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Chromatin remodeling and nucleoskeleton synergistically control osteogenic differentiation in different matrix stiffnesses

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ABSTRACT

Matrix stiffness plays an important role in determining cell differentiation. The expression of cell differentiation-associated genes can be regulated by chromatin remodeling-mediated DNA accessibility. However, the effect of matrix stiffness on DNA accessibility and its significance for cell differentiation have not been investigated. In this study, gelatin methacryloyl (GelMA) hydrogels with different degrees of substitution were used to simulate soft, medium, and stiff matrices, and it was found that a stiff matrix promoted osteogenic differentiation of MC3T3-E1 cells by activating the Wnt pathway. In the soft matrix, the acetylation level of histones in cells was decreased, and chromatin condensed into a closed conformation, affecting the activation of β-catenin target genes (Axin2, c-Myc). Histone deacetylase inhibitor (TSA) was used to decondense chromatin. However, there was no significant increase in the expression of β-catenin target genes and the osteogenic protein Runx2. Further studies revealed that β-catenin was restricted to the cytoplasm due to the downregulation of lamin A/C in the soft matrix. Overexpression of lamin A/C and concomitant treatment of cells with TSA successfully activated β-catenin/Wnt signaling in cells in the soft matrix. The results of this innovative study revealed that matrix stiffness regulates cell osteogenic differentiation through multiple pathways, which involve complex interactions between transcription factors, epigenetic modifications of histones, and the nucleoskeleton. This trio is critical for the future design of bionic extracellular matrix biomaterials.

1. Introduction

The extracellular matrix microenvironment influences various cell functions. Matrix stiffness can activate integrins and reorganize the cytoskeleton and other mechanosensors, thereby regulating the expression level or activity of mechanosensitive transcription factors, converting mechanical signals into biochemical signals and affecting cell gene expression and differentiation [1–3]. Recent studies have reported that matrix stiffness can remodel chromatin and affect DNA accessibility. DNA accessibility refers to the degree to which specific DNA segments on chromosomes can be accessed and contacted by nuclear proteins. Chromatin remodeling, by forming "open" or "closed" chromatin conformations, can affect the specific interaction between transcription factors and DNA, which is a key step in the regulation of gene transcription [4–8]. In addition, how matrix stiffness regulates chromatin remodeling and the importance of chromatin remodeling for differentiation remain to be elucidated [9,10].

In addition to regulating chromatin accessibility, ECM stiffness can also affect the mechanotransduction pathway, which involves the nucleus as a key mechanosensor in the regulation of gene expression [9,11,12]. Lamin A/C is a mechanosensitive protein located on the inner side of the nuclear membrane [13,14]. The expression level of lamin A/C was positively correlated with tissue stiffness in vivo [15], and patients with lamin A/C deficiency or mutation had skeletal developmental

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deformities [16]. Although lamin A/C was found to play an important role in regulating osteogenic differentiation, interestingly, the promotion of osteogenic differentiation by lamin A/C overexpression is dependent on the stiff matrix [14]. Recent literature has shown the importance of epigenetic regulation for the activation of mechanotransduction [17]. We speculated that matrix stiffness-mediated chromatin remodeling may affect the activation of lamin A/C-involved mechanotransduction.

In this study, we used GelMA with two degrees of substitution to simulate the extracellular matrix with different stiffnesses. First, we found that the cells formed a condensed chromatin structure in the soft matrix compared to the stiff matrix. This condensed chromatin structure was associated with a decrease in acetylated histone H3 (Ac-H3) and repression of transcription of β-catenin target genes. When we increased Ac-H3 by using TSA or β-catenin levels in cells by using Wnt-3a in the soft matrix, β-catenin target genes were still not significantly upregulated, and β-catenin remained restricted to the cytoplasm. We hypothesized that matrix stiffness regulates not only the accessibility of β-catenin to target genes but also the nuclear translocation of β-catenin. Further studies revealed that the downregulation of lamin A/C in the soft matrix restricts the nuclear translocation of β-catenin, and the "closed" chromatin conformation in the soft matrix affects the transcription of β-catenin target genes. Finally, we found that overexpression of lamin A/C and concomitant treatment of cells with TSA or Wnt-3a was more effective in activating β-catenin/Wnt signaling in cells in the soft matrix.

In conclusion, matrix stiffness mediates nuclear translocation of β-catenin by regulating lamin A/C expression, but transcriptional activation also depends on matrix stiffness-mediated changes in DNA accessibility. The results suggested that this dynamic chromatin remodeling mediated by three-dimensional (3D) matrix stiffness is closely related to cell fate, which is critical for understanding material/matrix rigidity-mediated osteogenic differentiation and the design of bionic extracellular matrix biomaterials.

2. Methods

2.1. Cell culture

MC3T3-E1 (Academy of Sciences Cell Bank, China) were cultured in α-MEM medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (HyClone, USA) at 37 °C and 5% CO2. When the culture reached 80–90% confluence, cells were digested with trypsin and collected by centrifugation, cells were then resuspended with GelMA (EFL, China) solutions at a density of 5×10^6 Cells/ml. Pipette 180–200 μL of GelMA/cell suspension into each curing ring (EFL, China) and cured with ultraviolet light (light wavelength, 405 nm; light intensity, 25 mW/cm^2). The cell-laden hydrogels were submerged in the medium and the medium was changed every 2 days.

2.2. Mechanical tests

Lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate (EFL, China) was dissolved in PBS (HyClone, USA) at a concentration of 0.25% weight/volume (w/v). Two different substitutions of GelMA macromers (10% w/v) were dissolved in LAP solution (0.25% w/v) at 60 °C. The solution was added to a PDMS module with a diameter of 25 mm and a height of 1 mm, the 30% substitution GelMA was cured with UV light for 40 s, and the 60% substitution GelMA was cured with UV light for 30 or 40 s, respectively, and then soaked in PBS solution for 24 h. Storage modulus and loss modulus were measured by shear rheology using a Physica MCR 302 rheometer (Anton Paar, Austria). The temperature of the gel solution was controlled with a Peltier element (T = 37 °C). Frequency sweep tests were performed in the linear regime for all samples. The frequency sweep was performed with a strain amplitude of $\gamma = 1\%$ over a frequency range of 0.1–100 rad s-1.

2.3. RNA isolation and gene expression analysis

The cell-laden hydrogel samples were washed three times with PBS, crushed with a cryogenic grinder, and then total RNA was isolated using Trizol reagent (Takara, Japan). The RNA was reverse-transcribed into cDNA and amplified using quantitative real-time PCR (q-PCR) in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The experiment was performed independently three times. Ribosomal protein L13a (Rpl13a) was used as the housekeeping gene, and the primer sequences for the genes were provided in Table S1.

2.4. Protein isolation and western blot

The cell-laden hydrogels were lysed by GelMA Lysis Buffer (EFL, China) at 37 °C for 30 min. Digestion was terminated using the complete medium, cells were washed with PBS. Whole-cell lysates was extracted with RIPA lysis buffer (Beyotime, China) containing protease and phosphatase inhibitors (Sigma, USA) and treated by ultrasonic cell disruption instrument for 15 min. For the separation of nuclear and cytoplasmic proteins, the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) was used and the extraction of nuclear and cytoplasmic fractions was performed according to the manufacturer's instructions. The protein concentration was determined by bicinchoninic acid (BCA) assay with Pierce BCA protein assay kit (Beyotime, China), and samples were normalized concerning protein content. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, USA). The membranes were blocked in 5% nonfat dry milk for 2 h at room temperature and then incubate with primary antibodies against lamin A/C (1:1000, CST, USA), Runx2 (1:1000, Abcam, USA), Osx (1:1000, Abcam, USA), lamin B1 (1:1000, Abcam, USA), Ac-H3 (1:2000, Millipore, USA), β-actin (1:1000, Proteintech, China), Active β-catenin (1:1000, CST, USA), HDAC3 (1:1000, Abcam, USA), CBP (1:1000, CST, USA), β-tubulin (1:1000, Proteintech, China), GAPDH (1:1000, Proteintech, China), Emerin (1:1000, Proteintech, China), Phospho-myosin light chain 2 (*p*-MLC) (1:1000, CST, USA), overnight at 4°. HRP-conjugated secondary antibodies (1:5000; Proteintech, China) were used to bind primary antibodies at room temperature for 2 h. The membranes were washed with TBST and imaged with the chemiluminescent reagent (Millipore, USA) and the imaging system (Bio-Rad, USA). The captured images were quantified in FIJI (NIH, USA).

2.5. Immunofluorescence

The cell-laden hydrogels were fixed with 4% paraformaldehyde for 15 min at room temperature, then the hydrogel was cut into pieces, washed with PBS, and permeabilized with 0.3% Triton X-100 for 30 min at room temperature. The sample was blocked with 5% donkey serum (Invitrogen) for 1 h at room temperature. Next, the cells were incubated with primary antibody against lamin A/C (1:200, CST, USA), Ac-H3 (1:500, Millipore, USA), Active β-catenin (1:200, CST, USA), HDAC3 (1:200, Abcam, USA), CBP (1:200, CST, USA), Emerin (1:50, Santa Cruz, USA) or Emerin (1:100, Proteintech, China). The secondary antibody (Invitrogen, USA) was incubated at a ratio of 1:200 for 2 h at room temperature. Filamentous actin (F-actin) was labeled using Alexa-Fluor 488 phalloidin (Invitrogen, USA) and DAPI (Beyotime, China) was diluted at a ratio of 1:1000 and incubated at room temperature for 10min. Immunofluorescence images were captured with a laser scanning confocal microscope (LCSM) (Leica, Germany).

2.6. siRNA transfection

Mouse emerin siRNAs were purchased from Santa Cruz Technology. The cells were cultured in the plate. When the cell confluence reached 70%–90%, the siRNAs were then transfected with Lipo 3000 (Thermo

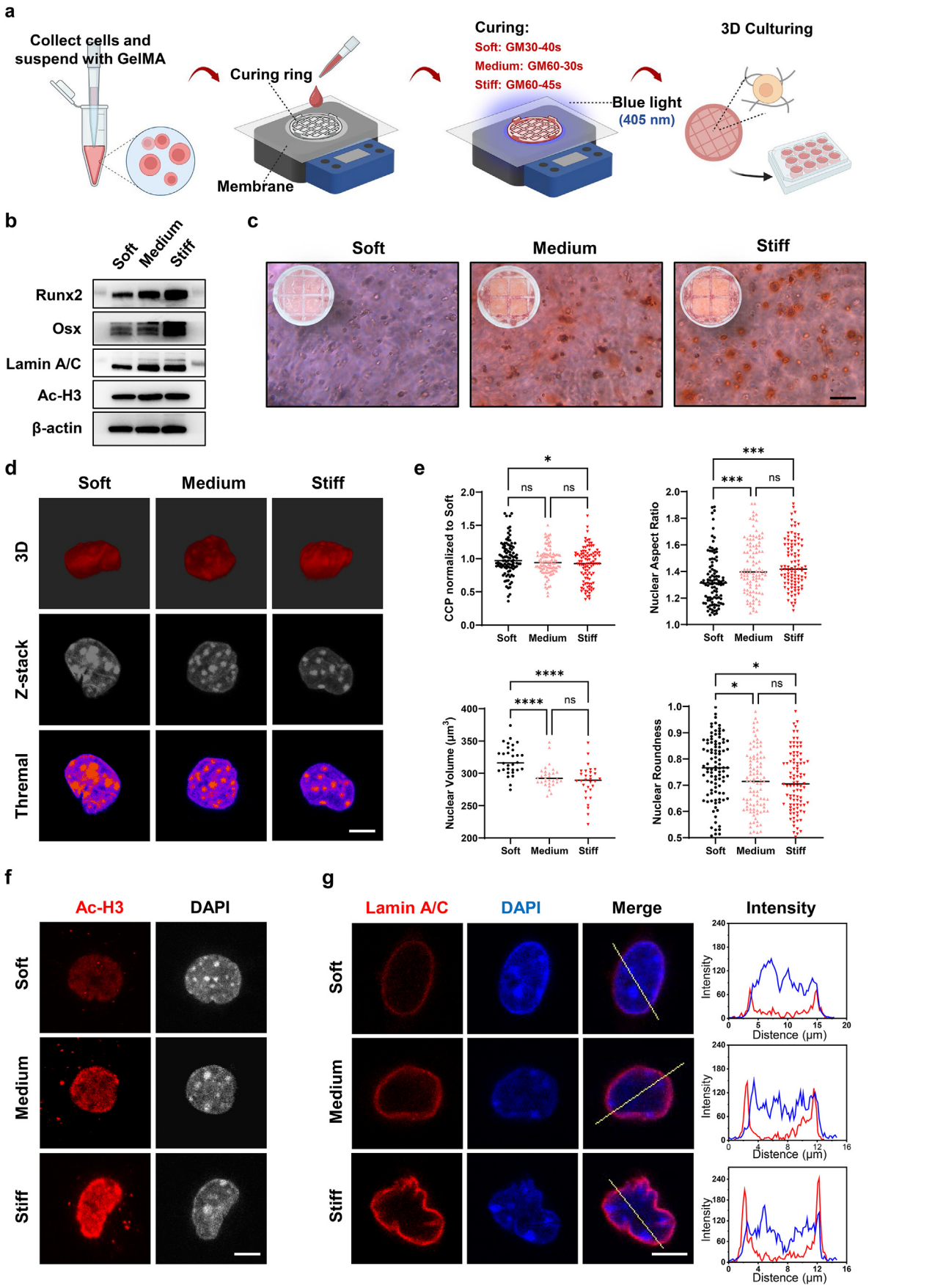

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Fig. 1. Stiff matrix stimulates osteogenic differentiation concomitant with the reduction of chromatin condensation and upregulation of lamin A/C. a) The flow of the 3D cell culture. **b)** Representative Western blots for Runx2, Osx, lamin A/C, Ac-H3, and the housekeeper protein β-actin of MC3T3-E1 cells that were cultured for 4 days in soft, medium, and stiff gels before lysis. **c)** Mineralized nodules formation of cells in gel were revealed by Alizarin red staining. Scale bar, 50 μm. **d)** Representative images of DAPI-stained nuclei in three different gels after a 2 days culture. Scale bar, 5 μm. **e)** CCP for cells cultured in hydrogels with different stiffness. The volume of the nucleus and nuclear roundness was quantified based on nuclear images after a 2 days culture. Mean ± SD (n = 100 images) by one-way ANOVA with Bonferroni's post-hoc test. **f)** Z-stack confocal imaging of Ac-H3 in MC3T3-E1, cultured in soft, medium, and stiff gels for 2 days. Scale bar, 5 μm. **g)** Z-stack confocal imaging of lamin A/C in MC3T3-E1, cultured in gels for 2 days. Scale bar, 5 μm. n = 3. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fisher, USA). Twenty-four after transfection, the cells were detached with trypsin, collected by centrifugation, and encapsulated in hydrogel.

2.7. Lentivirus infection

The overexpression and knockdown lentiviruses were purchased from Hanbio Biotechnology. To perform lentiviral infection, MC3T3-E1 were seeded into 12-well plates and cultured in medium until the cell reached 50% confluence. The cells were then incubated with lentiviral particles (MOI = 20) in medium supplemented with 5 μg/ml polybrene (Hanbio Biotechnology, China). After incubating the plates at 37 °C for 24 h, the medium was replaced. Stably transduced cells were selected with 5 μg/ml puromycin and maintained in medium containing 2 μg/ml puromycin. The efficacy of lentiviral infection was verified by quantitative real-time PCR.

2.8. Nuclear to cytoplasmic ratio quantification

Immunofluorescence images were acquired on the LCSM. To calculate the nuclear-to-cytoplasmic ratio of a protein, images with focal plane close to the middle of the nucleus were selected for further analysis. The nuclear and cytoplasmic regions were delineated using FIJI (NIH, USA) software. The fluorescence intensity of each region was measured to obtain the fluorescence signal intensity of the protein. The ratio of the fluorescence signal intensity of the protein in the nucleus to that in the cytoplasm was then calculated.

$$\text{Protein}\frac{\text{nuc}}{\text{cyt}}\text{ratio} = \frac{\frac{\text{integrated intensity of the protein in nuclear}}{\text{area of nucleus}}}{\frac{\text{integrated intensity of the protein in cytoplasm}}{\text{area of cytosol}}}$$

2.9. Inhibition experiments

All inhibitors were added to the culture medium 8 h after the cells were encapsulated in the hydrogel, at a concentration of 2 nM for TSA, 10 μM for Pu139, and 20 μM for blebbistatin (Bleb.), 1 μM for CHIR-99021 and 10 μM for MSAB. Treatment time was shown in each figure legend.

2.10. Chromatin condensation parameter (CCP) analysis

The nuclei were visualized by DAPI staining and imaged with the LCSM using a 63× oil objective. At least eight fields of view were imaged for each hydrogel. For analysis, individual nuclei for each field of view were isolated by cropping the image using FIJI and CCP was calculated as previously reported in the literature [18].

2.11. Statistical analysis

Data are presented as mean ± SD. All statistical analyses were performed with GraphPad Prism 9 software (GraphPad, USA). Equal variance data were analyzed by one-way ANOVA or Student's t-test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Stiff matrix stimulates osteogenic differentiation concomitant with a reduction in chromatin condensation and upregulation of lamin A/C

To simulate different extracellular matrix stiffnesses, we used GelMA with 30% and 60% substitution in this study. The 30% substitution degree of GelMA was cured with ultraviolet light for 40 s, and the 60% substitution degree of GelMA was cured under UV light for 30 and 40 s. The storage and loss moduli of the hydrogel are shown in Fig. S1. MC3T3-E1 cells were then encapsulated in the three hydrogels, and the flow of the 3D cell culture is shown in Fig. 1a. The osteogenic differentiation levels of the cells were tested by RT–qPCR (Figs. S2a and b), Western blots (Fig. 1b) and alizarin red staining (Fig. 1c). The results indicated that osteogenic differentiation was higher with increasing matrix stiffness.

Matrix stiffness can influence cell function by regulating chromatin remodeling in 2D conditions [5,6], however, the effects of matrix stiffness on the chromatin landscape in 3D conditions and the importance of chromatin remodeling on osteogenic differentiation remain unclear. To evaluate the state of chromatin under different stiffnesses, we labeled nuclear DNA with DAPI, and images were acquired for CCP analysis (Fig. 1d). The results showed a decreasing trend of chromatin condensation with increasing matrix stiffness (Fig. 1e). Nucleus morphology was also examined; the nucleus had the largest volume and a more rounded shape in soft hydrogels, while nuclei became increasingly irregular with increasing stiffness (Fig. 1e). Since the state of chromatin is associated with the covalent modification of histones [5], the level of Ac-H3 in cells cultured in different matrix stiffnesses was analyzed. The results showed that cells had the highest level of Ac-H3 in the stiff matrix, as determined by immunostaining or western blotting (Fig. 1b, f, Fig. S3). Regarding nuclear morphology, lamin A/C plays a crucial role in maintaining the shape of the nucleus as an important component of the nucleoskeleton [16]. The results showed that the expression level of lamin A/C was upregulated with increasing matrix stiffness (Fig. 1b, g).

3.2. Chromatin condensation correlates with histone acetylation and essentially influences osteogenic differentiation by activating the wnt/β-catenin pathway

To investigate whether chromatin remodeling is associated with changes in histone acetylation, we increased Ac-H3 levels in soft matrices with TSA and decreased Ac-H3 in stiff matrices with a histone acetyltransferase inhibitor (Pu139). CCP analysis showed that TSA treatment decreased chromatin condensation levels in soft gels, while Pu139 treatment increased chromatin condensation in stiff matrices (Fig. 2a). This finding suggests that matrix stiffness influences chromatin condensation by regulating the level of Ac-H3.

To elucidate the mechanism by which matrix stiffness-mediated chromatin remodeling affects osteogenic differentiation, we investigated the Wnt pathway, which is one of the most important pathways in regulating osteogenic differentiation [19]. Histone acetylation can activate the Wnt pathway by upregulating Wnt ligand and receptor expression [20]. We found that the stiff matrix upregulated nonphosphorylated (active) β-catenin, as well as the gene expression of Fzd-1, Fzd-7, Wnt-6,

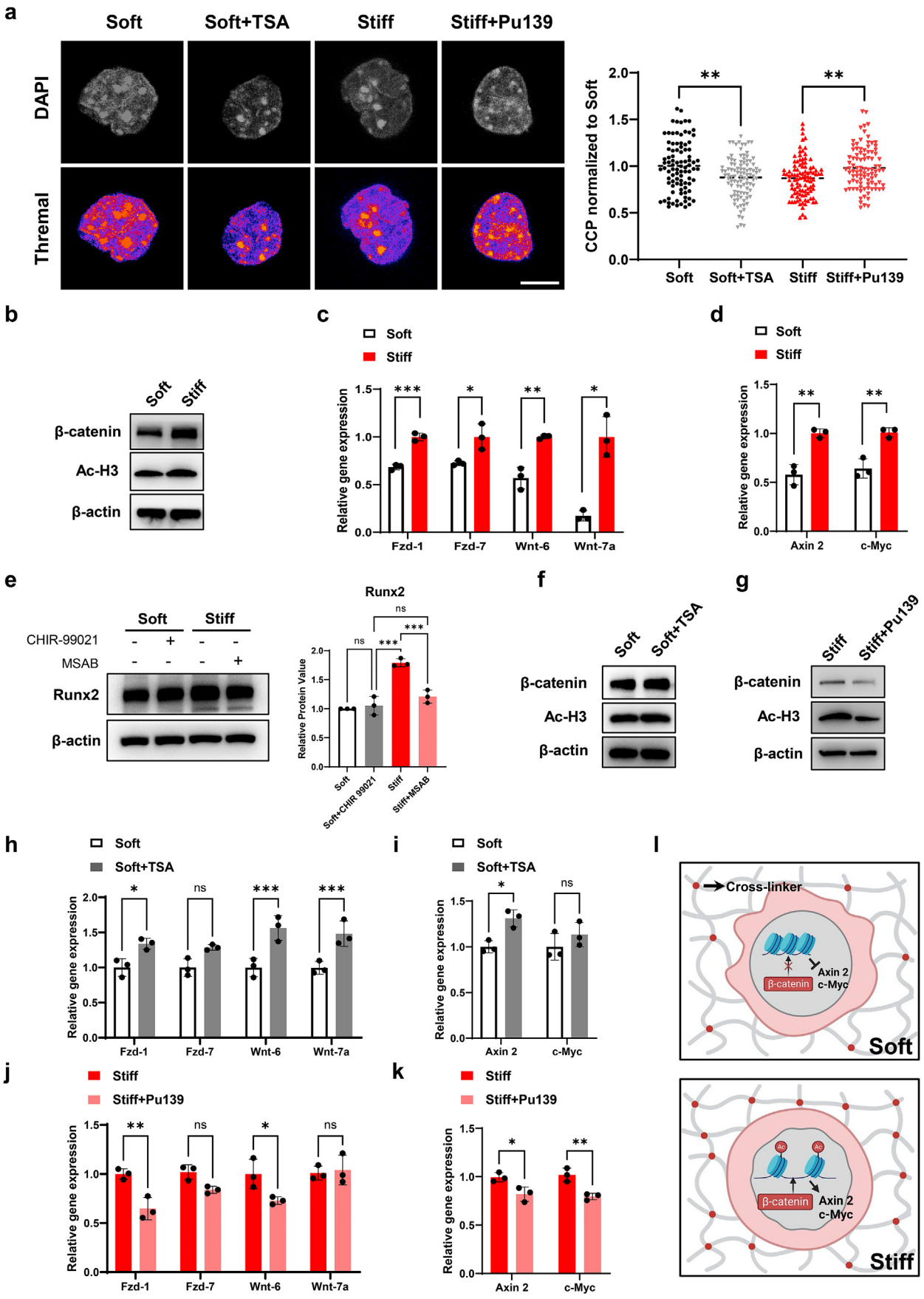

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Fig. 2. Chromatin condensation correlates with histone acetylation and essentially influences the osteogenic differentiation by activating Wnt/β-catenin pathway. **a**) Representative images of DAPI-stained nuclei after a 2 days culture subjected to CCP analysis. Scale bar, 5 μm. Right: quantification of chromatin compaction by CCP for cells in soft gel (Soft), TSA-treated cell in soft gel (soft + TSA), cells in stiff gel (Stiff), and Pu139-treated cell in stiff gel (stiff + Pu139) for 2 days (n = 100 images). **b)** Representative Western blots for active β-catenin, Ac-H3, and the housekeeper protein β-actin of MC3T3-E1 that were cultured for 3 days in the soft and stiff gel before lysis. **c)** Relative mRNA expression of Wnt-6, Wnt-7a, Fzd-1, Fzd-7 in MC3T3-E1 cultured in soft and stiff gels for 3 days. Mean ± SD (n = 3) by *t*-test. **d)** qPCR of Wnt-targeted genes in MC3T3-E1 from soft and stiff gels for 3 days. Mean ± SD (n = 3) by *t*-test. **e)** Representative Western blots for Runx2, and the housekeeper protein β-actin of cell in soft gel, CHIR-99021-treated cell in soft gel, cell in stiff gel, and MSAB-treated cell in the stiff gel for 3 days. Right: Quantification of Runx 2 on Western blot images; Mean ± SD (n = 3) by one-way ANOVA with Bonferroni's post-hoc test. **f)** Representative Western blots for active β-catenin, Ac-H3, and the housekeeper protein β-actin of MC3T3-E1 that were cultured for 3 days in soft and TSA-treated cells in soft before lysis. **g)** Representative Western blots for active β-catenin, Ac-H3, and the housekeeper protein β-actin of MC3T3-E1 that were cultured for 3 days in stiff and Pu139-treated cells in stiff before lysis. **h)** Relative mRNA expression of Wnt-6, Wnt-7a, Fzd-1, and Fzd-7 of cells in a soft gel and TSA-treated cells in soft gels for 3 days. **i)** qPCR of Wnt-targeted genes from the cell in soft gel and TSA-treated cell in the soft gel for 3 days. Mean ± SD (n = 3) by *t*-test. **j)** Relative mRNA expression of Wnt-6, Wnt-7a, Fzd-1, Fzd-7 of cells in stiff gel and Pu139-treated cell in stiff gels for 3 days. Mean ± SD (n = 3) by *t*-test. **k)** qPCR of Wnt-targeted genes from a cell in stiff gel and Pu139-treated cells in stiff gel for 3 days. **l)** The schematic illustration summarizing the effect of histone acetylation levels in different stiffness hydrogels on the expression of β-catenin target genes. ns: no significant difference, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

and Wnt-7a, in concert with increases in β-catenin target gene (Axin2 and c-Myc) expression (Fig. 2b–d). To further clarify the role of the Wnt pathway in osteogenic differentiation, we treated cells in stiff and soft hydrogels with an inhibitor of β-catenin (MSAB) and an activator of the Wnt pathway (CHIR-99021). Western blot analysis showed that MSAB significantly decreased the level of Runx2 in stiff gels, while CHIR-99021 did not significantly upregulate Runx2 (Fig. 2e). These results suggest that the stiff matrix regulates osteogenic differentiation by activating the Wnt pathway.

We then investigated whether the level of histone acetylation affects Wnt pathway activation. Treatment of cells in soft matrix with TSA resulted in an increase in active β-catenin levels, as well as gene expression of Fzd-1, Fzd-7, Wnt-6 and Wnt-7a and β-catenin target genes (Fig. 2f, h, i). Conversely, inhibition of histone acetylation with Pu139 decreased the levels of active β-catenin and the expression of these genes (Fig. 2g, j, k). These results suggest that matrix stiffness mediates changes in histone acetylation levels, which further regulates osteogenic differentiation by affecting Wnt pathway activation (Fig. 2l).

3.3. Lamin A/C mediates the nuclear localization of β-catenin to regulate the Wnt pathway and osteogenic differentiation

In Fig. 2e, CHIR-99021 was used to upregulate β-catenin and promote osteogenic differentiation of the cells, but the expression of Runx2 was not significantly upregulated. However, inhibition of β-catenin significantly suppressed the expression of Runx2. Similarly, when cells were treated with TSA in a soft matrix, β-catenin levels were increased to a level consistent with cells in the stiff matrix, but there was no significant increase in the expression of Runx2 (Fig. S4). We hypothesized that although TSA restored the expression level of β-catenin and DNA accessibility, β-catenin might still be restricted in the cytoplasm, thereby inhibiting the activation of the Wnt pathway and osteogenic differentiation of the cells. To test this hypothesis, we used Wnt-3a to increase the expression of β-catenin [21]. Immunofluorescence and western blot analyses revealed a higher nucleus/cytoplasm ratio of β-catenin in the stiff matrix in the presence of Wnt-3a (Fig. 3a and b). We then asked why the stiff matrix increased the nuclear translocation of β-catenin. A previous study reported that lamin A/C can regulate the activation of the Wnt pathway [22], and lamin A/C was shown to be upregulated in stiff hydrogels. To investigate this further, we generated a lentiviral shRNA vector of LMNA to knock down the expression of LMNA in MC3T3-E1 cells (Figs. S5a and b). The findings showed that knockdown of LMNA impaired osteogenic differentiation of the cells even in a stiff matrix (Figs. S5f–h) and resulted in reduced localization of β-catenin in the nucleus (Fig. 3c and d). Finally, TSA and Wnt-3a were used to upregulate β-catenin in sh-negative control (sh-NC) cells and sh-LMNA cells in the stiff matrix, and we found that the expression of β-catenin target genes showed significantly greater upregulation in the sh-NC cells (Fig. 3e). These results suggest that lamin A/C-mediated nuclear translocation of β-catenin is essential for the activation of the Wnt pathway (Fig. 3f).

3.4. Both histone acetylation and lamin A/C are required for activation of the Wnt pathway

To enhance the nuclear translocation of β-catenin in the soft matrix, LMNA was overexpressed in the cells (Figs. S5d and e). The osteogenic differentiation of the cells was partially restored (Figs. S5f and i), and the nuclear localization of β-catenin was increased (Fig. 4a). To assess the impact of LMNA overexpression on the activation of the Wnt pathway in the soft matrix, LMNA OE cells and negative control (NC) cells were encapsulated in soft gels and treated with Wnt-3a. Overexpression of LMNA could activate the transcription of β-catenin target genes more effectively in the presence of Wnt-3a than in NC cells (Fig. 4b). Similarly, NC and OE LMNA cells treated with TSA in soft matrix showed the same trend. These findings suggested that increased levels of LMNA and histone acetylation can synergistically promote the activation of the Wnt pathway (Fig. 4c and d).

3.5. Lamin A/C mediates the nuclear localization of β-catenin by emerin

Lamin A/C is a protein on the inner side of the nuclear membrane, and we then asked why lamin A/C can regulate the nuclear translocation of β-catenin. Previous studies have shown that emerin, a lamin-associated protein [23], can bind to β-catenin and inhibit its nuclear translocation [21]. We hypothesized that the decreased expression of lamin A/C in soft gel resulted in the diffusion of emerin in the cytoplasm, which restricts β-catenin entry into the nucleus. Immunofluorescence and western blotting showed that emerin was primarily located in the perinuclear region in the stiff matrix, and knockdown of lamin A/C resulted in increased expression of emerin in the cytoplasm (Fig. 5a and b). Similarly, the colocalization of emerin and lamin A/C was decreased in soft hydrogels, and more emerin was translocated to the cytoplasm (Fig. 5c and d). To verify whether the increase in emerin in the cytoplasm in the soft matrix led to a decrease in the nuclear translocation of β-catenin, we performed colocalization analysis of emerin and β-catenin by immunofluorescence. The results showed that the colocalization of emerin with β-catenin was indeed increased in the soft matrix (Fig. 5e). Furthermore, emerin knockdown in cells cultured in soft hydrogels resulted in an increase in the nuclear translocation of β-catenin (Figs. S6a and b, Fig. 5f).

3.6. Stiff matrix-mediated actin contractility limits the nuclear translocation of HDAC3 and induces a high level of histone acetylation

To explain the altered levels of histone acetylation in matrixes with different stiffnesses, we examined the expression of histone deacetylases (HDACs) and histone acetyltransferases (HATs). Western blot and

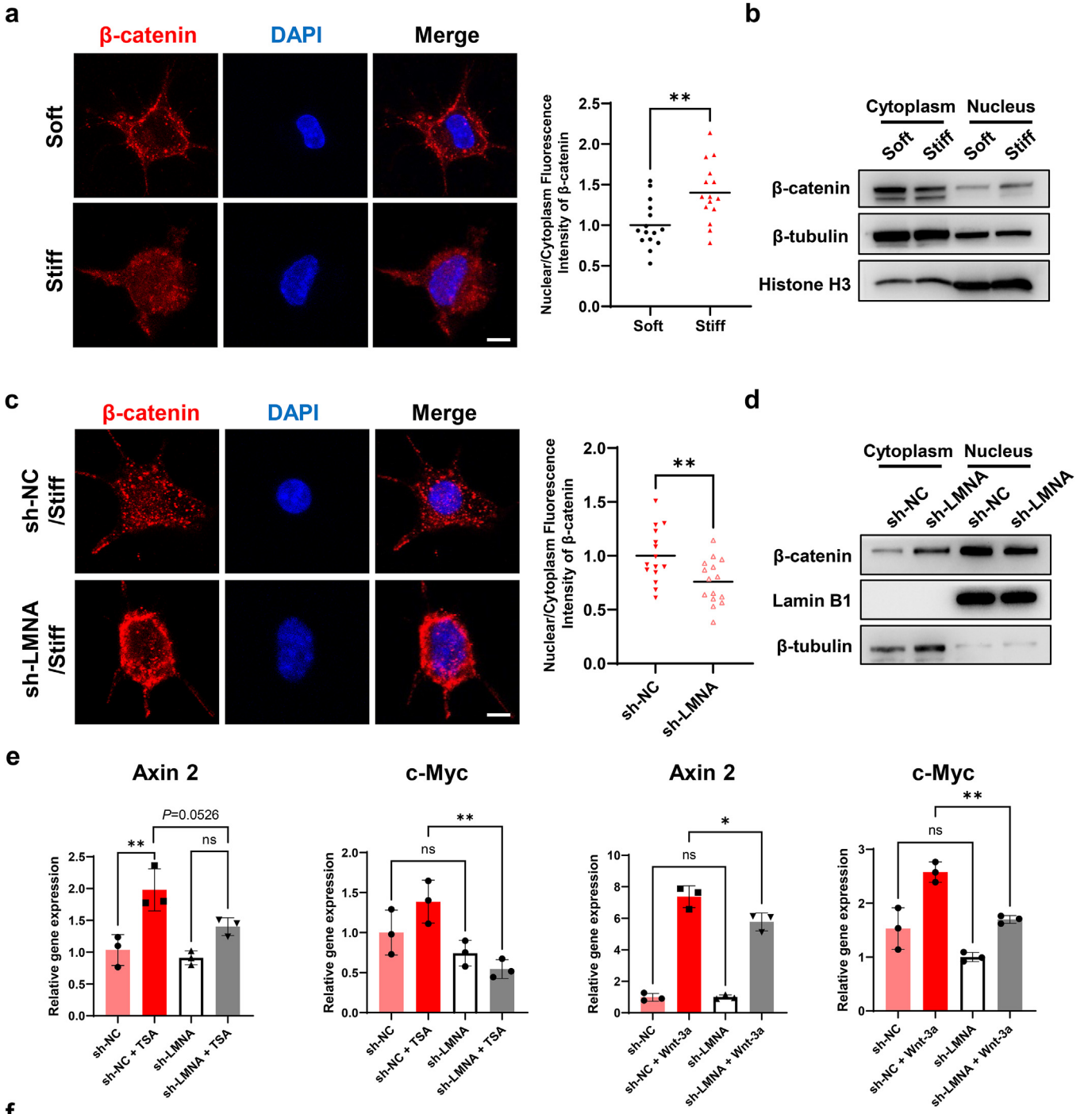

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Fig. 3. Lamin A/C mediates the nuclear localization of β-catenin to regulate the Wnt pathway and osteogenic differentiation. a) Confocal images of active β-catenin (Red) of cells cultured in the soft and stiff gel for 2 days, and then treated with Wnt-3a (50 ng/ml) for 4 h. Scale bar, 10 μm. Right: quantitation of active β-catenin levels n = 3 with 15 cells randomly scored/experiment. **b)** Representative Western blots for the cytoplasmic active β-catenin and the nuclear active β-catenin of cells in soft and stiff gels for 3 days. β-tubulin for the housekeeper of cytoplasmic protein and Histone H3 for the housekeeper of nuclear protein**. c)** Confocal images of active β-catenin (Red) in sh-NC cell and sh-LMNA cell cultured in the stiff gel for 2 days, and then treated with Wnt-3a (50 ng/ml) for 4 h. Scale bar, 10 μm. Right: quantitation of active β-catenin levels. Mean ± SD (n = 3 with 15 cells randomly scored/experiment) by *t*-test. **d)** Representative Western blots for the cytoplasmic active β-catenin and the nuclear active β-catenin of sh-NC cell and sh-LMNA cell in the stiff gel for 3 days. β-tubulin for the housekeeper of cytoplasmic protein and lamin B1 for the housekeeper of nuclear protein**. e)** Relative mRNA expression of Wnt-targeted genes of sh-NC cell and sh-LMNA cell treated or treated without TSA or Wnt-3a in the stiff gel for 3 days. **f)** The schematic showing the process by which stiffness mediated lamin A/C and regulated β-catenin nuclear translocation. Mean ± SD (n = 3) by one-way ANOVA with Bonferroni's post-hoc test. ns: no significant difference, *: $P < 0.05$, **: $P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

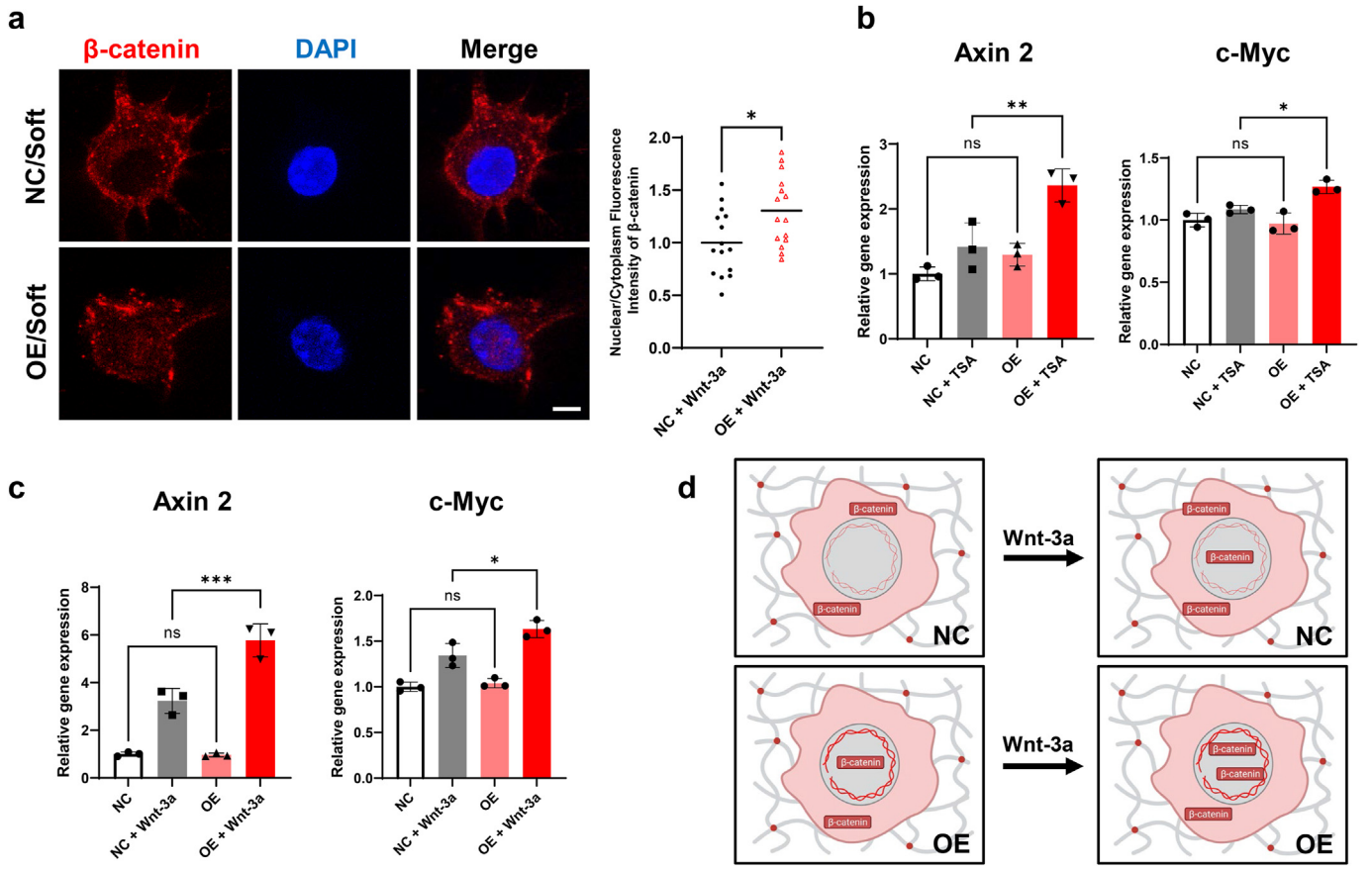

Fig. 4. Both histone acetylation and lamin A/C are required for activation of the Wnt pathway. a). Confocal images of active β-catenin (Red) in Negative control cell and LMNA overexpression cell cultured in the stiff gel for 2 days, and then treated with Wnt-3a (50 ng/ml) for 4 h. Scale bar, 10 μm. n = 3. Right: quantitation of active β-catenin levels. Mean ± SD (n = 3 with 15 cells randomly scored/experiment) by *t*-test. **b)** Relative mRNA expression of Wnt-targeted genes of sh-NC cell and sh-LMNA cell treated or treated without TSA in the stiff gel for 3 days. **c)** Relative mRNA expression of Wnt-targeted genes of Negative control cell and LMNA overexpression cell treated or treated without TSA in the soft gel for 3 days. Mean ± SD (n = 3) by one-way ANOVA with Bonferroni's post-hoc test. **d)** Schematic illustration showing that both histone acetylation and lamin A/C are required to activate the Wnt pathway. Ns: no significant difference, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

immunofluorescence analyses showed that compared to that in soft matrices, the expression of HDAC3 in the nucleus was lower in stiff matrices (Fig. 6a–c). Moreover, the total protein level of CREB-binding protein (CBP), a type of acetyltransferase, was increased with a higher proportion in the nucleus (Figs. S7a and b). It was reported that the decrease in actomyosin contractility can cause HDAC3 to shuttle from the cytoplasm to the nucleus [24,25]. Therefore, F-actin was labeled with phalloidin, and the fluorescence intensity was analyzed, revealing higher levels of actin polymerization in the stiff matrix (Fig. 6d). Additionally, *p*-MLC, a recognized marker of active myosin-II motors [24], was upregulated in the stiff matrix (Fig. 6e). Finally, to confirm that matrix stiffness regulates histone acetylation via actomyosin contractility, we used Bleb. to inhibit myosin-II. As a result, the nuclear translocation of HDAC3 was increased in the stiff matrix (Fig. 6a and b), accompanied by an increase in chromatin condensation and downregulation of Ac-H3 (Fig. 6f and g).

4. Discussion

Tissue regeneration relies on a suitable microenvironment that promotes its biological function [26]. Compared to biochemical signals, mechanical signals have the advantages of being direct, rapid,

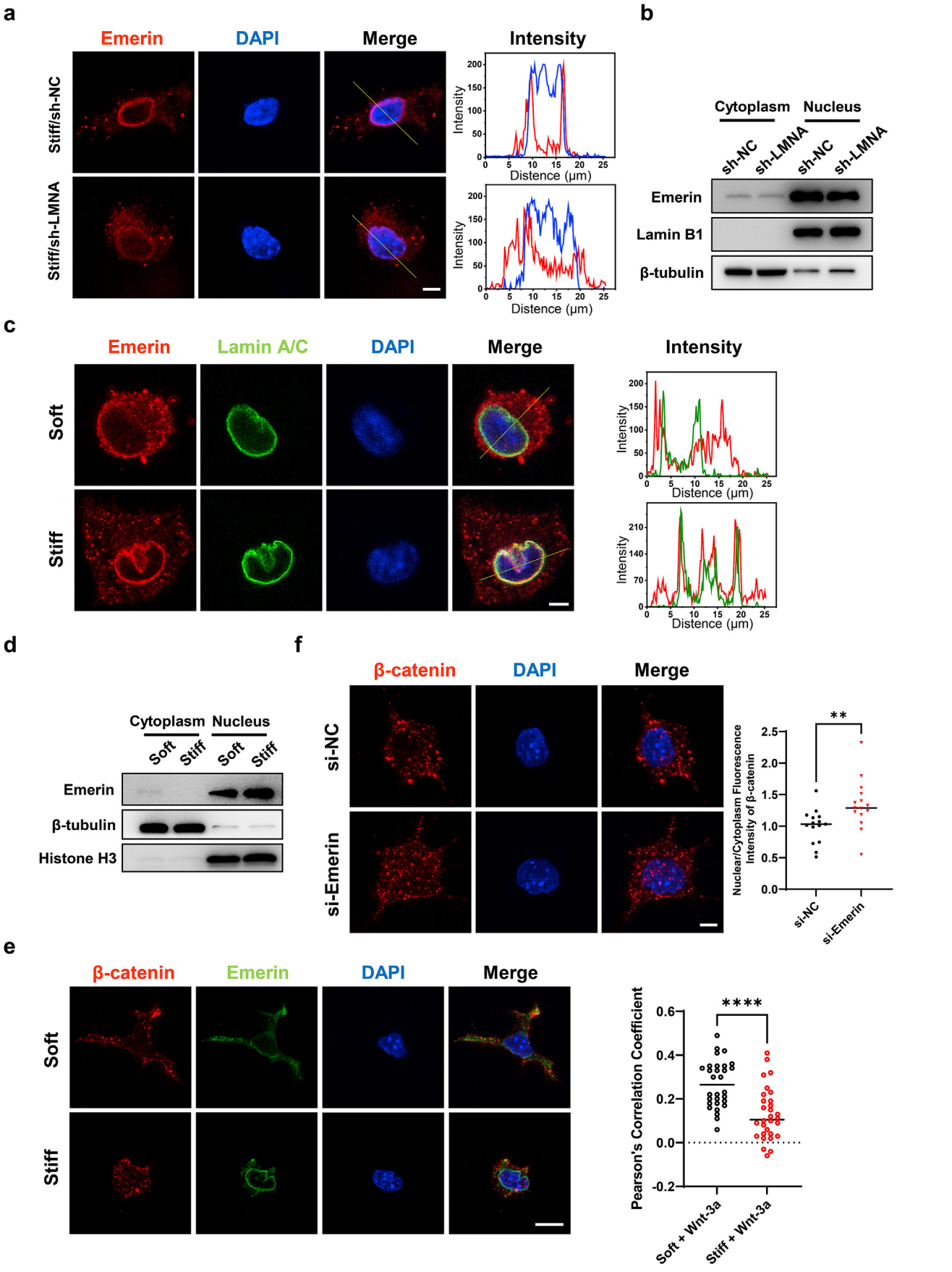

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Fig. 5. Lamin A/C regulates the nuclear localization of β-catenin by emerin. a) Confocal images of emerin (Red) in sh-NC cell and sh-LMNA cell cultured in the stiff gel for 2 days. Right: normalized fluorescence intensity profiles of lamin A/C (red) and DAPI (blue) along the yellow line crossing cells are shown. Scale bar, 5 μm. n = 3. **b)** Representative Western blots for the cytoplasmic emerin and the nuclear emerin of sh-NC cell and sh-LMNA cell in stiff gels for 2 days. β-tubulin for the housekeeper of cytoplasmic protein and lamin B1 for the housekeeper of nuclear protein. **c)** Confocal images of emerin (Red) and lamin A/C (Green) in MC3T3 cultured in the soft or stiff gel for 2 days. Right: normalized fluorescence intensity profiles of emerin (Red) and lamin A/C (Green) along the yellow line crossing cells are shown. Scale bar, 5 μm. n = 3. **d)** Representative Western blots for the cytoplasmic emerin and the nuclear emerin of si-Negative control cell and si-Emerin cell in soft gels for 2 days. β-tubulin for the housekeeper of cytoplasmic protein and Histone H3 for the housekeeper of nuclear protein. **e)** Confocal images of Active β-catenin (Red) and emerin (Green) in MC3T3 cultured in the soft or stiff gel for 2 days. Right: Quantification of co-localization of Active β-catenin and emerin in the soft or stiff gel for 2 days, and then treated with Wnt-3a (50 ng/ml) for 4 h. Scale bar, 10 μm. n = 3. Right: quantitation of active β-catenin levels. Mean ± SD (n = 3 with 30 images randomly scored/experiment) by *t*-test. **f)** Confocal images of active β-catenin (Red) in si-Negative control cell and si-Emerin cell in the soft gel for 2 days, and then treated with Wnt-3a (50 ng/ml) for 4 h. Scale bar, 5 μm. Right: quantitation of active β-catenin levels. Mean ± SD (n = 3 with 15 cells randomly scored/experiment) by *t*-test. Mean ± SD (n = 3) by *t*-test. ns: no significant difference, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

noninvasive, and long-lasting [1,24]. Therefore, understanding how mechanical cues regulate cell differentiation is important for the development of tissue engineering and biomaterials. Currently, most research conclusions regarding the regulation of cell differentiation by ECM stiffness are based on two-dimensional (2D) environments. However, in 2D culture, cells are significantly different from their natural growth state in vivo in terms of their morphology, structure, and function. In fact, several studies have reported that important pathways that regulate cell differentiation under 2D conditions are not applicable in 3D environments [25,27–31]. In fact, little is known about the mechanism of matrix stiffness-mediated osteogenic differentiation under 3D environments. This study aims to investigate how ECM stiffness affects gene transcription and expression patterns by modulating chromatin remodeling and how mechanosensitive transcription factors coordinate gene expression and osteogenic differentiation in response to ECM stiffness. We proposed that histone acetylation-mediated chromatin remodeling and lamin A/C-mediated mechanotransduction are involved in the regulation of osteogenic differentiation. The synergistic effects of these mechanisms are summarized in Fig. 7.

Various synthetic or natural materials have been used to manufacture hydrogels with controlled stiffness [26]. GelMA is synthesized from methacrylic anhydride (MA) and gelatin, and its chemical composition is similar to collagen, making it one of the most widely used basic biomaterials in tissue engineering [32]. GelMA is also used to study matrix-cell interactions because it can effectively simulate the extracellular matrix in vivo. While the stiffness of hydrogels can be changed by using different concentrations of macromolecules, it is difficult to separate the effects of stiffness from other interfering factors. To avoid the potential influence of macromolecular monomer concentration, RGD peptide density, and curing time on cells, we used commercial GelMA gels with varying degrees of methacryloyl substitution to alter the stiffness of the hydrogels.

Epigenetic regulation is important for cell differentiation and tissue formation. For example, increased tumor extracellular matrix can form an open chromatin conformation, upregulating gene transcription and further leading to the invasive phenotype of tumor cells [4]. However, the interaction between matrix stiffness-mediated chromatin remodeling and osteogenic differentiation remains unclear. Wnt signaling is an important pathway for regulating cell osteogenic differentiation. Recent literature has shown that a decrease in histone acetylation in bone marrow stromal cells (BMSCs) of osteoporosis patients leads to inhibition of the Wnt pathway, thereby affecting their osteogenic differentiation [20]. This finding indicates the important role of epigenetic regulation in regulating the activation of the Wnt pathway. Previous studies have shown that matrix rigidity affects the activation of the Wnt signaling pathway by regulating the integrin/focal adhesion kinase (FAK) signaling pathway. The results of this study suggested that matrix stiffness-mediated chromatin remodeling also plays an essential role in regulating the activation of the Wnt/β-catenin pathway.

The acetylation of histones is closely related to the expression of HATs and HDACs, which are enzymes involved in epigenetic modification. These enzymes also tend to respond to mechanical cues [9,33,34]. In our study, we examined the expression levels and subcellular localization of HDAC3 and CBP under different matrix stiffness conditions. Our findings confirm that actomyosin contractility plays a crucial role in regulating chromatin aggregation by mediating the subcellular localization of HDAC3. However, notably, our study did not directly investigate the regulatory effect of HDAC3 or CBP on the Wnt pathway. This issue is due to the complex regulatory mechanisms involved, which were not the main focus of our research.

Lamin A/C is a nuclear envelope protein that is an important component of the nuclear skeleton and is involved in maintaining the stability and morphology of the nucleus [35]. The expression level of lamin A/C is believed to play an important role in the activation of the Wnt pathway. Literature reports have shown that downregulation of lamin A/C expression in aging MSCs inhibits the nuclear translocation of β-catenin, leading to a decrease in osteogenic differentiation potential and an increase in adipogenic differentiation [22]. By overexpression or knockdown of LMNA, we further showed that ECM stiffness could regulate nuclear translocation of β-catenin through lamin A/C. Notably, overexpression of LMNA in the soft matrix increased β-catenin nuclear translocation but did not significantly increase the expression of β-catenin target genes, whereas coadministration of TSA resulted in significant upregulation of β-catenin target genes.

Lamin A/C is also involved in the regulation of other mechanotransduction pathways. For example, the nuclear translocation of YAP induced by the stiff matrix can be abolished by LMNA mutation [36]. Megakaryocytic leukemia factor 1 (MKL1) can sense mechanical signals via the aggregation of actin monomers (G-actin). Mechanical signal-mediated G-actin aggregation leads to the release of MKL1 from G-actin and increased nuclear expression. Fibroblasts with LMNA knockout or mutation impair the polymerization of G-actin, reducing the ability of MKL1 to enter the nucleus [23]. This phenomenon further inhibits the SRF pathway and leads to adipogenic differentiation of MSCs [37]. In addition to the regulatory mechanisms mentioned above, lamin A/C also directly binds to chromatin and forms lamina-associated domains (LADs), which are typically located at the periphery of the nucleus and are closely associated with the nuclear membrane. LADs are considered to be static regions of chromatin, where genes are typically silenced. The formation of LADs is closely related to the expression level of lamin. In cells lacking lamin, the number and size of LADs change, leading to abnormal chromatin structure and gene expression [38]. Lamin A/C has also been reported to regulate gene expression by binding to enhancers [39]. These studies indicate that lamin A/C can influence gene expression in multiple ways, although the biological importance and mechanisms of these regulatory effects are not yet clear. Due to the lack of standardized methods for cell recovery in 3D culture, it is difficult to study the direct regulation of chromatin and gene expression by lamin A/C. Nevertheless, it is an important regulatory mechanism that warrants further investigation in future studies.

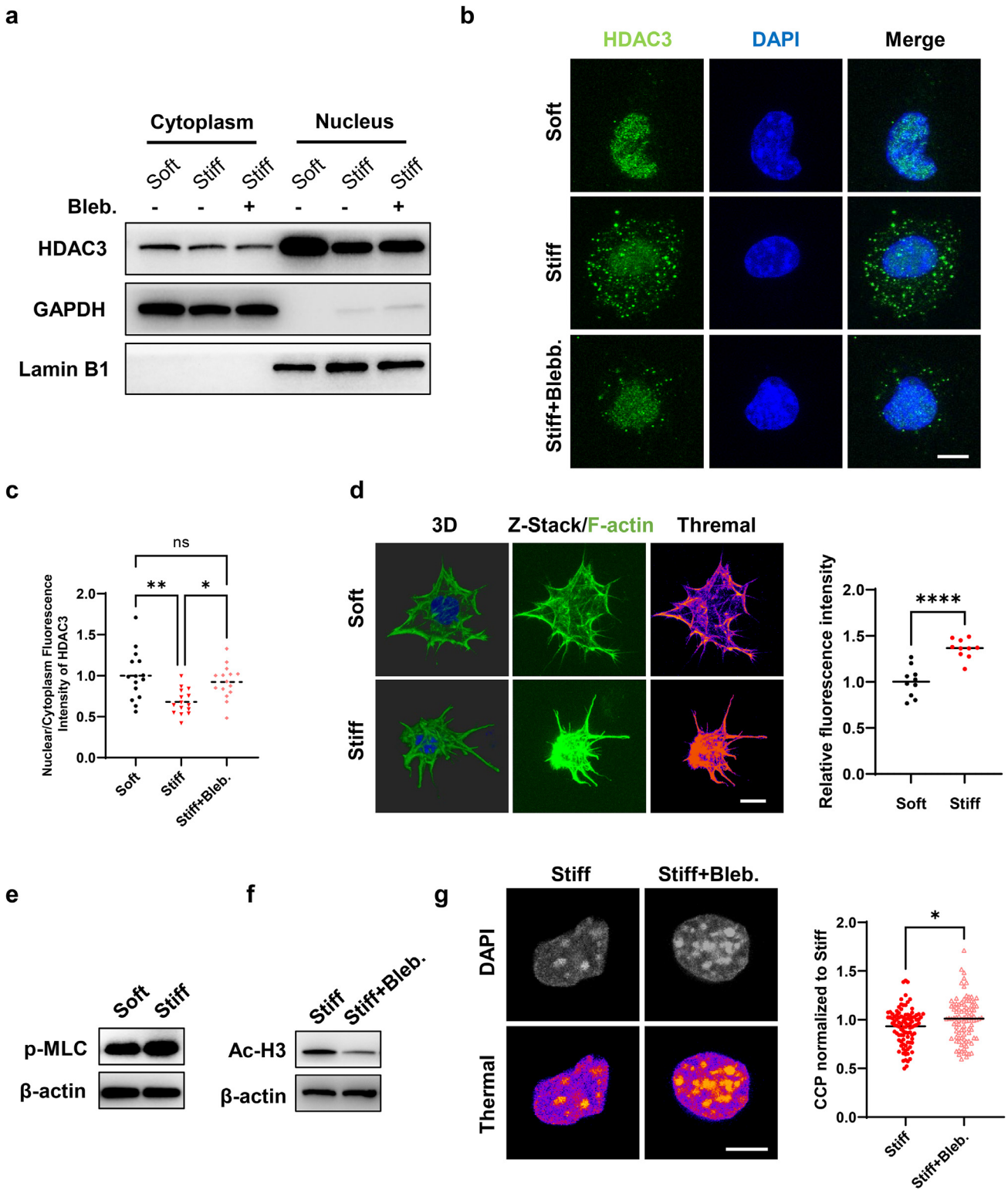

Fig. 6. Stiff matrix mediated actin contractility limits the nuclear translocation of HDAC3 and induced a high level of histone acetylation. a) Representative Western blots for the cytoplasmic HDAC3 and the nuclear HDAC3 of cells cultured in soft or stiff gel, and Bleb. treated cells in stiff gel for 2 days. Gapdh is for housekeeper of cytoplasmic protein and lamin B1 for housekeeper of nuclear protein. Right: Quantification of HDAC3 on Western blot images; Mean ± SD (n = 3) by one-way ANOVA with Bonferroni's post-hoc test. **b)** Confocal images of HDAC3 (Green) of cells cultured in soft or stiff gel, and Bleb. (20 μM) treated cells in stiff gel for 2 days. Scale bar, 5 μm. **c)** Quantification of HDAC3 level on fluorescence images of **(b).** n = 3 with 15 cells randomly scored/experiment. **d)** Z-stack confocal imaging of phalloidin labeled F-actin of cell in soft or stiff gel, the intensity was visualized by creating a heatmap. Scale bar, 10 μm. Right: Quantification of fluorescence intensity (n = 3 with 10 cells randomly scored/experiment). Significance of soft versus stiff based on *t*-test. **e)** Representative Western blots for *p*-MLC and the housekeeper protein β-actin of MC3T3-E1 cells that were cultured for 2 days in the soft and stiff gel before lysis. **f)** Representative Western blots for Ac-H3 and the housekeeper protein β-actin of cells and Bleb. treated cells in stiff gel for 2 days before lysis. **g)** CCP for cells and Bleb. treated cells cultured in stiff gel. Significance of stiff versus stiff + Bleb.: *, $P < 0.05$ based on *t*-test. ns: no significant difference, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

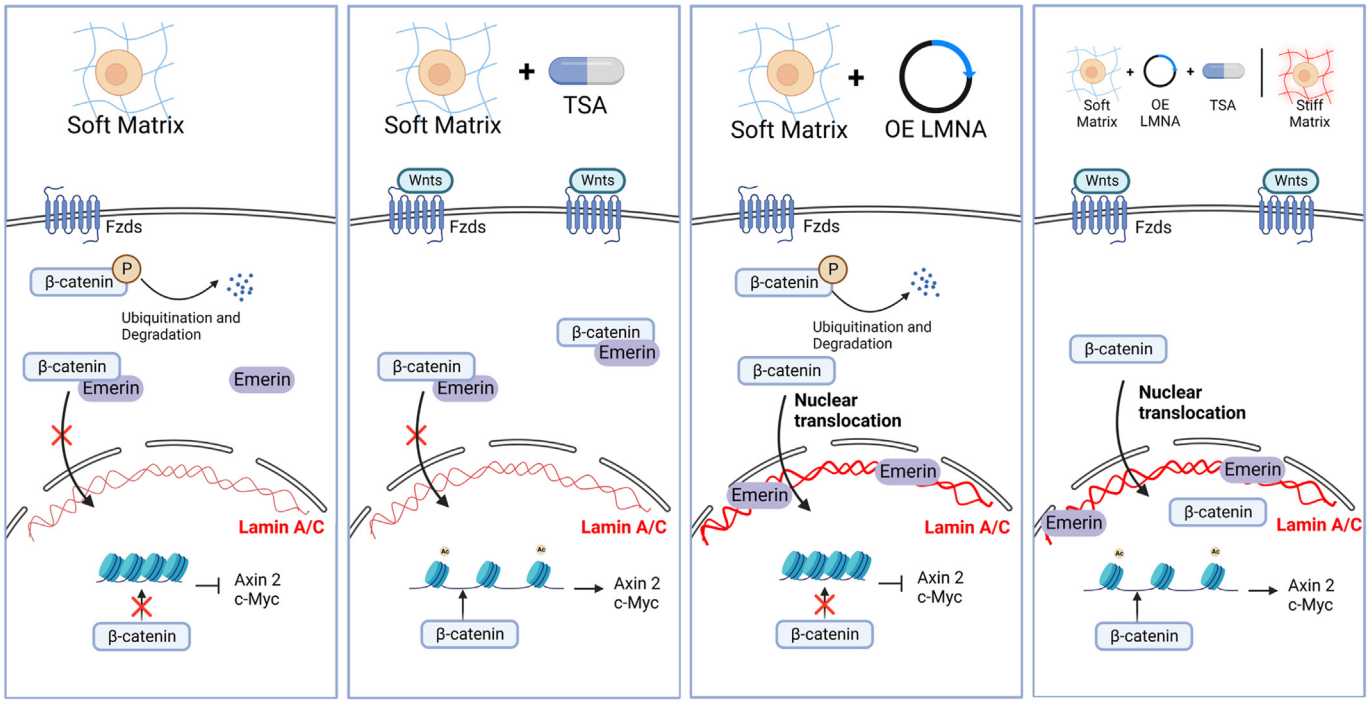

Fig. 7. Synergy between histone acetylation and lamin A/C-mediated mechanotransduction. From left to right. First, in soft matrix, due to the absence of lamin A/C and low levels of Ac-H3, no activated Wnt signaling or osteogenic differentiation was observed. Second, treatment with TSA can upregulate the expression of β-catenin and reduce the chromatin condensation. However, due to the low level of lamin A/C, emerin diffuses into the cytoplasm and binds to β-catenin, inhibiting its entry into the nucleus. Third, overexpression of lamin A/C enhances the nuclear localization of emerin. But due to the condensed state of chromatin, β-catenin is unable to bind to target genes and activate transcription. Finally, the simultaneous overexpression of lamin A/C and treatment with TSA in a soft matrix successfully activate the Wnt pathway.

Credit author statement

X.X., H.Z., and S.Y. designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. L.Y. assisted in the analysis of the nuclear chromatin condensation parameter. Z.J. and M.R. contributed to the experimental design and performed the experiments. T.C., Y·F., and Y·W assisted in data interpretation and drafted the manuscript. P.J. and S.Y. provided critical feedback and guidance during the writing and editing process.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2023.100661.

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