



Piezoelectric effect stimulates the rearrangement of chondrogenic cells and alters ciliary orientation via atypical PKC ζ

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ABSTRACT

Therapeutic ultrasound was administered to patients suffering from bone fracture with FDA approval. Bone and cartilage are piezoelectric materials. To investigate the effects of piezoelectricity on the cells of chondrogenic lineage, we applied ultrasound stimulation on an AT-cut quartz coverslip to generate electric field fluctuations. The bone-marrow-derived mesenchymal stem cells (BMMSC) and primary chondrocytes were cultured on either glass or quartz coverslips for ultrasound stimulation. The cells were immunofluorescent-labeled for the assessment of cell arrangement and ciliary orientation. Ultrasound and piezoelectricity both stimulate cell migration and disrupt ciliary orientation induced by directional migration. In particular, piezoelectric effects on cell rearrangement can be abolished by the inhibitor specifically targeting atypical Protein kinase C zeta (PKC ζ). Our findings shed light on the possibility of cellular modulation by using piezoelectric manipulation.

1. Introduction

Therapeutic ultrasound has been applied in bone healing and implicated in temporomandibular joint or even articular cartilage [1]. Ultrasound treatments in combination with regenerative stem cell therapy or ultrasound-enhanced *in vitro* preparations of stem cells show great potentials [2,3]. Collagen fibrils comprise many types of proteins which are arranged into a meshwork with regional parallel-aligned patterns [4–6]. Aligned collagen fibrils are piezoelectric materials [7] due to intrinsic shear mode piezoelectricity [8,9]. Chondrogenic lineages and the derivatives of chondrogenic differentiation are mostly embedded in a collagen-enriched matrix. Thus, ultrasound stimulation of cartilage or bones *in vivo* can naturally induce piezoelectric effects in addition to ultrasound stimulation. Piezoelectric effects in bone fracture healing have been reviewed [10]. Danti et al. (2013) [11] found that boron nitride nanotube ingestion by human osteoblast culture, when stimulated by low frequency (40 kHz) ultrasound, can help promote osteogenesis in terms of various osteogenic markers and mineral deposition. Genchi et al. (2018) [12] successfully stimulated SaOS-2

osteoblast-like cells cultured on poly (vinylidenedifluoride-trifluoroethylene) (P(VDF-TrFE)) and P(VDF-TrFE)/boron nitride nanotubes films with 1W/cm² ultrasound to induce voltages of 20–60 mV for the purpose of osteoblast differentiation. The molecular mechanisms that are involved in piezoelectric-induced osteogenesis are not completely clear, but we know that the electric stimulation on MC3T3-E1 osteoblasts induces calcium signaling that leads to the activation of cytoskeletal calmodulin [13].

In addition to osteoblasts, mesenchymal stem cells and chondrocytes play major roles before or during the endochondral stage of fracture healing. To study the difference of ultrasound and piezoelectrical stimulation on bone-marrow-derived mesenchymal stem cells (BMMSC), we have developed a device [14] in which the cells attached on either a glass or a quartz coverslip can be stimulated by either ultrasound or piezoelectrical stimulation. We found that the piezoelectric stimulation promotes the aggregation and chondrogenic differentiation of BMMSC although both ultrasound and piezoelectrical stimulations can up-regulate the SOX9 protein level [15].

Since the piezoelectric stimulation induced an aggregation of cells

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that most likely involves the migration and reorientation of the cells, we studied the literature of cartilage development to understand the current views on the orientation and arrangement of chondrocytes. The mutation or deletion of genes involving the ciliogenesis of chondrogenic lineage impacted the development of articular cartilages in mice [16, 17]. Chondrocytes proliferate in an orientation-specific manner and arrange themselves into columns in the growth plate [18,19]. The disruption of cilia alters the columnar arrangements of chondrocytes presumably by affecting the actin cytoskeleton, focal adhesion complexes, and extracellular matrix deposition [16,17]. Comparing the three-dimensional orientation of the primary cilia in the horse articular cartilage, the primary cilia orientation is more restricted in the mechanical load-bearing regions than the more randomly-oriented non-load-bearing regions [20]. In fact, primary cilia incidence, length, and orientation can be altered by mechanical stimuli such as low-intensity ultrasound of 5 MHz and pressure amplitude of 14–60 kPa [21].

In this study, we set out to investigate whether piezoelectric stimulation can influence cell migration and cell orientation from the perspective of primary cilia position relative to the nuclear position or relative to the direction of cell migration. We found that piezoelectric stimulation accelerates the cell migration of BMMSC significantly more than ultrasound stimulation. The clustering of BMMSC induced by piezoelectrical fields involves a polarization of primary cilia positions relative to the cell axis. This behavior can also be observed in primary chondrocytes isolated and cultured from the articular cartilages of neonatal mice. PKC ζ is known to interact with intraflagellar transport protein 20 (IFT20) that regulates cell polarity and affects the cell arrangement of differentiating osteoblasts [22]. Inhibiting PKC ζ can abolish the cell reorganization effect of piezoelectric fields. We believe these findings confirm the effects of certain aspects of ultrasound on bone fracture healing, in which the bone is piezoelectric material and the different stages of fracture healing involve cell migration and chondrogenesis.

2. Materials and methods

2.1. Stimulating chamber and method

In the experiment, a laboratory-developed ultrasonic stimulation chamber (LIC) was used as a device for stimulating cells [15]. Chondrocytes were seeded on glass slides or quartz slides, to prepare for ultrasound and piezoelectric stimulations respectively. The quartz/glass coverslip was sandwiched between the top cover and base plate to facilitate ultrasound propagation. <https://www.dropbox.com/s/972kvyneavbo2y/BBreports-revision.mov?dl=0>.

According to the piezoelectric characteristics of quartz slides, the small deformation of the quartz slides produced during ultrasonic stimulation causes the quartz to generate piezoelectric environment. The parameter of the stimulation is 12V (voltage), continuous wave, and the I_{SATA} measured is about 1.87–14.31 mW/cm².

2.2. Human Mesenchymal stem cell culture

Human Mesenchymal stem cells (hMSC, PT-2501, Lonza, Basel, Schweiz) was amplified in hMSC growing medium (MSCGM, PT-3238, Lonza, Basel, Schweiz). The cells designated for experimental procedures were seeded on glass slides to about 80%–90% confluency before wound healing assay.

2.3. Chondrocyte culture

The articular cartilages were isolated from the articular cartilages dissected from the postnatal day 5–7 ICR mice purchased from National Taiwan University School of Medicine, Taipei, Taiwan. Cartilages were immersed in F12K (30–2004, ATCC, Manassas, Virginia, USA) medium containing Collagenase Type II (17101-015, Gibco, Waltham, USA) and

incubated in a 37 °C tissue culture incubator (LEEC Culture Safe Touch 190 CO₂, Nottingham, UK). After 24 h, the cells were strained through a 40 μ m cell strainer (431750, Corning, Corning, New York, USA). After centrifugation, the cells were resuspended and seeded on glass and quartz slides (0.17 mm in thickness) that had been sterilized and plasma-treated. Cell cultures were maintained in Stem cell growth medium SF1 (ITRI, Hsinchu, Taiwan) for the experiments designated for Western or immunofluorescent analysis.

2.4. BMMSC wound healing assay

Confluent human BMMSC on glass slide was scratched using a 200 μ l tip. Images of the wound were recorded before stimulation. Subsequently, the glass slide was placed on a half-glass-half-quartz chamber to be stimulated for 5 min. The stimulated cells were incubated in a 37 °C incubator for 16 h before imaging for the quantification of wound healing under bright fields and fixation for immunofluorescence.

2.5. Stimulation and PKC ζ inhibitor treatment procedures

Four glass slides and two quartz slides were prepared for each set of experiments. 30,000 chondrocytes were seeded on each slide. Cells were cultured for 24 h before stimulations. Among the six groups, two glass slides and one quartz slide were used in the non-inhibitor treated groups. A glass or a quartz coverslip containing the cells was set up for LIC stimulation for 5 min, which was under ultrasound or piezoelectric stimulation respectively. The remaining unmedicated group of glass cell slides was designated the control group. The cells with inhibition were treated with 5 μ M pseudosubstrate peptide of PKC ζ (ZIP, ab120993, Abcam, Cambridge, UK) 40 min before the stimulation. After stimulations, media with inhibitor was rinsed off with sterile Phosphate buffered saline (PBS) with 1% Penicillin-Streptomycin (15140122, Gibco, Waltham, USA) three times, each time for 5 min. Culture medium SF1 was added subsequently for culture maintenance. The six slides were then put back into the incubator for 24 h, and the stimulation with or without inhibitor was performed until the day of confluency for immunofluorescence or Western analysis.

2.6. Immunofluorescence

When the cultures were confluent, cells were rinsed with PBS. Fixation was performed using 4% paraformaldehyde (PFA, 158127, Sigma, ST Louis, Missouri, USA) in 4 °C refrigerator for 30 min. PBS wash for three times of 5 min each was followed. Permeabilization of cells was achieved using PBS+0.4% Triton X-100 solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Subsequently, cells were blocked with PBS buffer containing 1% FBS (A4766801, Gibco, Waltham, USA) for an hour before Antibody incubation. Primary antibodies alpha Tubulin (acetyl K40) antibody (EPR16772, Abcam, Cambridge, UK) and ARL13B antibody (ab136648, Abcam, Cambridge, UK) were diluted in blocking buffer (PBS, 0.4%Triton, 1%FBS) and added to the fixed cells in 4 °C overnight. The primary antibodies were washed off the cells using PBS three times before adding the secondary antibodies, such as Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113, Abcam, Cambridge, UK), and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) (ab150080, Abcam, Cambridge, UK) or in some cases phalloidin (Phalloidin-iFluor 647 Reagent ab176759, Abcam, Cambridge, UK). Secondary antibodies were allowed 60 min at room temperature to bind to the primary antibodies. Subsequently, the non-binding secondary antibodies were washed off using PBS. Mounting Medium with DAPI (ab104139, Abcam, Cambridge, UK) was applied at the end for fluorescent microscopy. Images were analysed and quantified with the application of ImageJ.

3. Results

3.1. Quantitative analysis of piezoelectric stimulation with numerical simulation

To better understand how cells cultured on the quartz coverslip were influenced by piezoelectric stimulation, we built a 3D model of our setup (Fig. 1A) to compute the electric field distribution on the coverslip during ultrasound excitation. The AT-cut quartz coverslip is a thickness shear mode resonator [23] similar to a collagen membrane and can produce a localized electric field gradient vertical to the coverslip when stimulated by ultrasound. We first analyse the shear stress produced by ultrasound and estimate the associated electric field (Fig. 1B) based on piezoelectricity in quartz. With this overall distribution, we can obtain the electric field fluctuation across the distance (Fig. 1C) by cutting a cross section along the coverslip. This allows us to calculate the electric field gradient experienced by the cells during the stimulation. For example, a 100 μm wide cell would be exposed to an electric field gradient equivalent to 1 mV in membrane potential difference across the cell body (Fig. 1D). Since the electric field is mediated by ultrasound excitation, the electric field gradient synchronizes its fluctuation with ultrasound, moving from positive gradient to negative gradient in one resonance cycle (1 μs in this case, see Fig. 1D).

3.2. BMMSC cell re-arrangement induced by piezoelectric stimulation

We previously discovered that piezoelectric stimulation of BMMSC on AT-cut quartz by ultrasound facilitates the chondrogenesis detected by Alcian blue staining [15]. We also observed the aggregation of BMMSC induced by piezoelectric stimulation that was not happening in the cells stimulated by only ultrasound under the chondrogenic culture condition (Fig. 2A–F). The immunofluorescent staining of primary cilia revealed by Arl13b and K40-acetylated tubulin showed no significant difference in the controls compared to either ultrasound or piezoelectric stimulation groups in terms of primary cilia incidence and ciliary size (Fig. 2G–H), while there was a significant difference of the cilia formed in the cells cultured in stem cell media compared to those in chondrogenic media (Fig. 2G–H). The immunofluorescence also allowed us to quantify cell rearrangement and ciliary position (Fig. 2I). We found that the cell axis and the relative ciliary position were not changed in the ultrasound stimulation compared to the controls, whereas it was significantly rearranged on quartz after 3–4 days of consecutive piezoelectric stimulations (Fig. 2J). We compared the alignment between the cilia and the cell orientation in a R^2 analysis (Fig. 2K). It is clear that the differentiation media imposed a strong effect on cell polarity compared to the growth media. Interestingly, piezoelectric stimulation (either in growth $R^2 = 0.52$ or differentiation $R^2 = 0.63$ media) produced a similar R^2 value with cells in differentiation media ($R^2 = 0.42$) while all other groups produced a negligible value ($R^2 < 0.12$). This suggests that piezoelectric stimulation has a similar effect on cell polarity as the differentiation media.

3.3. Ultrasound and piezoelectric-stimulated ciliary polarity changes in wound-healing-directed migration

The aggregation of BMMSC induced by piezoelectric stimulation involves both cell migration and ciliary orientation. To tease apart the effects of ultrasound and piezoelectric stimulation on cell migration and ciliary orientation, we further performed a scratched wound assay to test whether the cell migration and ciliary orientation can be decoupled. The scratched wounds were partially healed at the time point of 16 h after a single ultrasound or piezoelectric stimulation (Fig. 3A–H). When comparing piezoelectric-stimulated cells to the non-stimulated control, there was a significant increase ($p = 0.0005$) of wound healing (Fig. 3I). However, there was no significant difference ($p = 0.147$) comparing the ultrasound and piezoelectric stimulation (Fig. 3I), indicating that the

increased cell migration is caused by ultrasound effect rather than by piezoelectric stimulation. The ultrasound stimulation alone could not cause a change in cell or ciliary polarity during the normal expansion or culture (Fig. 2B and E). However, ultrasound caused a significant disruption of ciliary polarity during the wound healing of the scratched BMMSC ($p = 0.038$) (Fig. 3J). Paradoxically, the disruption of ciliary polarity accelerates cell migration (Fig. 3I–J). Moreover, this data suggests that wound-induced ciliary reorientation can be overridden by both ultrasound and piezoelectric stimulations without compromising the degree of cell migration.

3.4. Piezoelectric stimulation but not ultrasound induces the cell rearrangement of chondrocytes, and inhibition of PKC ζ disrupts the rearrangement

Chondrocytes are the major component in the soft callus, or the endochondral stage of bone fracture healing. Thus, we also tested whether chondrocytes are regulated by piezoelectric or ultrasound in terms of cell arrangement and ciliary polarity. To test whether cell rearrangement induced by piezoelectric stimulation is mediated through the activity of PKC ζ , we performed the experiment using primary chondrocytes and treated the cells with 5 μM PKC ζ inhibitor (ZIP) 40 min prior to ultrasound or piezoelectric stimulations. The results showed that the piezoelectric-induced cellular rearrangement can be disrupted by the inhibition of PKC ζ activity (Fig. 4A–G). Statistical 2-way ANOVA revealed that the effect of ultrasound stimulation on cell rearrangement was not significant ($p = 0.65$) while piezoelectric stimulation effect was significant ($p < 0.0001$), and there was a significant interaction of ZIP inhibitory effect with piezoelectric effect ($p = 0.0047$) compared to the control group while the ZIP effect alone was not significant ($p = 0.28$). In addition, comparing the piezoelectric effect to ultrasound stimulation in 2-way ANOVA also yielded significant difference ($p = 0.0007$). In this case, the interaction of piezoelectric and ZIP inhibitory effect was also significant ($p = 0.029$) although the ZIP effect alone was not ($p = 0.094$). The changes in cell size and cell number were consistently observed upon piezoelectric stimulation in addition to cell rearrangement (Fig. 4H–I). This data suggests that PKC ζ inhibition only abolished the piezoelectric stimulation of cellular rearrangement but not the piezoelectric stimulation of cell size decrease and cell number increase.

4. Discussion

Therapeutic benefits by ultrasound on articular cartilage repair and regeneration have been studied in various animal models [24,25]. Ultrasound-induced chondrogenic progenitor cell migration has been characterized in both monolayer culture and fibrin hydrogel-embedded cartilage defects [26,27]. In these studies, the Integrin-mediated focal adhesion complex respond to ultrasound thus is assumed to be the mechanosensitive signaling mediating the cell migration effects. Our lab has also previously characterized the ultrasound-stimulated incidence and size of focal adhesion complex revealed by phosphorylated p130CAS signals in epithelial cells [28]. Mechanosensitive p130CAS can be phosphorylated by Src [29], and Src activation on one hand is correlated to a higher level of chondrogenic transcription factor SOX9 [30]. On the other hand, Src co-immunoprecipitated with PKC ζ in angiotensin II elicited extracellular signal regulated kinases [31].

Since bone and cartilage are collagen-enriched tissues, thus piezoelectric materials by nature [32], ultrasound treatment not only generates mechanical force but also electric fields due to piezoelectric effect, as we discovered in this study, contributing to the modulation of the cells involved. When we observed the re-arrangement of cells and re-orientation of primary cilia relative to nuclear position (Fig. 2), it occurred to us that PKC ζ is likely involved. In fact, it has been long established that PKC ζ is mediating Integrin signals to re-position the microtubule organizing center (MTOC) and microtubule cytoskeleton for directed cell protrusion in the wound healing process [33].

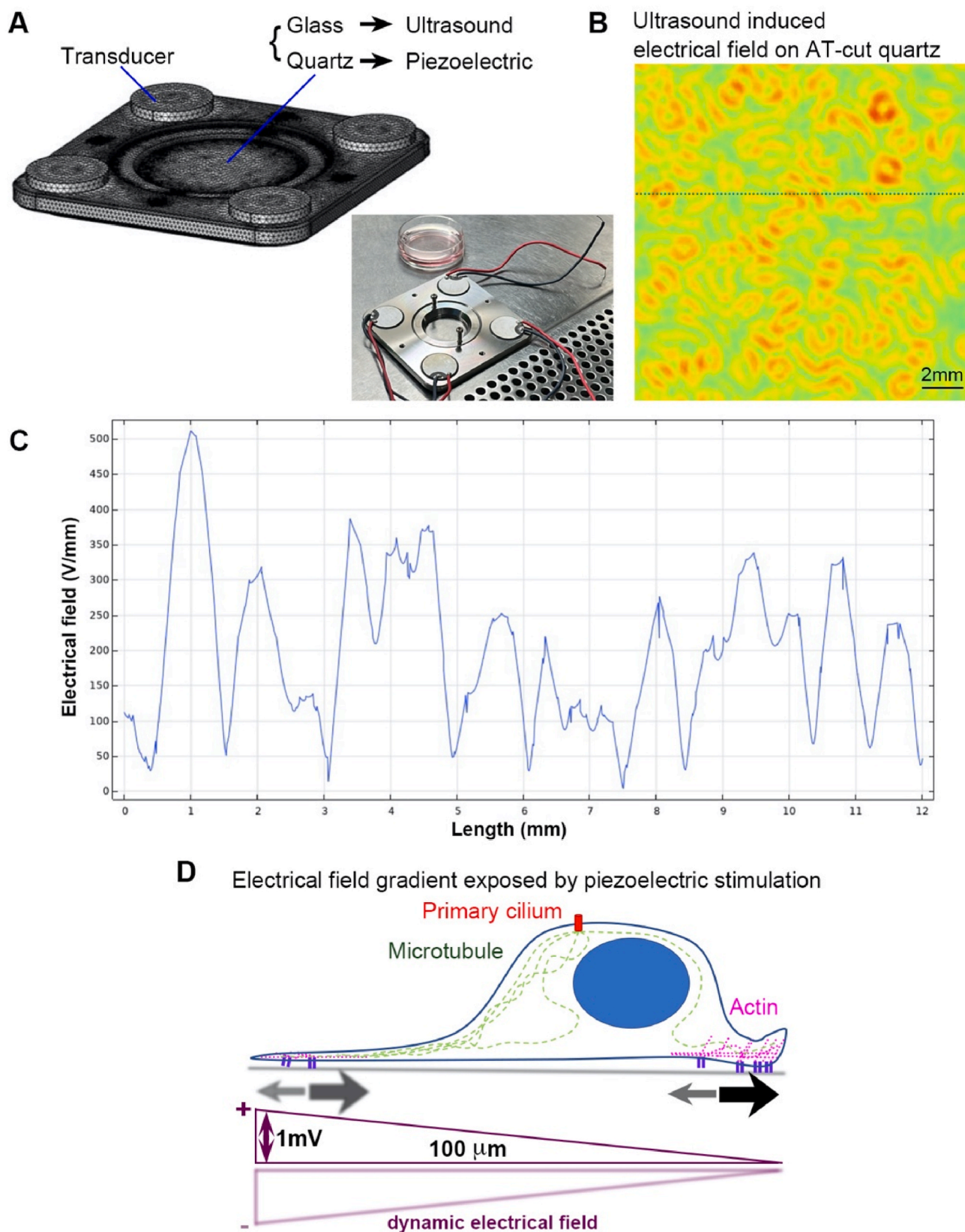


Fig. 1. Experimental device, method, and estimated magnitude of electrical field for piezoelectric stimulation. (A) Mesh diagram generated for a 3D model of our experimental setup. (B) Electric field distribution on the coverslip. (C) Electric field across a cross section of the quartz coverslip. The results derived from this graph indicated that the electrical field measurement on the cover slips was 1mV/100 µm. (D) Schematic diagram illustrating the electrical field gradient experienced by a cell during piezoelectric stimulation. The dynamic electrical field promotes cell migration and rearranges cell polarity.

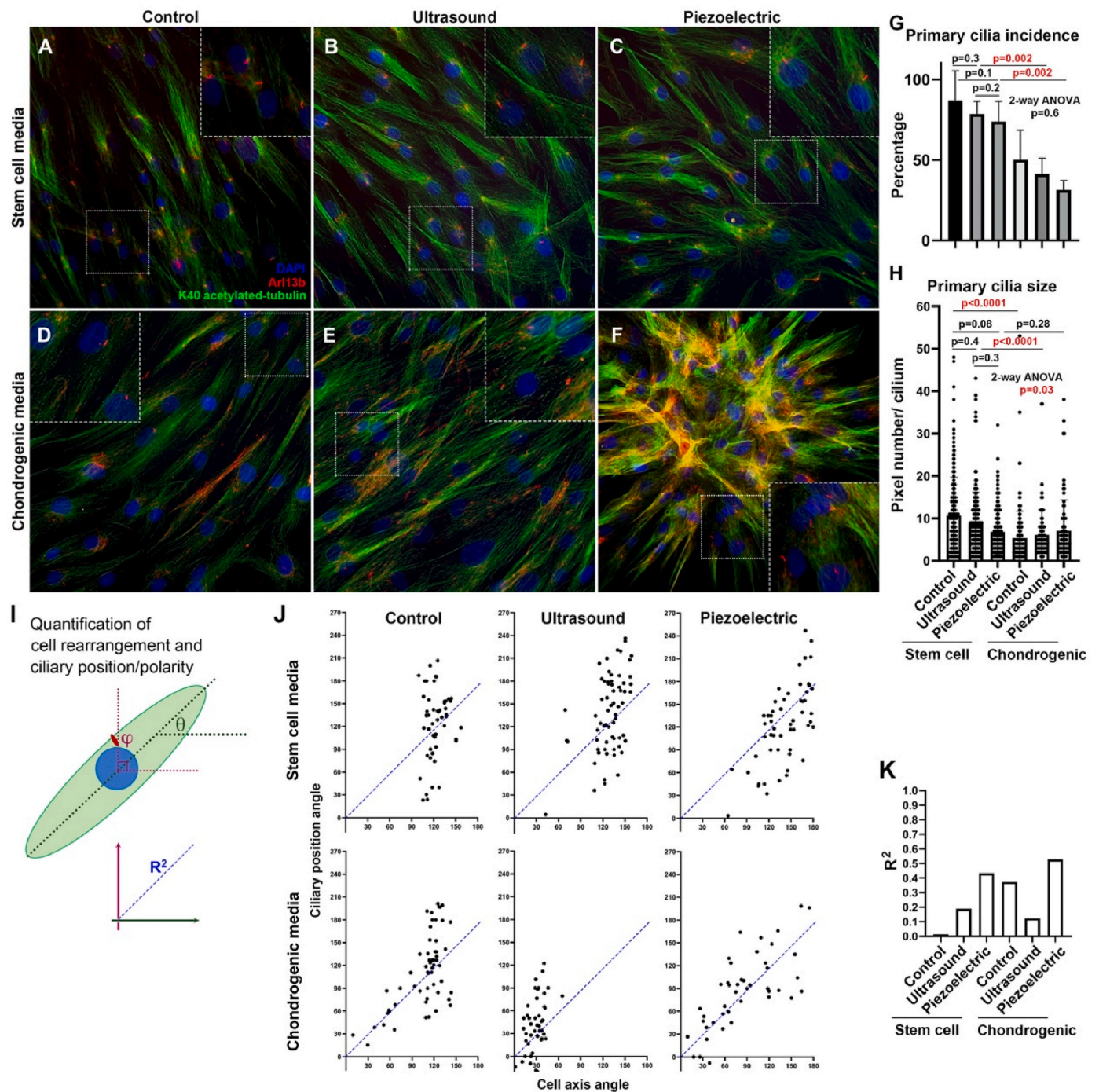


Fig. 2. Piezoelectric stimulation induces cellular rearrangement of BMMSC. Micrograph of BMMSC in stem cell media under control condition (A), ultrasound stimulation (B), and piezoelectric stimulation (C). Chondrogenic media cultured BMMSC under control (D), ultrasound stimulation (E), and piezoelectric stimulation (F). (G) Quantification of primary cilia incidence. (H) Quantification of primary cilia size. (I) Schematic diagram illustrating the analysis of cilia/cell alignment. (J) Quantification of cilia/cell orientation alignment. (K) R² analysis with respect to perfect alignment. These results indicated that the incidence and size of cilia are not significantly altered upon piezoelectric stimulations whereas ciliary position and cell polarity are affected in a statistically significant manner. On the contrary, ultrasound stimulation does not cause such cellular rearrangement.

Ultrasound and piezoelectric stimulation always accelerated the wound healing in scratch assay compared to the controls (Fig. 3). We were surprised that the wound healing rate seemed uncorrelated to the MTOC as indicated by the cilia staining revealed by Arl13b (Fig. 3). The general belief in the biology community is that free space in a wound healing assay induces cell polarity and this in turn guides cell migration. Our results agree with this principle. Intriguingly, we found that the free-space-induced frontal positioning of MTOC was not observed in

ultrasound and piezoelectric stimulations. However, the stimulations promoted a faster directional migration (Fig. 3). The results go against the conventional wisdom on MTOC polarity driven migration. Both ultrasound and piezoelectric stimulation have a stronger effect stimulating MTOC polarity than the free space and the migration speed is not necessarily controlled by free-space-induced polarity.

The fact that piezoelectric stimulation can induce ciliary polarity and cause cellular rearrangement of the primary chondrocytes and BMMSC

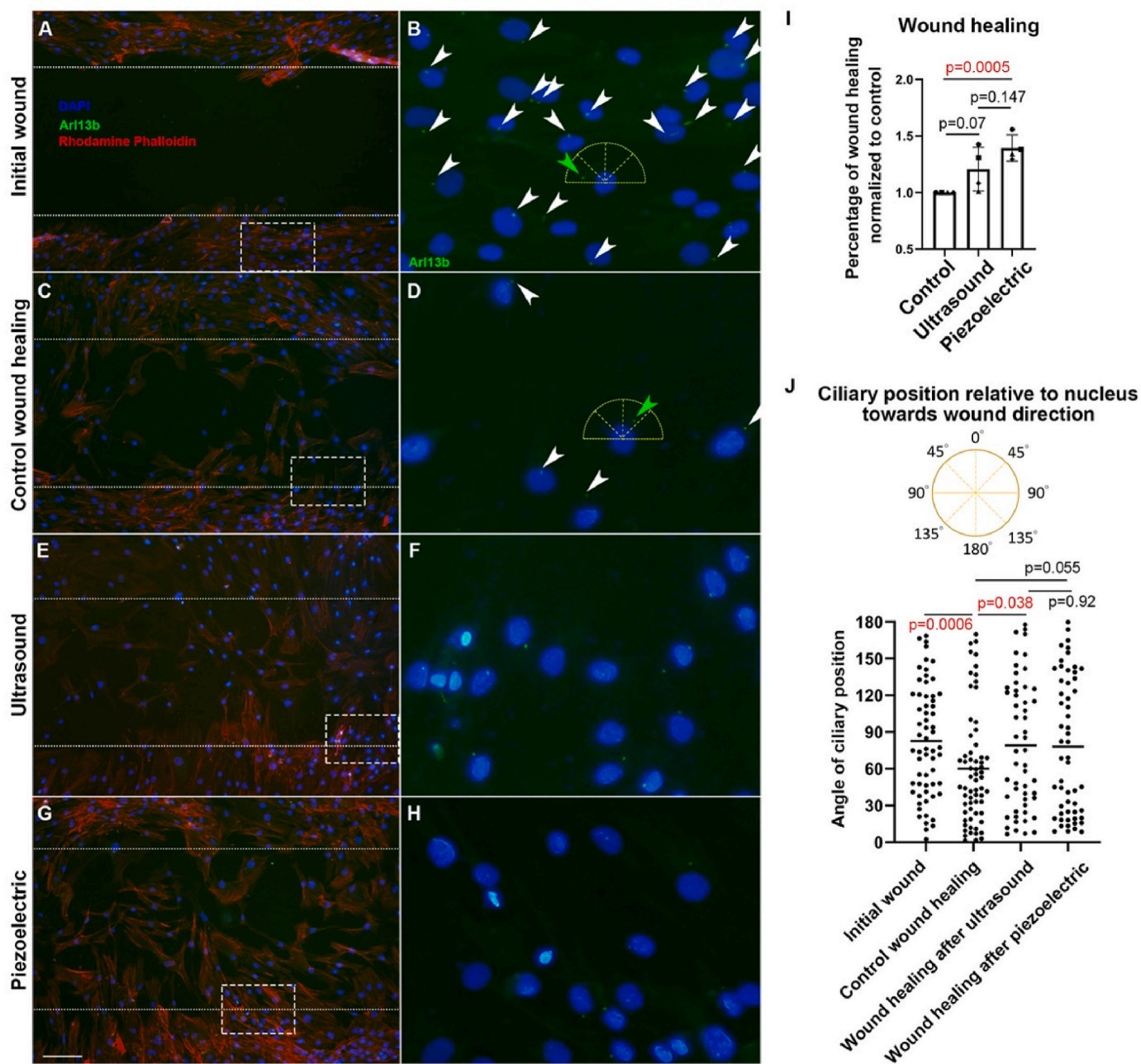


Fig. 3. Disruption of wound-space-induced ciliary re-positioning by ultrasound and piezoelectric stimulations. Micrographs of BMMSC in a wound healing assay initially in control (A, B) and 16 h later in control (C, D), ultrasound (E, F), and piezoelectric stimulation (G, H). (I) Comparison of the wound healing percentage normalized to control. (J) Ciliary position relative to nucleus with respect to the wound direction in different experimental groups. The results in A, B, C, D indicate that ciliary position is induced to direct cell migration towards the cell-free area. The results in E, F, G, H indicate that both ultrasound and piezoelectric override this ciliary orientation towards the cell-free area. Counterintuitively, the randomized ciliary position accelerates the migration.

deserves further elaboration. Linking these observations with the fact that the primary cilia orientation is more regulated in the mechanical load-bearing regions than the more randomly-oriented non-load-bearing regions [20] points to a possible role of piezoelectricity in bone physiology. As polarity is relevant to cell distribution regulation, collagen organization and distribution might play a role in guiding cell distribution in bones, especially in the developmental stage.

PKC ζ gene deletion in mice seems not to cause any obvious bone and cartilage phenotypes. Nevertheless, PKC ζ is expressed in primary chondrocytes cultures of chicks [34,35]. However, the phosphorylation of PKC ζ in certain circumstances may indicate an osteoarthritic inflammation signaling of interleukin-1 and tumor necrosis factor that

can cause a catabolic destruction of cartilage [36]. On the other hand, cell migration from the niche of stem cells to the fracture site is important for bone fracture healing, and PKC ζ has been reported to play a role in interleukin-8-induced cell migration in human BMMSC [37]. Known for its role in cell polarity regulation, PKC ζ can be recruited into primary cilia via IFT20 for the osteoblast and osteocyte cell arrangement in developing long bones, and the disruption of cell arrangement in bone may compromise its mechanical loading properties [22]. Intriguingly, PKC ζ also plays a role in fluid shear stress activated insulin growth factor-1 receptor signaling [38]. We discover that PKC ζ can also affect the piezoelectric-stimulated cell rearrangement in BMMSC and chondrocytes, suggesting multiple roles of PKC ζ in regulating chondrogenic

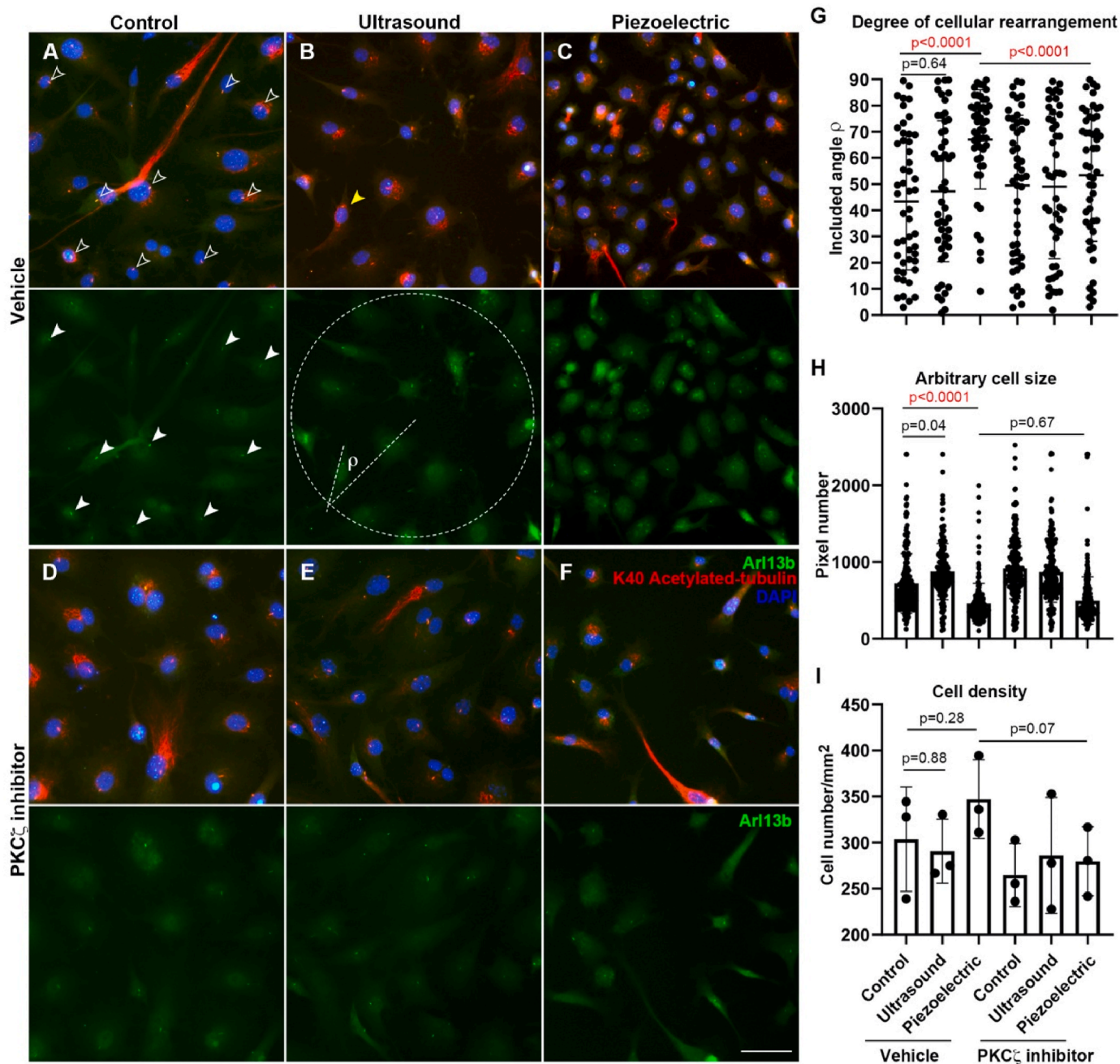


Fig. 4. Cellular rearrangement of primary chondrocytes can be abolished by the inhibition of PKC ζ . Micrographs of chondrocytes in regular control (A), ultrasound (B) and piezoelectric stimulation (C), and with ZIP, PKC ζ inhibitor among control (D), ultrasound (E), and piezoelectric stimulation (F). (G) Quantification of cellular orientation with respect to the radial direction of a defined circle separating the cells that are grouped into clusters, and arbitrary circle in the case of control and ultrasound. (H) Quantification of cell sizes. (I) Quantification of cell densities. The piezoelectric induced cellular rearrangement effect is also observed in chondrocytes. This rearrangement is abolished if pseudosubstrate of PKC ζ is added into the media during the piezoelectric stimulation.

or osteogenic lineages.

Recently, Özlem Şen et al. (2022) [39] has published that piezoelectric nanoparticles can be used as a material to hinder tumor-induced angiogenesis in combination with ultrasound stimulation. This occurred at first glance to contradict our findings. However, it is due to a completely different purpose and strategy that piezoelectric stimulation can hinder angiogenic behavior. In their study, Nutlin-3a-loaded PVDF-TrFE piezoelectric nanoparticles were surface-functionalized with a peptide, which mimics a fragment of apolipoprotein E. These nanoparticles were internalized by the cells. After a couple of days, cells were subjected to ultrasound stimulation with the dosage of 1 MHz frequency and 1 W/cm² intensity. Piezoelectric stimulation was thus

generated intracellularly. In our experiment, piezoelectric stimulations were applied from extracellular environment with ultrasound I_{SATA} measured of about 1.87–14.31 mW/cm². The difference of experimental outcomes could be due to a completely different purpose and strategy. In addition to the effects on the cells derived from the skeletal system, piezoelectric stimulation can also promote the neurite extensions of SH-SY5Y neuroblastoma cells, PC12 pheochromocytoma cells, and rat spinal cord neurons [40–42]. Note that the molecular machineries of cell migration front are in a lot of cases shared by the explorative growth cones of neurites.

The ultrasound and piezoelectric stimulations that lead to cell migration may involve signaling of mechanosensitive ion channels in

addition to focal adhesion signaling [43]. The ultrasound effects on different tissues, such as bone packed with densely-aligned type I collagen and other soft tissues such as brain will involve different intensities of piezoelectricity. This will impact the design of ultrasound parameters in different purposes, such as the stem cell preparations for regenerative medicine or the direct therapeutic ultrasound in pain management or transcranial stimulations.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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