Title: Epigenetic erasing and pancreatic differentiation of dermal fibroblasts into insulin-producing cells are boosted by the use of low-stiffness substrate

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Conflict of Interest: The authors declare that they have no conflict of interest.

ABSTRACT

Several studies have demonstrated the possibility to revert differentiation process, reactivating hypermethylated genes and facilitating cell transition to a different lineage. Beside the epigenetic mechanisms driving cell conversion processes, growing evidences highlight the importance of mechanical forces in supporting cell plasticity and boosting differentiation.

Here, we describe epigenetic erasing and conversion of dermal fibroblasts into insulin-producing cells (EpiCC), and demonstrate that the use of a low-stiffness substrate positively influences these processes.

Our results show a higher expression of pluripotency genes and a significant bigger decrease of DNA methylation levels in 5-azacytidine (5-aza-CR) treated cells plated on soft matrix, compared to those cultured on plastic dishes. Furthermore, the use of low-stiffness also induces a significant increased up-regulation of ten-eleven translocation 2 (*Tet2)* and histone aminotransferase1 (*Hat1)* genes, and more decreased histone deacetylase enzyme1 (*Hdac1)* transcription levels. The soft substrate also encourages morphological changes, actin cytoskeleton re-organization, and the activation of the Hippo signaling pathway, leading to yes-associated protein (YAP) phosphorylation and its cytoplasmic translocation. Altogether, this results in increased epigenetic conversion efficiency and in EpiCC acquisition of a mono-hormonal phenotype.

Our findings indicate that mechano-transduction related responsed influence cell plasticity induced by 5-aza-CR and improve fibroblast differentiation toward the pancreatic lineage.

Keywords: Cell plasticity, Epigenetic conversion, Hippo signaling pathway, Matrix elasticity, 5-azacytidine, Insulin-producing cells

INTRODUCTION

Development and phenotype definition are regulated by complex epigenetic mechanisms that control genomic imprinting, specific gene transcription programs and chromatin structure [1]. However, the differentiation process is reversible and may be altered by biochemical and biological manipulations, making it an attractive target to reactivate hypermethylated genes [2] and facilitate cell phenotype changes [3]. During the last years, the possibility to interact with the epigenetic signature of terminally differentiated cells and with a preexisting quiescent sub-population of pluripotent stem cells detected in different adult tissue, has been described as a possible tool for regenerative medicine [4-14]. In particular, we demonstrated that a short exposure to the epigenetic eraser 5-azacytidine (5-aza-CR) allows the acquisition of a transient high plasticity state [4-6, 9, 11], which is achieved through the well-known 5-aza-CR ability to deplete DNA methyltransferase (DNMT) 1 enzymatic activity[15, 16]. Furthermore, we recently demonstrated that these events are related to a direct and active ten-eleven translocation 2 (TET2)-mediated demethylating effect[11]. The increased high plasticity, induced by 5-aza-CR treatment, is transient but sufficient to allow a complete and direct differentiation into a new mature and functional cell type [4-6, 9].

Parallel studies addressed their attention to tissue architecture and mechanical forces and showed their involvement (together with chemical signals) in the control of cell plasticity and differentiation. In particular, it was demonstrated that the microenvironment, provided by the traditional polystyrene culture systems, fails to imitate the physiological and biochemical features of cells and can cause deviations in cell response. This is related to the significant differences between the stiffness of the original tissue and that of several GPa of the support used. In contrast, the use of a surface that matches the stiffness of native tissues, exerts a direct effect on lineage commitment, positively influences cell differentiation [17-22] and

might be crucial for specific cellular functions [23]. However, it is yet unclear if matrix elasticity is able to impact cell epigenetic profiles, inducing changes in methylation levels and/or in gene expression [23].

In the present work, we investigate whether matrix elasticity may affect the epigenetic conversion process. In particular, we focus our attention on the two main steps of the protocol, namely cell erasing and cell differentiation. In order to investigate whether a soft substrate may influence epigenetic erasing induced by 5-aza-CR treatment, we monitor DNA methylation changes and the expression levels of its regulatory genes, namely ten-eleven translocation 2 (*Tet2*), histone aminotransferase 1 (*Hat1*), and histone deacetylase enzyme 1 (*Hdac1*). We also analyze the expression of pluripotency-related genes and the morphological changes that take place in response to the exposure to the epigenetic eraser. We then examine the effect of the selected low-stiffness substrates on the differentiation efficiency of fibroblasts into insulin-producing cells (EpiCC) and monitor the impact of matrix elasticity on the acquisition of a mono-hormonal phenotype, which is distinctive of terminally differentiated pancreatic cells.

Finally, in order to better understand the mechanisms involved, we analyze the activation of the Hippo signaling mechano-transduction pathway along the processes.

MATERIALS AND METHODS

All reagents were purchased from Thermo Fisher Scientific unless otherwise indicated.

Ethics statement

Murine dermal fibroblasts were isolated from 7-week-old C57BL/6N male mice obtained from Charles River. All studies were reviewed and approved by the Ethical Committee of

the University of Milan. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH).

Isolation and culture of murine skin fibroblasts

Primary dermal fibroblast cultures were established using skin fresh biopsies from 7-week-old C57BL/6N mice. Fragments of skin tissue of approximately 2 mm^3 were transferred onto 0.1% gelatin (Sigma) pre-coated Petri dish (Sarstedt) and cultured in DMEM supplemented with 20% Fetal Bovine Serum (FBS), 2 mM glutamine (Sigma) and antibiotics. After 6 days of culture, fibroblasts started to grow out of the tissue fragments and the latter were carefully removed. Cells were maintained in the medium described above, grown in 5% CO_2 at 37°C, and passaged twice a week in a 1:4 ratio. All experiments were performed in triplicate on at least three different lines.

Substrate preparation

Based on data from the literature showing that *in vivo* beta-cells are surrounded by a rich network of soft tissue (0.1– 1kPa) [24, 25] and that islet encapsulation in 1 kPa silk hydrogel increased insulin secretion and expression of functional genes [26], here we selected thin polyacrylamide (PAA) gels with elastic modulus of 1 kPa.

PAA gels of controlled stiffness (1kPa) were produced according to a protocol previously described by Pelpham and Wang [27].

Briefly, a thin layer (approximately 100 µm thickness) of polyacrylamide was deposited onto a glass slide. The stiffness of the final gel was tuned by varying the percentage of acrylamide and the ratio acrylamide/bisacrylammide and measured by Atomic Force

Microscope (AFM). Aiming to allow cell attachment and proliferation, the polyacrylamide surface was activated by treatment with sulfosuccinimidyl 6 (49-azido-29-nitrophenyl-amino) hexanoate (Sulfo-SANPAH; Pierce) and coated by overnight incubation at 4°C with 0.2 mg/ml type I collagen (Worthington).

Treatment of murine dermal fibroblasts with 5-aza-CR

Murine dermal fibroblasts were plated either onto standard plastic plates or on PAA gels, at a concentration of 7.8 X 10^4 cells/cm^2. Twenty-four hours after plating, cells were exposed to 1 μM 5-aza-CR (Sigma) for 18 hours. Concentration and time of exposure were selected according to our previous works [4-6, 9]. At the end of the 18-hour exposure, cells were rinsed three times with PBS and incubated for 3 hours with ESC culture medium [28].

Pancreatic induction

Pancreatic differentiation was induced using the three-step protocol modified by Shi et al. [29]. 5-aza-CR treated cells were cultured in basal medium composed by DMEM/F12 supplemented with 1% N2, 1% B27, 0.1 mM β-mercaptoethanol (Sigma), 2 mM glutamine (Sigma), MEM Non-Essential Amino Acids and 0.05 mg/ml bovine serum albumin (BSA, Sigma). During the first 24 hours of pancreatic induction, the basal medium was supplemented with 30 ng/ml activin A. On the second day, 10 μM retinoic acid (Sigma) was added. From the day after onward, cells were cultured in basal medium supplemented with 1% B27, 5 ng/ml basic fibroblast growth factor (bFGF) and 1% insulin–transferrin–selenium (ITS) to further encourage differentiation. Medium was refreshed daily. Cells were differentiated for a total of 10 days.

Global methylation analysis

Genomic DNA was extracted with PureLink® Genomic DNA Kits according to the manufacturer's instructions. DNA was converted to single-stranded DNA by incubation at 95°C for 5 min, followed by rapid chilling on ice. Samples were then digested to nucleosides by incubating the denatured DNA with nuclease P1 for 2 h at 37°C in 20 mM sodium acetate (pH 5.2). Alkaline phosphatase was added and incubated for 1 hour at 37°C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation; CELL BIOLABS) according to the manufacturer's protocol.

Gene expression analysis

RNA was extracted using the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Quantitative PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using predesigned gene-specific primers and probe sets from TaqManGene Expression Assays (Thermo Fisher Scientific), listed in Table 1. *Gapdh* and *Rps18* were used as internal reference genes. CFX Manager software (Bio-Rad Laboratories) was used for target gene quantification.

Western blotting

Cells were lysed and constitutive proteins were extracted using a ReadyPrep Protein Extraction Kit (Bio-Rad). Protein concentration was assessed by Coomassie Blue-G Dye-binding method. 100 μg of proteins were resuspended in sample buffer (1:1) consisting of

4% (wt/vol) SDS, 10% 2-mercaptoethanol, 20% (wt/vol) glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl at pH 6.8. Equal amounts of total protein were loaded and electrophoresed on a SDS-polyacrylamide gels. Proteins were then transferred onto 0.45 μm pore size nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences) and probed with primary antibodies listed in Table 2. Protein bands were visualized by the WesternBreeze chemiluminescent kit. Densitometric analysis was performed with Image-J Software. The protein expression was normalized to GAPDH protein expression by calculating the optical density ratio.

Immunocitochemistry

Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), washed three times in PBS and permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) in PBS. Samples were treated with blocking solution containing 5% (vol/vol) BSA and 5% (vol/vol) not immuno serum in PBS. Primary antibodies were incubated over-night at +4°C and their working dilutions are listed in Table 2. Cells were incubated with suitable secondary antibodies (Alexa Fluor) for 45 min. Nuclei were stained with 4',6-diamidino-2- phenylindole (DAPI, Sigma). Samples were observed under a Nikon Eclipse TE200 and Zeiss Apotome.

Quantification of the nuclear/cytoplasmic ratio of YAP

The number of YAP immuno-positive cells was counted in 5 randomly selected fields at 200×total magnification. A minimum of 100 cells were counted in three independent replicates. YAP fluorescent signal was calculated using ImageJ software. YAP nucleus/cytoplasm ratio was obtained dividing the sum of the intensity values for the

pixels in the nuclear/nuclear corresponding area per the sum of the intensity values for the pixels in the cytoplasm/ cytoplasm corresponding area, as previously described [30].

Cell Counting

Cells were fixed, permeabilized and treated with blocking solution as described above. Sample were then incubated overnight at +4°C with primary antibodies specific for glucagone (GLUC), somatostatin (SOM) and C-peptide (C-PEP; see Table 2 for working dilutions) and, subsequently, with secondary antibodies (Alexa Fluor - 488; Alexa Fluor - 549; Alexa Fluor – 633). Nuclei were stained with 4',6-diamidino-2- phenylindole (DAPI, Sigma). Samples were observed Zeiss Apotome.

When cells formed spherical structures, these were dissociated with 0.25% trypsin-EDTA and Accutase (Innovative Cell Technologies) at 37°C for 10–15 min, and attached to slides, using a cytocentrifuge (Cytospin 4, Thermo Shandon).

The number of immuno-positive cells was counted in 10 randomly selected fields at 200× total magnification. A minimum of 500 cells were counted in three independent replicates. The number of positively stained cells was expressed as a percentage of the total cell counted.

Flow Cytometry

EpiCCg were dissociated with 0.25% trypsin-EDTA and Accutase (Innovative Cell Technologies) at 37°C for 10–15 min. Cells were washed and fixed with 2% (wt/vol) paraformaldehyde in PBS at room temperature for 45 min and permeabilized with 0.2% TRITON X- 100 in PBS for 15 min. Before incubation with primary antibodies, pellets were resuspended in blocking solution containing 10% (vol/vol) BSA in PBS and

incubated for 30 min. Cells were incubated with primary antibodies specific for GLUC, SOM and C-PEP (see Table 2 for working dilutions) and, subsequently, with appropriate secondary antibodies (Alexa Fluor - 488; Alexa Fluor - 549; Alexa Fluor – 633). Cells were then washed and resuspended in PBS. Samples incubated with primary isotypic antibodies were used as a control. Flow cytometry was carried out with a FACS Canto II (BD Bioscience) and analyzed with BD FACSDiva v6.1.3 software.

Confocal analysis

Confocal analysis was carried out under a LSM-710 Zeiss Confocal microscope. Images were acquired with a Z-stacking protocol. In order to ensure the quantification of each fluorescence channel and to determine the fluorescence intensity in the inner vs. the outer part of EpiCC aggregates, PMT voltages and digital image enhancement settings were set to register identical intensities in reference isolated cells showing equal staining levels for the three considered antigens. Z-stack images were then acquired using identical numbers of optical sections along Z-axis and using the same pinhole opening value. In order to obtain aggregate-specific fluorescence distribution patterns, the maximum intensity projection of each of the Z-Stacks was derived using ZEN software (Zeiss). The measures of the fluorescence intensity for the three antigen channels (Alexa Fluor - 488; Alexa Fluor - 549; Alexa Fluor - 633) were obtained by profiling the fluorescence along an arbitrarily set diameter-like axis on each of the images and then transformed into data tables where fluorescence intensity was plotted against the axis length by Graph Pad (Prism). Fluorescence density plots representing in a 2D dimension the intensity of the antigens fluorescence intensity were obtained by maximum intensity projection image post processing using Image-J software.

In vitro functional analysis

EpiCC functional activity was evaluated measuring insulin release in supernatant. Cells were stimulated for 1 hour with 20mM and 5 mM D-glucose (final concentration) in basal medium without ITS. Glucose-dependent insulin release was assessed with Mouse Insulin ELISA (Mercodia) following the manufacturer's instruction. Values were normalized against DNA content of the stimulated cells. DNA was extracted with PureLink® Genomic DNA Kits (Thermo Fisher Scientific) and its concentration was assessed using NanoDrop8000 (Theromoscientific).

Statistical analysis

Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

RESULTS

Isolation and characterization of murine dermal fibroblasts

Fibroblasts obtained from dorsal skin biopsies grew out of the original explants forming a monolayer (Fig. 1). They displayed a standard elongated morphology with a uniform immuno-positivity for the fibroblast specific marker vimentin (VIM) and a complete absence of the pancreatic markers (C-PEP, GLUC and SOM) (Fig. 1).

Effect of matrix elasticity on DNA methylation changes after 5-aza-CR exposure

After exposure to 5-aza-CR, both cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg) showed a significant decrease in global DNA methylation compared to untreated fibroblasts seeded on plastic plates (T0p) and PAA gels (T0g) (Fig. 2A). However, Post 5-aza-CRg cells exhibited a significantly lower methylation level compared to that of Post 5-aza-CRp. No methylation changes were observed in cells maintained in medium without 5-aza-CR (w/o 5-aza-CRp; w/o 5-aza-CRg), indicating that the substrate alone is not able to affect DNA methylation levels in 18 hours.

Effect of matrix elasticity on cell plasticity after 5-aza-CR exposure

5-aza-CR induced methylation changes were accompanied by the up-regulation of the ten-eleven translocation 2 (*Tet2*) and histone aminotransferase 1 (*Hat1*) genes. Moreover, we detected a parallel decreased in the histone deacetylase enzyme 1 (*Hdac1*) transcription levels (Fig. 2C). Increase of cell plasticity was also shown by the onset of pluripotency genes, namely POU class 5 homeobox 1 (*Oct4*), nanog homeobox (*Nanog*), ZFP42 zinc finger protein (*Rex1*), and sex determining region Y-box 2 (*Sox2*), which were undetectable in untreated fibroblasts (T0p, T0g; Fig. 2B) as well as in cells not exposed to 5-aza-CR (w/o 5-aza-CRp, w/o 5-aza-CRg; Fig. 2B). Interestingly, Post 5-aza-CRg cells showed higher expression levels of all the genes analyzed, when compared to those of Post 5-aza-CRp. No expression changes were observed between cells plated onto plastic and PAA gels not subjected to 5-aza-CR exposure (w/o 5-aza-CRp, w/o 5-aza-CRg), indicating that the substrate alone is not able to affect gene expression levels in 18 hours.

Effect of matrix elasticity on EpiCC morphological changes

No differences in cell morphology were observed between untreated fibroblasts plated onto plastic (T0p; Fig. 3A) and PAA gels (T0g; Fig. 3B). Cells appeared large, flat and elongated, regardless of the support used. Furthermore, no variations in filamentous actin distribution and organization were evident between them (T0p F-ACTIN DAPI; T0g F-ACTIN DAPI, Fig. 3A, B), with rhodamine-phalloidin stainings, showing well-spread F-actin bundles and fine filaments.

By contrast, morphological changes become evident at the end of the pancreatic induction, in cells grown on plastic (EpiCCp, Fig. 3A) as well as in those differentiated on gel (EpiCCg, Fig. 3B). Both cell populations lost the typical elongated shape of untreated fibroblasts and acquired an epithelioid morphology. However, EpiCCp mainly kept a reticular organization and formed only small aggregates (Fig. 3A), while EpiCCg were able to organize in distinct large three-dimensional spherical structures (Fig. 3B). Interestingly, EpiCC showed a striking re-organization of the actin cytoskeleton, with depolarized F-actin filaments. These changes were more evident in EpiCCg, where we detected an intense rhodamine-phalloidin staining at the cortical area, immediately adjacent to the plasma membrane (EpiCCg F-ACTIN DAPI, Fig. 3B).

Effect of matrix elasticity on mechano-responses and biochemical signals

Immunocytochemical studies revealed that yes-associated protein (YAP) was evenly distributed between cytoplasm and nucleus in untreated fibroblasts (T0p, T0g; Fig. 4A). No effect on its nuclear accumulation was detected after 18-hour exposure to 5-aza-CR either in cells plated onto plastic plates (Post 5-aza-CRp; Fig. 4A) or in those grown on PAA gels (Post 5-aza-CRg; Fig. 4A). In contrast, at the end of pancreatic induction, YAP localization markedly shifted into the cytoplasm (EpiCCp, EpiCCg; Fig. 4A), with a

304 significantly higher number of cells showing nuclear immuno-positivity exclusion in
305 EpiCCg (Fig. 4B).
306 Consistent with these observations, significant changes in YAP phosphorylation were
307 detected in EpiCCp and EpiCCg. More in detail, EpiCC showed significantly higher levels
308 of pYAP compared to untreated fibroblasts (T0p, T0g) and 5-aza-CR treated cells (Post 5-
309 aza-CRp, Post 5-aza-CRg; Fig. 4B). Interestingly, Hippo-pathway regulating kinases,
310 namely Large Tumor Suppressor 1 (LATS1) and MOB kinase activator 1 (MOB1),
311 changed their phosphorylation levels in parallel to those of YAP protein (Fig. 4C, D).
312
313 **Effect of matrix elasticity on conversion efficiency**
314 At the end of pancreatic induction, EpiCC obtained both on plastic plates (EpiCCp) and on
315 PAA gels (EpiCCg) displayed immuno-positivity for endocrine pancreatic hormones,
316 namely C-PEP, GLUC and SOM (Fig. 5A). Gene expression analysis confirmed these
317 results, showing the onset of active transcription for mature pancreatic specific genes,
318 namely *Ins*, *Gcg* and *Sst* (Fig. 5E).
319 Significant differences in conversion efficiency were observed, when comparing cells
320 differentiated onto standard plastic plates vs. PAA gels. In particular, the percentage of
321 hormone immuno-positive cells significantly increased from 26.86±5.8% in EpiCCp to
322 82.83±6.8% in cells differentiated onto the soft substrate (EpiCCg; Fig. 5B).
323
324 **Effect of matrix elasticity on EpiCC acquisition of a mono-hormonal phenotype**
325 EpiCCp displayed positivity for C-PEP, GLUC and SOM. In particular, in all immuno-
326 reactive cells we detected a co-localization of the three pancreatic hormones within each
327 single cell (EpiCCp, Fig. 5A, B).

Low stiffness PAA gels significantly promoted the acquisition of a mature pancreatic phenotype with 65.33±2.5% of EpiCCg showing a mono-hormonal staining distribution, while only 17.5±0.98% remained poly-hormonal (Fig. 5A, B). Furthermore, in depth analysis of these cells demonstrated that 13.57±1.1% were positive for SOM, 23.76±2.4% for GLUC, and 45.5±3.1% C-PEP (Fig. 5C, D).

The 3D spherical structures formed by EpiCCg were then analyzed using confocal microscopy. The results obtained showed poly-hormonal cells confined to the surface of the spheres, with mono-hormonal ones localized to the core (Fig. 6A). Moreover, fluorescence intensity measurement along the sphere diameter indicated SOM and C-PEP signal peaking on the external shell of the sphere, and glucagon expression homogeneously distributed along the diameter (Fig. 6B, C).

Effect of matrix elasticity on EpiCC insulin release

EpiCC grown on plastic plates and on low stiffness PAA gels were able to respond to 1-hour exposure to 20mM glucose and actively released insulin in cell supernatants. However, significantly higher concentrations of insulin were released by EpiCCg (4.15 ± 0.07 µg /µg DNA) compared to EpiCCp (1.91 ± 0.09 µg /µg DNA) (Fig. 5D).

DISCUSSION

The results obtained in the present study indicate that matrix elasticity may have a profound influence on the epigenetic conversion and differentiation of murine dermal fibroblasts into insulin-producing cells. In particular, the data obtained suggest that the presence of a soft surface is able to affect the different steps involved in the epigenetic

conversion protocol, influencing both the transient acquisition of cell plasticity and the efficiency of differentiation.

After treatment with 5-aza-CR, both cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg) showed a significant decrease in global DNA methylation, compared to untreated fibroblasts, seeded on plastic plates (T0p) and PAA gels (T0g) (Fig. 2A). However, Post 5-aza-CRg cells exhibited a significantly lower methylation level compared to that of Post 5-aza-CRp. Since no DNA methylation changes were observed in cells plated on PAA gels, without exposure to 5-aza-CR (w/o 5-aza-CRp and w/o 5-aza-CRg, Fig. 2A), the methylation decrease observed is unlikely to be accounted for the substrate and its elasticity "per sè". This is in line with Schellenberg et al. that previously demonstrated no influence by Young's moduli on mesenchymal cell methylation profiles [23]. In our understanding, the effect on methylation is more probably due to the combined actions of the matrix and the compound. On the other hand, it cannot be ruled out that the substrate alone may exert a direct effect, but it may require a longer time of exposure than the one described in these experiments.

Based on the results previously obtained in our laboratory [11], we also investigated whether the use of low-stiffness substrate may influence regulatory genes that are involved in DNA acetylation and methylation changes. The results here obtained showed a significant up-regulation of *Hat1* gene transcription in cells exposed to 5-aza-CR, which was increased by the use of soft substrate (Fig. 2C). This was paralleled by a significant decreased expression of *Hdac1* gene, that was lower in cells plated on PAA gels compared to that seeded on plastic dishes (Fig. 2C). These results are in agreement with our previous work demonstrating the key role played by these two genes in transcriptional regulation changes after 5-aza-CR treatment [11].

It is also tempting to speculate that the decrease in methylation may be the result of the upregulation of *Tet2*, which we have shown to play a direct and active demethylating action [11], and the expression of which was increased by the soft substrate (Post 5-aza-CRg, Fig. 2 B). TET family genes also have an essential role in pluripotency regulation of ESC [31, 32] and in the very early stage of somatic cell reprogramming toward iPSC [33]. Interestingly, our results show that epigenetic erasing through 5-aza-CR, caused an increase in *Tet2* (Fig. 2C), paralleled by the onset of other pluripotency-related genes (*Oct4*, *Nanog*, *Rex1*, and *Sox2*, Fig. 2B), confirming previous studies by our laboratory, that demonstrated induction of OCT4, NANOG, REX1, SOX2 and TET2 in species other than the mouse [4-6, 11]. The mechanisms driving epigenetic erasing have been previously investigated and a relation with 5-aza-CR demethylating ability, either by inhibiting DNMT activity and/or modulating TET protein transcription was demonstrated [11]. The resulting global demethylation is likely to lead to a decrease of energy gradients required for the transition of mature cells to a higher plasticity state [34]. On the other hand, multiple and synergistically acting mechanisms may be hypothesized: the involvement and recruitment of a preexisting quiescent sub-population of pluripotent stem cells, recently detected in different adult tissues [12, 13], can be a distinct possibility. Their existence in very few number [14] can explain the lack of positivity for pluripotency related genes that might be below detection levels in T0 cell population.

Oct4, *Nanog*, *Rex1*, and *Sox2* expression was detected in cells erased on plastic, but was boosted by the presence of the soft matrix, which induced a 19%, 14%, 23% and 25% increment in *Oct4*, *Nanog*, *Rex1*, and *Sox2* gene expression respectively. These observations are in agreement with previous data, reporting that iPSC cultured on soft PAA gels showed higher expression of pluripotency genes, than the same cells plated on

399 rigid plastic dishes [35] and are in line with the possibility to maintain and promote self-
400 renewal of murine ESC (mESC), in the absence of exogenous LIF, through the use of soft
401 substrates that match the intrinsic stiffness of the mESC [36].
402 Cell transition to pancreatic phenotype induced evident morphological rearrangements of
403 the cytoskeletal organization, that shifted from F-actin with fine filaments in untreated
404 fibroblasts (T0) to strikingly re-organized and depolarized actin in EpiCC. These changes
405 were, however, more evident in EpiCCg, where F-actin destabilization led to a clear
406 modification in its localization, that concentrated under the cortical area, immediately
407 adjacent to the plasma membrane. This is consistent with recent studies demonstrating the
408 presence of many stress fibers oriented along the line of applied force in cells cultured on
409 stiff surfaces, while showing, in contrast, random and depolarized actin networks in cells
410 grown on soft matrixes [37, 38].
411 Interestingly, the morphological changes described in the present manuscript, were also
412 accompanied by activation of the Hippo signaling pathway (Fig. 4C) and by modifications
413 in YAP cellular localization (Fig. 4A, B). Indeed, while fibroblasts showed an even
414 distribution of this protein between cytoplasm and nucleus, regardless of the substrate
415 utilized (T0p and T0g), EpiCC displayed a restricted YAP localization to the cytoplasmic
416 compartment (Fig. 4B) with a significantly higher number of cells showing nuclear
417 immuno-positivity exclusion in EpiCCg compared to EpiCCp (Fig. 4B). These data are
418 altogether in agreement with recent studies, demonstrating a correlation between
419 cytoplasmic retention, the subsequent complete nuclear exclusion of YAP and F-ACTIN
420 destabilization/disruption [39-41]. Notably, cytoplasmic confinement of YAP has been
421 reported to be distinctive of differentiating cells, while ESC showed the presence of the
422 protein in the nucleus as well as in the cytoplasm [34-37]. Similarly, in the experiments

here reported, we detected YAP equal localization in both compartments of cells exposed to 5-aza-CR (Fig. 4A, B), regardless of the matrix elasticity and, most likely related to the newly acquired high plasticity state. These results are well in line with recent work, demonstrating nuclear YAP essential role in ESC self-renewal and in the control of the levels of the pluripotency genes *Oct4*, *Nanog* and *Sox2* [42-45]. Nuclear/cytoplasmic translocation of YAP has also been described to be controlled by the phosphorylation levels of the protein, which are higher in differentiating cells, compared to undifferentiated ones [42]. In particular, phosphorylation via MOB and LATS kinases, that are core components of the Hippo signaling pathway, resulted in YAP phosphorylation and subsequent exclusion from the nuclear compartment [41]. Consistent with this, the data here presented, demonstrate the phosphorylation of the Hippo-pathway regulating kinases, that paralleled changes in the YAP protein phosphorylation levels (Fig. 4C) and in its nuclear/cytoplasmic distribution (Fig 4 A, B).

In this manuscript, we report for the first time the epigenetic conversion of dermal fibroblasts in the murine species. This result supports the robustness of the method, confirming and extending to the mouse our previous experiments carried out in human, pig, and dog [4, 5, 9]. On the other hand, some species-specific differences became evident. In contrast to what observed in the other species, murine converting cells showed a more limited tendency to form aggregates and organized in smaller and scattered clusters (Fig. 3A). Similarly, conversion efficiency was 26.86 ± 5.8% (Fig. 5B), while a significantly higher percentage of C-peptide immuno-positive cells was obtained in the human (35 ± 8.9%) [4], pig (38.1±9.2 %) [5], and dog (38±6.1%) [9]. Interestingly, the use of a substrate with lower than plastic elastic modulus, allowed mechanotransduction-related stimuli, that guided cells to rearrange into distinct large three-dimensional

spherical structures (Fig. 3B) and induced the morphological changes described, that were paralleled by a significantly higher pancreatic differentiation efficiency (82.83 ± 6.8% vs. 26.86 ± 5.8%) (Fig. 5B). This is consistent with recent studies that demonstrated soft gel encapsulation system ability to enhance cell differentiation towards the endodermal lineage [46-48].

In the experiment here described, epigenetic conversion of murine skin fibroblasts led to the acquisition of a pancreatic phenotype. Genuine differentiation was supported both by active transcriptional activity for pancreatic genes as well as immuno-positivity for C-PEP, SOM and GLUC. However, while EpiCCp displayed a co-localization of the three pancreatic hormones in all immuno-reactive cells, EpiCCg showed a mono-hormonal staining distribution in over 65% of the population (Fig. 5A, B). Interestingly, it has been observed that primitive endocrine cells, typical of early fetal stages, co-express insulin and glucagon, while they mature into a mono-hormonal phenotype later in development [49, 50]. Hormone compartmentalized localization of EpiCCg may therefore be suggestive of a more mature phenotype and to be distinctive of terminally differentiated cells.

Altogether, these results indicate that the use of a soft substrate has a general effect on the differentiation of epigenetically erased fibroblasts and indicate a positive impact both on efficiency and on the promotion of a mono hormonal mode. Furthermore, the low compliance substrate used in the present experiments appear to support a spatial-related maturation process, with a clear compartmentalization of poly-hormonal cells to the surface of the spheres, and of mono-hormonal ones in the core of the structures, as shown by the confocal analysis (Fig. 6). Although further studies are needed in order to better understand this aspect, a substrate-dependent maturation gradient could be hypothesized.

In vitro functional experiments, showed that, when challenged with 20mM D-glucose, EpiCC actively released insulin (Fig. 5D), demonstrating their ability to respond to the primary and physiological stimulus for insulin secretion, and supporting the achievement of a functional phenotype. However, it is interesting to note that a significantly higher amount of insulin was released by EpiCCg (Fig. 5D). This is likely to be related to the acquisition of a more mature/mono-hormonal phenotype which was obtained thanks to the use of a soft substrate. Needless to say that, although these results are very promising, more tests assessing the efficacy of EpiCCg in vivo are needed in order to further characterize these cells and the impact of the soft substrate on their functional activity.

In conclusion, in the present manuscript, we describe the epigenetic conversion of dermal fibroblasts in the murine species and demonstrate that cell mechano-sensing and biomechanical properties of the surrounding matrix, may influence the acquisition of cell plasticity and enhance tissue differentiation, increase conversion efficiency and encourage the acquisition of a mature pancreatic phenotype. The results confirm and strengthen previous data obtained in other species [4, 5, 9, 51], and expands our knowledge on the mechanisms underlying the epigenetic erasing and conversion processes. Furthermore, the data here obtained may have interesting technological impacts in order to increase reliability and increment efficiency of the conversion process. In our opinion, this aspect is crucial for clinical translation of the results, since it allows swift scale-up culture procedures that are essential for cell therapy and tissue engineering applied to human regenerative medicine.

ACKNOWLEDGEMENTS

This work was funded by Carraresi Foundation, by European Foundation for the Study of Diabetes (EFSD) and by Ricerca Corrente at Centro Cardiologico Monzino, IRCCS. The Laboratory of Biomedical Embryology is member of the COST Action CA16119 In vitro 3-D total cell guidance and fitness (CellFit), the COST Action BM1308 Sharing advances on large animal models (SALAAM) and the COST Action CM1406 Epigenetic Chemical Biology (EPICHEM).

The Authors are indebted with Drs Loredana Casalis and Denis Scaini at ELETTRA-Sincrotrone for helping with assessment of the substrates compliance with AFM-assisted nano-indentation methods.

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FIGURE LEGENDS

Figure 1. Characterization of murine skin fibroblasts. Fibroblasts obtained from murine skin biopsies form a monolayer and display a standard elongated morphology and uniform immuno-positivity for vimentin (VIM). No signals are detected for C-peptide (C-PEP), glucagon (GLUC) and somatostatin (SOM). Nuclei are stained with DAPI. Scale bars: 100 μm.

Figure 2. Effect of matrix elasticity on methylation and cell plasticity. (A) Global DNA methylation changes in cells plated on plastic dishes and PAA gels and exposed to 5-aza-CR. Highest level set to 1 and all other relative to this. Bars represent the mean ± SD of three independent replicates. Different superscripts denote significant differences between groups ($P<0.05$). **(B)** After 5-aza-CR treatment, cells show the onset of pluripotency genes, namely *Oct4*, *Nanog*, *Rex1* and *Sox2*. Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P<0.05$). **(C)** 5-aza-CR treatment also induce the up-regulation of *Tet2* and *Hat1* genes, and a decrease of *Hdac1* transcription. Gene expression levels are reported with the T0 expression set to 1 and all

other times relative to this. Different superscripts denote significant differences between groups ($P<0.05$).

Figure 3. Effect of matrix elasticity on cell morphology. (A) Untreated fibroblasts plated on plastic dish (T0p) appear large, flat and elongated, showing well-spread F-actin bundles and fine filaments (T0p F-ACTIN DAPI). At the end of pancreatic induction, cells differentiated on plastic (EpiCCp) acquire an epithelioid morphology and mainly kept a reticular organization, or aggregated in small and scattered clusters, displaying F-actin de-polymerized fibers (EpiCCp F-ACTIN DAPI). Scale bars: 100 μm. **(B)** Untreated fibroblasts plated on substrate with low mechanical compliance (T0g) appear large, flat and elongated, with well-spread F-actin bundles and fine filaments (T0g F-ACTIN DAPI). At the end of the conversion protocol, EpiCCg form large three-dimensional spherical structures and exhibit de-polymerized fibers, mainly localized at the cortical area (EpiCCg F-ACTIN DAPI). Scale bars: 100 μm.

Figure 4. Effect of matrix elasticity on mechanoresponses and biochemical signals. (A) YAP protein is evenly distributed between the cytoplasm and nucleus in untreated fibroblasts (T0p, T0g) and after 18-hour exposure to 5-aza-CR both in cells plated onto plastic plates (Post 5-aza-CRp) and PAA gels (Post 5-aza-CRg). At the end of pancreatic induction YAP staining is excluded from the nucleus and shifts to the cytoplasm (EpiCCp, EpiCCg). Nuclei are stained with DAPI. Scale bars: 50 μm. **(B)** Quantification of the nuclear/cytoplasmic ratio of YAP. Bars represent mean±SD of three independent replicates. Different superscripts (a, b, and c) denote significant differences between groups ($P<0.05$). **(C)** Representative western blot immuno-detection of MOB1, pMOB1,

LATS1, pLATS1, YAP, pYAP and GAPDH during epigenetic conversion. Full-length blots are included in the supplementary information. **(D)** Densitometric analysis of the Western blots. The values are reported as relative optical density of the bands normalized to GAPDH. Different superscripts denote significant differences between groups ($P<0.05$).

Figure 5. Effect of matrix elasticity on epigenetic conversion. (A) EpiCC show immuno-positivity for C-peptide (white), glucagon (green) and somatostatin (red). EpiCCp display a co-localization of the three pancreatic hormones within each single immuno-reactive cell, while EpiCCg exhibit a mono-hormonal staining distribution. Nuclei are stained with DAPI. **(B)** Hormone immuno-positive cell rate and poly/mono-hormonal cell percentages in EpiCCp and EpiCCg. Different superscripts denote significant differences between groups ($P<0.05$). **(C) S**omatostatin, glucagon and C-peptide positive cell rates in EpiCCg. **(D)** Representative output of flow cytometer analysis of EpiCCg showing somatostatin (SOM), glucagon (GLUC) and C-peptide (C-PEP) labeled cells. **(E)** Quantification of insulin release in cell supernatant in response to 5 mM and 20 mM D-glucose exposure for 1 h. Bars represent the mean ± SD of three independent replicates. Different superscripts denote significant differences between groups ($P<0.05$). **(F)** At the end of pancreatic induction cells show the up-regulation of *Ins*, *Gcg* and *Sst* genes. Gene expression levels are reported with the T0 expression set to 1 and all other times relative to this. Different superscripts denote significant differences between groups ($P<0.05$).

Figure 6. Confocal analysis of 3D spherical structures formed by EpiCCg. (A) EpiCCg spheres display immuno-positivity for glucagon, somatostatin and C-peptide. **(B)** Fluorescence intensity analysis, along an arbitrarily set diameter-like axis, shows poly-

hormonal cells confined to the surface of the spheres and mono-hormonal ones localized to the core. **(C)** Density plots representing glucagon, somatostatin and C-Peptide fluorescence intensity.