Ultrasonic stimulation in a 3D HA hydrogel accelerates cellular reprogramming for iPS generation

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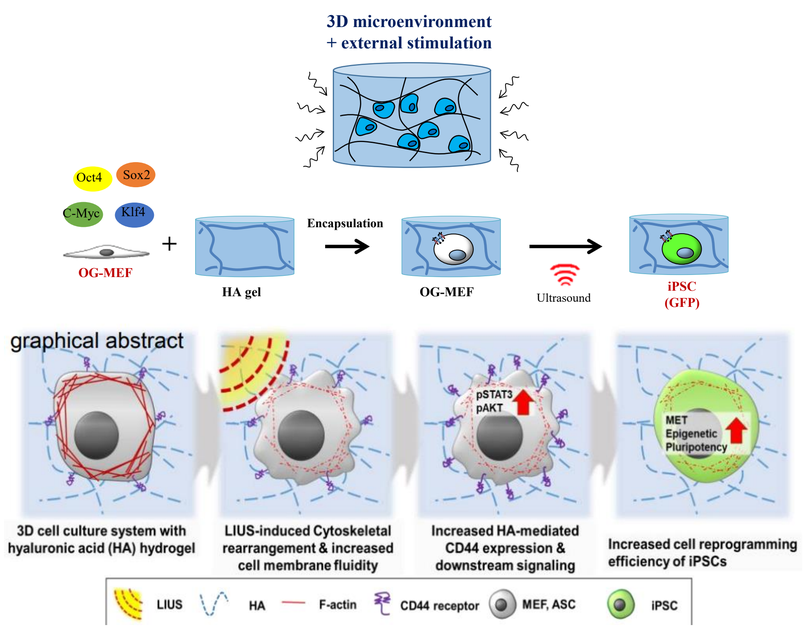
ABSTRACT

Induced pluripotent stem cells (iPS) could be used as a powerful source of tissue regeneration based on their pluripotency. Traditional methods for generating iPS have critical problems with low efficiency and slow speed. In our previous study, we developed a three-dimensional (3D) microenvironment system using hyaluronic acid (HA) hydrogel to improve the efficiency of iPS generation. In this study, we investigated whether the external stimulation of low-intensity ultrasound stimulation (LIUS) enhances the efficiency of iPS generation in HA hydrogel. As a result, we found that there is no cytotoxicity of LIUS within 20 min at the intensity of 300 mW/cm^2. In addition, the time-dependent manner of the LIUS treatment increased the reprogramming efficiency, and it was confirmed by the intensity of OCT4-GFP fluorescence, colony formation, and expression of pluripotency markers at the protein and gene levels. High expression of CD44 is related to increasing the reprogramming efficiency by activating the signal cascade. We found that LIUS increased CD44 expression by degrading cytoskeletal structures and increasing the fluidity of cell membranes. Consequently, LIUS increased the epigenetic modification markers such as Acetyl H3, H3K4me2, and H3K4me3. Based on these results, we suggested that new 3D external stimulation platform for advanced iPS generation.

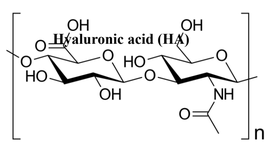
INTRODUCTION

Graphical Abstract



- The biophysical factor, **low-intensity ultrasound (LIUS),** integrated with **three dimensional hyaluronic acid hydrogel** promotes **cellular reprogramming** into **induced pluripotent stem cells.**
- LIUS initially modulates cellular changes in **cytoskeletal rearrangement** and **membrane fluidity**, and further **increases cell-ECM interaction** to increase expression of **CD44** and its **downstream signals.**

3D HA-LIUS system

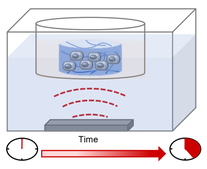


Why HA hydrogel?

Hyaluronic acid (HA), an immuno-neutral polysaccharide present in the human body. A previous study reported enhanced reprogramming efficiency of iPSCs under a HA based 3D hydrogel niche with controlled stiffness, initially upregulating the HA mediated CD44 expression. In the present study, we apply the different duration of LIUS during cellular reprogramming of iPSCs within a HA based 3D hydrogel, and the reprogramming efficiency of the iPSCs is assessed.

Why ultrasound?

Ultrasound, a non-invasive acoustic wave force, is another biophysical factor capable of applying mechanical stress in a non-contact way. Low-intensity ultrasound (LIUS) has lower intensity than conventional ultrasound energy (less than 3 w/cm^2). We expect that LIUS will affect the cytoskeleton of cells, which will result in upregulated CD44 expression.



Low-intensity ultrasound stimulation (LIUS)
Frequency: 40 kHz
Intensity: 300 mW/cm^2
Stimulate every other day

RESULTS

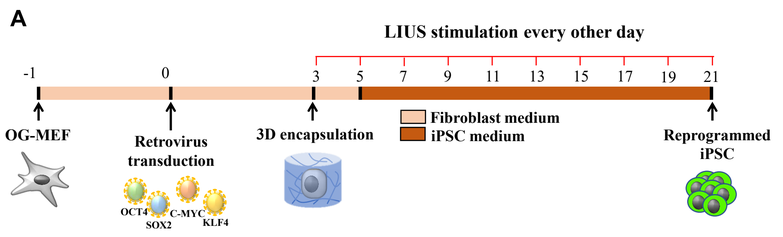
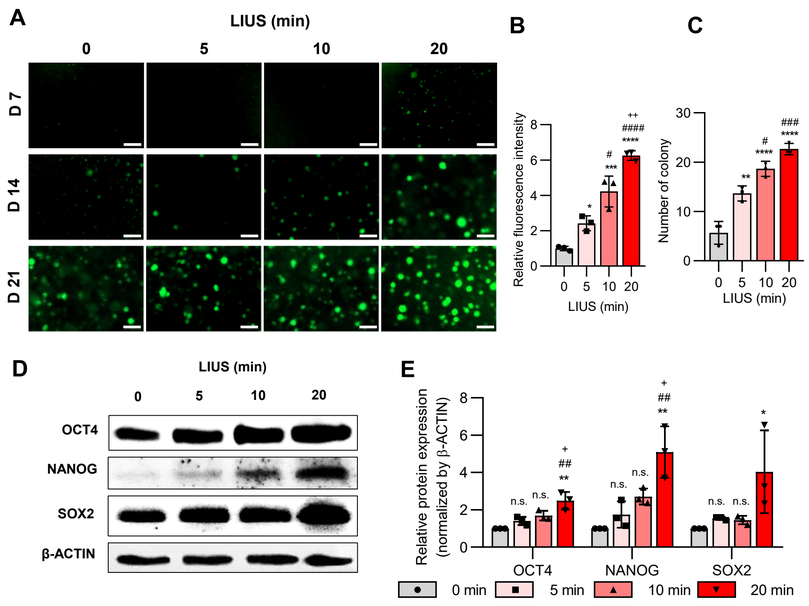


Figure 1. Schematic representation of the reprogramming of OCT4-GFP (OG)-MEFs into iPSCs under LIUS stimulation.



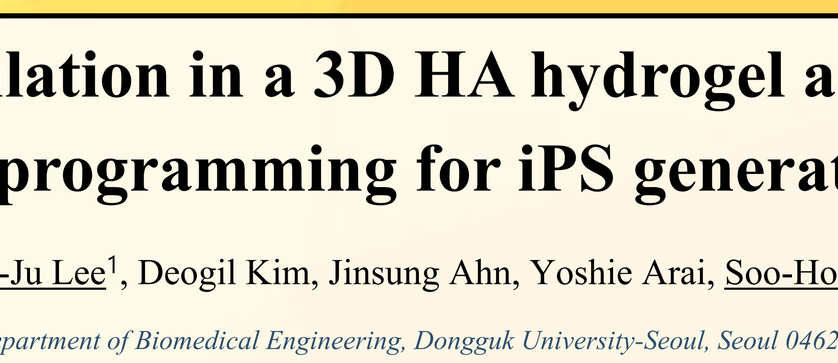


Figure 2. LIUS enhances reprogramming efficiency in 3D hydrogel. (A) OCT4 expression of iPSCs derived from OG-MEFs under various LIUS times in MAHA hydrogel. Scale bars: 300 μm. Quantitative analysis of (B) OCT4-GFP intensity and (C) a number of iPSC colonies derived from OG-MEFs within MAHA hydrogel with various LIUS times. (D) western blot analysis (OCT4, NANOG, and SOX2). (E) Quantitative analysis of immunoblotted protein band intensity by NIH ImageJ software. Immunofluorescence images of (F) NANOG and (G) SOX2 under LIUS stimulated OG-MEFs after the reprogramming process. Scale bars: 300 μm. Analysis of pluripotent markers by (H) qRT-PCR (*Pou5f1, Nanog,* and *Sox2*)

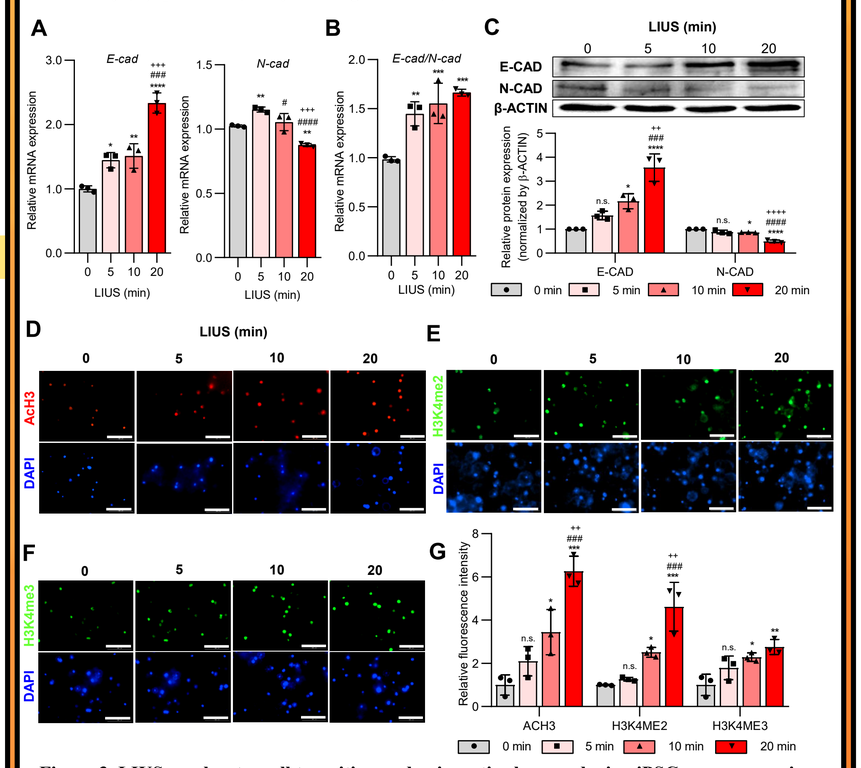


Figure 3. LIUS accelerates cell transition and epigenetic changes during iPSC reprogramming in 3D hydrogel. (A) qPCR analysis of MET markers, E-cad and N-cad, and (B) ratio of Cdh1/Cdh2 expression and (C) western blot analysis of the MET markers (E-cadherin and N-cadherin) of OG-MEFs under LIUS (D1). Immunofluorescence staining of (D) Acetyl H3 (ACH3), (E) dimethyl H3K4 (H3K4ME2), and (F) trimethyl H3K4 (H3K4ME3) of OG-MEFs stimulated with LIUS (D1; scale bar, 100 μm). (G) Quantification of fluorescence intensity in each immunofluorescence stained cell sample. All data are expressed as the mean ± s.d. The statistical significance was determined with one-way ANOVA followed by Tukey's multiple comparison post test.

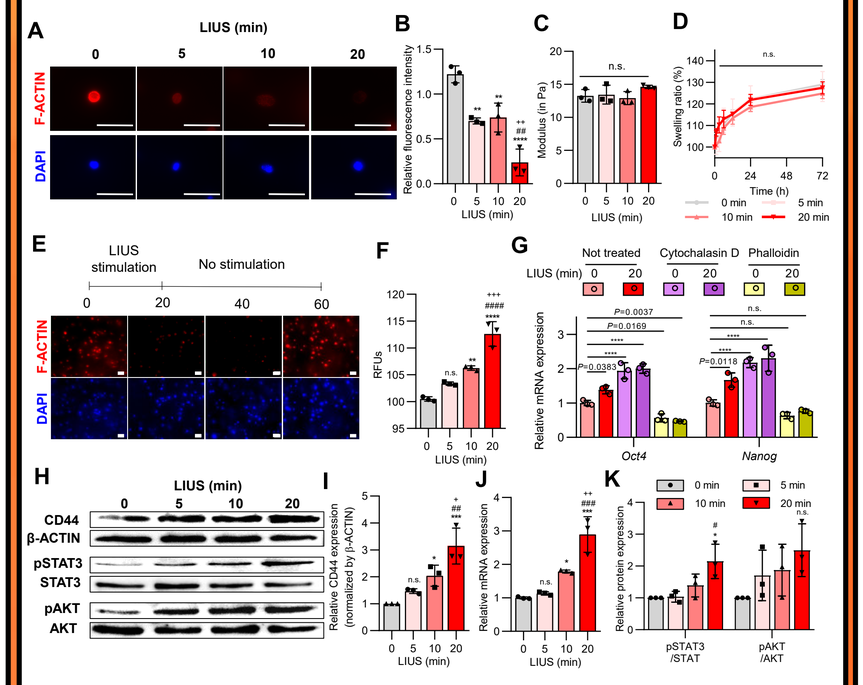


Figure 4. LIUS modulates cytoskeletal rearrangement and CD44 interaction. a, Immunofluorescence staining of F-actin after LIUS stimulation (scale bar, 50 μm) and **b,** its graphically quantified intensity. Mechanical properties of hydrogel by LIUS application were analyzed by **c,** shear modulus and **d,** swelling ratio. **e,** Time-dependent expressions of F-actin from LIUS were analyzed by the fluorescence intensities of F-actin (scale bar, 50 μm). **f,** Cell membrane fluidity of LIUS was measured by the relative fluorescence intensity of excimer to monomer ratio (n=3). **i,** Expressions of CD44 under LIUS were analyzed by qPCR. **j,** Protein expression of CD44 and signaling molecules were analyzed with western blotting. Relative protein expressions of **k,** CD44 and **l,** signaling molecules, STAT3 and AKT, were measured.

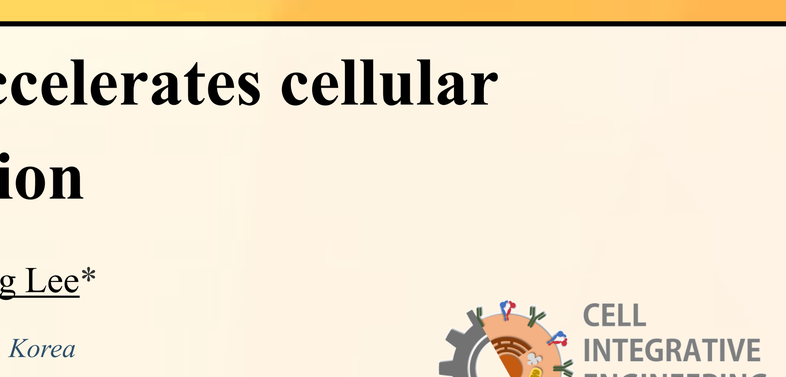


Figure 5. The reprogramming efficiency of LIUS system under various hydrogels. The reprogramming efficiency of LIUS system under different hydrogels were analyzed by immunofluorescence expression of pluripotent markers, (A) OCT4-GFP, (B), NANOG, and (C), SOX2 (scale bar, 300).). (D) Quantification of fluorescence intensity in each immunofluorescence stained cell sample. All data are expressed as the mean ± s.d. The statistical significance was determined with two-sided t-tests for comparisons between two experimental groups. n.s., not significant, ****P <0.0001

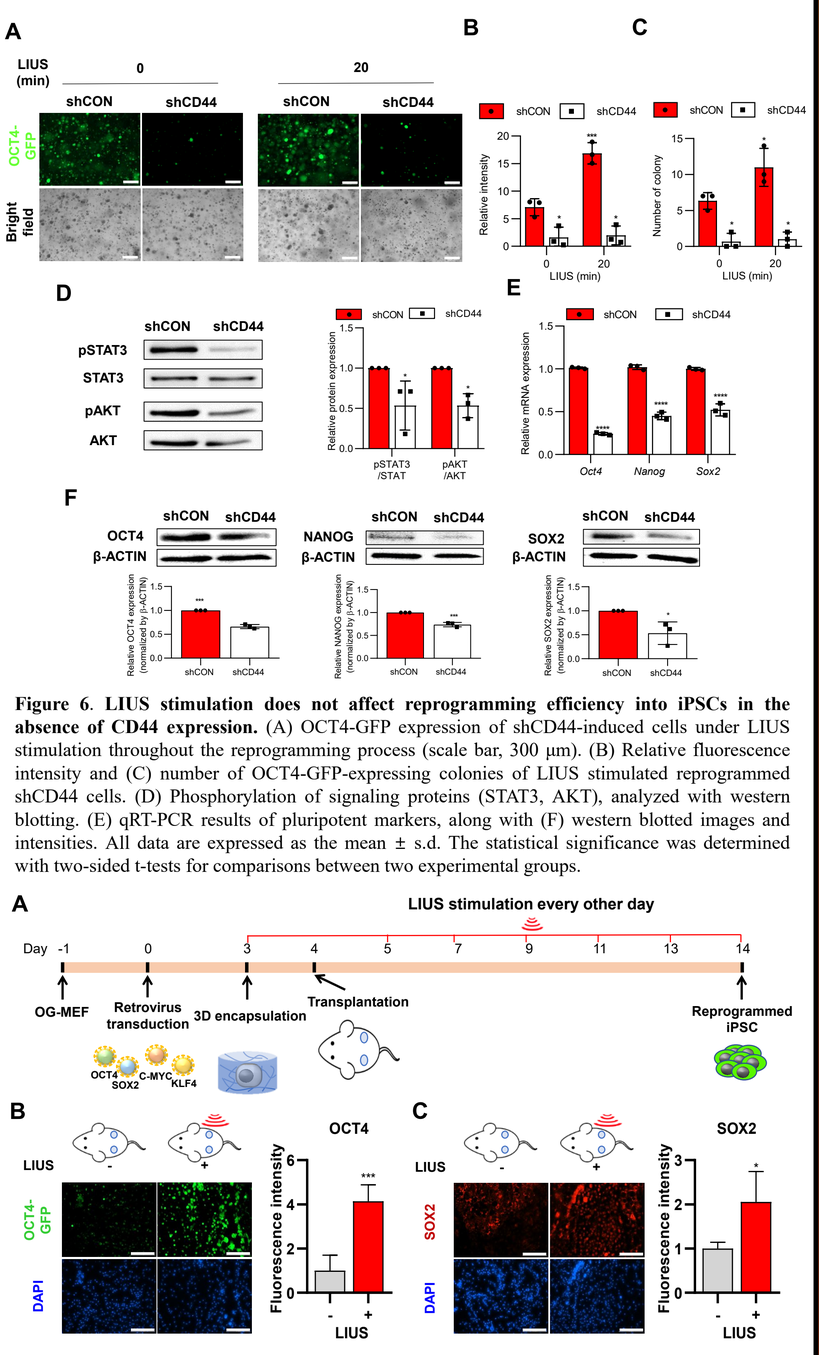


Figure 6. LIUS stimulation does not affect reprogramming efficiency into iPSCs in the absence of CD44 expression. (A) OCT4-GFP expression of shCD44-induced cells under LIUS stimulation throughout the reprogramming process (scale bar, 300 μm). (B) Relative fluorescence intensity and (C) number of OCT4-GFP-expressing colonies of LIUS stimulated reprogrammed shCD44 cells. (D) Phosphorylation of signaling proteins (STAT3, AKT), analyzed with western blotting. (E) qRT-PCR results of pluripotent markers, along with (F) western blotted images and intensities. All data are expressed as the mean ± s.d. The statistical significance was determined with two-sided t-tests for comparisons between two experimental groups.

Figure 7. 3D HA-LIUS system also enhances reprogramming efficiency in mouses in vivo. (A) Sc hematic representation of the reprogramming of OG-MEFs into iPSCs under ultrasound stimulation *in vivo*. (B) OCT4-GFP expression of iPSCs derived from OG-MEFs under LIUS stimulation in HA hydr ogel and its fluorescence intensity. (C) Immunofluorescence staining of SOX2 and its fluorescence int ensity. Scale bars: 100 μm.

CONCLUSIONS

We demonstrated a novel reprogramming strategy involving non-contact ultrasonic stimulation in a 3D hydrogel system to improve the efficiency of reprogramming. Incorporating LIUS into an established 3D hydrogel system improves reprogramming efficiency because more ultrasound stimulation leads to higher cellular reprogramming of iPSCs. LIUS degrades the cytoskeletal structure and increases the fluidity and mobility of cell membranes. These changes increase the expression of CD44, epigenetics, and pluripotency during the reprogramming process. The figure above shows the overall research scheme. In conclusion, our study provides an entirely new kind of 3D external stimulation platform for advanced iPS generation. Thus, the strategy presented here could be beneficial for a variety of biological and biomedical applications.

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