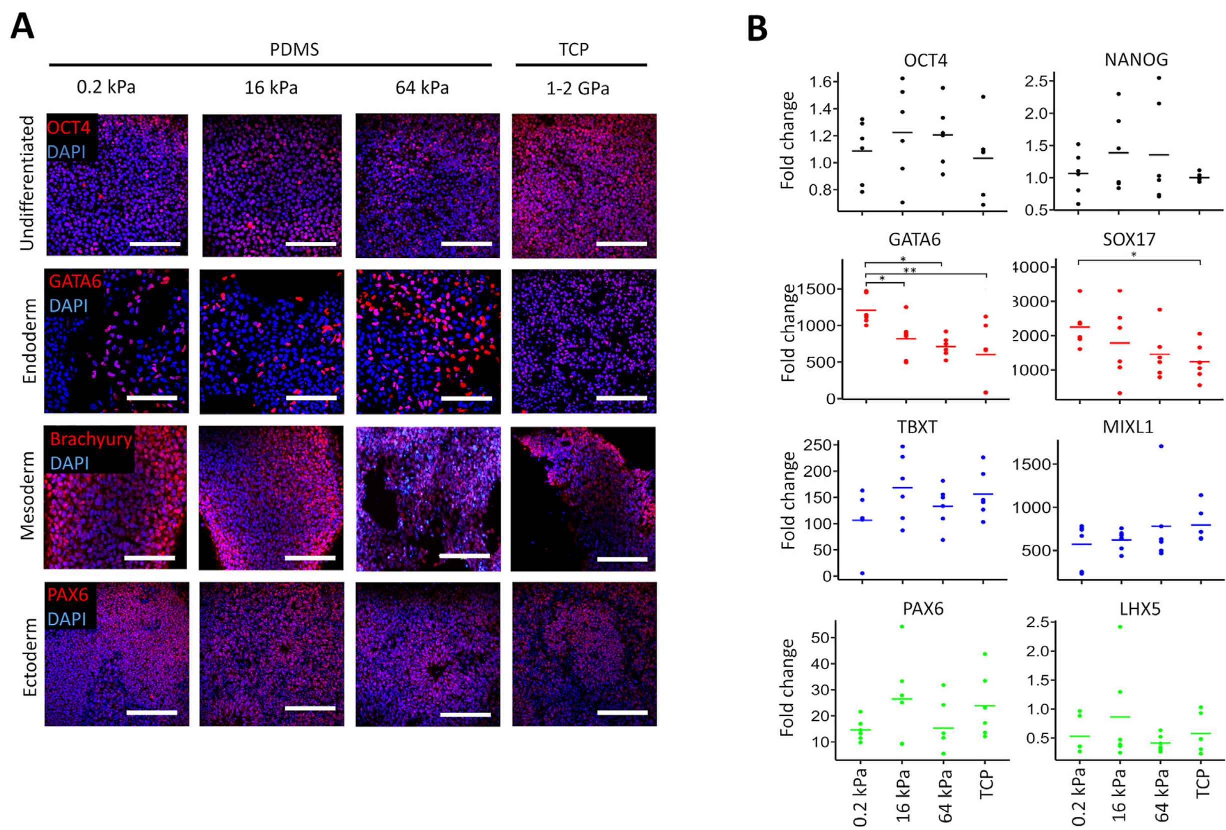

Fig. 1. Early germ layer specification of iPSCs on different substrates. (A) Immunofluorescence images of cells in PDMS substrates with different elasticities (0.2 kPa, 16 kPa and 64 kPa) or TCP. Undifferentiated iPSCs stained with OCT4, endoderm differentiation stained with GATA6, mesoderm differentiation stained with Brachyury and ectoderm differentiation stained with PAX6. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Images show no significant differences between undifferentiated or differentiated iPSCs on different substrate elasticities (scale bar: 50 μm). (B) Gene expression analysis with reverse transcription quantitative polymerase chain reaction of candidate genes for lineage-specific differentiation. Data are normalized to undifferentiated cells on TCP (n = 6; significance measured with unpaired *t*-test; *$P < 0.05$, **$P < 0.01$). (Color version of figure is available online.)

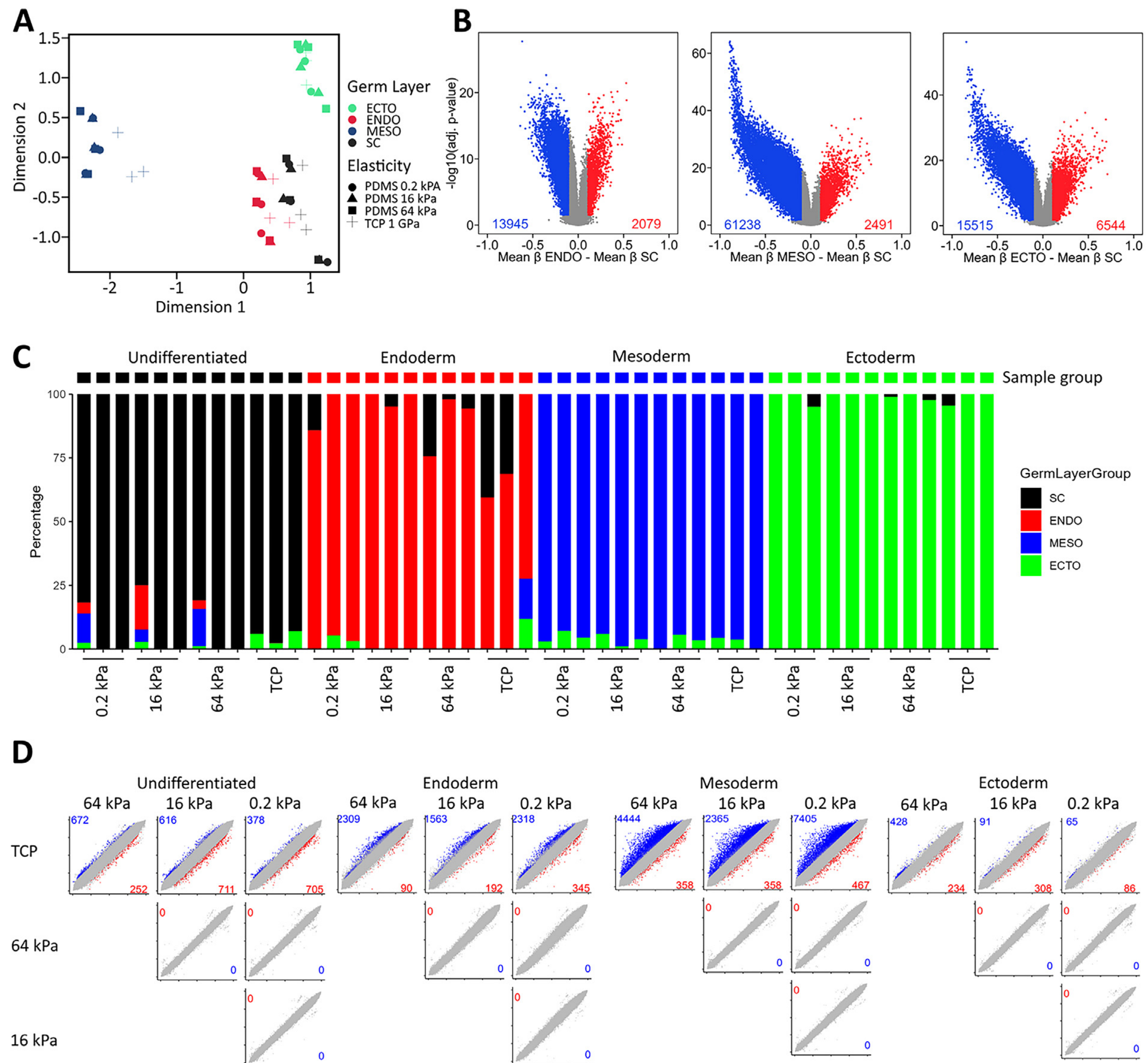

Fig. 2. DNA methylation changes during germ layer specification on different elasticities. (A) MDS dimensional reduction plot for top 10 000 variable CpG sites for ectoderm (ECTO), endoderm (ENDO), mesoderm (MESO) and iPSCs (SC). Cells were cultured on substrates with different Young's modulus on PDMS (0.2 kPa, 16 kPa and 64 kPa) and TCP (1 GPa). (B) Volcano plot showing significantly hypermethylated (red) or hypomethylated (blue) CpGs after differentiation into endoderm, mesoderm and ectoderm, respectively (Adj. $P < 0.05$; diff. in mean DNAm (beta-value) as compared with iPSCs >10%). (C) Estimation of the composition of germ layers based on epigenetic signatures of 12 CpGs of GermLayerTracker (PluripotencyScreen) [19]. (D) Pairwise comparison of differentiated CpGs between cells culture on different substrates. This analysis was performed for undifferentiated iPSCs, endoderm, mesoderm, and ectoderm differentiation. The scatter plots depict mean DNAm values on the corresponding substrates and the number of significantly hypo- and hypermethylated CpGs are indicated (Adj. $P < 0.05$, diff. mean DNAm >10%). (Color version of figure is available online.)

some differences between TCP and each of the PDMS substrates. However, there were no significant differences upon culture on the different PDMS elasticities (Figure 2D). This was unexpected, as PDMS substrate covered a broad range of elasticities (from 0.2 to 64 kPa). Thus, the different substrate elasticities in PDMS did neither affect DNAm patterns in undifferentiated iPSCs nor the DNAm changes during directed differentiation.

Different materials can evoke DNAm changes

To better understand the aforementioned differences between PDMS and TCP, we compared all PDMS substrates versus TCP. Even under non-differentiation culture conditions, we observed 522 hypomethylated and 514 hypermethylated CpGs on PDMS (Adj. P value < 0.05; mean DNAm difference > 10%). Particularly on mesodermal differentiation there were marked epigenetic differences between PDMS and TCP: 25 254 CpGs became significantly hypomethylated (Figure 3A). We then analyzed whether these substrate-associated DNAm changes were related to the germ layer−specific DNAm changes. In fact, the vast majority of the CpGs that were hypomethylated on PDMS were also hypomethylated during the course of mesodermal differentiation (Figure 3B). Thus, the choice of PDMS and TCP affects the DNAm profiles and may point toward enhanced mesodermal differentiation on PDMS.

Discussion

Within the last 15 years, it became almost a dogma that stiffness plays a central role in directing cell-fate decisions. Thus, it was unexpected that the broad range of substrate elasticities on PDMS did neither clearly affect differentiation of iPSCs nor their DNAm patterns. It is conceivable that some of the matrix stiffness−related effects are masked by the interaction with proteins that are absorbed to the surface for coating or secreted by the stem cells [28,29]. Yet, culture of

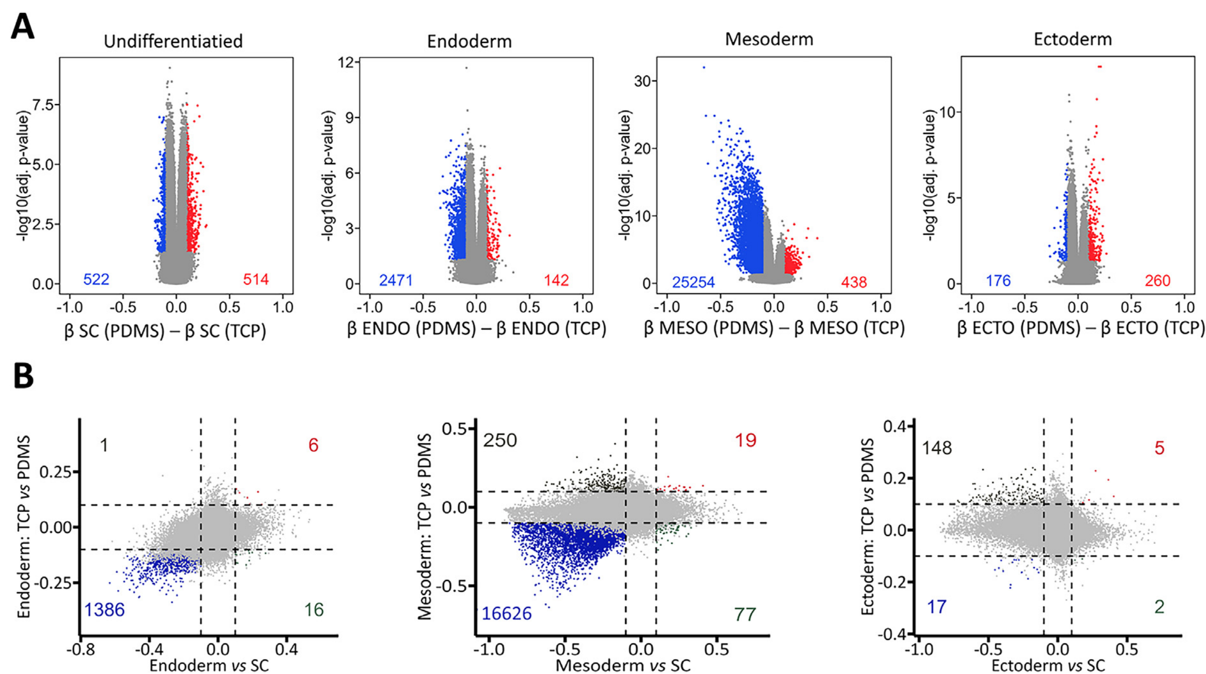

Fig. 3. Differentially methylated CpGs on PDMS versus TCP. (A) Volcano plots showing differentially methylated CpGs between samples cultured on TCP or PDMS for undifferentiated iPSCs, endodermal-, mesodermal- and ectodermal-differentiated cells. The numbers of significantly hypomethylated (blue) and hypermethylated (red) CpGs on PDMS are indicated (Adj. $P < 0.05$, diff. mean DNAm (beta-value) >10%). (B) Scatter plots showing comparison of DNA methylation changes during differentiation toward endoderm, mesoderm and ectoderm, respectively (x-axis), against the DNA methylation changes between TCP and PDMS (y-axis). Many CpGs that are hypomethylated during mesodermal differentiation are also hypomethylated on PDMS. (Color version of figure is available online.)

iPSC is not possible without suitable coating [30], and we did not observe differences on Matrigel- or vitronectin-coated substrates. Furthermore, in our previous work, we did not observe significant epigenetic differences in iPSC-derived mesenchymal stromal cells that were either generated on tissue culture plastic or a collagen-based hydrogel [11]. It is plausible that some of the phenotypic stiffness-related effects are only transient—while the cells are on the substrate—but not reflected in epigenetic changes, which ultimately determine cell fate.

In this study, iPSCs were kept on the different substrates for entire differentiation process (5−7 days), which is in line with many previous investigations that addressed the effect of biomaterials on differentiation of iPSCs [8,9,12]. In contrast, it is conceivable that iPSCs need to be exposed to the substrates for longer time, e.g., for acclimatization to the soft PDMS substrates for several passages before initiating the differentiation, to yield more pronounced epigenetic changes. Virdi and Pethe [10] used such extended culture of iPSCs on soft PDMS substrates before differentiation, but they did not observe significant results between iPSC differentiation on different elasticities, too. In the future, it will be interesting to explore not only the epigenetic effect of biomaterials on iPSCs but also how extended exposure to biomaterials may enhance such effects and might even reveal an epigenetic impact of substrate elasticity.

As already mentioned, modulation of the physical parameter elasticity is often interwoven with other relevant factors, such as surface topography or surface chemistry [31,32]. This might explain why we observed some epigenetic differences on TCP versus PDMS. Cells committed to mesoderm and endoderm were shown to undergo widespread coordinated epigenetic rearrangements driven by ten-eleven translocation−mediated demethylation [33]. It needs to be further explored whether the increased hypomethylation events on PDMS substrates are really associated with enhanced mesodermal differentiation—at least with regards to immunophenotype or expression of marker genes, we did not observe such differences.

Taken together, a wide range of elasticities of PDMS substrates did not affect differentiation of iPSC. Albeit TCP has a very high non-physiological Young's modulus, epigenetic impact of TCP versus PDMS is not necessarily related to differences in elasticity—they may rather be attributed to many other physical or chemical parameters that differ between these materials.

Declaration of competing interest

WW is a founder of Cygenia GmbH, which provides services for various epigenetic signatures (www.cygenia.com). The medical faculty of RWTH-Aachen has claimed patent application for GermLayer-Tracker and MEM, KZ and WW are co-applicants for the patent.

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Author Contributions

Conception and design of the study: WW. Acquisition of data: MEM, KZ, ACH and CM. Analysis and interpretation of data: MEM. Drafting or revising the manuscript: MEM and WW. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2024.03.485. Supplementary materials include supplemental figures 1-3 and supplemental table 1.

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