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Low intensity pulsed ultrasound (LIPUS) maintains osteogenic potency by the increased expression and stability of Nanog through spleen tyrosine kinase (Syk) activation

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ABSTRACT

Mesenchymal stem cells (MSCs) are a powerful tool for cell-based, clinical therapies like bone regeneration. Therapeutic use of cell transplantation requires many cells, however, the expansion process needed to produce large quantities of cells reduces the differentiation potential of MSCs. Here, we examined the protective effects of low intensity pulsed ultrasound (LIPUS) on the maintenance of osteogenic potency. Primary osteoblastic cells were serially passaged between 2 and 12 times with daily LIPUS treatment. We found that LIPUS stimulation maintains osteogenic differentiation capacity in serially passaged cells, as characterized by improved matrix mineralization and Osteocalcin mRNA expression. Decreased expression of Nanog, Sox2, and Msx2, and increased expression of Pparg2 from serial passaging was recovered in LIPUS-stimulated cells. We found that LIPUS stimulation not only increased but also sustained expression of Nanog in primary osteoblasts and ST2 cells, a mouse mesenchymal stromal cell line. Nanog overexpression in serially passaged cells mimicked the recuperative effects of LIPUS on osteogenic potency, highlighting the important role of Nanog in LIPUS stimulation. Additionally, we found that spleen tyrosine kinase (Syk) is an important signaling molecule to induce Nanog expression in LIPUS-stimulated cells. Syk activation was regulated by both Rho-associated kinase 1 (ROCK1) and extracellular ATP in a paracrine manner. Interestingly, the LIPUS-induced increase in Nanog mRNA expression was regulated by ATP-P2X4-Syk Y323 activation, while the improvement of Nanog protein stability was controlled by the ROCK1-Syk Y525/526 pathway. Taken together, these results indicate that LIPUS stimulation recovers and maintains the osteogenic potency of serially passaged cells through a Syk-Nanog axis.

1. Introduction

Mesenchymal stem cells (MSCs) have multipotent capacity, allowing for their differentiation into osteoblasts, adipo-progenitor cells, and chondroblasts. In the field of hard tissue-regenerative medicine, MSCs have emerged as a promising tool for the clinical and commercial applications of cell transplantation and cell-based therapies such as bone regeneration, cartilage regeneration, spinal fusion, disc regeneration, and tendon healing [1,2]. Although the therapeutic effects of MSCs have been demonstrated through clinical trials across a variety of disease models [3], there are several problems in the practical application of MSCs. A major issue in clinical MCS use is that transplantation of MSCs requires a great number of cells, usually ranging from 10^7 to 10^8 MSCs [4]. Because higher proliferation of MSCs leads to improved regeneration and healing in the tissues to which the MSCs are applied [5], serial passaging is required to prepare sufficient cell

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numbers for therapeutic use. However, this MSC expansion process causes the loss of proliferation capacity and differentiation potential, which begins from the moment an in vitro culture is started, despite being largely undetectable [6]. Accordingly, higher passages of murine MSCs and human adipose-derived stem cells display significantly lower osteogenic potential compared to cells with fewer passage iterations [7,8]. It has also been shown that the preservation of osteogenic potential is reduced through adipogenesis in MSCs, suggesting that MSCs at early passages must be used for effective osteogenic differentiation [9]. Therefore, in order to utilize the beneficial effects of MSC transplantation on bone regeneration, the loss of stemness as a result of serial passaging needs to be overcome.

The fate and function of MSCs depends on many external factors such as plating density, plastic surface quality, cell culture media and its supplements, cytokines, and oxygen concentration [5]. Importantly, mechanical stimulation is also reported as an effective regulator of differentiation potency in MSCs. Previous studies have shown that cyclic mechanical stretch maintains self-renewal and multipotency of MSCs and embryonic stem (ES) cells [10,11]. Similarly, oscillatory shear stress has been shown to maintain the multi-differentiation potential of MSCs by upregulating the expression of multipotency genes, including Nanog, octamer-binding transcription factor 4 (Oct4), and sex determining region Y-box 2 (Sox2) [12]. Interestingly, another recent report showed that mechanical stretch enhanced the reprogramming efficiency of human dermal fibroblasts by upregulating four reprogramming factors (OCT4, SOX2, Kruppel-like factor 4 (KLF4), and c-MYC) without affecting the viral transduction rate during the establishment of pluripotency-induced stem cells [13]. Since Nanog, Oct4, and Sox2 are known to improve cell expansion and increase stemness in MSCs [14–17], mechanical stress-induced activation of these multipotency genes is a promising approach to prevent the loss of MSC function during therapeutic manipulation.

While promising, the functional role of mechanical stimulation in the maintenance of MSC property often appears to be inconsistent [18]. For example, the properties of mechanically-induced cellular stretch systems, such as the biomaterial substrates of the cultured cells and the parameters of mechanical stimulation, play a role in pluripotency and MSC differentiation lineage, but may differ between systems [19]. Inconsistencies in cell differentiation upon application of shear stress are also partly explained by the fact that not all stem cells equally express non-motile cilia, a mechanosensory organelle that reaches from the cell surface into the local environment [20,21]. In addition, custom-designed systems and substrates for the delivery of mechanical-stimulation might be difficult to engineer and standardize. If MSC potency can be regulated by a mechanostimulatory system consisting of simple, flat materials that allow for the easy change of cellular parameters, the result may be significantly improved opportunities for MSC use in stem cell biology and regenerative medicine.

Low-intensity pulsed ultrasound (LIPUS) is a form of mechanical micro-wave stimulation that is often applied clinically to promote the healing of recent bone fractures [22]. Previous studies, including our own, have reported that multipotent cells such as mouse MSCs [23], human amnion or bone marrow-derived MSCs [24,25], and human periodontal ligament-derived stem cells, are highly responsive to the regulatory effects of LIPUS on MSC differentiation. The use of LIPUS stimulation in vitro is very simple and allows for easy manipulation of the cell culture parameters. In addition, a previous study verified the methodology behind the experimental application of LIPUS to improve reproducibility and ensure its successful transfer to other experimental conditions [26]. These studies suggest that LIPUS may be a simple and effective tool to prevent the loss of stemness during serial passaging of MSCs.

In this study, we show that LIPUS stimulation maintains the differentiation potency of serially-passaged primary osteoblastic cells from newborn mice. We found that LIPUS-induced Nanog expression is involved in the recovery of osteogenic potency. We also show that spleen tyrosine kinase (Syk) is an important signaling molecule involved in the increased mRNA expression and protein stability of Nanog. Our data indicate that LIPUS may provide an efficient and simplified method for improving the multipotency of high passaged MSCs, improving their ability to be applied clinically.

2. Materials and methods

2.1. Reagents and antibodies

Piceatannol, a Syk-specific inhibitor, was purchased from Enzo Life Science (Plymouth Meeting, PA). SB203580, a p38-specific inhibitor; U0126, a MEK-specific inhibitor; A438079, a P2X7-specific receptor blocker; 5-BDBD, a P2X4-specific receptor blocker; and PPADS, a P2X1, 2, 3, 5 receptor blocker, were all purchased from Funakoshi (Tokyo, Japan). Cycloheximide was purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against Syk, ERKs, p38, ROCK1, and Nanog, along with phospho-specific antibodies against Syk Y323, Syk Y525/526, ERKs, and p38 were purchased from Cell Signaling Technology (Danvers, MA). The antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific antibody against ROCK1 was purchased from Bioss (Woburn, MA).

2.2. Cell culture

Primary osteoblasts were isolated from newborn C57BL/6 mouse calvariae (Japan Clea, Tokyo, Japan) as previously described [27]. Osteogenic differentiation of primary osteoblasts was induced by the addition of 280 μM L-ascorbic acid 2-phosphate trisodium and 5 mM β-glycerophosphate in Eagle's α-MEM containing 10% FBS, 50 units/ml penicillin, and 50 mg/ml streptomycin. ST2 cells, a mouse mesenchymal stromal cell line, were obtained from RIKEN Cell Bank (Tsukuba, Japan).

2.3. Ultrasound application

Cells were stimulated using a LIPUS-generating system (Teijin Pharma Ltd., Tokyo, Japan), which has been previously described [23,28]. The LIPUS signal consisted of a series of 1.5-MHz and 200 μs burst sine waves at 1.0 kHz. The LIPUS signal pattern employed in this study was essentially the same as those used in clinical practice and in animal model experiments. In some experiments, LIPUS intensity was altered: ranging between 7.5 and 120 mW/cm^2.

Following Isolation, 1×10^6 primary osteoblasts were cultured in 6 well plates. Cells were incubated with or without daily LIPUS (30 mW/cm^2, 60 min) for 2 days after the initial plating, and then 1×10^6 cells were passaged to new plates. Continued LIPUS stimulation occurred during serial passaging, with experimental measurements being performed at passages 2, 6, and 12.

2.4. Quantitative polymerase chain reaction analysis

Total RNA collection and real-time PCR were conducted as previously described [29]. Relative mRNA expression levels were compared with ribosomal protein L13a (Rpl13a) as internal control. The primer sequences were: 5′-CAG CAG ATG CAA GAA CTC TCC-3′ and 5′-GTG CTG AGC CCT TCT GAA TC-3′ for Nanog, 5′-GCT CGC AGA CCT ACA TGA AC-3′ and 5′-TGG AGT GGG AGG AAG AGG TA-3'for Sox2, and 5′-CTG CTC TCA AGG TTG TTC GGC T-3′ and 5′-CCT TCC GTT TCT CCT CCA GAG T-3'for Rpl13a. Other sequences have been previously described [23,30].

2.5. Western blot analysis

Cells were lysed in PLC lysis buffer. Immunoblotting was performed as previously described [23].

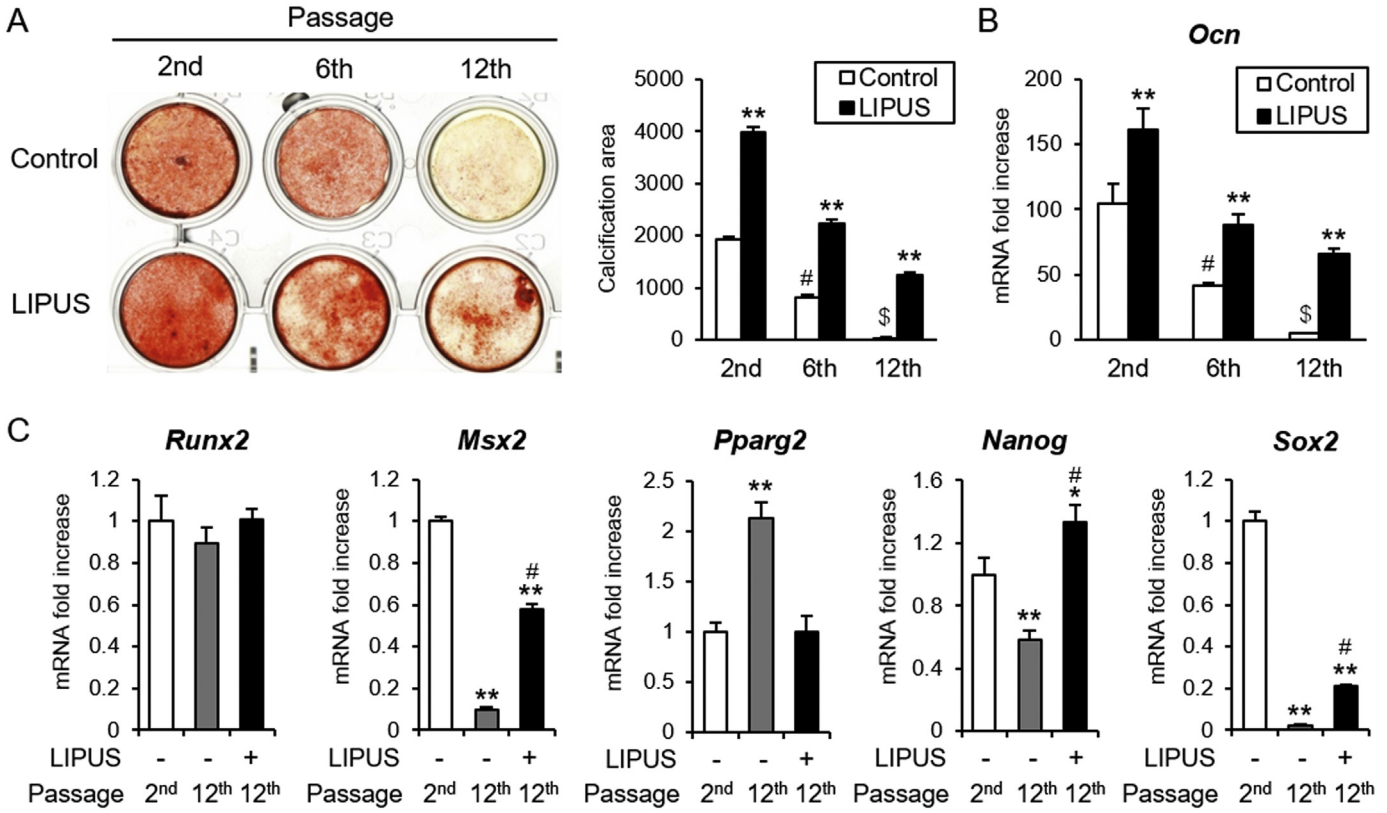

Fig. 1. LIPUS maintains the differentiation potency of serially passaged-primary osteoblastic cells. A & B. Primary osteoblasts were serially passaged with or without daily LIPUS stimulation (30 mW/cm^2) for 60 min. After 2nd, 6th, and 12th passages, the cells were differentiated by the addition of 280 μM ascorbic acid and 5 mM β-glycerophosphate for 18 days. Cell calcification area was determined by staining with Alizarin Red S and photographical analysis with ImageJ software (A). Osteocalcin mRNA expression was analyzed by real-time RT-PCR. Relative mRNA expression levels compared with ribosomal protein L13a *(Rpl13a)* are shown. Experiments were repeated at least 3 times to ensure consistent results. Error bars represent ± 1 s.d (**$P < .01$; vs Control of each passage, #$P < .01$; 2nd control vs 6th control, \$$P < .01$; 6th control vs 12th control). C. After the serial passages of osteoblasts as in A, the gene expression in each passaged cells was analyzed as in B (**$P < .01$; vs Control, #$P < .01$; 12th control vs 12th LIPUS). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Plasmid transfection

The cDNAs for mouse Nanog were amplified by RT-PCR with Nanog-specific primers using total RNA from primary osteoblasts as the template. After confirmation of the DNA sequences, these cDNAs were cloned into a pcDNA3.1(−) vector, producing pcDNA3.1-Nanog. The vector was transiently transfected into primary osteoblasts by HilyMax (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

2.7. RNA interference

Small interfering RNA (siRNA) duplexes specific for murine Syk (SASI_Rn01_00069533) and ROCK1 (SASI_Mm01_00077172), along with non-targeting control siRNA (MISSION® siRNA Universal Negative Control #1) duplexes, were obtained from Sigma Aldrich (St. Louis, MO). The duplex siRNAs were transfected into cultured cells using HilyMax according to the manufacturer's instructions.

2.8. Tet-on 3G inducible expression system

The pCMV-Tet3G vector (Clontech, Mountain view, CA) was stably transfected into ST2 cells by HilyMax according to the manufacturer's instructions. This was followed by selection with 500 μg/ml G418. Amplified cDNAs of murine Syk were cloned into a pTRE3G vector (Clontech). After verification by restriction mapping and sequencing, a linear hygromycin marker (Clontech) and the constructed inducible expression plasmid (termed as pTRE3G-Syk) were stably transfected into the ST2 pCMV-Tet3G cell line by HilyMax. To establish a negative control cell line, the pTRE3G vector was stably transfected into the ST2 pCMV-Tet3G cell line. After selection with 150 μg/ml hygromycin B, the resistant clones were isolated and tested for inducible protein expression from the inserted cDNAs using 2 μg/ml doxycycline (DOX). For each construct, three individual cell lines with good inducible protein expression were analyzed.

2.9. Measurement of ATP levels in cell culture media

ST2 cells were seeded at a density of 1.0×10^5 cells/ml 24 h prior to stimulation. The culture medium was replaced with fresh medium and the cells were allowed to incubate for 1 h, after which cells were stimulated with LIPUS (30 mW/cm^2) for the indicated times. The concentration of extracellular ATP in the cell culture medium was measured using an ENLITEN ATP Assay System (Promega, San Luis Obispo, CA) according to the manufacture's instruction.

2.10. Statistical analyses

We performed statistical analyses using a Student's *t*-test or two-way ANOVA. When the ANOVA indicated a significant difference, the specific difference was identified using Tukey and Bonferroni post hoc analysis ($*p < .01$). All data are expressed as the mean ± SEM. Results represent more than two independent experiments.

3. Results

LIPUS stimulation maintains the differentiation potency of serially-passaged primary osteoblastic cells.

To explore the beneficial effects of LIPUS on osteogenic differentiation potency, primary osteoblasts from mouse calvariae were serially passaged between 2 and 12 times with or without daily LIPUS (30 mW/cm^2) treatment. After preparation of the 2nd, 6th, and 12th passages, osteoblastic cells were cultured in osteogenic differentiation medium to confirm the degree of differentiation potency at each passage, using matrix mineralization and mRNA expression level of Osteocalcin as metrics. Cell calcification area was inversely related to the passage number in the untreated control cultures. We observed gradual decreases in calcification with each cell passage, leaving the calcification area completely diminished by the 12th passage (Fig. 1A). However, daily LIPUS stimulation during cell passages significantly increased matrix mineralization, recovering a great degree of calcification in the highly passaged cells. LIPUS also blocked the suppressive effect of cell passaging on Osteocalcin expression, relative to the untreated controls (Fig. 1B). This finding indicates that daily LIPUS stimulation may be able to maintain the differentiation potency of osteoblasts during serial passaging.

In order to analyze the characteristics of the passaged cells treated with LIPUS stimulation, we evaluated the mRNA expression of differentiation-related transcriptional factors in each of the passage conditions (Fig. 1C). Although the expression of Runt-related transcription factor 2 (*Runx2*), a master regulator of osteogenic differentiation [31], was not changed by cell passage number or LIPUS treatment, the expression of Msh Homeobox 2 (*Msx2*), the regulator of Runx2 [32], showed significant recovery in LIPUS-treated, 12th-passaged osteoblastic cells. Conversely, the increased expression of peroxisome proliferator-activated receptor γ2 (*Pparg2*), a master transcriptional factor of adipogenesis [33], that was seen in highly-passaged control cells, was prevented by LIPUS treatment. Interestingly, the expression of Nanog and *Sox2*, which suppress cell determination factors in pluripotent stem cells [34], were also increased by LIPUS in 12th passaged cells. These data suggest that daily LIPUS stimulation during serial passages affects the basal expression of osteogenic and multipotency-related transcriptional factors.

Nanog induction is involved in the recovery of osteogenic potency through LIPUS stimulation.

We next examined the stimulatory effects of differential LIPUS treatment duration on the expression of transcriptional regulators in ST2 cells (Fig. 2A) and mouse primary osteoblasts (Fig. 2B). Nanog mRNA expression was significantly increased by LIPUS stimulation (30 mW/cm^2) for 60 and 120 min in both ST2 cells and primary osteoblasts, however shorter LIPUS stimulation, ranging between 10 and 30 min, had no effect on Nanog mRNA expression. *Sox2* and *Msx2* mRNA expression were not changed by LIPUS stimulation, regardless of the duration. Oct4, a regulator of pluripotency in stem cells [34], was not expressed in either ST2 cells or primary osteoblasts (data not shown).

Following our investigation of LIPUS stimulation duration, we evaluated the effect of differential LIPUS intensity on Nanog expression in ST2 cells. Using a uniform 60 min LIPUS time frame, we found that both 30 and 60 mW/cm^2 stimulation effectively promoted mRNA expression of Nanog (Fig. 2C). LIPUS stimulation at 120 mW/cm^2 also showed promotive effects on Nanog expression, however induction was significantly lower than the 30 and 60 mW/cm^2 intensities. We also found that the increase in Nanog expression was sustained for several hours following 30 mW/cm^2 LIPUS stimulation (Fig. 2D). In the case of 60 mW/cm^2 stimulation, Nanog mRNA induction showed a similar increase immediately following treatment, however this effect was not sustained beyond the first hour post-treatment (Fig. 2E).

In order to validate the functional involvement of Nanog in the osteogenic recovery of serially passaged cells, we overexpressed Nanog in 6th passaged osteoblastic cells (Fig. 2F). The decreased expression of Nanog in 6th passaged control cells was rescued through transient transfection with a Nanog overexpression vector, resulting in upregulated matrix mineralization (Fig. 2G) and mRNA expression of osteocalcin (Fig. 2H) in the passaged cells. These results support the possibility that the promotive effect of LIPUS on Nanog expression is one mechanism for maintaining the osteogenic differentiation potency in serially passaged cells.

Syk is a crucial signaling molecule in the induction of Nanog expression by LIPUS.

Our previous study demonstrated that Syk is abundantly expressed in mesenchymal stromal cells and is involved in the balance between osteogenic and adipogenic differentiation [30]. Accordingly, we examined the involvement of Syk in LIPUS stimulation of ST2 cells (Fig. 3A) and primary osteoblasts (Fig. 3B). Induction of Syk phosphorylation at Y323 and Y525/526 occurred 20 min after a 20-min bout of LIPUS stimulation. In contrast with the delayed Syk phosphorylation, LIPUS treatment rapidly induced the phosphorylation of extracellular signal-regulated kinase (ERK) in ST2 cells: a finding that is consistent with our previous study [23]. To confirm the role of Syk in Nanog induction, we treated ST2 cells with either piceatannol, a Syk-specific inhibitor, or U0126, an ERK-specific inhibitor. We found that Syk inhibition significantly blocked LIPUS-induced mRNA expression of *Nanog* (Fig. 3C). Transient transfection with Syk-specific siRNA effectively inhibited Syk protein expression (Fig. 3D) and suppressed LIPUS-induced Nanog mRNA expression (Fig. 3E). Additionally, we prepared ST2 Tet-on 3G Syk cells which inducibly overexpress Syk upon DOX stimulation using the Tet-on inducible expression system (Fig. 3F). LIPUS-induced Nanog expression was significantly promoted by the DOX-induced overexpression of Syk (Fig. 3G). These results show that Syk plays an important role in the induction of Nanog expression.

LIPUS-induced Syk activation is partially regulated by Rho-associated kinase 1 (ROCK1).

Our previous study showed that ROCK1 is a crucial signaling molecule in the LIPUS-induced signaling pathway of ST2 cells [23] and human periodontal ligament fibroblasts [35]. To explore the involvement of ROCK1 in the Syk - Nanog axis, we stimulated ST2 cells with LIPUS, following transfection of these cells with ROCK1-specific siRNA. ROCK1 protein expression was highly suppressed by siRNA transfection (Fig. 4A). LIPUS-induced Syk phosphorylation at Y525/526, but not Y323, was inhibited by ROCK1 knockdown (Fig. 4B), however, Nanog induction was not affected (Fig. 4C). These results suggest that ROCK1 - Syk Y525/526 signaling is not involved in LIPUS-induced Nanog expression.

LIPUS induces ATP paracrine signaling for Syk phosphorylation by the P2X4 receptor.

Previous studies have reported that LIPUS and other shockwave-based treatments induce the extracellular release of ATP from MC3T3-E1 [36], a mouse osteoblastic cell line, human MSCs [37], and human Jurkat T-cells [38]. Because ATP is reported to activate Syk signaling pathways [39], we examined the effect of LIPUS on the extracellular release of ATP from ST2 cells. Extracellular ATP release was significantly increased in cells treated with 1, 2, and 5 min of LIPUS stimulation compared to untreated cells (Fig. 5A). Extracellular ATP treatment of ST2 cells induced p38 phosphorylation and Syk phosphorylation at Y323 residues, but not at Y525/526 (Fig. 5B). Treatment of the ST2 cells with variable ATP concentrations yielded a dose-

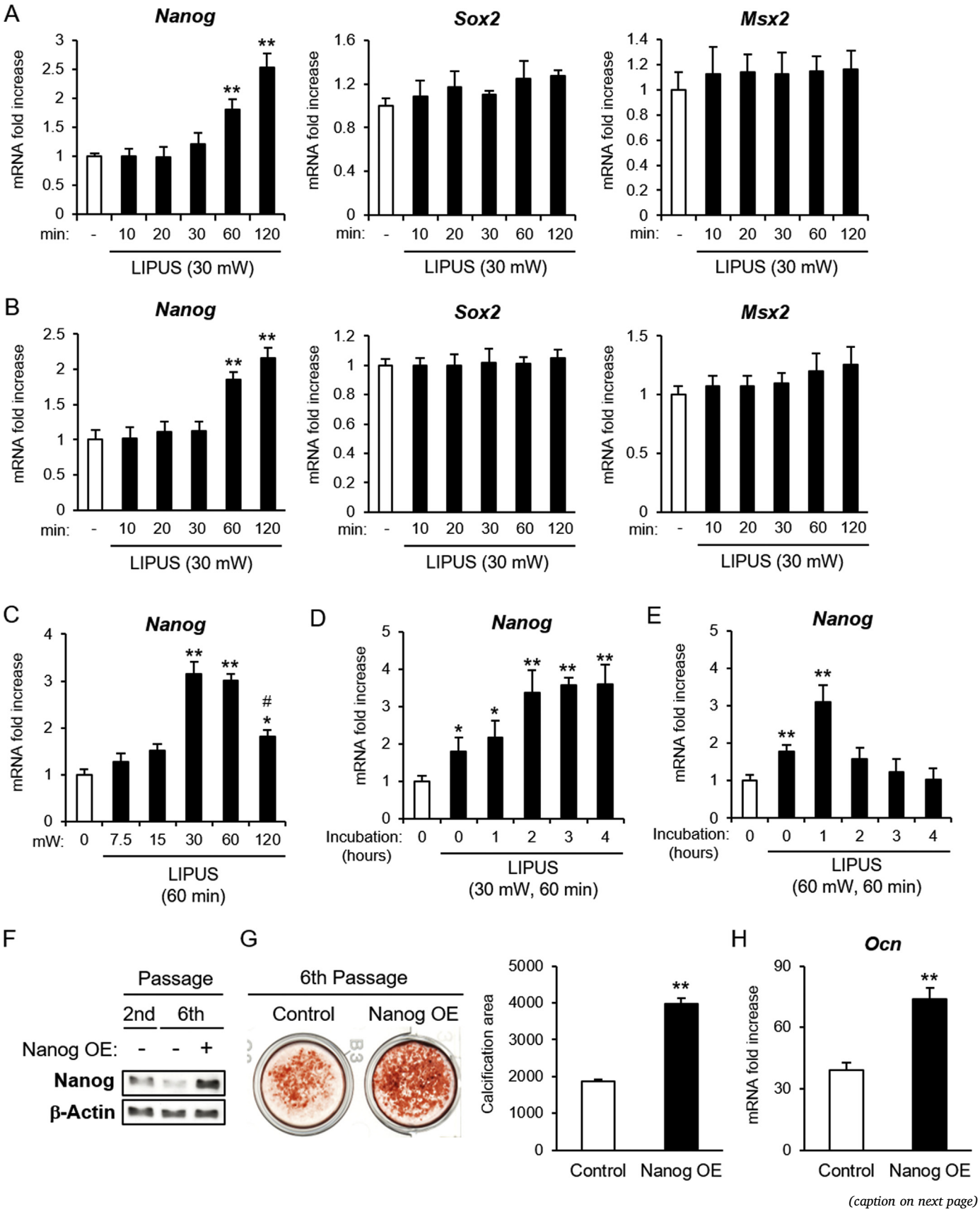

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Fig. 2. LIPUS-induced Nanog expression is involved in the recovery of osteogenic potency. A & B. ST2 cells (A) and primary osteoblasts (B) were stimulated with LIPUS (30 mW/cm^2) for the indicated times. Gene expression was analyzed by real-time RT-PCR. Relative mRNA expression levels compared with *Rpl13a* are shown. Experiments were repeated at least 3 times to ensure consistent results. Error bars represent ± 1 s.d. (**$P < .01$; vs Control). C. ST2 cells were stimulated by LIPUS at the indicated intensities (mW/cm^2) for 60 min. Nanog mRNA expression was analyzed as in A. (#P < .01; 60 mW control vs 120 mW) D & E. ST2 cells were stimulated by LIPUS (D: 30 mW/cm^2, E: 60 mW/cm^2) for 60 min. After the stimulation, cells were incubated for the indicated times. Nanog mRNA expression was analyzed as in A. F—H. Primary osteoblasts were serially passaged 6 times. 6th passaged cells were transiently transfected with pcDNA3.1 Nanog for 24 h. The protein expression of Nanog was analyzed by western blotting (F). After transfection, cells were differentiated by the addition of 280 μM ascorbic acid and 5 mM β-glycerophosphate for 18 days. Cell calcification area was determined by staining with Alizarin Red S and photographical analysis with ImageJ software (G). Osteocalcin mRNA expression was analyzed as in A (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

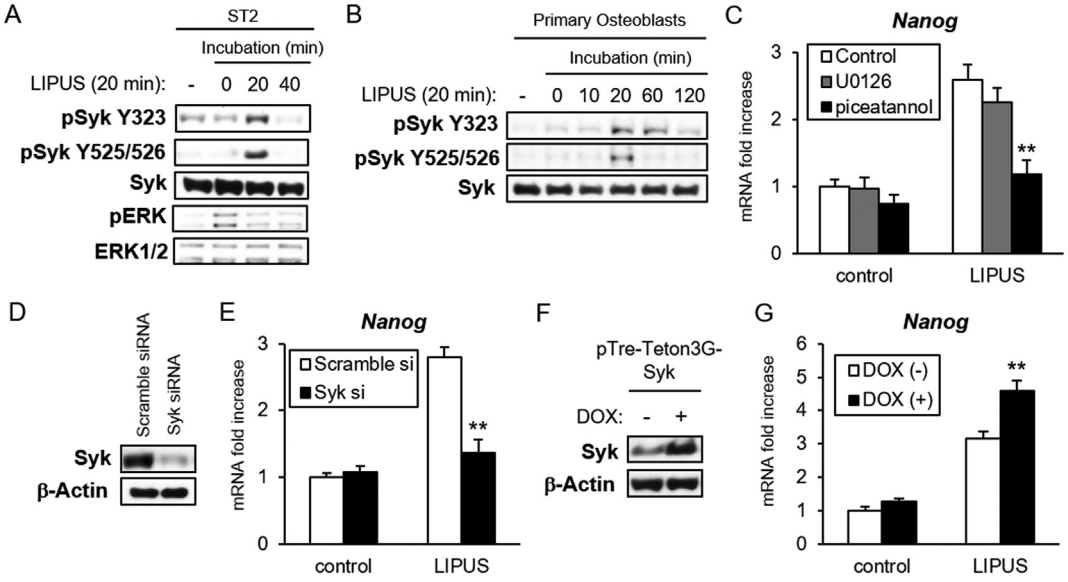

Fig. 3. Syk mediates LIPUS-induced Nanog expression. A & B. ST2 cells (A) and primary osteoblasts (B) were stimulated with LIPUS (30 mW/cm^2) for 20 min. After stimulation, cells were incubated for the indicated times. Cells were lysed in PLC lysis buffer. Cell lysates were separated by SDS-PAGE, and western blotting was performed with the indicated antibodies. C. ST2 cells were treated with 2.5 μM U0126, an ERK-specific inhibitor, and 5 μM piceatannol, a Syk-specific inhibitor, for 6 h. After the treatment, cells were stimulated with LIPUS (30 mW/cm^2) for 2 h. Nanog mRNA expression was analyzed by real-time RT-PCR. Relative mRNA expression levels compared with *Rpl13a* are shown. Experiments were repeated at least 3 times to ensure consistent results. Error bars represent ± 1 s.d. (**P < .01; vs Control). D. ST2 cells were transiently transfected with Syk siRNA for 24 h. After incubating for 24 h, cell lysates were collected in PLC buffer. Syk protein expression was analyzed by western blotting. E. After siRNA transfection as in D, ST2 cells were stimulated with LIPUS, and *Nanog* mRNA expression was analyzed as in C. F. ST2 Tet-on 3G Syk cells were treated with 2 μg/ml of doxycycline (DOX) for 24 h. Syk protein expression was analyzed by western blotting. G. After DOX treatment as in F, ST2 Tet-on 3G Syk cells were stimulated with LIPUS, and Nanog mRNA expression was analyzed as in C.

dependent response in Nanog expression, with significant increases in gene expression being seen at 20 μM and 50 μM ATP concentrations (Fig. 5C). ATP-induced Nanog mRNA expression was also significantly suppressed following ST2 treatment with piceatannol, but not SB203580, a p38-specific inhibitor (Fig. 5D). To clarify which type of ATP receptor is involved in Nanog induction, ST2 cells were treated with A438079, a P2X7-specific receptor blocker; 5-BDBD, a P2X4-specific receptor blocker; and PPADS, a P2X1, 2, 3, 5-receptor blocker. Inhibition of the P2X4 receptor was the only treatment to significantly reduce ATP-induced mRNA expression of Nanog (Fig. 5E). Interestingly, LIPUS-induced Syk Y323 phosphorylation (Fig. 5F) and Nanog mRNA expression (Fig. 5G) were also inhibited by 5-BDBD treatment in the ST2 cells. These findings collectively suggest that LIPUS-induced ATP release activates Syk Y323 phosphorylation and promotes Nanog expression.

LIPUS increases the protein stability of Nanog through ROCK1-Syk activation.

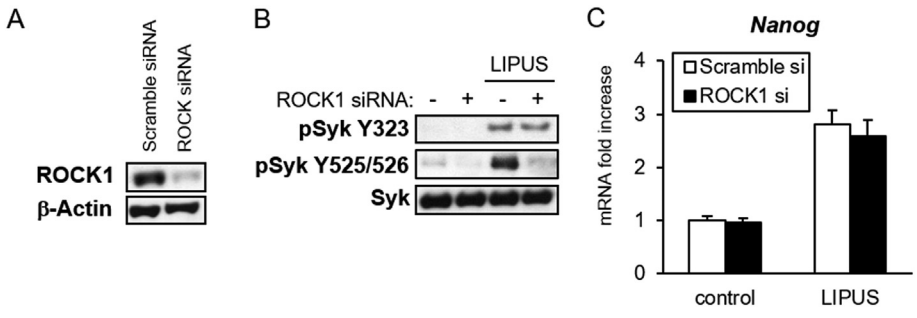

Fig. 4. The knockdown of ROCK1 inhibits Syk phosphorylation at Y525/526 sites but does not alter Nanog expression. A. ST2 cells were transiently transfected with ROCK1 siRNA for 24 h. After incubating for 24 h, cell lysates were collected in PLC buffer. ROCK1 protein expression was analyzed by western blotting. B. After siRNA transfection as in A, ST2 cells were stimulated by LIPUS (30 mW/cm^2) for 2 h. Nanog mRNA expression was analyzed by real-time RT-PCR (B). Relative mRNA expression levels compared with *Rpl13a* are shown. Experiments were repeated at least 3 times to ensure consistent results. Error bars represent ± 1 s.d. (*$p < .01$). C. After siRNA transfection as in A, ST2 cells were stimulated by LIPUS (30 mW/cm^2) for 20 min. After the stimulation, cells were incubated for 20 min. Cells were lysed in PLC lysis buffer. Cell lysates were separated by SDS-PAGE, and western blotting was performed with the indicated antibodies.

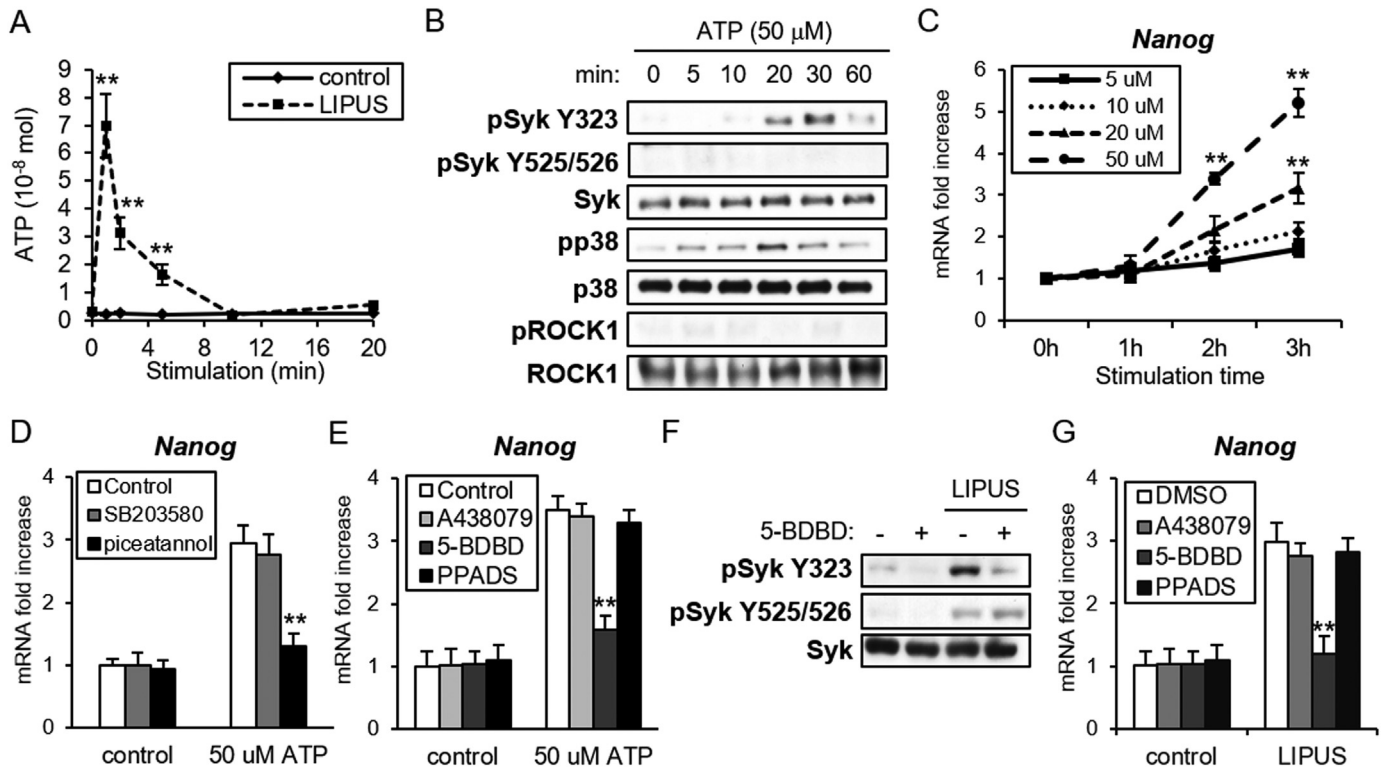

Fig. 5. Paracrine release of ATP by LIPUS induces Syk activation and Nanog expression. A. ST2 cells were seeded at a density of 1.0×10^5 cells/ml for 24 h prior to the stimulation. The culture medium was replaced with fresh medium, and after 1 h incubation, cells were stimulated with LIPUS (30 mW/cm^2) for the indicated times. The concentration of extracellular ATP in cell culture medium was measured using ENLITEN ATP Assay System. Error bars represent ± 1 s.d. (**P < .01; vs 0 min). B. ST2 cells were stimulated with 50 μM ATP for the indicated times. Cells were lysed in PLC lysis buffer. Cell lysates were separated by SDS-PAGE, and western blotting was performed with the indicated antibodies. C. ST2 cells were stimulated by ATP (5, 10, 20 or 50 μM) for the indicated times. Nanog mRNA expression was analyzed by real-time RT-PCR. Relative mRNA expression levels compared with *Rpl13a* are shown. Experiments were repeated at least 3 times to ensure consistent results. Error bars represent ± 1 s.d. (**P < .01; vs 0 h). D. ST2 cells were treated with 5 μM SB203580, a p38-specific inhibitor, and 5 μM piceatannol, a Syk-specific inhibitor, for 6 h. After the treatment, cells were stimulated with 50 μM ATP for 2 h. Nanog mRNA expression was analyzed as in B (**P < .01; vs Control). E - G. ST2 cells were stimulated with 50 μM ATP (E) or 30 mW/cm^2 LIPUS (F & G) with or without 10 μM A438079, a P2X7-specific receptor blocker, 10 μM 5-BDBD, a P2X4-specific receptor blocker, and 10 μM PPADS, a P2X1, 2, 3, 5 receptor blocker, for 2 h. *Nanog* mRNA expression was analyzed as in B (E & G). Western blotting was performed with the indicated antibodies (F). (**P < .01; vs Control).

In order to examine whether ATP treatment can mimic the beneficial effects of LIPUS on osteogenic potency, we serially passaged primary osteoblasts between 2 and 12 times with daily additions of either BzATP, a P2X4 agonist [40], or ATP. However, neither treatment was able to recover the loss of osteogenic potency after serial passaging (data not shown). In order to determine the functional differences between LIPUS stimulation and ATP treatment in the restoration of osteogenic potency, we analyzed the protein stability of Nanog in LIPUS or ATP-treated ST2 cells using cycloheximide. LIPUS stimulation proved to be protective against degradation of Nanog after cycloheximide treatment, while ATP treatment had no effect on Nanog stability (Fig. 6A). Supporting these results, we found that the treatment of serially passaged ST2 cells with a Syk-specific inhibitor, but not an ERK-specific inhibitor, abrogated the protective effects of LIPUS on the stability of Nanog (Fig. 6B). Furthermore, the beneficial effect of LIPUS on Nanog stability was blocked by a ROCK1 knockdown (Fig. 6C). Collectively, these findings suggest that LIPUS-induced improvement of Nanog protein stability is involved in the enhanced osteogenic potency that results from the ROCK1-Syk signaling axis.

4. Discussion

Cell amplification is an essential process in the utilization of MSCs for cell transplantation and cell-based therapies like bone regeneration. Unfortunately, serial passaging of MSCs causes the loss of proliferation capacity and stemness, which adversely affects their differentiation potential and induces the loss of MSC function in transplanted areas [6–8]. The conflict between serial passaging and potency maintenance on the therapeutic potential of MSCs necessitates the development of new methods to improve the efficiency of regenerative medicine. Therefore, it is promising that new cell culture methods show potential for overcoming the adverse effects of serial passaging on MSC potency. Previous studies have reported that mechanical microwave stimulation, including LIPUS [24,25] and shockwave treatments [41,42], have beneficial effects on self-renewal and differentiation potency of MSCs, suggesting that microwave-type mechanical stress is useful in maintaining the multipotency of stem cells. In the present study, we have demonstrated that daily LIPUS stimulation protects against the loss of osteogenic potency in serially passaged primary osteoblastic cells, as represented by improved cell calcification and Osteocalcin mRNA expression (Fig. 1A & B).

We found that serially passaging osteoblastic cells in combination with daily LIPUS treatment resulted in significantly increased expression of *Msx2*, Nanog, and *Sox2* (Fig. 1C). Of these LIPUS-induced, potency-regulating genes, our study showed that the expression of Nanog, but not *Sox2* or *Msx2*, was significantly promoted in response to a single bout of LIPUS stimulation in ST2 cells and primary osteoblastic cells (Fig. 2A-E). We further demonstrated the functional importance of Nanog in passaged cells through vector-induced overexpression of Nanog, where the result was increased Osteocalcin mRNA expression and matrix mineralization (Fig. 2G & H). The relationship between Nanog and Sox2 induction in response to LIPUS stimulation appears

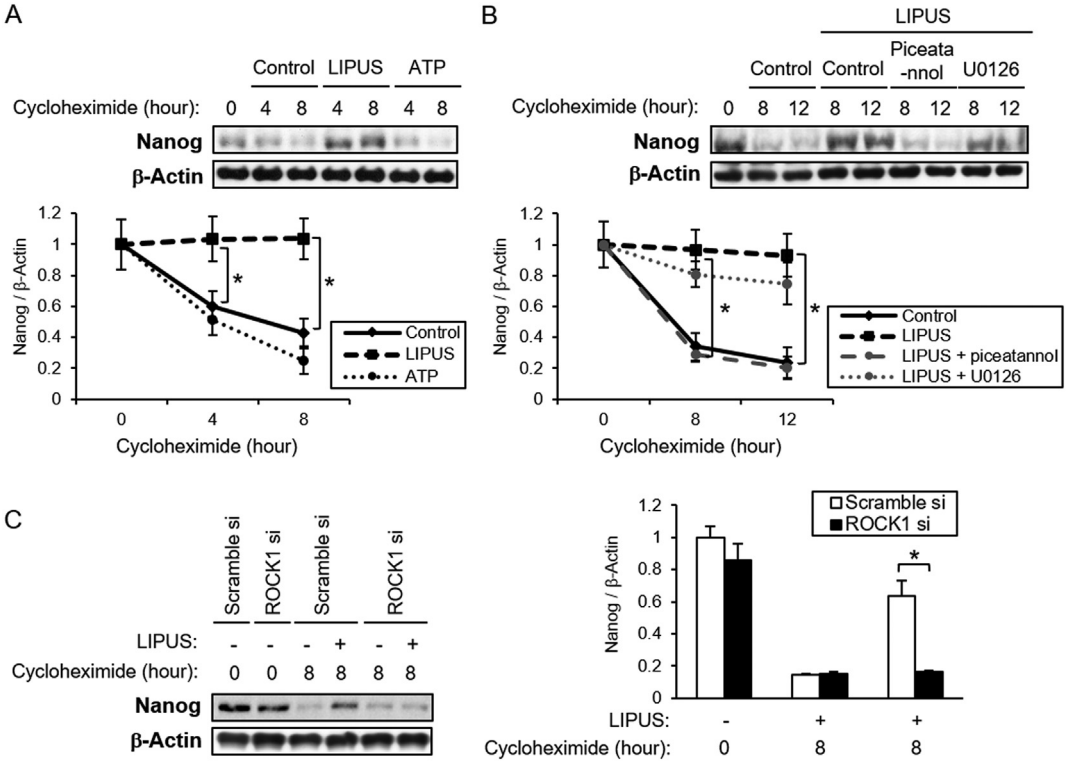

Fig. 6. LIPUS promotes protein stability of Nanog through a ROCK1-Syk signaling axis. A. ST2 cells were stimulated with LIPUS (30 mW/cm^2) or 50 μM ATP for 1 h. After the stimulation, cells were treated with 10 μg/ml of cycloheximide for the indicated times. Cells were lysed in PLC lysis buffer. Cell lysates were separated by SDS-PAGE, and western blotting was performed with the indicated antibodies. The blot images were photographically analyzed using ImageJ software. Experiments were repeated at least 3 times to ensure consistent results. Error bars represent ± 1 s.d. (*$p < .01$). B. ST2 cells were pretreated with 5 μM piceatannol or 2.5 μM U0126 for 6 h. After changing cell culture medium, LIPUS stimulation and cycloheximide treatment was performed as in A. C. ST2 cells were transiently transfected with ROCK1 siRNA for 24 h. After transfection, LIPUS stimulation and cycloheximide treatment was performed as in A.

indirect, as illustrated by our results (Figs. 1C, 2A & B). One previous study showed that mechanical stimulation using oscillatory shear stress upregulated the expression of NANOG and SOX2 in human MSCs [12]. Sox2 is known as a major target of Nanog [43]; yet while Nanog expression levels fluctuate extensively in undifferentiated stem cells, Sox2 levels remain relatively stable [44]. Our data mirrored these results, where both Nanog and Sox2 were increased under LIPUS stimulation in 12th passaged cells (Fig. 1C), despite showing very different responses to changes in LIPUS stimuli duration (Fig. 2A & B). Accordingly, mechanically induced Sox2 expression may be mediated by Nanog induction.

Our data also showed that increases in Nanog expression were sustainably upregulated even after LIPUS stimulation ceased (Fig. 2D). Another group also reported that cyclic strain maintains Nanog expression after termination of mechanical stimulation through PI3K-Akt signaling in mouse ES cells under the withdrawal of leukemia inhibitory factor [45]. These results suggest that the effects of mechanical stimuli on multipotency may be prolonged well beyond the actual administration of LIPUS treatment. Collectively, our findings suggest that LIPUS-induced upregulation of Nanog leads to recovery and sustained maintenance of osteogenic potency in serially passaged osteoblastic cells.

One of our principle findings is that LIPUS stimulation activates Syk to induce Nanog expression in ST2 cells and primary osteoblastic cells (Fig. 3). The mechano-sensitivity of Syk is reported in fluid shear stress-stimulated platelets [46,47] and B cells [48]. Although one previous study showed that zeta-chain-associated protein kinase-70 (Zap70), a tyrosine kinase which is structurally homologous to Syk, plays important roles in modulating the balance between self-renewal capacity and multipotent differentiation ability in mouse ES cells [49], our study is the first to show the role of Syk in the regulation of stemness. Interestingly, our recent report showed that Syk is abundantly expressed in undifferentiated MSCs, however, the expression levels of Syk protein gradually decreased during the courses of adipogenic and osteogenic differentiation in MSCs [30]. This decreased expression of Syk during differentiation may play a role in the loss of stemness in MSCs.

ROCK1 is cytoskeleton-anchoring molecule that is activated by dynamic remodeling of the actin cytoskeleton [50]. Our previous study found that ROCK1 is an upstream activator of cancer Osaka thyroid oncogene/tumor progression locus 2 (Cot/Tpl2) - mitogen-activated protein kinase kinase (MEK) - ERK pathway in LIPUS-stimulated MSCs [23]. Consistent with our previous work, our current data show that LIPUS-induced Syk Y525/526 phosphorylation is inhibited by the knockdown of ROCK1 (Fig. 4B). This indicates that ROCK1 is a central signaling molecule that not only activates the ERK pathway, but also activates Syk signaling in the mechanical stress response.

Alternatively, we found that LIPUS-induced Syk Y323 phosphorylation is mediated in a paracrine manner through an ATP - P2X4 axis (Fig. 5F). One study has reported that LIPUS treatment induces ATP release from MC3T3-E1 cells [36], while other studies have demonstrated the stimulatory effects of extracellular ATP on Syk activation in osteoclasts [39] and thrombin-activated platelets [51]. It is well documented that transient Ca^{2+} ion channel activation and cytoskeleton reorganization are important intracellular events that are influenced by mechanical stimuli like ultrasound, microwave, and cyclic stretch in osteoblasts and MSCs [52,53]. These two mechanisms upregulate each other and synergistically lead to functional signaling cascades, which regulate adhesion, proliferation, and differentiation in MSCs. Because P2X4 is a typical P2X-family receptor with trimeric ATP-gated cation channels [54], both the ROCK1 pathway and the ATP-P2X4 axis are able to play a role in Syk activation. Recognizing the duality of these processes in Syk activation is necessary to facilitate an understanding of

the broad effects that LIPUS stimulation has on MSC function.

P2X4 is one of the most sensitive ATP receptors (at nanomolar ATP concentrations), and is about one thousand times more than sensitive than P2X7 [54]. We reported that LIPUS-stimulated ST2 cells release 7×10^{-8} mol of ATP (Fig. 5A), which is enough for P2X4 activation. The role of P2X4 in MSCs is not well-studied, however previous reports indicate that the effects of electrical stimulation on ATP oscillations and chondrogenesis are dependent on extracellular ATP signaling via P2X4 [55]. Interestingly, a recent study reported that P2X4 has a role as an ATP-independent receptor and mechanotransducer in podocytes [56]. Further studies are needed to better understand the stimulatory effects of LIPUS and other mechanical stimuli on P2X4 in MSCs.

Our data indicate that the site-specific difference between ROCK1 and ATP-induced Syk phosphorylation leads to functional differences in Syk. LIPUS-induced *Nanog* expression was inhibited by a P2X4-specific blocker (Fig. 5G), but not ROCK1 siRNA treatment (Fig. 4C). Similarly, ATP treatment mimicked LIPUS-induced Syk Y323 phosphorylation and increased *Nanog* mRNA expression (Fig. 5B-E). On the other hand, ROCK1 - Syk Y525/526 signaling promoted the stability of Nanog protein (Fig. 6). With respect to Syk phosphorylation, previous studies have shown that the functional activities of Syk were differentially regulated by its several phosphorylation sites. Syk Y352 phosphorylation can transiently induce downstream signaling in the phospholipase C γ (PLCγ) pathway, however signal intensity is not long-lived [57]. Syk Y323 activation is required for the recruitment of the phosphatidylinositol 3-kinase (PI3K) p110δ subunit and the accumulation of PI3K products [58]. In contrast, the Y525/526 phosphorylation site is in the activation loop of the kinase domain, and its phosphorylation is directly related to the enzymatic activity of Syk [59]. Phosphorylation at Y525/526 greatly enhances the signaling capacity of Syk in B-cells [57]. Our previous study also showed that Syk Y352 phosphorylation is important for regulating adipogenic or osteogenic differentiation of MSCs [30], however Syk Y352 was not affected by LIPUS stimulation (data not shown). Therefore, site-specific phosphorylation of Syk as a result of MSC differentiation, or by way of MSC response to LIPUS, may be responsible for the ultimate differences in MSC destiny. Since Syk phosphorylation is regulated by adaptor proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs), Src family kinases [60], protein kinase C-δ (PKC-δ) [61], and phosphatidylinositol 3-kinase (PI3-K) [62], the downstream molecules of ROCK1 or Ca^{2+} channel activation may be involved in distinct types of Syk phosphorylation and its subsequent function in LIPUS-stimulated cells.

Nanog is a key transcription factor for maintaining multipotency in both ES cells and MSCs. Functionally, Nanog cooperates with Oct4 and Sox2 to establish the multipotent property of stem cells [34]. Since Nanog is an unstable protein, as exhibited by its short half-life [63], both mRNA expression level and post-transcriptional modification is crucial for Nanog function. Our study showed that LIPUS-induced activation of the ROCK1-Syk Y525/526 signaling axis improved the stability of Nanog (Fig. 6). Previous studies have indicated that Nanog is phosphorylated at multiple serine and threonine residues by ERK1/2 and cyclin-dependent kinase 1 (Cdk1) [64–66]. This phosphorylation of Nanog promotes the interaction between Nanog and coupling proteins, which leads to Nanog stabilization by suppressing its ubiquitination. Our data showed that LIPUS stimulation induced ERK phosphorylation (Fig. 3A), however, treatment with an ERK inhibitor did not affect the LIPUS-improved stability of Nanog (Fig. 6B). Therefore, it is possible that direct or indirect phosphorylation of Nanog by Syk has a central role in the regulation of Nanog function in LIPUS-stimulated cells. Further study is needed to elucidate the mechanisms behind Nanog protection resulting from mechanical stimulation.

In summary, our data show that daily LIPUS stimulation prevents the loss of osteogenic potency during serial passaging in osteoblastic cells. These LIPUS-induced effects are mediated by increased Nanog expression and stability through both ROCK1-Syk and ATP-P2X4-Syk signaling pathways. These results suggest the possibility of using mechanical stimuli, including LIPUS, to improve regenerative therapies like MSC transplantation. This study also provides new insights into the molecular mechanisms behind the effects of LIPUS and other mechanical stimuli on cellular function.

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Author contributions

JK performed most of the experiments, interpreted the results and prepared the manuscript. CS performed the experiments shown in Fig. 2. NSM assisted with data interpretation and prepared the manuscript. TN and KB provided technical assistance for analysis and interpretation of the data shown in Fig. 5. KS and IS provided technical help on the experiments shown in Fig. 1. TM directed and supervised the project.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

References

[1] C.C. Wei, A.B. Lin, S.C. Hung, Mesenchymal stem cells in regenerative medicine for musculoskeletal diseases: bench, bedside, and industry, Cell Transplant. 23 (4–5) (2014) 505–512.
[2] M.N. Knight, K.D. Hankenson, Mesenchymal Stem Cells in Bone Regeneration, Adv. Wound Care 2 (6) (2013) 306–316.
[3] S. Razzouk, R. Schoor, Mesenchymal stem cells and their challenges for bone regeneration and osseointegration, J. Periodontol. 83 (5) (2012) 547–550.
[4] S. Tsutsumi, A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi, Y. Kato, Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF, Biochem. Biophys. Res. Commun. 288 (2) (2001) 413–419.
[5] S. Sisakhtnezhad, E. Alimoradi, H. Akrami, External factors influencing mesenchymal stem cell fate in vitro, Eur. J. Cell Biol. 96 (1) (2017) 13–33.
[6] M.M. Bonab, K. Alimoghaddam, F. Talebian, S.H. Ghaffari, A. Ghavamzadeh, B. Nikbin, Aging of mesenchymal stem cell in vitro, BMC Cell Biol. 7 (2006) 14.
[7] J.D. Kretlow, Y.Q. Jin, W. Liu, W.J. Zhang, T.H. Hong, G. Zhou, L.S. Baggett, A.G. Mikos, Y. Cao, Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells, BMC Cell Biol. 9 (2008) 60.
[8] J.A. Di Battista, W. Shebaby, O. Kizilay, E. Hamade, R. Abou Merhi, S. Mebarek, D. Abdallah, B. Badran, F. Saad, K.A. E, W.H. Faour, Proliferation and differentiation of human adipose-derived mesenchymal stem cells (ASCs) into osteoblastic lineage are passage dependent, Inflam. Res. 63 (11) (2014) 907–917.
[9] Y.K. Yang, C.R. Ogando, C. Wang See, T.Y. Chang, G.A. Barabino, Changes in phenotype and differentiation potential of human mesenchymal stem cells aging in vitro, Stem Cell Res Ther 9 (1) (2018) 131.
[10] G. Song, Y. Ju, H. Soyama, T. Ohashi, M. Sato, Regulation of cyclic longitudinal mechanical stretch on proliferation of human bone marrow mesenchymal stem cells, Mol. Cell. Biomech. 4 (4) (2007) 201–210.
[11] S. Saha, L. Ji, J.J. de Pablo, S.P. Palecek, Inhibition of human embryonic stem cell differentiation by mechanical strain, J. Cell. Physiol. 206 (1) (2006) 126–137.
[12] Y.C. Kuo, T.H. Chang, W.T. Hsu, J. Zhou, H.H. Lee, J. Hui-Chun Ho, S. Chien, O.K. Lee, Oscillatory shear stress mediates directional reorganization of actin cytoskeleton and alters differentiation propensity of mesenchymal stem cells, Stem Cells (Dayton, Ohio) 33 (2) (2015) 429–442.
[13] Y.M. Kim, Y.G. Kang, S.H. Park, M.K. Han, J.H. Kim, J.W. Shin, Effects of mechanical stimulation on the reprogramming of somatic cells into human-induced pluripotent stem cells, Stem Cell Res Ther 8 (1) (2017) 139.
[14] D.S. Yoon, Y.H. Kim, H.S. Jung, S. Paik, J.W. Lee, Importance of Sox2 in maintenance of cell proliferation and multipotency of mesenchymal stem cells in low-density culture, Cell Prolif. 44 (5) (2011) 428–440.
[15] S.M. Han, S.H. Han, Y.R. Coh, G. Jang, J. Chan Ra, S.K. Kang, H.W. Lee, H.Y. Youn, Enhanced proliferation and differentiation of Oct4- and Sox2-overexpressing human adipose tissue mesenchymal stem cells, Exp. Mol. Med. 46 (2014) e101.
[16] C.C. Tsai, P.F. Su, Y.F. Huang, T.L. Yew, S.C. Hung, Oct4 and Nanog directly

regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells, Mol. Cell 47 (2) (2012) 169–182.
[17] B. Gharibi, S. Farzadi, M. Ghuman, F.J. Hughes, Inhibition of Akt/mTOR attenuates age-related changes in mesenchymal stem cells, Stem Cells (Dayton, Ohio) 32 (8) (2014) 2256–2266.
[18] R. Goetzke, A. Sechi, L. De Laporte, S. Neuss, W. Wagner, Why the impact of mechanical stimuli on stem cells remains a challenge, Cell. Mol. Life Sci. 75 (18) (2018) 3297–3312.
[19] M.N. Andalib, Y. Dzenis, H.J. Donahue, J.Y. Lim, Biomimetic substrate control of cellular mechanotransduction, Biomater. Res. 20 (2016) 11.
[20] D.A. Hoey, S. Tormey, S. Ramcharan, F.J. O'Brien, C.R. Jacobs, Primary cilia-mediated mechanotransduction in human mesenchymal stem cells, Stem Cells (Dayton, Ohio) 30 (11) (2012) 2561–2570.
[21] T.R. Coughlin, M. Voisin, M.B. Schaffler, G.L. Niebur, L.M. McNamara, Primary cilia exist in a small fraction of cells in trabecular bone and marrow, Calcif. Tissue Int. 96 (1) (2015) 65–72.
[22] C.L. Romano, D. Romano, N. Logoluso, Low-intensity pulsed ultrasound for the treatment of bone delayed union or nonunion: a review, Ultrasound Med. Biol. 35 (4) (2009) 529–536.
[23] J. Kusuyama, K. Bandow, M. Shamoto, K. Kakimoto, T. Ohnishi, T. Matsuguchi, Low intensity pulsed ultrasound (LIPUS) influences the multilineage differentiation of mesenchymal stem and progenitor cell lines through ROCK-cot/Tpl2-MEK-ERK signaling pathway, J. Biol. Chem. 289 (15) (2014) 10330–10344.
[24] L. Ling, T. Wei, L. He, Y. Wang, X. Feng, W. Zhang, Z. Xiong, Low-intensity pulsed ultrasound activates ERK1/2 and PI3K-Akt signalling pathways and promotes the proliferation of human amnion-derived mesenchymal stem cells, Cell Prolif. 50 (6) (2017).
[25] V. Costa, V. Carina, S. Fontana, A. De Luca, F. Monteleone, S. Pagani, M. Sartori, S. Setti, C. Faldini, R. Alessandro, M. Fini, G. Giavaresi, Osteogenic commitment and differentiation of human mesenchymal stem cells by low-intensity pulsed ultrasound stimulation, J. Cell. Physiol. 233 (2) (2018) 1558–1573.
[26] K. Hensel, M.P. Mienkina, G. Schmitz, Analysis of ultrasound fields in cell culture wells for in vitro ultrasound therapy experiments, Ultrasound Med. Biol. 37 (12) (2011) 2105–2115.
[27] J. Kusuyama, K. Bandow, T. Ohnishi, M. Hisadome, K. Shima, I. Semba, T. Matsuguchi, Osteopontin inhibits osteoblast responsiveness through the down-regulation of focal adhesion kinase mediated by the induction of low-molecular weight protein tyrosine phosphatase, Mol. Biol. Cell 28 (10) (2017) 1326–1336.
[28] J. Nakao, Y. Fujii, J. Kusuyama, K. Bandow, K. Kakimoto, T. Ohnishi, T. Matsuguchi, Low-intensity pulsed ultrasound (LIPUS) inhibits LPS-induced inflammatory responses of osteoblasts through TLR4-MyD88 dissociation, Bone 58 (2014) 17–25.
[29] J. Kusuyama, A. Komorizono, K. Bandow, T. Ohnishi, T. Matsuguchi, CXCL3 positively regulates adipogenic differentiation, J. Lipid Res. 57 (10) (2016) 1806–1820.
[30] J. Kusuyama, A. Kamisono, S. ChangHwan, M.S. Amir, K. Bandow, N. Eiraku, T. Ohnishi, T. Matsuguchi, Spleen tyrosine kinase influences the early stages of multilineage differentiation of bone marrow stromal cell lines by regulating phospholipase C gamma activities, J. Cell. Physiol. 233 (3) (2018) 2549–2559.
[31] T. Komori, Signaling networks in RUNX2-dependent bone development, J. Cell. Biochem. 112 (3) (2011) 750–755.
[32] T. Komori, Regulation of osteoblast differentiation by transcription factors, J. Cell. Biochem. 99 (5) (2006) 1233–1239.
[33] U.A. White, J.M. Stephens, Transcriptional factors that promote formation of white adipose tissue, Mol. Cell. Endocrinol. 318 (1–2) (2010) 10–14.
[34] A. Saunders, F. Faiola, J. Wang, Concise review: pursuing self-renewal and pluripotency with the stem cell factor Nanog, Stem Cells (Dayton, Ohio) 31 (7) (2013) 1227–1236.
[35] J. Kusuyama, T. Nakamura, T. Ohnishi, B.G. Albertson, Y. Ebe, N. Eiraku, K. Noguchi, T. Matsuguchi, Low-intensity pulsed ultrasound promotes bone morphogenic protein 9-induced osteogenesis and suppresses inhibitory effects of inflammatory cytokines on cellular responses via Rho-associated kinase 1 in human periodontal ligament fibroblasts, J. Cell. Biochem. (2019) (Epub ahead of print).
[36] S. Manaka, N. Tanabe, T. Kariya, M. Naito, T. Takayama, M. Nagao, D. Liu, K. Ito, M. Maeno, N. Suzuki, M. Miyazaki, Low-intensity pulsed ultrasound-induced ATP increases bone formation via the P2X7 receptor in osteoblast-like MC3T3-E1 cells, FEBS Lett. 589 (3) (2015) 310–318.
[37] D. Sun, W.G. Junger, C. Yuan, W. Zhang, Y. Bao, D. Qin, C. Wang, L. Tan, B. Qi, D. Zhu, X. Zhang, T. Yu, Shockwaves induce osteogenic differentiation of human mesenchymal stem cells through ATP release and activation of P2X7 receptors, Stem Cells (Dayton, Ohio) 31 (6) (2013) 1170–1180.
[38] T. Yu, W.G. Junger, C. Yuan, A. Jin, Y. Zhao, X. Zheng, Y. Zeng, J. Liu, Shockwaves increase T-cell proliferation and IL-2 expression through ATP release, P2X7 receptors, and FAK activation, Am. J. Physiol. 298 (3) (2010) C457–C464.
[39] R. Hazama, X. Qu, K. Yokoyama, C. Tanaka, E. Kinoshita, J. He, S. Takahashi, K. Tohyama, H. Yamamura, Y. Tohyama, ATP-induced osteoclast function: the formation of sealing-zone like structure and the secretion of lytic granules via microtubule-deacetylation under the control of Syk, Genes Cells 14 (7) (2009) 871–884.
[40] M.F. Jarvis, B.S. Khakh, ATP-gated P2X cation-channels, Neuropharmacology 56 (1) (2009) 208–215.
[41] C.M. Schuh, P. Heher, A.M. Weihs, A. Banerjee, C. Fuchs, C. Gabriel, S. Wolbank, R. Mittermayr, H. Redl, D. Runzler, A.H. Teuschl, In vitro extracorporeal shock wave treatment enhances stemness and preserves multipotency of rat and human adipose-derived stem cells, Cytotherapy 16 (12) (2014) 1666–1678.
[42] H. Zhang, Z.L. Li, F. Yang, Q. Zhang, X.Z. Su, J. Li, N. Zhang, C.H. Liu, N. Mao, H. Zhu, Radial shockwave treatment promotes human mesenchymal stem cell self-renewal and enhances cartilage healing, Stem Cell Res. Ther. 9 (1) (2018) 54.
[43] Y.H. Loh, Q. Wu, J.L. Chew, V.B. Vega, W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K.Y. Wong, K.W. Sung, C.W. Lee, X.D. Zhao, K.P. Chiu, L. Lipovich, V.A. Kuznetsov, P. Robson, L.W. Stanton, C.L. Wei, Y. Ruan, B. Lim, H.H. Ng, The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells, Nat. Genet. 38 (4) (2006) 431–440.
[44] I. Chambers, J. Silva, D. Colby, J. Nichols, B. Nijmeijer, M. Robertson, J. Vrana, K. Jones, L. Grotewold, A. Smith, Nanog safeguards pluripotency and mediates germline development, Nature 450 (7173) (2007) 1230–1234.
[45] R. Horiuchi, T. Akimoto, Z. Hong, T. Ushida, Cyclic mechanical strain maintains Nanog expression through PI3K/Akt signaling in mouse embryonic stem cells, Exp. Cell Res. 318 (14) (2012) 1726–1732.
[46] J.H. Haga, L.K. Jennings, S.M. Slack, Inhibition of shear-stress-induced platelet aggregation and phosphotyrosine signaling by GPIIb-IIIa antagonists, Ann. Biomed. Eng. 30 (10) (2002) 1262–1272.
[47] H.E. Speich, S. Grgurevich, T.J. Kueter, A.D. Earhart, S.M. Slack, L.K. Jennings, Platelets undergo phosphorylation of Syk at Y525/526 and Y352 in response to pathophysiological shear stress, Am. J. Physiol. 295 (4) (2008) C1045–C1054.
[48] F. Apoorva, A.M. Loiben, S.B. Shah, A. Purwada, L. Fontan, R. Goldstein, B.J. Kirby, A.M. Melnick, B.D. Cosgrove, A. Singh, How biophysical forces regulate human B cell lymphomas, Cell Rep. 23 (2) (2018) 499–511.
[49] Y. Cha, B.H. Moon, M.O. Lee, H.J. Ahn, H.J. Lee, K.A. Lee, A.J. Fornace Jr., K.S. Kim, H.J. Cha, K.S. Park, Zap70 functions to maintain stemness of mouse embryonic stem cells by negatively regulating Jak1/Stat3/c-Myc signaling, Stem Cells (Dayton, Ohio) 28 (9) (2010) 1476–1486.
[50] S.T. Boyle, M.S. Samuel, Mechano-reciprocity is maintained between physiological boundaries by tuning signal flux through the Rho-associated protein kinase, Small GTPases 7 (3) (2016) 139–146.
[51] X. Liu, Y. Yan, L. Bao, B. Chen, Y. Zhao, R. Qi, Ginkgolide B inhibits platelet release by blocking Syk and p38 MAPK phosphorylation in thrombin-stimulated platelets, Thromb. Res. 134 (5) (2014) 1066–1073.
[52] S. Zhang, J. Cheng, Y.X. Qin, Mechanobiological modulation of cytoskeleton and calcium influx in osteoblastic cells by short-term focused acoustic radiation force, PLoS One 7 (6) (2012) e38343.
[53] D. Liu, C. Yi, K. Wang, C.C. Fong, Z. Wang, P.K. Lo, D. Sun, M. Yang, Reorganization of cytoskeleton and transient activation of Ca2+ channels in mesenchymal stem cells cultured on silicon nanowire arrays, ACS Appl. Mater. Interfaces 5 (24) (2013) 13295–13304.
[54] J. Suurvali, P. Boudinot, J. Kanellopoulos, S. Ruutel Boudinot, P2X4: a fast and sensitive purinergic receptor, Biom. J. 40 (5) (2017) 245–256.
[55] H.J. Kwon, G.S. Lee, H. Chun, Electrical stimulation drives chondrogenesis of mesenchymal stem cells in the absence of exogenous growth factors, Sci. Rep. 6 (2016) 39302.
[56] A.L. Forst, V.S. Olteanu, G. Mollet, T. Wlodkowski, F. Schaefer, A. Dietrich, J. Reiser, T. Gudermann, M. Mederos y Schnitzler, U. Storch, Podocyte purinergic P2X4 channels are mechanotransducers that mediate cytoskeletal disorganization, J. Am. Soc. Nephrol. 27 (3) (2016) 848–862.
[57] L. Carsetti, L. Laurenti, S. Gobessi, P.G. Longo, G. Leone, D.G. Efremov, Phosphorylation of the activation loop tyrosines is required for sustained Syk signaling and growth factor-independent B-cell proliferation, Cell. Signal. 21 (7) (2009) 1187–1194.
[58] J. Schymeinsky, C. Then, A. Sindrilaru, R. Gerstl, Z. Jakus, V.L. Tybulewicz, K. Scharffetter-Kochanek, B. Walzog, Syk-mediated translocation of PI3Kdelta to the leading edge controls lamellipodium formation and migration of leukocytes, PLoS One 2 (11) (2007) e1132.
[59] J. Zhang, M.L. Billingsley, R.L. Kincaid, R.P. Siraganian, Phosphorylation of Syk activation loop tyrosines is essential for Syk function. An in vivo study using a specific anti-Syk activation loop phosphotyrosine antibody, J. Biol. Chem. 275 (45) (2000) 35442–35447.
[60] A. Mocsai, J. Ruland, V.L. Tybulewicz, The SYK tyrosine kinase: a crucial player in diverse biological functions, Nat. Rev. Immunol. 10 (6) (2010) 387–402.
[61] K.M. Bijli, F. Fazal, M. Minhajuddin, A. Rahman, Activation of Syk by protein kinase C-delta regulates thrombin-induced intercellular adhesion molecule-1 expression in endothelial cells via tyrosine phosphorylation of RelA/p65, J. Biol. Chem. 283 (21) (2008) 14674–14684.
[62] J.C. Resendiz, S. Feng, G. Ji, K.A. Francis, M.C. Berndt, M.H. Kroll, Purinergic P2Y12 receptor blockade inhibits shear-induced platelet phosphatidylinositol 3-kinase activation, Mol. Pharmacol. 63 (3) (2003) 639–645.
[63] S. Ramakrishna, B. Suresh, K.H. Lim, B.H. Cha, S.H. Lee, K.S. Kim, K.H. Baek, PEST motif sequence regulating human NANOG for proteasomal degradation, Stem Cells Dev. 20 (9) (2011) 1511–1519.
[64] M. Moretto-Zita, H. Jin, Z. Shen, T. Zhao, S.P. Briggs, Y. Xu, Phosphorylation stabilizes Nanog by promoting its interaction with Pin1, Proc. Natl. Acad. Sci. U. S. A. 107 (30) (2010) 13312–13317.
[65] S.H. Kim, M.O. Kim, Y.Y. Cho, K. Yao, D.J. Kim, C.H. Jeong, D.H. Yu, K.B. Bae, E.J. Cho, S.K. Jung, M.H. Lee, H. Chen, J.Y. Kim, A.M. Bode, Z. Dong, ERK1 phosphorylates Nanog to regulate protein stability and stem cell self-renewal, Stem Cell Res. 13 (1) (2014) 1–11.
[66] J. Brumbaugh, J.D. Russell, P. Yu, M.S. Westphall, J.J. Coon, J.A. Thomson, NANOG is multiply phosphorylated and directly modified by ERK2 and CDK1 in vitro, Stem Cell Rep. 2 (1) (2014) 18–25.