




# Cyclic mechanical stretch enhances BMP9-induced osteogenic differentiation of mesenchymal stem cells

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## Abstract

**Purpose** The purpose of this study was to investigate whether mechanical stretch can enhance the bone morphogenetic protein 9 (BMP9)-induced osteogenic differentiation in MSCs.

**Methods** Recombinant adenoviruses were used to overexpress the BMP9 in C3H10T1/2 MSCs. Cells were seeded onto six-well BioFlex collagen I-coated plates and subjected to cyclic mechanical stretch [6% elongation at 60 cycles/minute (1 Hz)] in a Flexercell FX-4000 strain unit for up to 12 hours. Immunostaining and confocal microscope were used to detect cytoskeleton organization. Cell cycle progression was checked by flow cytometry. Alkaline phosphatase activity was measured with a Chemiluminescence Assay Kit and was quantified with a histochemical staining assay. Matrix mineralization was examined by Alizarin Red S Staining.

**Results** Mechanical stretch induces cytoskeleton reorganization and inhibits cell proliferation by preventing cells entry into S phase of the cell cycle. Although mechanical stretch alone does not induce the osteogenic differentiation of C3H10T1/2 MSCs, co-stimulation with mechanical stretch and BMP9 enhances alkaline phosphatase activity. The expression of key lineage-specific regulators (e.g., osteocalcin (OCN), SRY-related HMG-box 9, and runt-related transcription factor 2) is also increased after the co-stimulation, compared to the mechanical stretch stimulation along. Furthermore, mechanical stretch augments the BMP9-mediated bone matrix mineralization of C3H10T1/2 MSCs.

**Conclusions** Our results suggest that mechanical stretch enhances BMP9-induced osteoblastic lineage specification in C3H10T1/2 MSCs.

**Keywords** Mechanical stretch · BMP9 · Osteogenic differentiation · Mesenchymal stem cells

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## Introduction

As an attractive cell source for tissue engineering and regenerative medicine, mesenchymal stem cells (MSCs) can be isolated from different tissues to give rise to several lineages (e.g., bone, adipose, cartilage, and muscle) [1]. Osteogenic differentiation is a complex but well-regulated process controlled via a series of chemical and physical factors [2]. Therefore, a better understanding of the mechanisms underlying osteogenic differentiation will be instrumental for the clinical use of stem cells.

Bone morphogenetic proteins (BMPs) play important roles in the regulation of stem cell proliferation and osteogenic differentiation [3]. BMPs belong to the transforming growth factor  $\beta$  superfamily; its members are crucial for cell proliferation, adhesion, and differentiation, skeletal development, and bone formation [4]. BMPs are highly conserved secretory

multifunctional proteins, with at least 14 members in humans [5]. Among these members, BMP2, BMP4, BMP6, BMP7, and BMP9 can effectively induce the osteogenic differentiation of MSCs *in vitro* and *in vivo* [6]. BMP9 is one of the most osteogenic yet least studied BMPs.

Mechanical stimulation also has remarkable effects on the regulation of stem cell fate. Recent studies have shown that mechanical stimulation induces changes in stem cell behaviour such as adhesion, migration, proliferation, differentiation, and survival [7]. Previous studies of MSCs have focused on the cellular responses to chemical and biological factors. However, information about the synergistic effects of physical cues and biochemical factors on osteogenesis is limited. To determine whether mechanical stretch acts synergistically with the BMP9-induced osteogenic differentiation of MSCs, we first tested whether C3H10T1/2 MSCs are responsive to mechanical stretch. We then examined the effects of BMP9 and mechanical stretch co-stimulation on cell proliferation, the expression of osteogenic genes, and bone matrix mineralization in C3H10T1/2 MSCs.

## Materials and methods

### Cell culture

Human embryonic kidney 293 (HEK293) and C3H10T1/2 MSCs were obtained from the American Type Culture Collection (Manassas, VA, USA). C3H10T1/2 cells are commonly used and well characterized MSCs [5, 6]. Cells were maintained in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), 100 U/mL of penicillin, and 100 g/mL of streptomycin (Beyotime, Shanghai, China) in a humidified 37 °C incubator with 5% CO<sub>2</sub>.

### Recombinant adenoviruses

Recombinant adenoviruses are generated based on a replication-defective adenoviral vector that provides a versatile system for gene expression studies. Recombinant adenoviruses were produced following the previously described AdEasy protocols [8]. The coding regions of human BMP9, green fluorescence protein (GFP), and red fluorescence protein (RFP) were amplified by polymerase chain reaction (PCR) and cloned into an adenoviral shuttle vector that was subsequently used to generate recombinant adenoviruses in HEK293 cells. The adenoviruses were designated AdBMP9 or AdR-BMP9 (AdBMP9 expressed GFP, whereas AdR-BMP9 expressed RFP as a marker to monitor the infection efficiency). Adenoviruses expressing only monomeric RFP (AdRFP) or GFP (AdGFP) were used as controls [9].

### Application of mechanical stretch

A Flexcell Strain Unit Fx-4000 (Flexcell Corp., Burlington, NC, USA) was used to apply mechanical stretch to C3H10T1/2 MSCs. Cells were seeded onto six-well BioFlex collagen I-coated plates (Flexcell Corp.) at 10% confluence for cytoskeletal analysis and at 30–40% confluence for other studies. Four hours after seeding, cells were infected with adenoviruses. Thirty hours after infection, cells were subjected to serum starvation overnight and cyclic mechanical stretch [6% elongation at 60 cycles/minute (1 Hz)] for up to 12 hours. Static control cells were subjected to the same conditions, except mechanical stretch was not applied.

### Cytoskeletal analysis

Actin, a cellular skeleton protein, was visualized with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Enzo Life Sciences, Lausen, Switzerland), which was added immediately after cells were stretched with mechanical stimuli. The medium was removed from each sample, which was then washed twice with phosphate-buffered saline (PBS). Samples were fixed with 4% paraformaldehyde in PBS for 10 min and washed three times with PBS. Fixed cells were permeabilized with Triton X-100 (0.1%) in PBS and blocked with 5% bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO, USA) at room temperature, followed by incubation in 10 µg/mL of fluorescent phalloidin-conjugate solution in PBS overnight at 4 °C. After washing, the cells were stained with 4',6-diamidino-2-phenylindole (Beyotime) for ten minutes protected from light at room temperature. The cells were then analyzed on an inverted confocal microscope (Leica SP8; Leica, Wetzlar, Germany) with a long working distance lens. Cell F-actin responses to mechano-stimuli are semi-quantified by the aspect ratio and the number of branch points of the cells by using ImageJ software.

### Flow cytometry

Samples were harvested after trypsinization, fixed with cold 70% ethanol overnight, and resuspended in PBS followed by incubation with RNase A in propidium iodide staining solution (Suzhou New Med Cytomics Biotechnology Co., Ltd., Suzhou, China) for 15 minutes at room temperature. The DNA content was measured on a flow cytometer (BD Influx™; BD Biosciences, San Jose, CA, USA). Data were analyzed using ModFit LT software (Verity Software House Inc., Topsham, ME, USA).

### ALP assay

ALP activity was measured quantitatively with a Great Escape SEAP Chemiluminescence Assay Kit (BD Clontech, Palo

Alto, CA, USA) and was quantified with a histochemical staining assay using a mixture of 0.1 mg/mL of naphthol AS-MX phosphate and 0.6 mg/mL of Fast Blue BB Salt (Sigma-Aldrich Co.) as described previously [10, 11]. ALP activity was normalized to the total cellular protein concentration. Calcium mineral deposits were examined under bright field microscopy.

### RNA isolation and reverse transcription qRT-PCR

To determine whether C3H10T1/2 MSCs are sensitive to mechanical stretch, total RNA was isolated from mechanically stimulated and unstimulated cells at early time points (0, 0.5, 1, 2, or 4 h). RNA was isolated using TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instructions. RNA was reverse-transcribed using a PrimeScript™ II First Strand cDNA Synthesis Kit (Takara). Real-time quantification of mRNA was performed in a total volume of 25  $\mu$ L using the Bio-Rad CFX Connect PCR system with SYBR Premix ExTaq™ II kits (Takara) according to the manufacturer's instructions. Target gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). RT-PCR primers for mouse osteocalcin (OCN; 5'-CCT TCA TGT CCA AGC AGG A-3' and 5'-GGC GGT CTT CAA GCC ATA C-3'), runt-related transcription factor 2 (Runx2; 5'-CCG GTC TCC TTC CAG GAT-3' and 5'-GGG AAC TGC TGT GGC TTC-3'), SRY-related HMG-box 9 (Sox9; 5'-AGC TCA CCA GAC CCT GAG AA-3' and 5'-TCC CAG CAA TCG TTA CCT TC-3'), and GAPDH (5'-GGC TGC CCA GAA CAT CAT-3' and 5'-CGG ACA CAT TGG GGG TAG-3') transcripts were designed using Primer3 Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).

### Alizarin Red S staining

AdBMP9- or AdGFP-infected cells were first exposed to cyclic mechanical stretch and then cultured in the presence of 50 mg/mL of ascorbic acid (Sigma-Aldrich Co.) and 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich Co.). After 14 days of culture, mineralized matrix nodules were stained for calcium precipitation by means of Alizarin Red S staining as described previously [10, 11]. Cells were fixed with 0.05% (*v/v*) glutaraldehyde at room temperature for ten minutes and washed with PBS. Fixed cells were then incubated with 0.4% Alizarin Red S (Sigma-Aldrich Co.) for 5 min, followed by washing with PBS. Calcium mineral deposits were examined under bright field microscopy.

### Statistical analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data are expressed as the mean  $\pm$

standard deviation. Comparisons between two groups were analyzed using the two-tailed unpaired Student's *t* test. A value of  $p < 0.05$  was considered to indicate statistical significance.

## Results

### Mechanical stretch-induced actin cytoskeleton reorganization in MSCs

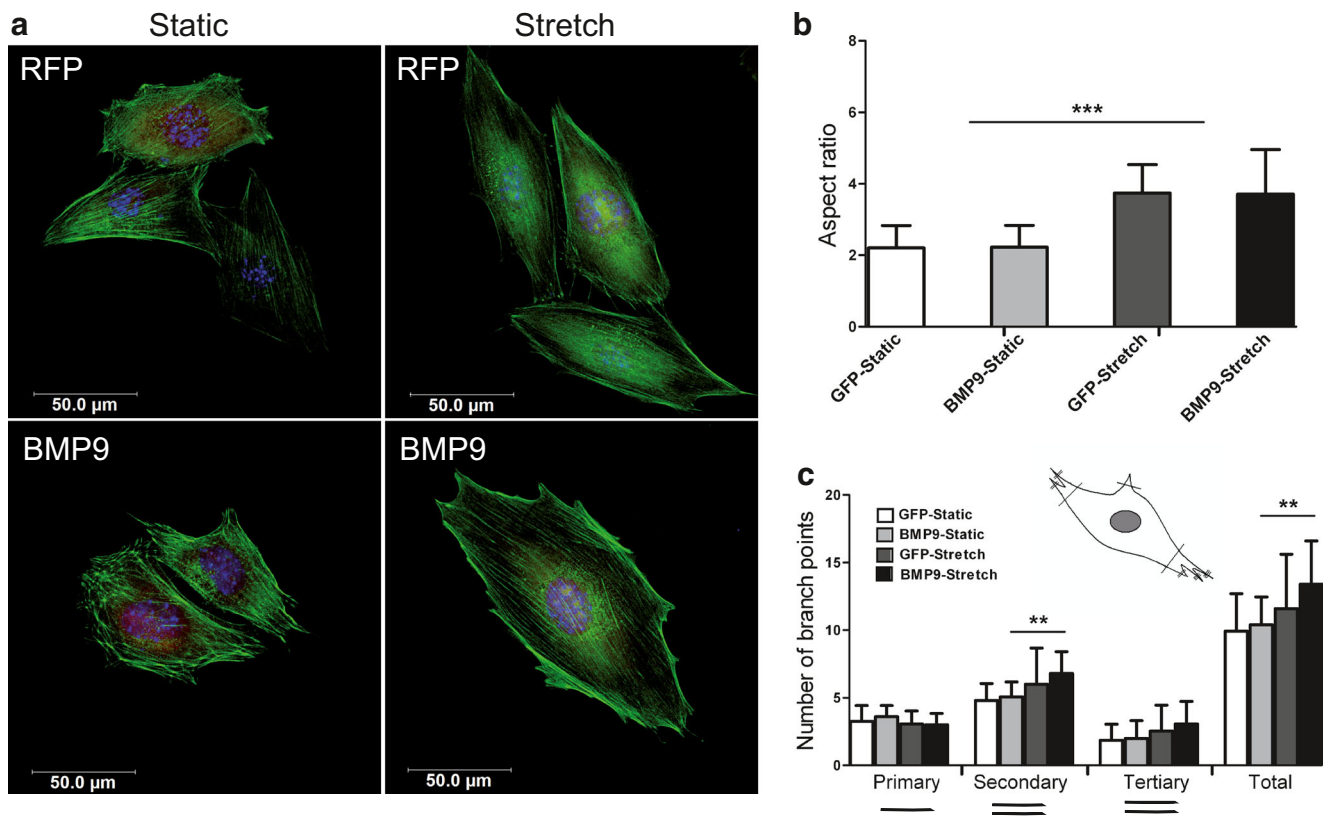
Cells infected with Ad-RFP or AdR-BMP9 were subjected to cyclic mechanical stretch and then stained with phalloidin-FITC to observe the organization of actin filaments. Static cells were polygonal and displayed randomly oriented stress fibers, whereas stretched cells were fusiform, exhibiting realigned stress fibers orientated with the direction of the longitudinal axis (Fig. 1a). AdR-BMP9-infected cells exhibited thicker and more differentiated stress fibers than Ad-RFP control cells. Furthermore, the stretched cells transduced with AdBMP9 had higher aspect ratio and more branching than static cells (Fig. 1b, c). These data suggest that both mechanical stretch and BMP9 are involved in actin cytoskeleton reorganization in MSCs.

### Effects of mechanical stretch on the cell cycle in MSCs

To assess the effects of mechanical stretch and BMP9 on the cell cycle, cell cycle profiles were examined by flow cytometry after DNA content staining (Fig. 2). After applying mechanical stretch for 12 hours, the accumulation of cells in G1 phase increased sharply (approximately 34% and 25% in GFP- and BMP9-infected groups, respectively). Meanwhile, the stretched cells in S phase decreased by almost 35% compared to static cells in both the GFP- and BMP9-infected groups. These data indicate that mechanical stretch inhibits cell proliferation by arresting the DNA content in G1 phase of the cell cycle.

### Mechanical stretch enhances the BMP9-induced early osteogenic differentiation of MSCs

To determine the effect of mechanical stretch on BMP9-induced early osteogenesis, subconfluent C3H10T1/2 MSCs were infected with adenoviruses and mechanical stretch was applied. Successful infection of C3H10T1/2 MSCs by the adenoviruses was validated by examining green fluorescence (Fig. 3a). ALP activity was quantitatively determined and histochemical staining was conducted on days three, five and seven after applying mechanical stretch. Mechanical stretch alone did not induce ALP activity, whereas BMP9 alone significantly induced ALP activity in C3H10T1/2 MSCs. Interestingly, BMP9 and mechanical stretch co-stimulation further induced ALP activity in C3H10T1/2 MSCs relative



**Fig. 1** Mechanical stretch remodeled actin filaments in C3H10T1/2 MSCs. **a** Microfilaments in stretched cells were remodeled and well organized compared to static cells. Actin filaments (green), BMP9 (red), RFP (red), and nuclei (blue). Scale bars represent 50.0  $\mu$ m. All

images were obtained under the same magnification. **b**, **c** Cell morphology quantified for aspect ratio (**b**), and the number of primary, secondary, tertiary, and total branch points (**c**) using ImageJ software. Fifteen cells were analyzed for each treatment ( $n = 15$ ).  $^{***}p < 0.01$

to BMP9 alone (Fig. 3b). Similar results were obtained from ALP histochemical staining (Fig. 4a–d). These findings indicate that mechanical stretch enhances the BMP9-induced osteoblast lineage commitment of C3H10T1/2 MSCs.

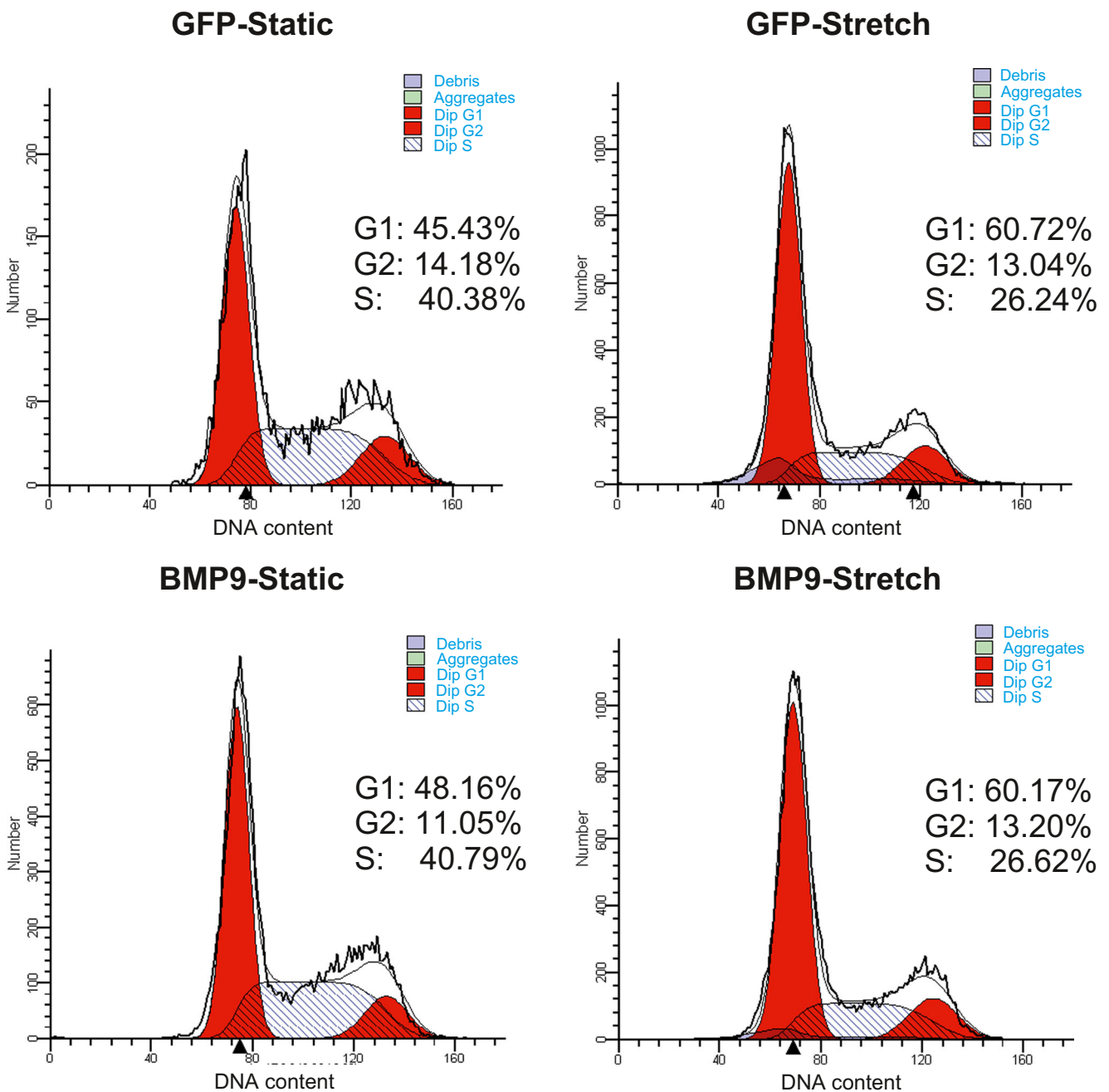
### Mechanical stretch enhances BMP9-induced late osteogenic differentiation and matrix mineralization in MSCs

To investigate the effect of mechanical stretch on late osteogenic and chondrogenic differentiation induced by BMP9, total RNA was collected at the indicated time points. Three chondro-osteogenic genes were analyzed by qRT-PCR: OCN, Runx2, and Sox9. When C3H10T1/2 MSCs were transduced with AdBMP9, all three genes were significantly upregulated compared to the AdGFP group (Fig. 5a–c). Furthermore, the expression of OCN and Runx2 was significantly increased in C3H10T1/2 MSCs after applying loading tension for two and four hours compared to the BMP9 alone group (Fig. 5a, b). Sox9, a key chondrogenic lineage-specific regulator, was also significantly upregulated in C3H10T1/2 cells after applying loading tension for four hours compared to the control group (Fig. 5c). These results confirm that mechanical stretch

enhances BMP9-induced late osteogenic differentiation. Lastly, the effects of mechanical stretch and BMP9 on cell matrix mineralization were analyzed. Co-stimulation with mechanical stretch and BMP9 resulted in stronger Alizarin Red S staining indicating matrix mineralization in C3H10T1/2 MSCs compared to the static control group (Fig. 5d, e). Taken together, these results demonstrate that mechanical stretch enhances the BMP9-induced osteogenic differentiation of MSCs.

### Discussion

We previously reported that BMP9 is a central BMP family member. BMP9 induces the osteogenic differentiation of MSCs by synergizing with a set of downstream targets, including growth hormone, retinoic acid, insulin-like growth factor 2, and hypoxia-inducible factor 1 $\alpha$  [9–12]. Moreover, recent evidence revealed that the BMP signaling pathway is directly regulated by mechanotransduction [13]. However, it is unclear whether mechanical stretch affects BMP-induced cell fate. In this study, we applied cyclic mechanical stretch to C3H10T1/2 MSCs that were infected with BMP9-overexpressing adenoviruses. Mechanical stretch inhibited



**Fig. 2** Mechanical stretch prevented C3H10T1/2 MSCs from entering S phase of the cell cycle by arresting the cells in G1 phase. Cell cycle analysis of untreated cells or cells treated with tension. Flow cytometric

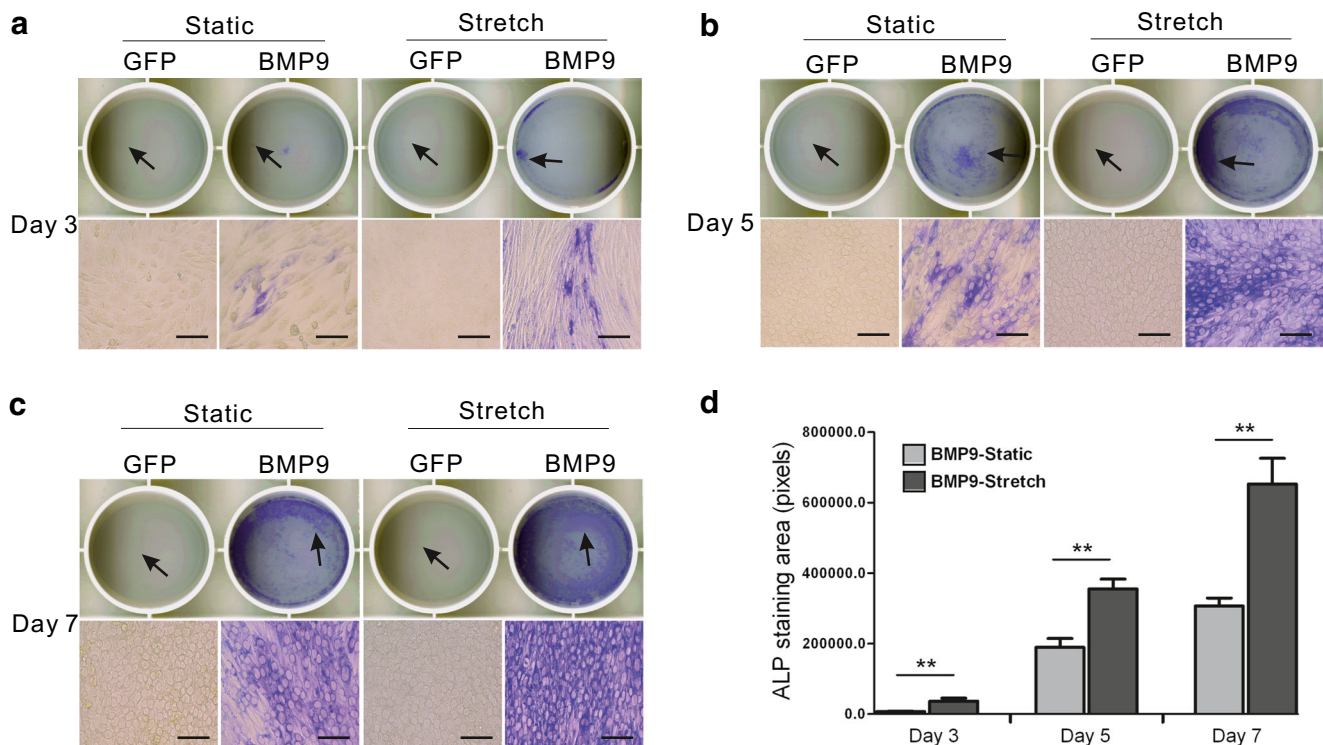
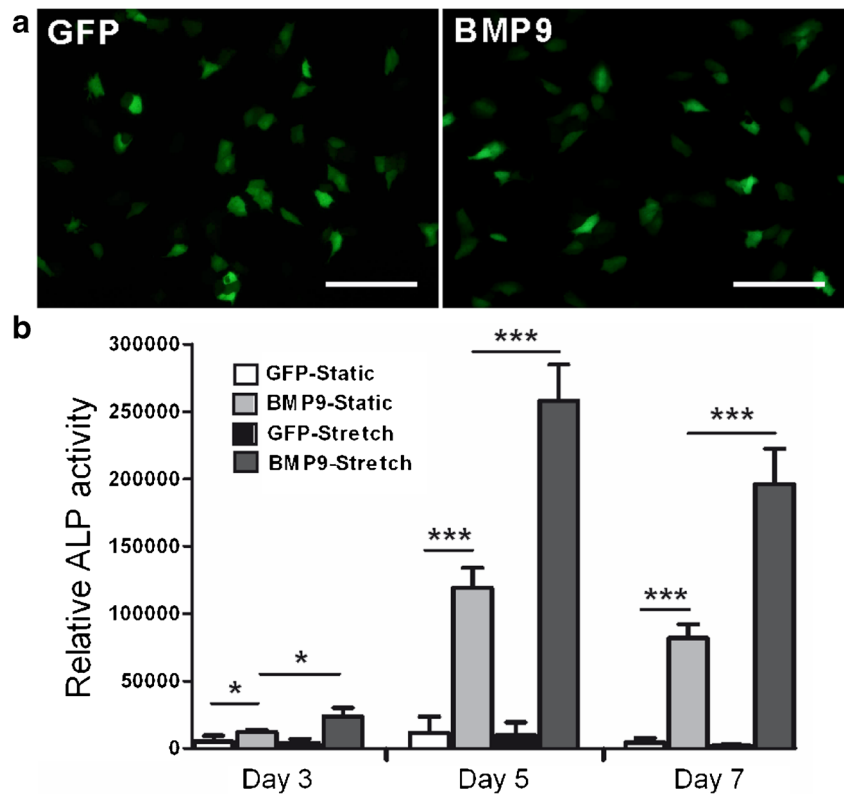
data were analyzed using ModFit LT software. The percentage of cells in each cell cycle phase (G1, G2, and S) is reported in the boxed areas within each panel

cell proliferation by arresting the cell cycle at G1 phase and acted synergistically with BMP9 to alter the cytoskeleton and promote osteogenesis in MSCs.

Putative mediators of mechanosensation include ion channels, integrins, surface receptors, cell adhesion, and the actin cytoskeleton [2, 14]. The actin cytoskeleton is a composite network that not only plays an important role in cell morphology, size, and movability, but it also acts as a mechanosensor in response to mechanical stimuli, reorganizes the cellular structure, and activates associated signaling pathways [15, 16]. Actin

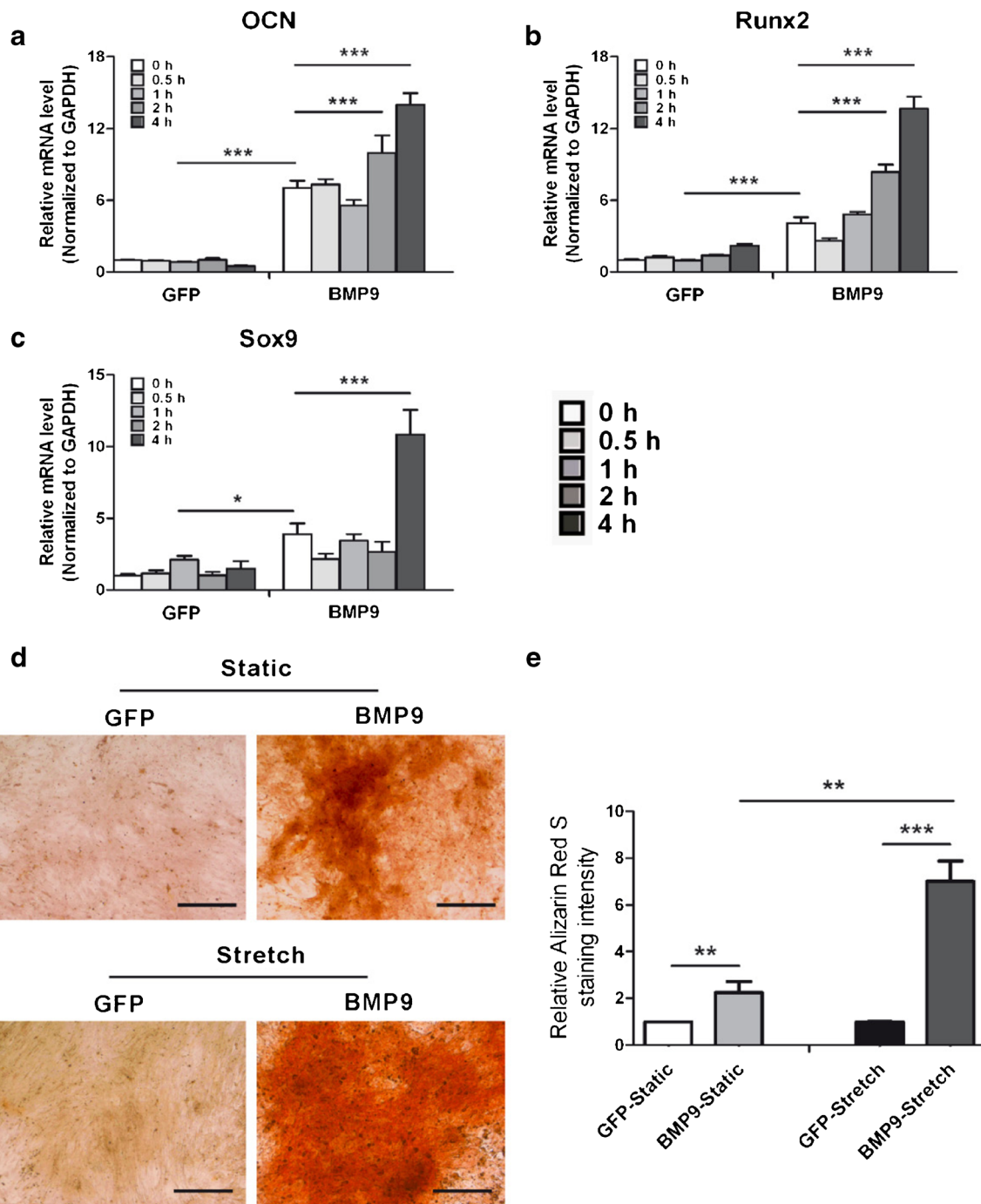
cytoskeleton functions as a coordinator in controlling cell proliferation, differentiation, and migration [17]. Recent reports have demonstrated that the cytoskeletal contractility and actin polymerization resulted in osteogenic differentiation on 2D substrates [18]. Our results indicate that, after mechanical stretch, the actin cytoskeleton in C3H10T1/2 MSCs was remodeled dramatically, being arranged regularly with the longitudinal axis. Moreover, the synergistic effects of mechanical stretch and BMP9 induced a thicker and polymerized actin network, higher aspect ratio, and more branch points.

**Fig. 3** **a** GFP shows efficient infection of C3H10T1/2 MSCs by adenovirus. Scale bars = 200  $\mu$ m. **b** A quantitative ALP activity assay was performed at the indicated time points ( $n = 4$ ). ALP activity was significantly higher in cells subjected to co-stimulation with mechanical stretch and BMP9 than in the BMP9-alone group. ALP activity was normalized to total cellular protein. \* $p < 0.05$ , \*\*\* $p < 0.001$



**Fig. 4** ALP staining of untreated cells or cells treated with mechanical stretch. **a–c** Overall ALP staining of the wells (top row) and microscopic images (bottom row) are shown in each panel. Scale bars = 100  $\mu$ m. Positive staining is labeled in purple/blue. Arrows indicate the location

of microscopic images. **d** Quantification of ALP staining area (pixels) is based on images from BMP9-infected groups ( $n = 3$ ). \*\* $p < 0.01$ . Mechanical stretch enhanced BMP9-induced ALP activity in MSCs, consistent with the results of the quantitative ALP activity assay



**Fig. 5** Mechanical stretch enhanced BMP9-induced late osteogenic differentiation and matrix mineralization in C3H10T1/2 MSCs. **a–c** qRT-PCR analysis of OCN, Runx2, and Sox9 expression ( $n = 3$ ). Cultured cells were infected with adenoviruses and subjected to mechanical stretch. Samples were collected at 0 (static control), 0.5, 1, 2, or 4 hours for qRT-PCR analysis. Results were normalized to GAPDH and are expressed as

the mean  $\pm$  standard error of the mean ( $*p < 0.05$ ,  $***p < 0.001$ ) relative to the GFP control group. **d–e** At 14 days post-infection, cells were fixed and stained with Alizarin Red S solution. Cells co-stimulated with mechanical stretch, and BMP9 exhibited more bone mineralization than the BMP9 infection group. Scale bar = 500  $\mu\text{m}$ .  $**p < 0.01$ ,  $***p < 0.001$

Cell cycle regulation is required for stem cells to remain in a quiescent state or for entry into proliferation. Flow cytometric analysis showed that the percentage of cells in S phase was dramatically decreased and that cells in G1 phase were largely accumulated in the mechanical stress-treated group compared with the static control group. However, the present study

shows that BMP9 has no significant effect on cell cycle transitions. These results indicate that mechanical stretch inhibits C3H10T1/2 MSC proliferation. Our results are in agreement with earlier findings that MSC proliferation can be inhibited by mechanical stretch [19]. Conversely, other researchers found that appropriate mechanical stretch treatment promoted

the proliferation of MSCs [20]. These differing results may be due to differences in methods, time, frequency, and/or level of mechanical force loading [21].

The osteogenic potency of MSCs can be characterized by the expression of ALP as an early marker and key lineage-specific regulators, as well as matrix mineralization [6, 12]. Quantitative and qualitative measurements of ALP activity showed that mechanical stretch alone did not induce significant ALP activity, whereas co-stimulation with BMP9 and mechanical stretch induced ALP activity significantly in C3H10T1/2 MSCs. Our findings demonstrate that mechanical stretch is necessary but insufficient to induce MSCs to enter the early stage of osteogenesis without BMP9 stimulation. In accordance with these results, the qRT-PCR data also indicate that late osteogenic markers were significantly upregulated in C3H10T1/2 MSCs under mechanical stretch and BMP9 co-stimulation. The expression of OCN and Runx2 was significantly increased in C3H10T1/2 MSCs following co-treatment with mechanical stretch and BMP9 compared to the BMP9-induced group. OCN, also known as bone  $\gamma$ -carboxyglutamate protein, is the most abundant noncollagenous protein in bone and a key regulator of osteogenic maturation [22]. Runx2 is an essential regulator of osteoblast differentiation, chondrogenesis, and endochondral ossification. The expression of Runx2 can be induced by BMP2 or BMP7 in mouse calvarial mesenchymal progenitor cells and myoblast precursor cells [23, 24]. Interestingly, mechanical stretch and BMP9 also caused Sox9 mRNA upregulation four hours after exposure to force. Sox9 is mainly characterized as a key regulator in chondrogenesis. Previous study has demonstrated that mechanical load alone has no effect on stimulating the expression of cartilage-specific target genes (COL2A1 and ACAN). However, mechanical load enhances chondrogenesis in the presence of additional chondrogenic factors, such as dexamethasone, in a dose-dependent manner. This indicates that additional chondrogenic factors were required for chondrogenesis under *in vitro* conditions [25]. At present, the role of Sox9 in directing osteogenesis remains largely unknown. Since Sox9 is activated through BMP signaling pathway [26], we determined whether Sox9 can be induced by BMP9 and stretch treatments in our study. Our results indicate that Sox9 can be regulated by both chemical factors and physical cues. This is consistent with a report that Sox9 expression is increased in human inner meniscus cells which were treated by stretch for four hours. Sox9 expression is even more increased in these cells after stretch treatment for eight hours, indicating that stretch induces Sox9 expression in a time-dependent manner [27]. Although Sox9 expression can be regulated by both stretch and BMP9 at the early stage of stimulation, no further appreciable chondrogenesis is observed with the approaches used in our study. The reason is that stretch and BMP9 induce the differentiation of C3H10T1/2 MSCs into osteoblast lineage rather than other lineages. With respect to matrix mineralization, co-stimulation

with BMP9 and mechanical stretch resulted in maximum Alizarin Red S staining in C3H10T1/2 MSCs, whereas mechanical stretch alone did not induce any detectable mineralization.

BMP ligands bind to type I and type II BMP receptors to activate BMP signaling pathways. After binding with BMP ligand, homomeric type II receptors form a tetrameric complex with homomeric type I receptors, which transphosphorylates homomeric type I receptor to trigger signal transduced through either Smads or MAPKs. The activation of Smads further activates the transcription of specific target genes involved in skeletal development and bone formation. In the BMP signaling pathway, Runx2 is a key transcriptional factor. The coordinated activity of Runx2 and activated Smads is critical for BMP9-mediated osteogenic differentiation and bone formation [26]. The way how mechanical stretch synergizes with BMP9 to induce osteogenic differentiation of MSCs remains further investigation. One possible mechanism is that the interaction between stretch and BMP9 increases the expression of Runx2, which has been found to regulate downstream targets of BMP signaling pathway.

In conclusion, the present study demonstrates that C3H10T1/2 MSCs are mechanosensitive. The tension-induced cytoskeleton reorganization in C3H10T1/2 MSCs inhibited cell proliferation by preventing DNA entry into S phase of the cell cycle. Mechanical stretch synergizes with BMP9 to induce the expression of key lineage-specific regulators (e.g., OCN, Sox9, and Runx2) and enhance matrix mineralization in MSCs. Stretch alone appears to be insufficient for inducing appreciable osteogenesis in MSCs without BMP9 stimulation, whereas BMP9 treatment is a more potent stimulus than stretch to initiate MSC differentiation. In addition, stretch can be effective at enhancing BMP9 induced MSC osteogenic differentiation. Our study expands the methods used to promote osteogenesis of MSCs and provides an opportunity for cell-based therapies in bone regenerative medicine.

**Author contributions** Yang Song and Yinhong Tang contributed equally to this paper.

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## Compliance with ethical standards

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

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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