Histone deacetylase inhibitor overrides the effect of soft hydrogel on the mechanoresponse of human mesenchymal stem cells

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Abstract:

Human Mesenchymal stem cells (hMSCs) are promising in regenerative medicine for their multi-lineage differentiation capability. It has been demonstrated that lineage specification is governed by both chemical and mechanical cues. Among all the different mechanical cues known to control hMSCs fate, substrate stiffness is the most well-studied. It has been shown that the naive mesenchymal stem cells when cultured on soft gel, they commit towards adipogenic lineage while on stiff gel they become osteogenic. Soft substrates also cause less cell spreading, less traction, less focal adhesion assembly and stress fibre formation. Furthermore, chromatin condensation increases when cells are cultured on soft substrates. As the nucleus has been postulated to be mechanosensor and mechanotransducer, in this paper we asked the question how mechanosensing and mechanoresponse process will be influenced if we reduce the chromatin condensation in hMSCs cultured on soft substrates by using an external reagent. To address this question, we treated hMSCs cultured on soft polyacrylamide (PA) gels with a histone deacetylase inhibitor (HDACi) called Valproic Acid (VA) which decondense the chromatin by hyperacetylation of histone proteins. We found that the treatment with VA overrides the effect of soft substrates on hMSCs morphology, cellular traction, nuclear localization of mechnosensory protein YAP (yes-associated protein), and differentiation. VA treated cells behaved as if they are on stiff substrates in all aspects tested here. In contrast, when we increase chromatin condensation using histone acetyl transferase inhibitor (HATi), adipogenic differentiation on soft gel increases. Furthermore, we have shown that VA controls hMSCs differentiation via activation of ERK/MAPK pathway by increasing the p-ERK expression. Collectively, these findings demonstrate for the first time that inhibiting histone deacetylase can override the mechanoresponse of hMSCs. This

work will help us to fundamentally understand the mechanosignalling process and to control the hMSCs differentiation in tissue engineering and regenerative medicine.

Key words: hMSCs, soft hydrogel, mechanoresponse, HDACi, p-ERK

Significance

To harness the clinical potential of Human mesenchymal stem cells (hMSCs), it is important to understand the working mechanism of various signals in deciding cell fate. It has been shown in the recent past that the substrate rigidity influence hMSCs differentiation. Though the mechanism is not fully understood, the mechanosignals are known to alter the cell fate via reorganizing the chromatin packing. In this work, we ask if altering chromatin packing can change the cell fate as dictated by the mechanical properties of the substrates. We have shown that almost all the known effector of mechanosignals get modified when we tweak the chromatin packing via histone deacetylase inhibitor (HDACi). This study is helpful for developing a better understanding of the mechano-signalling process. It gives us a tool to override the effect of the substrates, particularly when the mechanical properties of biomaterials are not conducive for the desired differentiation outcome. Additionally, as HDACi are being used in the therapy of cancer and different neurodegenerative diseases, our understanding of their crosstalk with tissue stiffness in controlling cell fate will help to understand any unknown and undesirable side effects of using HDACi as therapeutic agent.

Introduction:

Substrate stiffness controls many different cellular behaviors such as cell morphology, maturation of focal adhesion, and formation of stress fibers (1, 2). Cells sense the mechanical properties of their microenvironment by deforming the substrate via applying traction force. The information is then transduced to the nucleus via stress fibers and other mechano-transducing proteins(3–6). It has been observed that on softer substrates the cells spread less, apply less traction, and the chromatin remains in a more condensed state. Recently, YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif), the main transcriptional effector molecules of hippo signaling pathway, have been identified as a key mechanosensor and mechanotransducers of mechanical cues (7, 8). Depending on substrate rigidity YAP/TAZ is known to shuttle between nucleus and cytoplasm. On soft substrates YAP translocates more into the cytoplasm (inactivated) but on a rigid substrate, it localizes more in the nucleus (activated) and works as a transcriptional co-activator (8–10).

Not just the morphological changes, substrate rigidity also controls other critical cellular functions such as differentiation in the stem cells (11–13). It has been shown that human mesenchymal stem cells (hMSCs) favor adipogenic differentiation on soft substrate, while osteogenic differentiation is preferred on rigid substrates(14–16). Hence, understanding the mechano-signaling process is an active area of research in the field of regenerative medicines, stem cell biology, and tissue engineering.

Differentiation process is always linked with epigenetic modifications (17–19). During differentiation of hMSCs, genes responsible for self-renewal are turned off and genes related to lineage specifications are activated. Such controls are exerted via various epigenetic changes including histone modifications. Acetylation of histones is one such important modification that makes the genes available for transcription by opening or decondensing the chromatin(20–22). Histone acetylation is controlled by two sets of modifiers, HATs (histone acetyltransferases) and HDACs (histone deacetylases). While HATs decondense the chromatin and make the genes transcriptionally more accessible by acetylating the histone proteins, HDACs work in the opposite manner by removing the acetyl groups from the histones and thus causing the chromatin to condense and making the genes less accessible for transcriptional activities (23). In this context, it has been shown that a class of compounds known as histone deacetylase inhibitors (HDACi) which hyperacetylates histone proteins, promote osteogenic differentiation of hMSCs (24–28). However, there are a few contradictory reports as well showing HDACi, sodium butyrate stimulating adipogeneic gene expression and adipocyte differentiation (29, 30). A well-established HDACi called valproic acid (VA) has been shown to promote neuronal and hepatic differentiation of hMSCs (31–33). It also increases the anti-tumor effect of hMSCs in the gene therapy of glioma. Hence, it is important to understand the role of epigenetic modification such as histone acetylation/deacetylation in a specific context, mechanosensing being one. It is known that substrate rigidity controls hMSC differentiation and change chromatin packing (34). However, the interplay of epigenetic modifications using chemical inhibitor and mechanical cues is unexplored and not known if chromatin reorganization by the former can overwrite the signals coming from the latter.

To fill this gap, we modified chromatin compaction of hMSCs with valproic acid (VA) (35, 36) when the cells were cultured on soft (E = 3 KPa) polyacrylamide (PAA) gels. It is known that soft substrate causes chromatin condensation and inhibits osteogenic differentiation (34). On addition of VA, we found a significant reduction in chromatin condensation, as found by other researchers but on non-physiologically rigid substrates such as plastic (37). Concurrently, VA also modified many known effects of soft substrates on cellular morphology and functions such as cell spreading, cellular traction, expression of focal adhesion and stress fibres, nuclear localization of YAP, ERK phosphorylation, and differentiation. Soft substrates are known to promote adipogenic and supress osteogenic

differentiation (38). However, in the presence of VA, this fate was reversed. Furthermore, the expression level of p-ERK in the cell was higher upon addition of VA, which is known to promote osteogenic differentiation (39). When phosphorylation of ERK was inhibited by using ERK pathway inhibitor PD 98059, the effect of VA on hMSC differentiation was nullified. However, PD didn't have any effect on chromatin condensation, in presence or absence of VA. Altogether, our work shows that acetylation status of chromatin overrides the effect of substrate stiffness via phosphorylation of ERK.

These findings will help to fill the critical gap in our basic understanding of how substrate dependent mechanosignalling process can be altered through changing chromatin condensation. This work will help to gain broader insights of how modifying chromatin compaction by integrating chemo-mechanical cues can be used for controlling hMSCs fate in tissue engineering and regenerative medicine.

Material and methods

Substrate preparation.

Polyacrylamide gel having elastic modulus of ~3 kPa was prepared by crosslinking 40% poly-acrylamide and 2% bis-acrylamide solution. Protocol for substrate preparation and Elastic modulus value was adopted form previously reported work(40). Briefly, the gel solution for desired stiffness (~3 kPa, Table S1) was mixed with ammonium per sulphate (1:100) and TEMED (1:1000) and a drop of 130 microliters was placed between two glass coverslips, one coated with 3-APTMS (Sigma) and other with hydrophobic coating. After polymerization, hydrophobic coverslip was removed. The gel was coated with type I collagen (25 µg/ml) (Invitrogen; A1048301) using Sulfo-SANPAH based conjugation and kept at 4°C overnight(41).

Cell culture

Bone marrow-derived hMSCs were purchased from Lonza (Cat.No. #PT-2501). hMSCs were cultured in Low glucose DMEM (Himedia; AL006) supplemented with 16% FBS (Himedia ; RM9955), 1% Antibacterial-Antimycotic (Himedia; A002) and 1% Glutamax (Gibco;35050) under humidified conditions at 37°C with 5% CO2. The cells were trypsinized with TrypLE™(Gibco;12604021) once they reached the confluence of 70%. The cells were seeded on polyacrylamide gels with 2000 cells/cm^2 seeding density in 50 µl of media and flooded after 45 mins.

Treatment with HDACi

To check the effect of HDACi on soft substrate0.5mM of Valproic acid (PHR 1061) was added to the growth media at the time of flooding. For differentiation experiments, VA was added with differentiation media. For the experiments with Sodium Butyrate (SB, B5887), 0.5mM of SB was added in the similar manner.

Differentiation assays

hMSCs were seeded at 2000 cells/cm^2 in a 12-well culture plate in growth medium for 24h followed by differentiation media. Adipogenic (Invitrogen, A10410) and osteogenic (Invitrogen, A10069) differentiation kits were used. Cells were incubated for 9 days in adipo and 14 days for osteo induction media before quantitative assays. Differentiation media change was given after every third day. After the completion of differentiation duration, cells were fixed with 4% PFA for 30min at room temperature followed by staining with Oil Red O (SigmaO0625) for adipogenic differentiation and Alizarin Red (Sigma A5533) for osteogenic differentiation. After incubating with staining solution for 20 min samples are washed thrice with DPBS (adipo) or MilliQ (osteo). Images were captured for quantitative analysis using EVOS inverted microscope (Invitrogen) in bright-field colour channel.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature (RT) then washed with PBS thrice. Cells are then permeabilized with permeabilizing buffer (0.5% Triton X-100-Sigma Aldrich in CSB) for 10 min and blocked with BSA (4% Bovine serum albumin in PBS) for 30 min to minimize nonspecific protein binding. Anti-YAP (1:500, rabbit, Abcam, Cat. No. 52771), anti-PPAR-γ (1:500, rabbit, Abcam, Cat. No. 59256), anti-RUNX2 (1:500, rabbit, Abcam, Cat. No. 23981), anti-p-ERK (1:500, rabbit, Abcam, Cat. No. 65142), anti-OPN (1:500, rabbit, Abcam, Cat. No. 8448) primary antibodies in 4% BSA were added to the samples and incubated overnight at 4°C. Primary antibodies were removed and samples are rinsed with PBS two times for 10 min. Samples were then incubated at room temperature with secondary antibodies (1:500, donkey anti-rabbit AlexaFlour 568 Cat. No. 75470, goat anti rabbit AlexaFlour 488 Cat. No. 411034) phalloidin (1:400, AlexaFlour 532, Cat. No. A22282 and AlexaFlour 488, Cat. No. A12379) with Hoechst 33342 (Cat. No. H3570) with dilution of 1:5000 in 4% BSA for 2 hrs at room temperature. Thereafter the samples were rinsed twice with PBS. All immunostained samples were stored in PBS at 4°C until Imaging. All samples are imaged at 63X (oil) magnification using laser scanning Confocal Microscope (LSM, Carl Zeiss).

For vinculin staining hMSCs are fixed with ice cooled mixture of 1:1 (v/v) (4% PFA: permeabilizing buffer (1% Triton-X-100-Sigma Aldrich) for one minute on ice. Samples are rinsed with cytoskeleton stabilizing buffer (CSB) (60 mM PIPES, 27 mM HEPES, 10 mM EGTA, 4 mM magnesium sulphate, pH 7) and fixed again with 4% PFA in ice for 5 min. After fixing, samples were rinsed with CSB and blocked

with 1.5% BSA supplemented with 0.5% Triton-X-100 for 30 min on ice. Anti-vinculin (1:500, rabbit Abcam Cat. No. ab129002, mouse monoclonal, Sigma) primary antibody in 4% BSA were added to the samples and incubated overnight at 4°C. Primary antibody was removed and samples are rinsed with PBS two times for 10 min. Sample was then incubated at room temperature with secondary antibody (1:500, goat anti rabbit AlexaFlour 488 Cat. No. 411034), phalloidin (1:400, AlexaFlour 532, Cat. No. A22282) with Hoechst 33342 (Cat. No. H3570) with dilution of 1:5000 in 4% BSA for 2 hrs at room temperature. All immunostained samples are imaged as previously mentioned.

Analysis of chromatin condensation parameter (CCP)

DAPI stained nuclei were imaged with laser scanning confocal microscope (LSM 780; Carl Zeiss) at 63X (oil). For analysis, individual nuclei were cropped using FIJI to get only one nucleus per image. CCP was calculated using a MATLAB script as previously described (42) in which a gradient based Sobel edge detection algorithm was used to measure the edge density of individual nuclei.

Traction force microscopy (TFM)

Gels of 3 kPa were made on 22 × 22 mm^2 coverslips, once gels were solidified, 25µl drop of 3 kPa solution having 1 µm fluorescent beads (Fluka with a final concentration of 1:50) was added on the hydrophobic plate and then the solidified gel was inverted into it and allowed to solidify. Gels are then treated with Sulfo-SANPHA and coated with collagen as mentioned above. Cells were seeded with the seeding density of 1000 cells/well. After 24h of cell seeding, cells were lysed using 100µl of1% Triton-X in 2ml of complete media. Images of the stressed (before lysing) and unstressed (after lysing) gels were captured by the EVOS FL Auto cell imaging system (Invitrogen). An average of 10 cells was analysed per gel. The code from J.P.Butler was used to calculate the traction force.

Statistical analysis

Data are presented as means ± standard deviation (otherwise mentioned) and were analysed using Microsoft Excel software. Data was plotted using OriginLab software (IIT Bombay License). Statistically significant differences were claimed at: $*p < 0.05$.

Results

Effect of substrate stiffness and HDACi on chromatin packing

Mechanical properties such as substrate stiffness are known to influence hMSCs morphology, fate and chromatin packing. We asked if the mechanical influence of the substrate can be overwritten by actively remodelling the chromatin. For that purpose, we first cultured hMSCs on stiff (E = 34kPa; Table

S1) and soft polyacrylamide (PAA) gel (E = 3 kPa; Table S1) for 48 hrs and estimated the chromatin packing using chromatin condensation parameter (CCP) (Fig 1a) (see materials and methods for more details). In brief, images of the DAPI stained nuclei were captured at high magnification (Fig 1B-D). The bright DAPI stained regions indicate densely packed chromatin (Fig 1 E-G) (42). We observed that hMSCs cultured on soft hydrogel have significantly higher chromatin condensation (~ 2 times) as compared to stiff hydrogel as shown in fig (B-C & E-F). This observation matches well with the results obtained by earlier researchers (43). Next, to modify chromatin organization, we treated the cells on soft gel with valproic acid (VA) (0.5mM) for the period of 24 hours. Valproic acid is a histone deacetylase inhibitor (HDACi) which inhibits histone deacetylase enzyme (HDAC) promoting hyperacetylation of chromatin resulting into chromatin de-condensation. The concentration of VA was selected after confirming no considerable cell death at that concentration (data not shown). Addition of HDACi on soft hydrogels decreased chromatin condensation and restored the level to that on stiff gels (as shown in the figure 1D, G, and H).

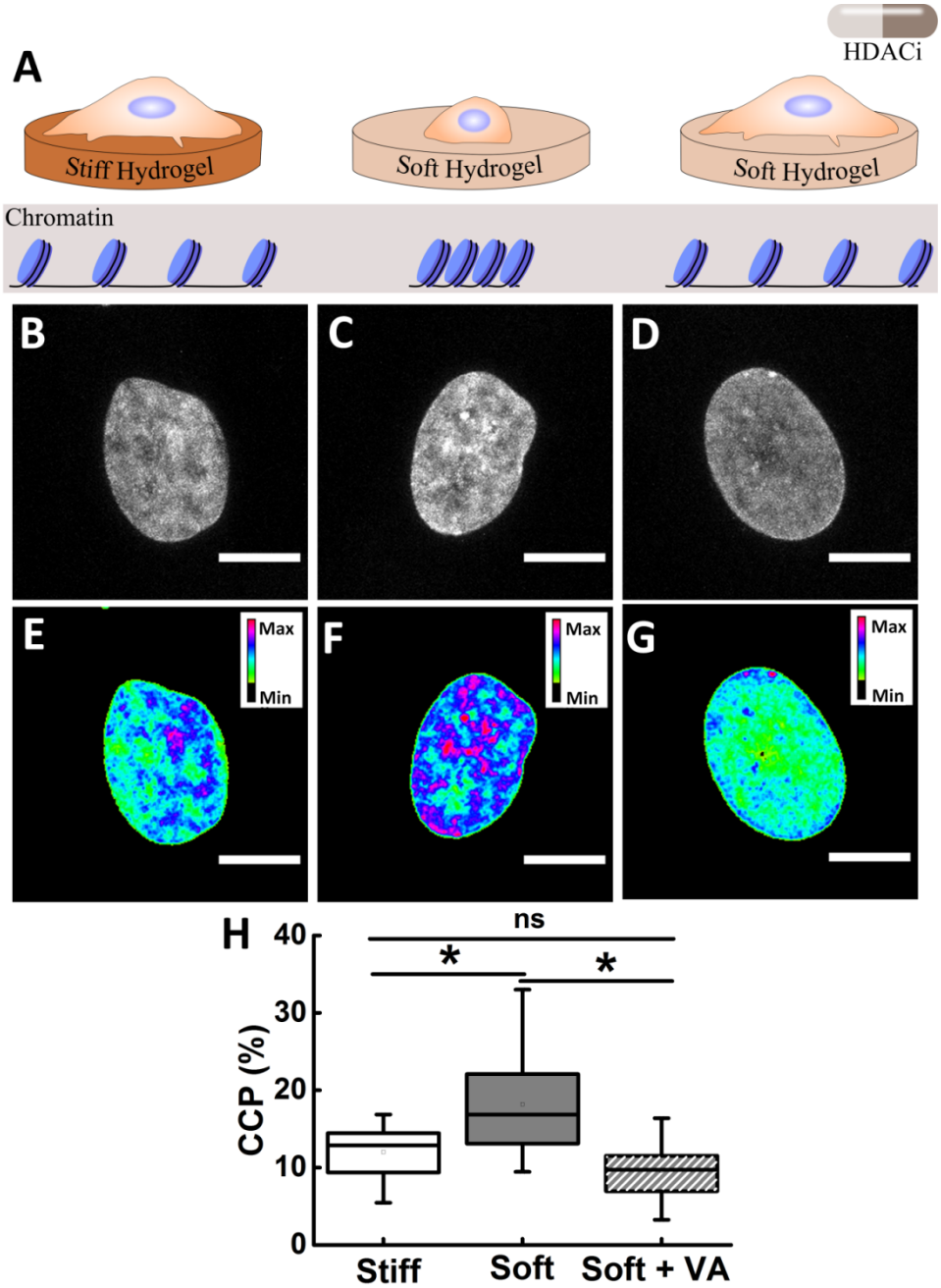

Fig 1. Influence of substrate stiffness and histone deacetylase inhibitor (HDACi) on chromatin condensation. (A) A schematic showing changes in chromatin condensation due to substrate stiffness and HDACi (Valproic acid, VA). (B-D) Representative images of DAPI-stained nuclei (8 bit) from stiff (34 kPa), soft (3 kPa) and soft + VA. (E-G) The corresponding heatmap of DAPI intensity. (H) Chromatin condensation parameter (CCP) for the nuclei from stiff, soft and soft + VA demonstrating that while soft substrate increases chromatin condensation, the same can be brought down to the level of stiff substrates using VA. *$p<0.05$, N=3, n>30. Scale Bar: 10 µm

HDACi increases cell spreading, focal adhesion maturation and actin stress fibre formation

of hMSCs cultured on soft substrates

Next, we asked the question how the chromatin remodelling with hDACi influences other mechano-responsive phenotypes of hMSCs cultured on soft substrates. Differential spreading of cells depending on substrate rigidity is one of the first observable mechano-response in hMSCs. They spread less on the substrates with low elastic moduli (13, 16) Hence, we checked the effect of VA on the spreading of hMSCs when cultured on the soft gels. We found that upon addition of VA, the cell spreading on 3kPa gels increased significantly (~50%) compared to the control as shown in the fig 1 (A-C). We have also observed that in the presence of VA, cells show more protrusions (data not shown). To confirm that the effect is not VA specific, we used another histone deacetylase inhibitor sodium butyrate (SB) and found the similar outcome (fig. S1).

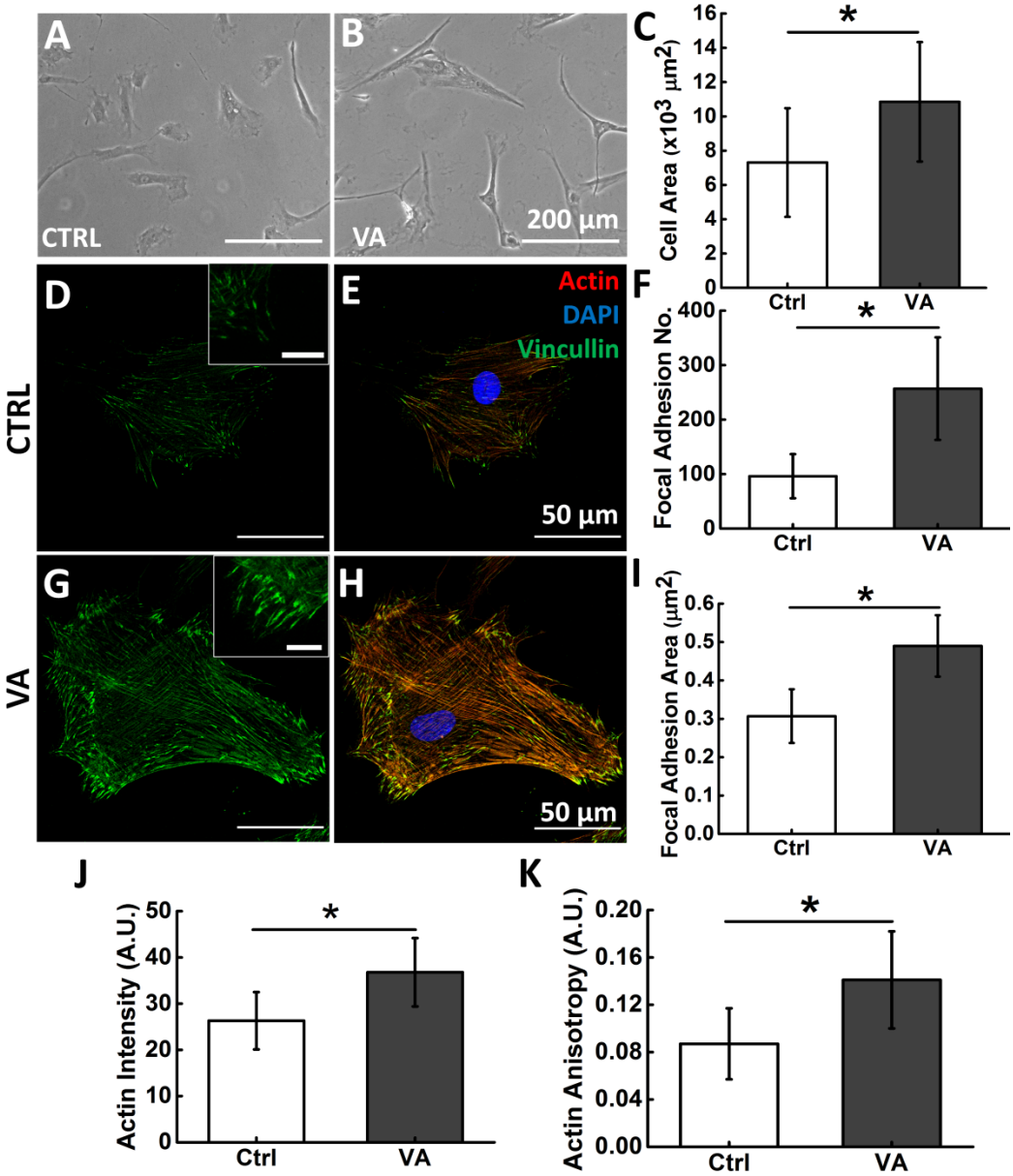

Fig 2. Effect of HDACi on cell spreading, focal adhesions and actin of hMSCs cultured on soft hydrogel. Representative phase contrast images of hMSCs cultured on 3 kPa gel (A) without VA and (B) with VA show that cell spreading increases with VA as quantified in (C). *$p<0.05$, N=3, n=100, scale bar: 200 µm. (D and G, and insets) hMSCs on 3kPa gel were immunostained for vinculin (green) and (G and H) actin (red) with (G and H) and without (D and E) Valproic acid. (F) and (I) show increase in focal adhesion number and area respectively when treated with VA. (J & K) show increase in actin intensity and anisotropy when the cells on soft gels were treated VA. *$p<0.05$, N=2, n=16. Scale Bar: 50 µm. Inset Scale Bar: 5 µm

As Cell spreading is known to be strongly associated with formation of matured focal adhesion and actin assembly (44). Hence, we examined the effect of VA on formation of focal adhesions (FAs) and actin stress fibre assembly by staining FA protein vinculin and F-actin respectively. Immunofluorescence images showed that cells treated with VA had significantly higher number of FAs (256 $\pm$ 94) as compared to control (94 $\pm$ 40) (Fig 2D, G &insets D & G). The average area of FAs indicating maturation were also significantly higher in VA treated cells (Fig 2I). Similarly, we observed significantly higher amount and assembly of actin stress fibres when treated with VA (Fig 2E, H, J& K), an atypical observation for cells cultured on soft substrates.

HDACi increases cellular traction of hMSCs cultured on soft substrates

From our earlier results, it was clear that HDACi treatment overrides the effect of soft gel (3 kPa) on hMSC morphology, focal adhesion maturation and actin assembly. As all these cellular properties are strongly associated with cellular traction (45, 46), we investigated how HDACi and substrate rigidity together influence cellular contractility. We cultured hMSCs for 24hrs in PAA hydrogel (E = 3 kPa) embedded with fluorescent beads (Fig. 3A). Subsequently, traction forces are calculated using Traction force microscopy (TFM). TFM revealed that VA significantly increases the traction force generated by hMSCs cultured on soft substrate (from ~ 152 Pa$\pm$ 32.05 to ~ 235 Pa$\pm$ 39) where they are known to be less contractile (45) as shown in the fig 3B-D. We also compared the interrelationship between cell spread area and traction. We found the cell spreading and traction holds a positive correlation as shown by others (47–49) for both with and without VA, albeit with different Pearson correlation

coefficient (0.69 for control and 0.47 with VA), as shown in figure 3E. We used another histone deacetylase inhibitor sodium butyrate (SB) and found the similar result (fig. S2).

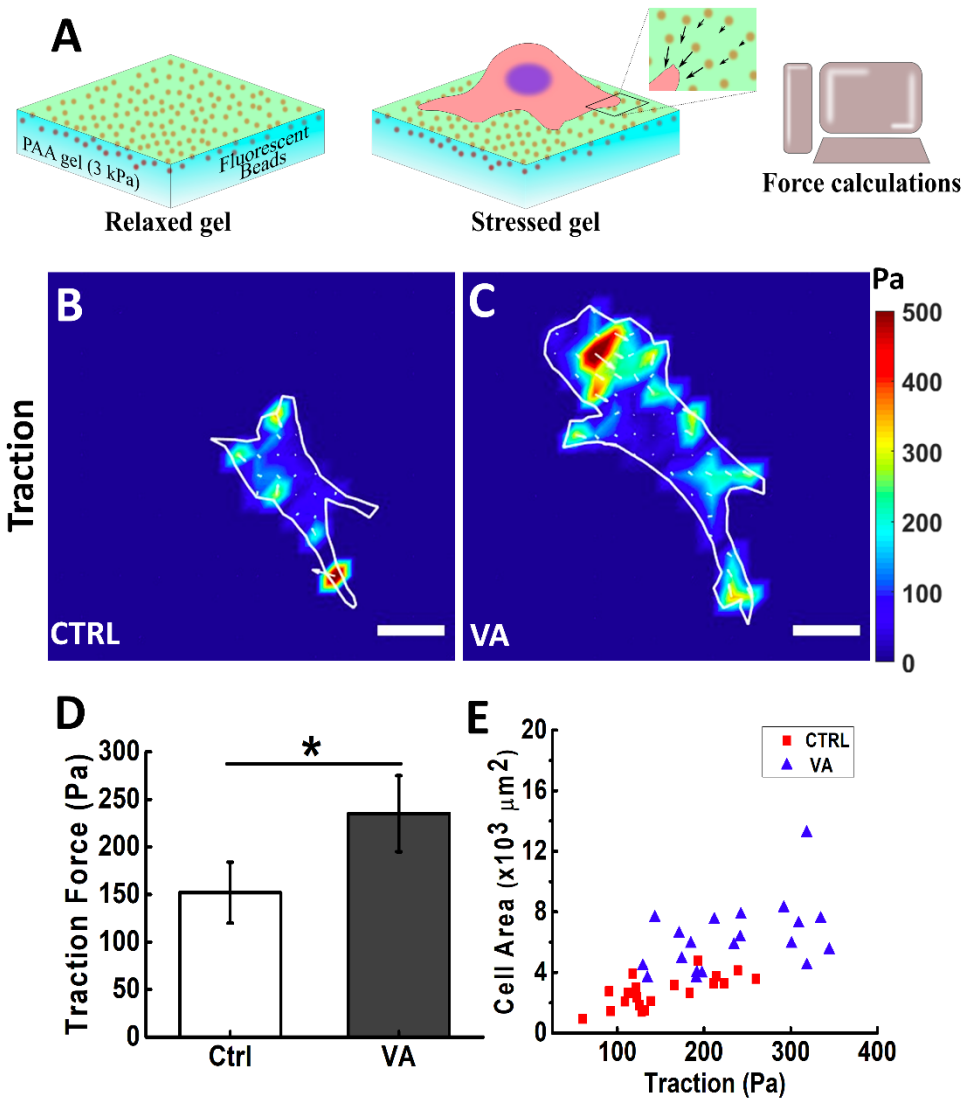

Fig 3. HDACi increase hMSCs traction on soft hydrogel. A) Schematic for PAA hydrogel-based traction force microscopy (TFM) in which cellular traction was estimated from the displacement of the embedded fluorescent beads. Heat map of hMSCs traction force B) without VA, and C) with VA. Colours correspond to magnitudes of forces as indicated in the colour bar. Graph D) shows increase in traction force in addition of valproic acid with respect to control. E) shows positive correlation between cell spread area and cellular traction *$p<0.05$, N=3, n >20. scale bar: 20 µm

Effect of HDACi on YAP nuclear translocation of hMSCs cultured on soft substrates

YAP (Yes-associated protein) is a cellular mechanosensor that translocates between nucleus and cytoplasm depending on substrate rigidity (8). This balance of nuclear to cytoplasmic ratio plays a crucial role in hMSCs fate determination. When hMSCs are cultured on a stiff substrate, YAP translocate more into the nucleus promoting osteogenesis while on the softer substrate, nuclear localization is lesser promoting adipogenesis. As this is a key molecule in the mechanosensing process, it was imperative to check the effect of hDACi on substrate mediated YAP translocation. We found that when hMSCs were cultured on the soft gel (3kPa) in presence of valproic acid, nuclear localization of YAP increased by ~1.5 times as compared to control (Fig. 4 A-E). We also checked for nuclear to cytoplasmic ratio and found that in the presence of valproic acid nuclear to cytoplasmic ratio increased by ~ 1.6 times as compared to control (Fig. 4F).

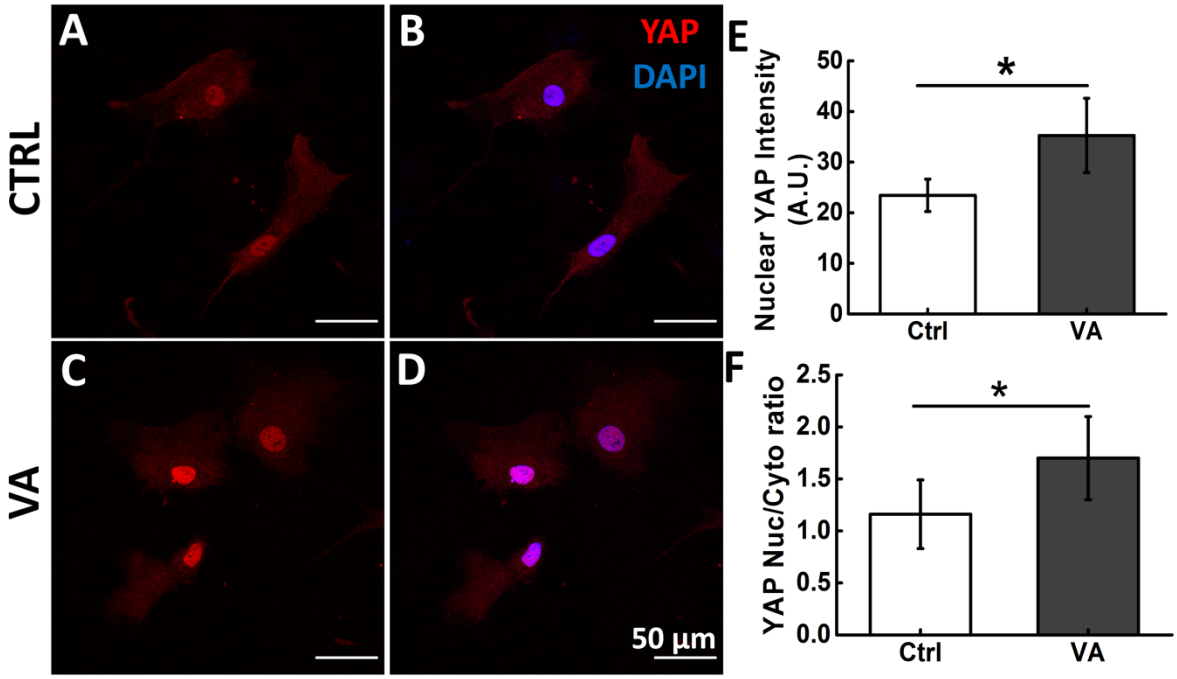

Fig 4. HDACi increases YAP nuclear translocation in hMSC cultured on soft hydrogel: Representative fluorescence images of YAP (red) and nucleus (DAPI:blue) on 3kPa gel with (C & D) and without (A & B) Valproic acid. Graph E) shows increase in Nuclear YAP intensity upon addition of valproic acid w.r.to control. (F) shows increase in nuc/cyto ratio upon addition of valproic acid w.r.to control. *$p<0.05$, N=3, n=30, Scale Bar: 50 µm

HDACi suppresses adipogenic differentiation and promotes osteogenic differentiation of hMSCs on soft gel

One of the most important characteristics of hMSCs is their multilineage potential which makes them an attractive choice for tissue engineering. It has been widely reported that soft substrates promote adipogenic differentiation and suppress osteogenic differentiation of hMSCs (11–15). To explore the combinatorial effect of substrate stiffness and HDACi on adipogenic/osteogenic differentiation of hMSCs, we cultured them on collagen coated PAA gels of 3 kPa stiffness with and without VA in presence of adipogenic/osteogenic differentiation media. We checked the formation of lipid oil droplets and expression of PPAR-γ which are the markers for adipogenic differentiation. Similarly, to assess the osteogenic differentiation, we checked the expression level of RUNX2 and osteopontin which are the markers for osteogenic differentiation. We found that addition of VA reduces the formation of lipid oil droplets at 3kPa substrate stiffness by almost one third. (Fig. 5A-C). Similar results were observed from different HDACi i.e., sodium butyrate (Fig. S3). We also checked for PPAR-γ which is a key transcriptional factor that regulates the expression of gene responsible for adipogenesis. We found that in the presence of VA PPAR-γ expression decreases ~28% compared to control. On the other hand, the expression of RUNX2 and osteopontin, two key transcriptional factors for osteogenesis, increases by ~26% and ~ 77% respectively in hMSCs cultured on soft gel in presence of VA as compared to control (Fig. 5G-L). These data together clearly shows that HDACi interferes with the mechanosignaling process in hMSCs and supersedes the effect of soft substrate on adipo/osteo differentiation.

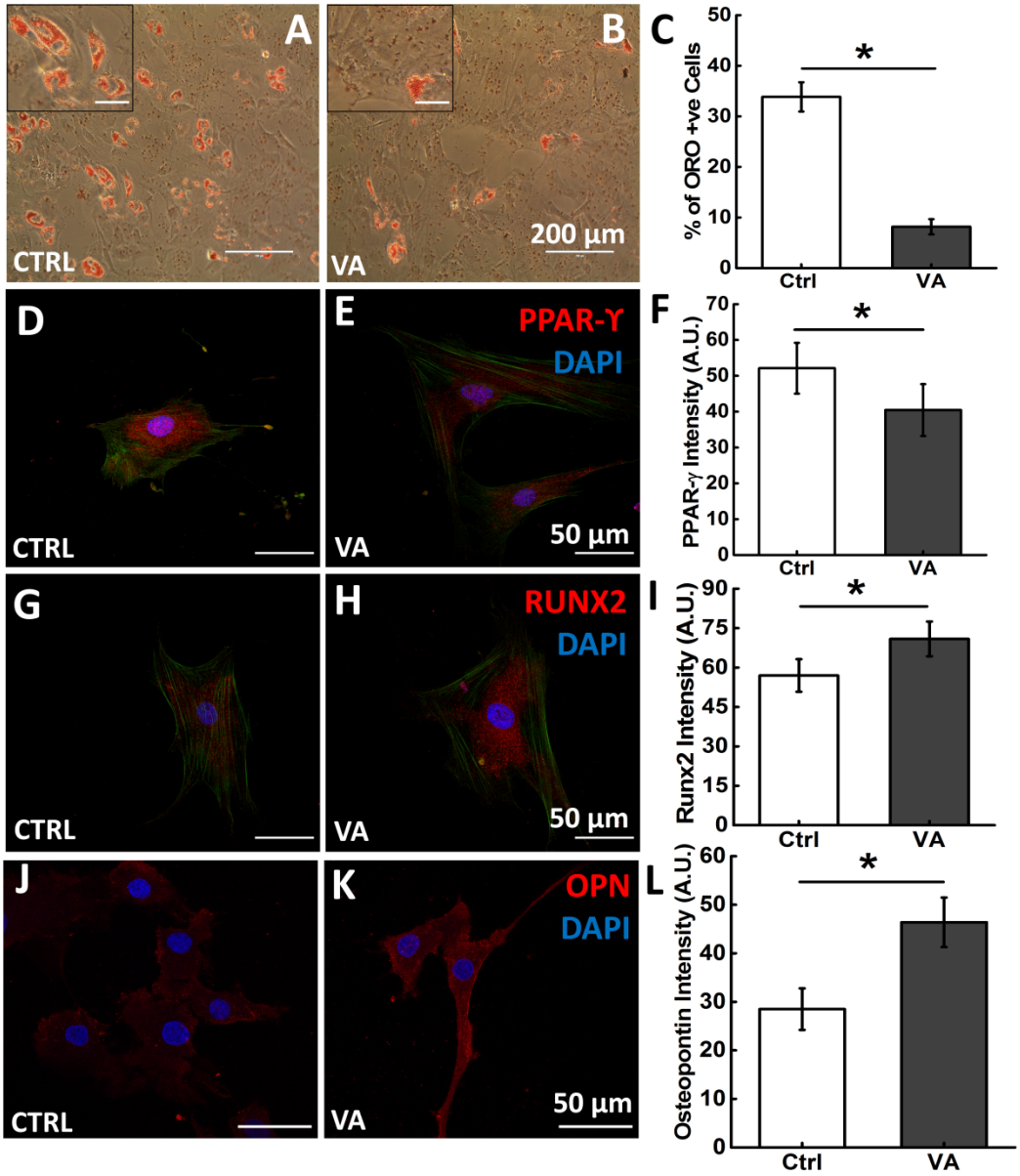

Fig. 5 HDACi inhibits adipogenesis of hMSCs cultured on soft hydrogels (3 kPA) and enhances osteogenesis. Oil red O staining after ten days of culture of hMSCs in adipogenic media and on soft gel (A) without VA, and (B) with VA, and (C) the corresponding quantification show significant drop in adipogenesis upon treatment with VA. (D and E) The same has been further demonstrated by staining adipogenic marker protein PPAR-gamma (red) and (F) corresponding quantification. When the cells were cultured with osteogenic induction media, VA enhanced osteogenesis as shown by staining (G-I) RUNX2and (J-L) osteopontin (OPN). For all the results, cells without VA but in corresponding differentiation induction media was considered as control. *$p<0.05$ N=3 n>25.

Effect of HDACi on p-ERK expression on soft substrate

Our results suggest that in the presence of VA, adipogenic differentiation is inhibited even on compliant substrate. Earlier studies have shown that ERK/MAPK signalling plays a key role in regulation of mesenchymal stem cell differentiation (50). It is now known that stiff substrate promotes osteogenesis by activating ERK signalling pathway while soft substrate downregulates activated ERK Signalling pathway and promotes adipogenesis (51). Hence, we Investigated if VA overwrites the effect of substrate stiffness on osteo/adipo lineage specification via regulating ERK. We checked for the expression of p-ERK in the presence of VA by immunostaining and found it to be increased by ~35 % as compared to the control (fig.6A, B and E). To further investigate the essentiality of ERK activation in the VA induced lineage specification, p-ERK pathway inhibitor PD 98059 (20µM) was used (Fig.6C, D, and E). Our results show that in the presence of PD 98059 there is an ~25 % decrease in the p-ERK expression. However, when VA and PD are used together p-ERK level is restored.

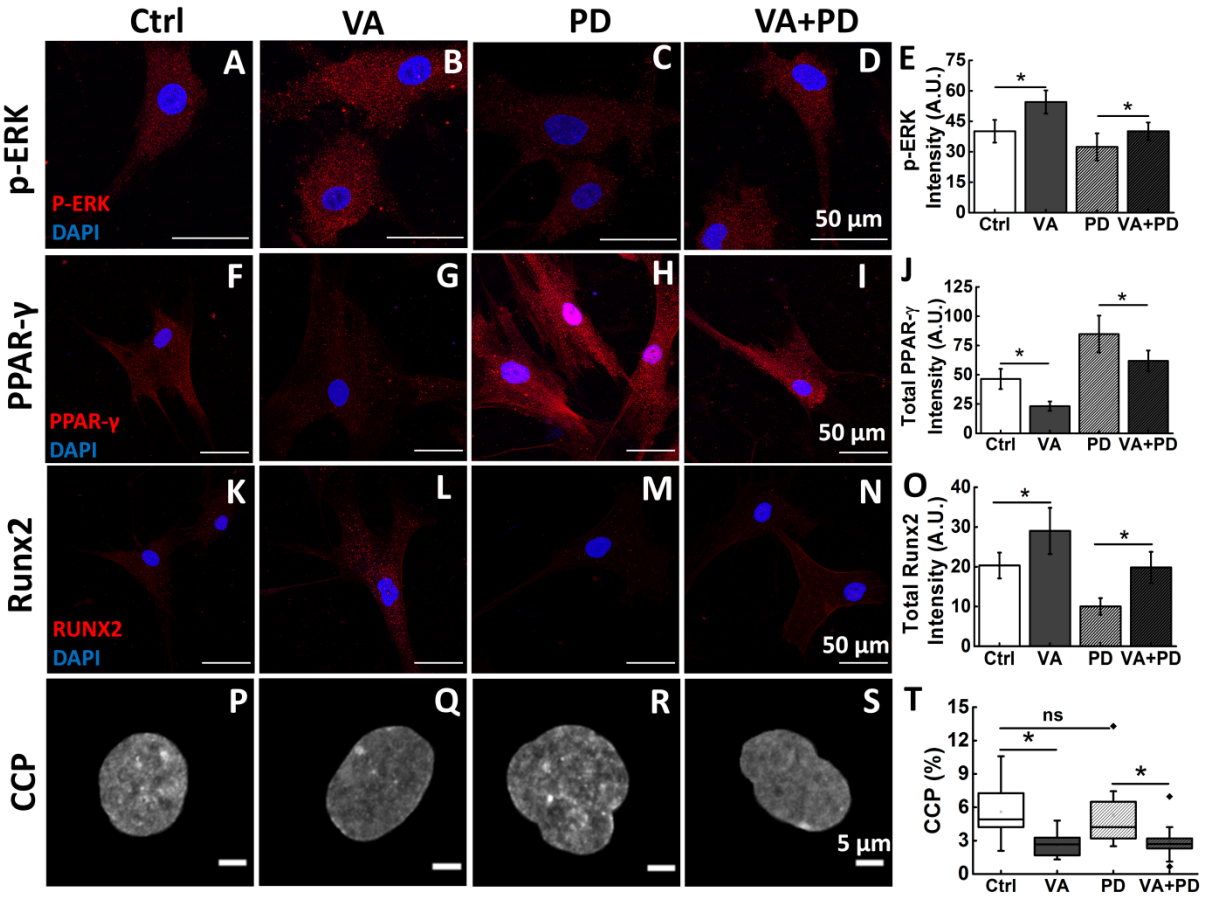

Fig. 6 HDACi overwrites the effect of substrate stiffness via p-ERK: (A-E) On soft gel, VA increases the expression of p-ERK (red). (C) A p-ERK inhibitor PD98059 when (D) added along with VA, brings p-ERK level back to the level of control (without any inhibitor). (F-J) The expression of adipogenic marker PPAR-γ (red) and (K-O) osteogenic marker RUNX2 are rescued upon inhibiting p-ERK with PD indicating

that HDACi overwrites the effect of substrate stiffness via p-ERK. Images (P-T) show that inhibition of p-ERK does not interfere with the chromatin condensation effect of VA.*$p<0.05$, N=3 n=25

We also checked the effect of p-ERK inhibition on PPAR-γ and RUNX2 expression. We found that the loss of adipogenic differentiation on soft substrate by VA (Fig. 6G) is restored when simultaneously also treated with PD (Fig. 6 I and J) which by itself promotes adipogenesis by blocking p-ERK (Fig 6H and J). As expected, an opposite trend was observed for osteogenic differentiation of hMSCs on soft substrates, as shown by the expression of RUNX2 (Fig. 6 K-O). We also checked the chromatin condensation parameter to understand the condensed state of chromatin. We found that VA (2.6 ± 1.02) significantly reduces chromatin condensation compared to the control (5.6 ± 2.26). However, application of PD98059 does not have any effect on CCP in presence or absence of VA, indicating phosphorylation of ERK does not interfere with the change in chromatin condensation by acetylation/deacetylation.

Increasing Chromatin condensation promotes adipogenic differentiation potential of hMSCs on soft hydrogel.

In the previous sections, we have shown that chromatin decondensation through HDACi decreases the adipogenic differentiation and promotes osteogenic differentiation of hMSCs on soft hydrogel. If the substrate stiffness is controlling the differentiation via chromatin packing as we have suggested here, the adipogenic differentiation should increase on soft hydrogel upon further condensing the chromatin. To verify that possibility, we treated hMSCs on soft gel with Anacardic Acid (30 μM) (ANA), a well-known histone acetyl transferase (HAT) inhibitor (52, 53). Exposure to ANA results in further chromatin condensation via decreased acetylation of histone proteins. Our results show that treating hMSC on soft PAA gel with ANA significantly increases (~24 %) the formation of lipid oil droplet as compared to control (Fig.7A, B and C).

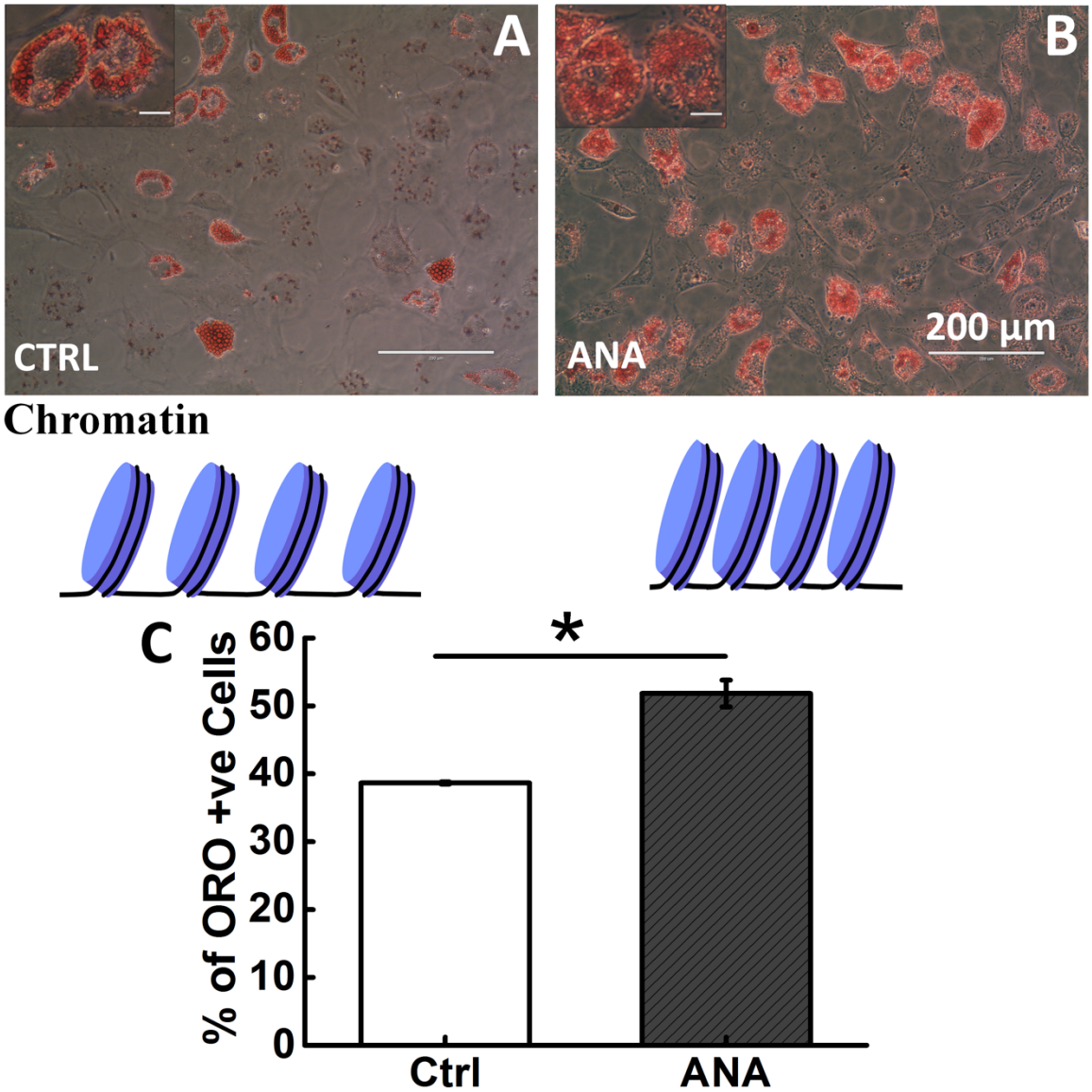

Fig.7 Chromatin condensation promotes osteogenesis of hMSCs cultured on soft hydrogel: Oil red O staining after ten days of culturing hMSCs in adipogenic induction media (AIM) and on soft gel (3 kPa) (A) AIM, (B) AIM with anacardic acid. Graph C) shows quantification of percentage of lipid oil droplets in presence of AIM and AIM with ANA. *$p<0.05$ N= 3 gels.

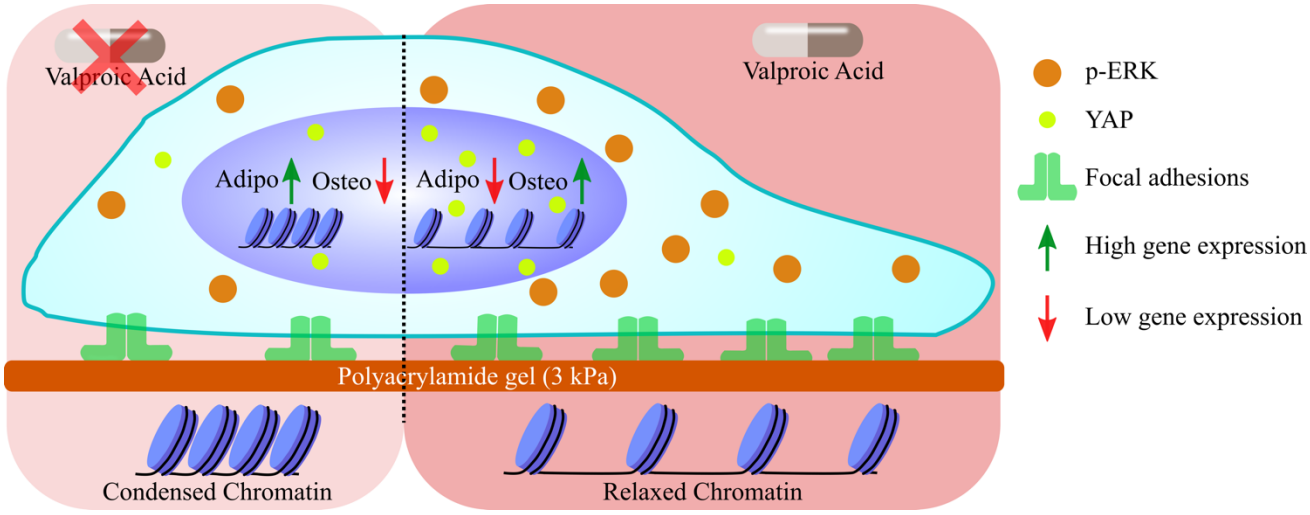

Fig. 8 Schematic diagram summarizing the effect of valproic acid (HDACi) on the mechanoresponse of hMSCs cultured on soft hydrogel.

Discussion

In this work, we have found that inhibition of histone deacetylation overrides the effect of substrate stiffness on the behaviour of hMSCs when they are cultured on soft hydrogels (Fig 8). In the presence of VA, a well-established HDACi, hMSCs on soft gels spread more (Fig.1), generate higher traction (Fig.3), and express higher maturation of focal adhesion and formation of stress fibres (Fig.2). These are some of the critical hallmarks that the cells exhibit when cultured on the rigid substrates but not on the soft substrates. These observations indicate that the effect of substrate rigidity on cellular behaviour can be modulated by modulating chromatin packing. Further, we looked at the mechanosensory protein YAP and found it to localize more in the nucleus as generally seen on stiff substrates(8). Finally, we found that HDACi not only modifies the morphological phenotypes but also control the hMSC differentiation (Fig. 5). While soft substrates promote adipogenic differentiation, in the presence of VA, osteogenic differentiation is preferred. We have further shown that HDACi control differentiation via phosphorylation of Extracellular Regulated Kinase (ERK). If phosphorylation of ERK is inhibited, the chromatin stays in its condensed state but its effect on differentiation disappears and the adipogenic differentiation is restored (Fig. 6).

hMSCs are the popular choice of stem cells in tissue engineering and regenerative medicines due to their tri-lineage differentiation potential(54, 55). However, to achieve a desired differentiation outcome, it is important to understand the role of various cues in governing the lineage specification. Matrix stiffness is one of such crucial cues that has been shown to control the differentiation of

hMSCs(14). When cultured on soft gel that mimic stiffness of fat tissue, hMSCs commit towards adipogenic lineage. Similarly, when cultured on rigid substrates, osteogenic differentiation is preferred(11, 56). Hence, while designing tissue scaffolds, such mechanical control on cellular behaviour needs to be taken into consideration. However, for various other practical reasons, the mechanical properties of the scaffold may not match the desired differentiation outcome. For example, in a bone scaffold, compliant materials might be preferred to faithfully match the defect shape. However, a compliant substrate is unsuitable for osteogenic (16, 38)Hence, it is needed to know how such mechanical controls of cellular differentiation can be overwritten by comparing the potency of conflicting cues and understanding the sequence of molecular players.

In this context, we have investigated the effect of histone deacetylase inhibitor HDACi on hMSCs cultured on soft polyacrylamide (PA) hydrogel substrates. HDACi are the group of molecules which are used in chemotherapy. In this work, we have used Valproic Acid which is an FDA approved anti-cancer agent. It has been used in breast cancer, head and neck cancer, cervical cancer etc (57–60). VA has also been used in various neurological disorders such as delirium, agitation, epilepsy(61). Due to its known inhibitory effect in higher concentration (~4mM) (62), in this work concentration of VA was kept at its minimal level (0.5mM).

As already mentioned, soft substrates promote adipogenic differentiation of hMSCs which is associated with epigenetic modification of the chromatin. Recently, Anseth's group have shown that substrate stiffness influences chromatin remodelling and epigenetic modification(28, 34). hMSCs cultured on stiff substrate have increased histone acetylation due to decreased expression of HDACs and increase expression of HATs leading to more decondensed chromatin state as compared to the cells on soft substrates(34). So, we asked what the cell fate will be if two contradictory signals are provided to the cells, namely mechano-signals from the soft gel which condense the chromatin and promotes adipogenic differentiation and chemical signals from HDACi which decondense the chromatin and promotes osteogenic differentiation. We have found that all the known downstream effect of the soft substrates on hMSCs can be changed by changing acetylation status of the chromatin. The role of the chromatin condensation on differentiation potential of hMSCs on soft hydrogel was further probed by using histone acetyl transferase inhibitor (HATi). Our results show that HATi concurrently increases the chromatin condensation and adipogenic differentiation.

In summary, our results suggest that if histone deacetylation is inhibited then hMSCs on soft gel behave as if they are on a stiff substrate. In other words, effect of mechanosignals can be completely masked by using a chromatin modifier. However, in future it is to be checked if same conclusion can be drawn by activating/inhibiting HATs on soft/stiff substrates. It is also to be seen if other known

lineage specifications of hMSCs that is chondrogenic, myogenic, and neurogenic differentiation can be achieved on substrates of different rigidities by simply changing the overall chromatin condensation state by using a cocktail of chromatin modifiers. Overall, the results presented in this paper advance our general understanding of the working of mechanosignals in the context of stem cell differentiation and regenerative medicines.

Author Contribution:

A.M. and R.J. conceptualized and designed the research; A.M. supervised the project. R.J., P.M., P.Y. and V.D. performed the experiments. R.J., P.M., P.Y. and V.D. analyzed the data. R.J. prepared the figures. R.J. and A.M. interpreted the results. R.J., A.M. and P.M wrote the paper. P.M., P.Y., V.D. provided the critical inputs on the manuscript.

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SUPPLEMENTARY INFORMATION

Table S1

Acrylamide from 40% stock solution (ml)	Bis-acrylamide from 2% Stock solution (ml)	Water (ml)	E ± St. Dev. (kPa)
1	1.125	7.875	3.13 ± 0.42

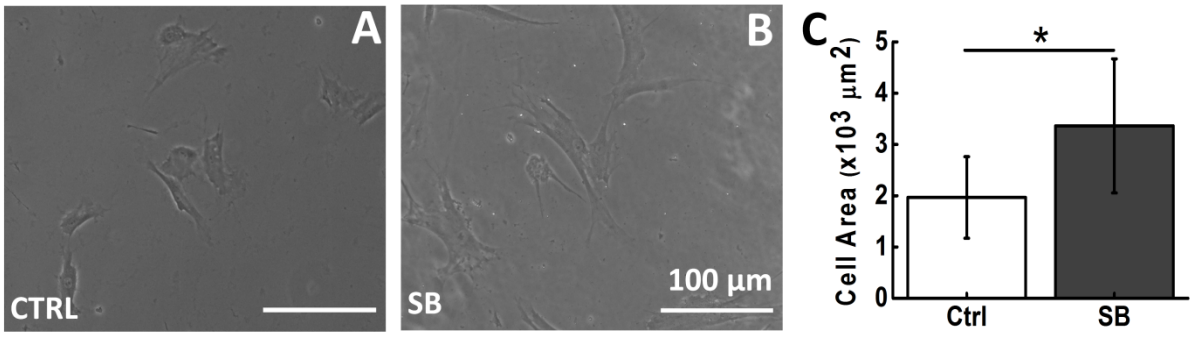

Fig. S1. HDACi increases cell spread area on soft hydrogel. Representative images of hMSCs cultured on 3 kPa gel (A) without SB and (B) with SB. Graph (C) show quantitative analysis of change in projected cell area on 3 kPa gels with and without SB. *p<0.001 n > 50 cells, scale bar: 100 µm.

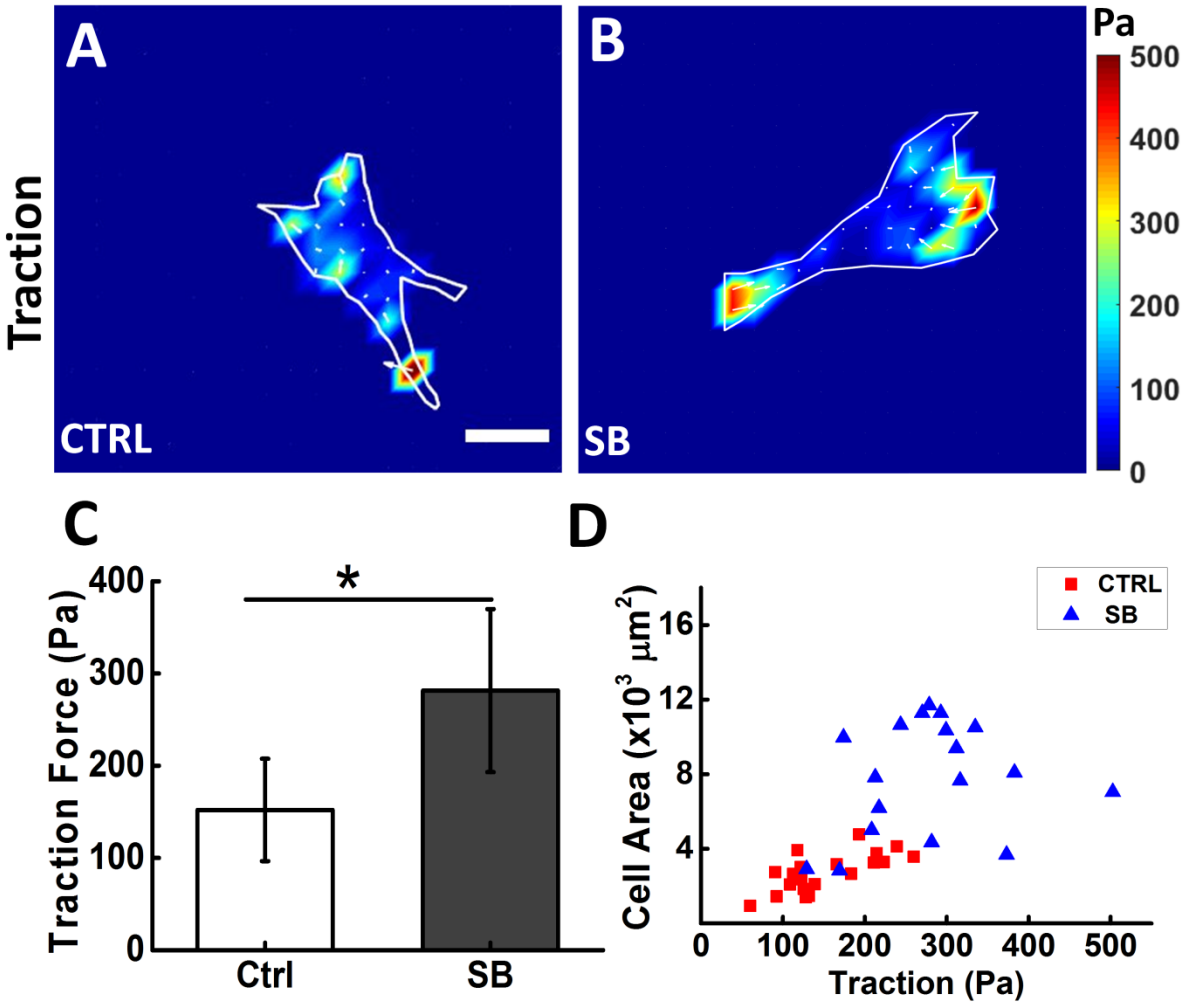

Fig. S2. HDACi increases hMSCs traction on soft hydrogel. Heat map of hMSCs traction stress (A) without SB, (B) with SB. Colours corresponds to magnitude of stresses as indicated in the colour bar. Graph (C) shows quantitative analysis of traction force on 3 kPa gel with and without SB (Ctrl). Scatter plot (D) shows positive correlation between cell spread area and cellular traction. *p<0.001 n= 18 cells scale bar: 100 µm.

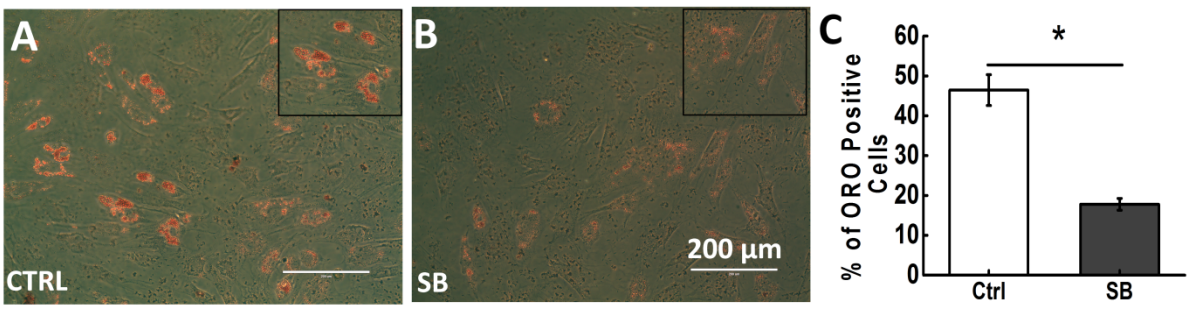

Fig. S3. HDACi inhibit adipogenesis of hMSCs cultured on soft hydrogel. Representative images of Oil Red O staining on 3 kPa gel without SB (A) and with SB (B). The insets are the

magnified local image to show Oil Red O formation. Graph (C) shows quantitative analysis of change in percentage of 0RO positive cells with and without SB. *p<0.001 scale bar: 100 µm.