Am J Stem Cells 2024;13(3):143-151
www.AJSC.us /ISSN:2160-4150/AJSC0156130

Original Article

Acoustic vibration promotes in vitro expansion of human embryonic stem cells

Xiangyue Hu[1,2], Haoyun Duan[2], Dulei Zou[2,3], Chunxiao Dong[2,3], Yani Wang[2], Yao Wang[2], Zongren Li[4], Zongyi Li[1,2]

[1]Shandong First Medical University (Shandong Academy of Medical Sciences), Jinan 250000, Shandong, China; [2]Eye Institute of Shandong First Medical University, State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Qingdao 266071, Shandong, China; [3]Eye Institute of Shandong First Medical University, Eye Hospital of Shandong First Medical University (Shandong Eye Hospital), Jinan 250021, Shandong, China; [4]970 Hospital of Chinese PLA Joint Logistic Support Force, Weihai 264200, Shandong, China

Received February 26, 2024; Accepted May 6, 2024; Epub June 15, 2024; Published June 30, 2024

Abstract: Objectives: This study aimed to investigate the effect of acoustic vibration on the pluripotency of human embryonic stem cells (hESCs) and evaluate cell proliferation and self-renewal ability post-treatment. Methods: The human ES cell line H1 was used for the experiments. hESCs were treated with an acoustic vibration device. Their proliferative ability was subsequently detected using a colony formation assay, while the expression of pluripotency-related markers was detected via immunofluorescence staining. Finally, changes in gene expression levels were examined using quantitative polymerase chain reaction (qPCR) in the presence of appropriate primers. Results: Compared with normal cells in the control group, the morphology of experimental cells subjected to acoustic vibration did not significantly change. Contrastingly, the colony-forming efficiency of the experimental cells significantly increased. Immunofluorescence staining results showed the cells in experimental group were positive for the pluripotency markers NANOG, octamer-binding transcription factor 4 gene (OCT4), and SRY (sex determining region Y)-box 2 (SOX2). In addition, the expression levels of pluripotency genes NANOG, OCT4, SOX2, and Yes-associated protein (YAP)-related genes were up-regulated following acoustic vibration. Conclusions: Our results revealed that acoustic vibration enhanced the proliferative ability of hESCs and increased the expression levels of NANOG, OCT4, SOX2, and YAP-related genes, indicating that acoustic vibration can optimize the self-renewal ability of hESCs and that the YAP signaling pathway may play a critical role in the functional process of acoustic vibration.

Keywords: Human embryonic stem cells, acoustic vibration, pluripotency, proliferation, YAP

Introduction

Human embryonic stem cells (hESCs) exhibit remarkable self-renewal capacity and potential to differentiate into various cell types, rendering them highly promising for the research of tissue development, pathological mechanisms, and human therapeutics [1, 2]. As is well documented, the pluripotency of hESCs is intricately regulated by epigenetic modifications, core stemness transcription factors, signaling pathways, and histone modifications [3-5]. Its maintenance relies on key transcription factors, such as SRY (sex determining region Y)-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4), and NANOG [6]. Despite extensive research, the mechanisms governing pluripotency and self-renewal of embryonic stem cells remain elusive. Various approaches, including modification of culture protocols [7], incorporating different factors [8-10], alteration of metabolic patterns [11], manipulation of RNA processing, and implementation of reprogramming techniques [12, 13], have been explored to sustain the pluripotency of embryonic stem cells in vitro.

The application of acoustic vibrations as a mechanical stimulus has emerged as a promising approach for mediating complex cell behaviors and directing stem cell differentiation [14]. Indeed, it has been extensively applied in cellular research and disease treatment [15, 16], including the generation of pluripotent stem

https://doi.org/10.62347/PJFC2708

cells using ultrasound [17], induction wound healing in vitro [18], promotion of mesenchymal stem cell proliferation, differentiation, and migration [19], as well as exosome secretion through low-intensity pulsed ultrasound (LIPUS) [20, 21], the acceleration of spinal healing [22], promotion of nerve regeneration and recovery [23, 24], the induction of neurogenesis, and the treatment of traumatic brain injury, mental disorders, and Alzheimer's disease [25, 26]. Notably, sound waves exert their influence on tissue homeostasis and regeneration by modulating intercellular communication, thereby impacting the onset and progression of diseases [27]. Acoustic vibration offers numerous advantages, such as streamlined instrumentation and experimental setup, enhanced operational resolution, and flexibility. This versatile and non-invasive technique represents an ideal method for conducting cellular research [18, 28-30].

The Yes-associated protein (YAP) pathway functions as a sensor and mediator of mechanical signals in the cell microenvironment. It is highly sensitive to a wide range of mechanical stimuli, enabling the transduction of these signals into cell-specific biological signals and consequent modulation of cellular responses that promote the expression of target genes by activating the transcription factor TEAD PDZ binding motif (TAZ) [31-33]. Specifically, YAP1 is vital for the self-differentiation and renewal of stem cells, as well as the long-term proliferation and survival of hESCs in vitro [34, 35].

This study aimed to investigate the impact of acoustic vibration treatment on hESCs culture and elucidate the underlying mechanism. We hypothesized that acoustic vibration could enhance hESCs pluripotency and promote proliferation and thus provide novel promising strategies for stem cell culture.

Material and methods

Cell culture

Human embryonic stem cell line H1 was donated by Professor Zhengqin Yin of Army Medical University (Chongqing, China). H1 cells were seeded onto plates coated with Matrigel (Corning 354277) and incubated in mTeSR1 medium (STEMCELL Technology) until the next passage, which was performed every 5-7 days using ReLeSR™ (STEMCELL Technology, 05872). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was renewed daily.

Cells were treated with acoustic vibration

This experiment was carried out using an acoustic vibration device (Beijing Yishengkang, China) comprising a regulating acoustic display, an acoustic transmission box, and an infinite input sound head. The acoustic output frequency of the device ranged from 20 Hz to 4000 Hz, whilst the acoustic intensity ranged from 0 to 100 dB. In the present study, the mode was employed at 3 intensity levels, with an acoustic output frequency of 450-500 Hz and an acoustic intensity of 30 dB.

After three days of passaging hESCs, the acoustic vibration device was utilized to stimulate the cells. The sonic wave intensity was set following the manufacturer's instructions, and the treatment lasted for 30 minutes. This treatment was administered twice daily, with a 10-hour interval between consecutive passages.

Total RNA preparation and quantitative real-time PCR

The cell samples underwent total RNA extraction using the Primescript First-Strand cDNA Synthesis kit (TaKaRa). Following this, cDNAs were synthesized from the extracted total RNA using the HiScript®III for qPCR (+gDNA wiper) kit (Vazyme). Real-time PCR was conducted on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using SYBR Green reagents. The PCR cycling conditions consisted of an initial denaturation step at 95°C for 10 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Quantification data were analyzed using Applied Biosystems' Sequence Detection System (SDS) software, with GAPDH serving as the internal control (**Table 1**).

Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized using 0.1% Triton X-100 (Beyotime Biotechnology). Next,

Table 1. List of primers

Gene name	Forward	Reverse
OCT4	CTGGGGGTTCTATTTGGGAAG	GTTGCCTCTCACTCGGTTCT
NANOG	ACCTCAGCTACAAACAGGTGAAG	AGAGTAAGGCTGGGGTAGGT
SOX2	GAGAACCCCAAGATGCACAAC	TTCTTCATGAGCGTCTTGGTTTT
YAP1	GCAGGATGGTGGGACTCAAA	CTGCTCATGCTTAGTCCACTGTCT
TEAD1	AGGCAAGACGAGGACCAGAA	ACGATCTGGGCTGAGGACTA
GAPDH	CATGTTCGTCATGGGTGTGAA	GGCATGGACTGTGGTCATGAG

Table 2. List of antibodies

Antibody	Company	Catalog no.	Dilution
Rabbit polyclonal to OCT4	Abcam	ab18976	1:200
Rabbit polyclonal to NANOG	Abcam	ab80892	1:200
Rabbit polyclonal to SOX2	Abcam	ab137385	1:200
Donkey anti-mouse IgG-AF594 (H+L)	Thermo Fisher	SA5-10168	1:400

the samples were blocked with 10% normal donkey serum for 30 minutes at room temperature and then incubated with the primary antibodies overnight at 4°C (**Table 2**), followed by incubation with Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) at 37°C for 1 hour. Lastly, the cell nuclei were stained with DAPI (Solarbio) and visualized under an Echo Revolve microscope (Echo Laboratories, San Diego, California).

Colony-forming assays (CFA)

Human embryonic stem cell line H1 cells were incubated in Matrigel-plated dishes. On the first day of inoculation, Rho inhibitor Y27632 was introduced into the culture medium, and then growth was maintained in the mTeSR1 medium until the next passage. Colony formation was initiated by seeding 10000 cells in 35-mm dishes with Matrigel and maintaining growth in mTeSR1 for 5-10 days at 37°C in a 5% CO_2 humidified incubator. Crystal violet staining was performed upon the appearance of macroscopic colonies. Afterward, the colonies were fixed with 4% paraformaldehyde for 10 minutes at room temperature and then stained with Crystal Violet Staining Solution (Beyotime Biotechnology) according to the manufacturer's protocol. Colony formation was assessed using a Gel Imager System (Tanon 1600, Shanghai, China), repeated three times, and the number of macroscopic clones was counted, plotted, and statistically analyzed using GraphPad Prism 8 to determine colony-forming efficiency (CFE).

Statistical analysis

The Statistical Package for the Social Sciences version 17.0 software and GraphPad Prism 8.0 software were utilized for statistical analysis. A two-tailed Student's t-test was employed to compare the two experimental groups. The data presented in this study were derived from at least three independent experiments and expressed as mean ± SEM. $P < 0.05$ was considered statistically significant.

Result

Treatment of hESCs with acoustic vibration stimulation

To investigate the effects of acoustic vibration stimulation on hESCs, an acoustic vibration device was employed for hESC stimulation. The experimental protocol is illustrated in **Figure 1A**. Briefly, hESCs were randomly divided into two groups after passage. After 3 days of natural growth, the experimental group was subjected to vibration treatment twice daily for 30 minutes each session, whereas the control group was allowed to grow naturally. After five to six days, the cells were passaged and vibrated for three consecutive generations. As displayed in **Figure 1B**, light microscopy images of hESCs undergoing acoustic vibration showed that acoustic vibration did not induce significant changes in cell morphology, and both two groups presented compact cells and large nucleocytoplasmic ratios (**Figure 1B**).

Acoustic vibration stimulation promoted the colony formation of hESCs

To examine the effects of acoustic vibration on hESCs, colony formation experiments of hESCs were carried out in the presence or absence of acoustic vibration. Rho-associated kinase (ROCK) inhibitor Y-23632 can promote hESC survival, inhibit dissociation-induced apoptosis, and improve cloning efficiency [36]. In the present study, the dissociated cells were seeded in the absence of Y-27632. The results of colony-forming assays exposed that the number and size of colonies in the

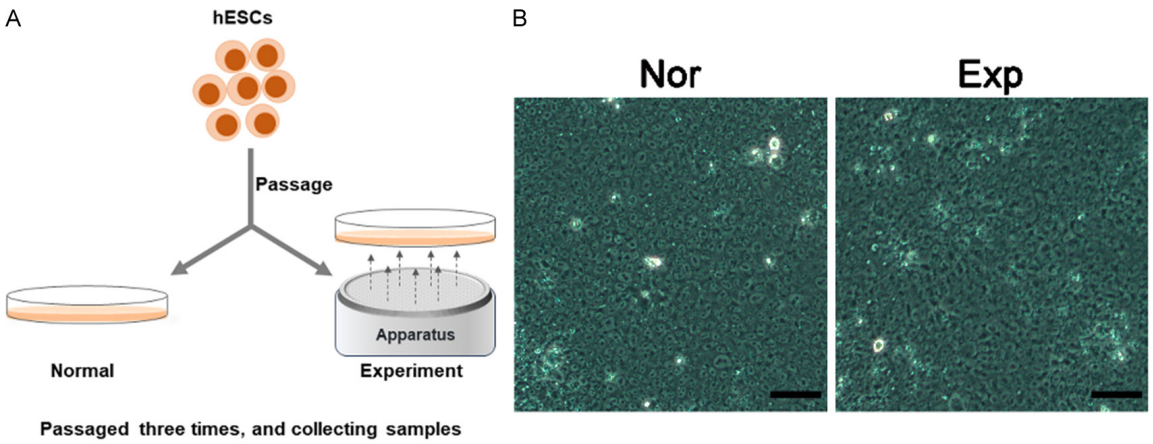

Figure 1. Schematic representation of cells treated with acoustic vibration is shown. A. Flowchart illustrating the process of sound wave vibration on human embryonic stem cells. Three days after passage, hESCs were randomly assigned to two groups: a control group and an acoustic treatment group. Three consecutive generations were treated accordingly, and samples were collected at each generation. B. Images depicting the control group and treatment group. hESCs: human embryonic stem cells, Nor: normal, Exp: experimental. The following is the same. Scale bar: 100 μm.

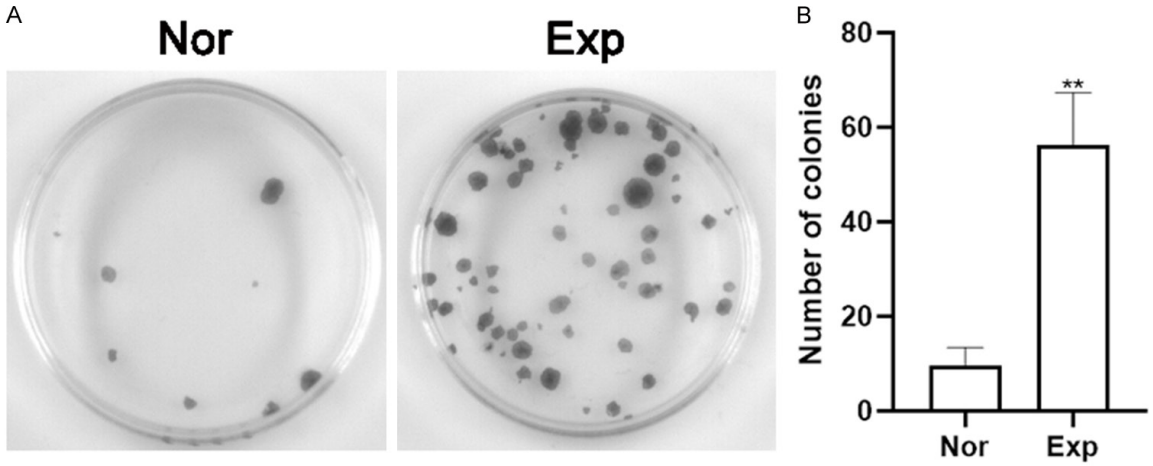

Figure 2. Acoustic vibration promoted the proliferation of hESCs. A. Colony-forming assays (CFA) of each group cells (n=3). B. Colony-forming efficiency (CFE) of normal hESCs and treaded hESCs (n=3). Nor: normal, Exp: experimental.

treated cells were significantly higher (**Figure 2A**). Moreover, statistical analysis determined that the colony formation rate of treated hESCs was significantly higher than of the control group (***P* < 0.01) (**Figure 2B**). These results collectively suggested that acoustic vibration treatment enhanced the proliferation and survival of hESCs.

Acoustic vibration stimulation enhanced the pluripotency and self-renewal abilities of hESCs

To evaluate the effects of acoustic vibration treatment on the pluripotency of hESCs, the expression of core stemness markers in hESCs with or without acoustic vibration treatment was detected. The immunostaining assay demonstrated that both two group cells exhibited the positive expressions of the markers SOX2, OCT4, and NANOG (**Figure 3A**). Likewise, PCR analysis indicated that the expression levels of stemness genes were significantly higher in the treated group compared to the control group (**Figure 3B**).

YAP and transcriptional regulators play a vital role in sustaining the pluripotent state of embryonic stem cells [37]. To investigate the involvement of the YAP pathway in the effect of acous-

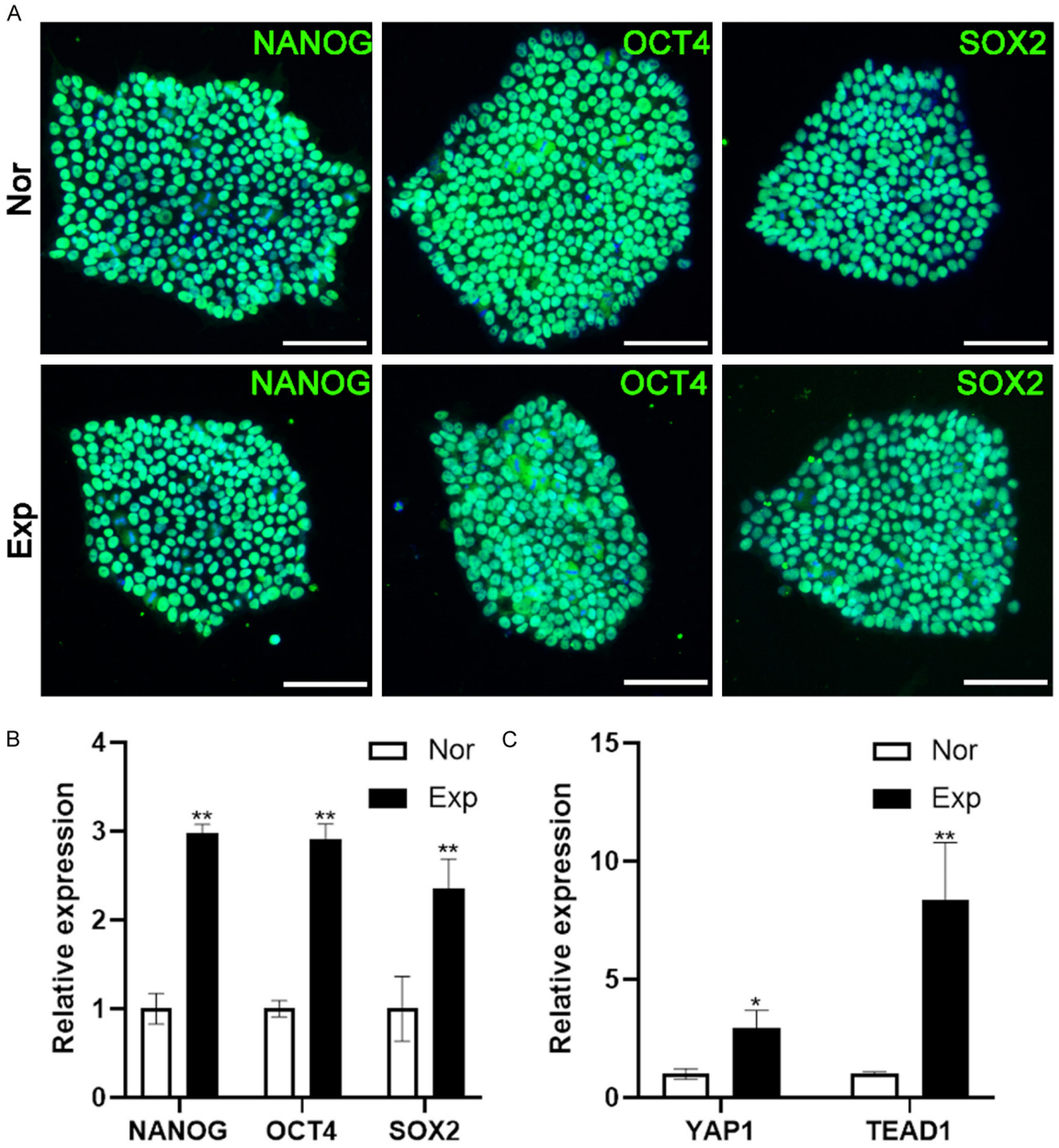

Figure 3. Acoustic vibration enhanced the stemness of hESCs and promoted the overexpression of YAP. A. Representative immunofluorescence staining of pluripotency markers NANOG, OCT4 and SOX2 (n > 3). B. Relative gene expression of NANOG, OCT4 and SOX2 (n=3). C. Relative gene expression of YAP1 and TEAD1 (n=3). Nor: normal, Exp: experimental. Scale bar: 100 μm.

tic stimulation on the pluripotency and proliferation of hESCs, the expression levels of YAP and TEAD1, key components of the YAP pathway, were detected. PCR analysis unveiled that the expression levels of YAP and TEAD1 were significantly increased in treated hESCs (**Figure 3C**), signifying that acoustic vibration stimulation promoted hESC stemness, possibly via modulating the YAP pathway.

Discussion

Stem cells hold promise for a wide range of applications, including studying early developmental events, providing potential sources for treating acquired or inherited diseases, and regenerating damaged tissues. They hold significant implications in cell therapy, regenerative medicine, disease modeling, and drug dis-

covery and screening [38]. At present, researchers are actively investigating the molecular mechanisms underlying embryonic stem cells differentiation and their response to diverse stimuli. Clinical trials are also using hESC-derived cells [1, 39-41]. Maintaining hESCs in an undifferentiated state is crucial for their widespread application [5]. While significant advances in hESC culture and derivation techniques, the absence of a universally accepted xeno-free and definitive method to maintain their undifferentiated state across multiple laboratories poses challenges [42]. In the present study, the effect of a non-invasive physical stimulation on the proliferation and pluripotency of hESCs was assessed.

Acoustic vibration exerts a gentle massaging effect on cells, thereby modulating cell function by modifying cell volume and cell membrane permeability and promoting metabolite exchange. Moreover, the application of acoustic tweezers in flow cytometry enhances cell survival and differentiation [43, 44]. This study demonstrated that acoustic vibration promotes the expression of the core stemness transcription factors NANOG, OCT4, and SOX2, thereby maintaining stem cells in a more naïve state. This finding holds significant implications for the application and investigation of hESCs. To optimize the in vitro culture system of embryonic stem cells, several strategies have been proposed, including changing the culture composition, manipulating RNA processing and reprogramming, and altering the feeding layer. Although these strategies have shown certain advantages in maintaining the proliferation and stemness of embryonic stem cells, multiple drawbacks persist. For example, adjusting the basic components of the medium, such as transcription factors, growth factors, or inhibitors, to improve the pluripotency of embryonic stem cells inhibits cell proliferation and compromise chromosome integrity, which is essential for the developmental potential of pluripotent cells [7, 45]. Deriving naive hESC by regulating RNA, including mRNA, lncRNA, etc., necessitates extensive data screening for target identification and requires appropriate control for RNA knockdown or overexpression [13, 46, 47]. Besides, uncertainties remain regarding off-target effects at the target location. Manipulating the feeding layer, such as with mitomycin C, irradiation, and ethanol treatment, may introduce residues that affect the proliferation and stemness of stem cells [48-50]. In comparison, acoustic vibration offers numerous advantages over alternative treatments, including a lower risk of infection or tissue damage, a high safety profile, cost-effectiveness, and convenience.

Of note, the maintenance of stemness function is closely intertwined with cell-cell interactions, protein factor secretion, and other cellular alterations [51]. Stem cell populations possess the unique ability to sense and modulate their responses to various external stimuli, encompassing both physical forces and chemical signals [52]. Acoustic vibration can influence both the viability and metabolic activity of cells. Additionally, variations in sound pressure can induce higher levels of cellular stress, which may impact cellular functions and overall cell health [53]. The YAP pathway serves as both a sensor and mediator of mechanical signals within the cellular microenvironment. It demonstrates outstanding sensitivity to a wide range of mechanical stimuli, facilitating the transduction of these signals into cell-specific biosignals and consequent modulation of cellular responses [54, 55]. YAP plays a critical role in maintaining the self-renewal abilities and survival of hESCs [56, 57]. Specifically, YAP overexpression promotes a primitive state in embryonic stem cells [58]. By inhibiting the expression of WNT2, YAP effectively suppresses differentiation, thereby preserving the pluripotency of hESCs [59]. On the other hand, YAP depletion results in the loss of pluripotency, contributing to cell differentiation. Our study uncovered the overexpression of both YAP and TEAD1, highlighting that our approach enhances cell pluripotency and proliferation through the YAP pathway.

Herein, a physical treatment method was developed to enhance the in vitro expansion of hESCs, offering a novel approach for experimental investigations and large-scale culture in cell factories. Nevertheless, there remain limitations, including its impact on the differentiation potential of hESCs. Furthermore, it is necessary to investigate whether similar effects of acoustic vibration stimulation can be observed in the in-vitro expansion of other pluripotent stem cells or adult stem cells.

Conclusion

An in vitro expansion method for hESCs was pioneered through the use of acoustic vibration, which can improve the pluripotency, proliferative capacity, and clone formation rate of hESCs, possibly mediated by the YAP signaling pathway. Overall, this study provides a novel and promising strategy for the expansion of hESCs in vitro and for large-scale culture in stem cell factories.

Acknowledgements

This work was supported by the funds from the National Natural Science Foundation of China (NSFC 81900834 to ZL) and the Taishan Scholar Program (tsqn202312370).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zongyi Li, Eye Institute of Shandong First Medical University, State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, No. 5 Yan'erdao Road, Qingdao 266071, Shandong, China. Tel: +86-18563971595; Fax: +86-0532-85891110; E-mail: lizongyi119@163.com

References

[1] Damdimopoulou P, Rodin S, Stenfelt S, Antonsson L, Tryggvason K and Hovatta O. Human embryonic stem cells. Best Pract Res Clin Obstet Gynaecol 2016; 31: 2-12.

[2] Dupont G, Yilmaz E, Loukas M, Macchi V, De Caro R and Tubbs RS. Human embryonic stem cells: distinct molecular personalities and applications in regenerative medicine. Clin Anat 2019; 32: 354-360.

[3] Warrier S, Taelman J, Tilleman L, Van der Jeught M, Duggal G, Lierman S, Popovic M, Van Soom A, Peelman L, Van Nieuwerburgh F, Deforce D, Chuva de Sousa Lopes SM, De Sutter P and Heindryckx B. Transcriptional landscape changes during human embryonic stem cell derivation. Mol Hum Reprod 2018; 24: 543-555.

[4] Varzideh F, Gambardella J, Kansakar U, Jankauskas SS and Santulli G. Molecular mechanisms underlying pluripotency and self-renewal of embryonic stem cells. Int J Mol Sci 2023; 24: 8386.

[5] De Los Angeles A. Frontiers of pluripotency. Methods Mol Biol 2019; 2005: 3-27.

[6] Yu L, Ji KY, Zhang J, Xu Y, Ying Y, Mai T, Xu S, Zhang QB, Yao KT and Xu Y. Core pluripotency factors promote glycolysis of human embryonic stem cells by activating GLUT1 enhancer. Protein Cell 2019; 10: 668-680.

[7] Hassani SN, Moradi S, Taleahmad S, Braun T and Baharvand H. Transition of inner cell mass to embryonic stem cells: mechanisms, facts, and hypotheses. Cell Mol Life Sci 2019; 76: 873-892.

[8] Lynch CJ, Bernad R, Calvo I and Serrano M. Manipulating the Mediator complex to induce naive pluripotency. Exp Cell Res 2020; 395: 112215.

[9] Zhao S, Xu J, Liu S, Cui K, Li Z and Liu N. Dppa3 in pluripotency maintenance of ES cells and early embryogenesis. J Cell Biochem 2019; 120: 4794-4799.

[10] Wang X, Wang X, Zhang S, Sun H, Li S, Ding H, You Y, Zhang X and Ye SD. The transcription factor TFCP2L1 induces expression of distinct target genes and promotes self-renewal of mouse and human embryonic stem cells. J Biol Chem 2019; 294: 6007-6016.

[11] Ware CB, Jonlin EC, Anderson DJ, Cavanaugh C, Hesson J, Sidhu S, Cook S, Villagomez-Olea G, Horwitz MS, Wang Y and Mathieu J. Derivation of naive human embryonic stem cells using a CHK1 inhibitor. Stem Cell Rev Rep 2023; 19: 2980-2990.

[12] Guo CJ, Ma XK, Xing YH, Zheng CC, Xu YF, Shan L, Zhang J, Wang S, Wang Y, Carmichael GG, Yang L and Chen LL. Distinct processing of lncRNAs contributes to non-conserved functions in stem cells. Cell 2020; 181: 621-636, e622.

[13] Liu R, Wubulikasimu Z, Cai R, Meng F, Cui Q, Zhou Y and Li Y. NAT10-mediated N4-acetylcytidine mRNA modification regulates self-renewal in human embryonic stem cells. Nucleic Acids Res 2023; 51: 8514-8531.

[14] Tani K, Fujiwara K and Koyama D. Adhesive cell patterning technique using ultrasound vibrations. Ultrasonics 2019; 96: 18-23.

[15] Amini A, Chien S and Bayat M. Impact of ultrasound therapy on stem cell differentiation - a systematic review. Curr Stem Cell Res Ther 2020; 15: 462-472.

[16] de Lucas B, Perez LM, Bernal A and Galvez BG. Ultrasound therapy: experiences and perspectives for regenerative medicine. Genes (Basel) 2020; 11: 1086.

[17] Lee YS, Heo H, Lee J, Moon SU, Jung WY, Park YK, Park MG, Oh SH and Kim S. An ultra-effective method of generating extramultipotent cells from human fibroblasts by ultrasound. Biomaterials 2017; 143: 65-78.

[18] Brugger MS, Baumgartner K, Mauritz SCF, Gerlach SC, Roder F, Schlosser C, Fluhrer R,

Wixforth A and Westerhausen C. Vibration enhanced cell growth induced by surface acoustic waves as in vitro wound-healing model. Proc Natl Acad Sci U S A 2020; 117: 31603-31613.
[19] Xia P, Shi Y, Wang X and Li X. Advances in the application of low-intensity pulsed ultrasound to mesenchymal stem cells. Stem Cell Res Ther 2022; 13: 214.
[20] Li X, Zhong Y, Zhou W, Song Y, Li W, Jin Q, Gao T, Zhang L and Xie M. Low-intensity pulsed ultrasound (LIPUS) enhances the anti-inflammatory effects of bone marrow mesenchymal stem cells (BMSCs)-derived extracellular vesicles. Cell Mol Biol Lett 2023; 28: 9.
[21] Xia P, Wang Q, Song J, Wang X, Wang X, Lin Q, Cheng K, Chen A and Li X. Low-intensity pulsed ultrasound enhances the efficacy of bone marrow-derived MSCs in osteoarthritis cartilage repair by regulating autophagy-mediated exosome release. Cartilage 2022; 13: 19476035221093060.
[22] Ning GZ, Song WY, Xu H, Zhu RS, Wu QL, Wu Y, Zhu SB, Li JQ, Wang M, Qu ZG and Feng SQ. Bone marrow mesenchymal stem cells stimulated with low-intensity pulsed ultrasound: better choice of transplantation treatment for spinal cord injury: treatment for SCI by LIPUS-BMSCs transplantation. CNS Neurosci Ther 2019; 25: 496-508.
[23] Xia B, Chen G, Zou Y, Yang L, Pan J and Lv Y. Low-intensity pulsed ultrasound combination with induced pluripotent stem cells-derived neural crest stem cells and growth differentiation factor 5 promotes sciatic nerve regeneration and functional recovery. J Tissue Eng Regen Med 2019; 13: 625-636.
[24] Ye K, Li Z, Yin Y, Zhou J, Li D, Gan Y, Peng D, Xiao M, Zhao L, Dai Y and Tang Y. LIPUS-SCs-Exo promotes peripheral nerve regeneration in cavernous nerve crush injury-induced ED rats via PI3K/Akt/FoxO signaling pathway. CNS Neurosci Ther 2023; 29: 3239-3258.
[25] Wang W, Li Z, Yan Y, Wu S, Yao X, Gao C, Liu L and Yu Y. LIPUS-induced neurogenesis: a potential therapeutic strategy for cognitive dysfunction in traumatic brain injury. Exp Neurol 2024; 371: 114588.
[26] Shimokawa H, Shindo T, Ishiki A, Tomita N, Ichijyo S, Watanabe T, Nakata T, Eguchi K, Kikuchi Y, Shiroto T, Takahashi J, Yasuda S and Arai H. A pilot study of whole-brain low-intensity pulsed ultrasound therapy for early stage of Alzheimer's disease (LIPUS-AD): a randomized, double-blind, placebo-controlled trial. Tohoku J Exp Med 2022; 258: 167-175.
[27] Jiang D, Liu J, Pan Y, Zhuang L and Wang P. Surface acoustic wave (SAW) techniques in tissue engineering. Cell Tissue Res 2021; 386: 215-226.
[28] Guo F, Li P, French JB, Mao Z, Zhao H, Li S, Nama N, Fick JR, Benkovic SJ and Huang TJ. Controlling cell-cell interactions using surface acoustic waves. Proc Natl Acad Sci U S A 2015; 112: 43-48.
[29] Shi J, Ahmed D, Mao X, Lin SC, Lawit A and Huang TJ. Acoustic tweezers: patterning cells and microparticles using standing surface acoustic waves (SSAW). Lab Chip 2009; 9: 2890-2895.
[30] Kang JH, Miettinen TP, Chen L, Olcum S, Katsikis G, Doyle PS and Manalis SR. Noninvasive monitoring of single-cell mechanics by acoustic scattering. Nat Methods 2019; 16: 263-269.
[31] Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N and Piccolo S. Role of YAP/TAZ in mechanotransduction. Nature 2011; 474: 179-183.
[32] Wang H, Zhang W, Cai Y, Guo Q, Pan L, Chu G, Chen J, Yuan Z and Li B. Moderate mechanical stimulation antagonizes inflammation of annulus fibrosus cells through YAP-mediated suppression of NF-kappaB signaling. J Orthop Res 2023; 41: 2667-2684.
[33] Heng BC, Zhang X, Aubel D, Bai Y, Li X, Wei Y, Fussenegger M and Deng X. An overview of signaling pathways regulating YAP/TAZ activity. Cell Mol Life Sci 2021; 78: 497-512.
[34] Kim YJ, Go YH, Jeong HC, Kwon EJ, Kim SM, Cheong HS, Kim W, Shin HD, Lee H and Cha HJ. TPX2 prompts mitotic survival via the induction of BCL2L1 through YAP1 protein stabilization in human embryonic stem cells. Exp Mol Med 2023; 55: 32-42.
[35] Ohgushi M, Minaguchi M and Sasai Y. Rho-signaling-directed YAP/TAZ activity underlies the long-term survival and expansion of human embryonic stem cells. Cell Stem Cell 2015; 17: 448-461.
[36] Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K and Sasai Y. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 2007; 25: 681-686.
[37] Di Benedetto G, Parisi S, Russo T and Passaro F. YAP and TAZ mediators at the crossroad between metabolic and cellular reprogramming. Metabolites 2021; 11: 154.
[38] Orozco-Fuentes S, Neganova I, Wadkin LE, Baggaley AW, Barrio RA, Lako M, Shukurov A and Parker NG. Quantification of the morphological characteristics of hESC colonies. Sci Rep 2019; 9: 17569.
[39] Filipovic R, Kumar SS, Bahr BA and Loturco J. Slice culture method for studying migration of neuronal progenitor cells derived from human

embryonic stem cells (hESC). Curr Protoc Stem Cell Biol 2014; 29: 1H.7.1-14.
[40] de Klerk E and Hebrok M. Stem cell-based clinical trials for diabetes mellitus. Front Endocrinol (Lausanne) 2021; 12: 631463.
[41] Yamasaki S, Tu HY, Matsuyama T, Horiuchi M, Hashiguchi T, Sho J, Kuwahara A, Kishino A, Kimura T, Takahashi M and Mandai M. A genetic modification that reduces ON-bipolar cells in hESC-derived retinas enhances functional integration after transplantation. iScience 2022; 25: 103657.
[42] Hewitt ZA, Amps KJ and Moore HD. Derivation of GMP raw materials for use in regenerative medicine: hESC-based therapies, progress toward clinical application. Clin Pharmacol Ther 2007; 82: 448-452.
[43] Chen D, Sun Y, Deng CX and Fu J. Improving survival of disassociated human embryonic stem cells by mechanical stimulation using acoustic tweezing cytometry. Biophys J 2015; 108: 1315-1317.
[44] Topal T, Hong X, Xue X, Fan Z, Kanetkar N, Nguyen JT, Fu J, Deng CX and Krebsbach PH. Acoustic tweezing cytometry induces rapid initiation of human embryonic stem cell differentiation. Sci Rep 2018; 8: 12977.
[45] Theunissen TW, Powell BE, Wang H, Mitalipova M, Faddah DA, Reddy J, Fan ZP, Maetzel D, Ganz K, Shi L, Lungjangwa T, Imsoonthornruksa S, Stelzer Y, Rangarajan S, D'Alessio A, Zhang J, Gao Q, Dawlaty MM, Young RA, Gray NS and Jaenisch R. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell 2014; 15: 471-487.
[46] Li D, Kishta MS and Wang J. Regulation of pluripotency and reprogramming by RNA binding proteins. Curr Top Dev Biol 2020; 138: 113-138.
[47] Wang Y, Guo B, Xiao Z, Lin H, Zhang X, Song Y, Li Y, Gao X, Yu J, Shao Z, Li X, Luo Y and Li S. Long noncoding RNA CCDC144NL-AS1 knockdown induces naive-like state conversion of human pluripotent stem cells. Stem Cell Res Ther 2019; 10: 220.
[48] Huang B, Ning S, Zhuang L, Jiang C, Cui Y, Fan G, Qin L and Liu J. Ethanol inactivated mouse embryonic fibroblasts maintain the self-renew and proliferation of human embryonic stem cells. PLoS One 2015; 10: e0130332.
[49] Wang L, Zhang R, Ma R, Jia G, Jian S, Zeng X, Xiong Z, Li B, Li C, Lv Z and Bai X. Establishment of a feeder and serum-free culture system for human embryonic stem cells. Zygote 2020; 28: 175-182.
[50] Zhao Z, Ma Y, Chen Z, Liu Q, Li Q, Kong D, Yuan K, Hu L, Wang T, Chen X, Peng Y, Jiang W, Yu Y and Liu X. Effects of feeder cells on dopaminergic differentiation of human embryonic stem cells. Front Cell Neurosci 2016; 10: 291.
[51] Rao S and Orkin SH. Unraveling the transcriptional network controlling ES cell pluripotency. Genome Biol 2006; 7: 230.
[52] Chen X, Li J, Huang Y, Liu P and Fan Y. Insoluble microenvironment facilitating the generation and maintenance of pluripotency. Tissue Eng Part B Rev 2018; 24: 267-278.
[53] Levario-Diaz V, Bhaskar P, Carmen Galan M and Barnes AC. Effect of acoustic standing waves on cellular viability and metabolic activity. Sci Rep 2020; 10: 8493.
[54] Mosaddad SA, Salari Y, Amookhteh S, Soufdoost RS, Seifalian A, Bonakdar S, Safaeinejad F, Moghaddam MM and Tebyanian H. Response to mechanical cues by interplay of YAP/TAZ transcription factors and key mechanical checkpoints of the cell: a comprehensive review. Cell Physiol Biochem 2021; 55: 33-60.
[55] Li Y, Wang J and Zhong W. Regulation and mechanism of YAP/TAZ in the mechanical microenvironment of stem cells (Review). Mol Med Rep 2021; 24: 506.
[56] Choe MS, Bae CM, Kim SJ, Oh ST, Kown YJ, Choi WY, Han HJ, Baek KM, Chang W, Kim JS, Lim KS, Yun SP and Lee MY. Human embryonic stem cell-specific role of YAP in maintenance of self-renewal and survival. Cell Mol Life Sci 2022; 79: 544.
[57] Shi J, Farzaneh M and Khoshnam SE. Yes-associated protein and PDZ binding motif: a critical signaling pathway in the control of human pluripotent stem cells self-renewal and differentiation. Cell Reprogram 2020; 22: 55-61.
[58] Qin H, Hejna M, Liu Y, Percharde M, Wossidlo M, Blouin L, Durruthy-Durruthy J, Wong P, Qi Z, Yu J, Qi LS, Sebastiano V, Song JS and Ramalho-Santos M. YAP induces human naive pluripotency. Cell Rep 2016; 14: 2301-2312.
[59] Estaras C, Hsu HT, Huang L and Jones KA. YAP repression of the *WNT3* gene controls hESC differentiation along the cardiac mesoderm lineage. Genes Dev 2017; 31: 2250-2263.