Induction of hair growth in hair follicle cells and organ cultures upon treatment with 30 kHz frequency inaudible sound via cell proliferation and antiapoptotic effects

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Abstract. Androgenic alopecia is a hair loss disease mediated by dihydrotestosterone (DHT) and is currently treated using minoxidil, finasteride, or low-level laser therapy. However, these treatments have side-effects, indicating the need for an alternative treatment. In the present study, it was demonstrated that inaudible sound at 30 kHz significantly induced proliferative and anti-apoptotic effects in human dermal papilla cells (hDPCs) and outer root sheath keratinocytes. Cell viability assay, ELISA, reverse transcription quantitative PCR and TUNEL assays were performed to evaluate the effect of inaudible sound. Inaudible sound was also demonstrated to significantly inhibit the hair loss signals induced by DHT treatment in hDPCs. Furthermore, inaudible sound significantly induced hair follicle (HF) elongation and hair matrix keratinocyte proliferation in human HF organ culture. Overall, the results suggested that inaudible sound may be effective in treating hair loss and could be used to develop a new hair loss treatment approach.

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Introduction

Androgenic alopecia, or male pattern hair loss, is a hair loss disease mediated by dihydrotestosterone (DHT), an active type of testosterone, which induces hair follicle (HF) shrinkage and converts terminal hair into vellus hair (1). Without treatment, patients experience gradual hair loss (2). Male-type alopecia is the most common cause of hair loss and its incidence increases with age. In the proportion of the population older than 80 years, ~73% of men and ~57% of women suffer from male-type alopecia and ~58% of men over the age of 50 years suffer from male-type alopecia (3-5). Male pattern hair loss can cause negative psychological effects, including obsessive self-doubt, aging-related anxiety and lethargy, in both men and women. Furthermore, these effects are more pronounced in women (6-8).

At present, minoxidil (MNX) and finasteride are the only Food and Drug Administration (FDA)-approved drugs for hair loss treatment that exhibit significant efficacy. However, MNX has been reported to cause pruritus and contact dermatitis (9) and finasteride has been reported to cause sexual dysfunction in a small number of patients (10). Patients are also reluctant to use finasteride because of concerns regarding systemic side effects, such as the headache, dizziness, skin rash and sexual dysfunction, from oral administration (10-13). Alternatively, low level laser therapy (LLLT) is an FDA-approved hair loss treatment with significant hair loss inhibitory efficacy (14). However, previous studies have described the limitations of this treatment, including a small number of responsive patients (15,16), short experimental periods (17) and lack of global phototrichograms (16). LLLT therefore has a lower success rate compared with orally administered hair loss drugs. Consequently, researchers are focusing on finding therapeutics that are more effective and have fewer side effects.

Ultrasound is used in various ways in both the medical and industrial fields. In medicine, ultrasounds are used as an *in vivo* contrast diagnosis technology as well as for increasing drug delivery efficiency (sonophoresis), fracture treatment

and neoplasm treatment (18-22). In general, when ultrasounds are applied to the human body, a medium such as a gel is used to increase their transmission efficiency. However, Oohashi et al (23), demonstrated that an inaudible frequency just above the audible sound frequency, which transmits through air to subjects, may alter the brain waves and hormonal levels of subjects (23-26). Moreover, these effects occur not via the ears, but only when inaudible sound is transmitted to the skin. Furthermore, a study by Denda and Nakatani (27), indicated that wound recovery was accelerated in mice when inaudible sound directly reached the wound area. These data suggest that inaudible sound can affect the skin surface and subsequently the whole body.

Based on these observations, we hypothesized that inaudible sound could also affect the scalp. In the present study, the effect of inaudible sound on human dermal papilla cells (hDPCs) was explored and whether inaudible sound could inhibit hair loss signals derived from DHT treatment in hDPCs was investigated. The efficacy of inaudible sound in treating the human scalp was assessed by observing the changes in hair growth rate in HF organ cultures treated with inaudible sound.

Materials and methods

Inaudible sound. Electrical sine waves were generated using a function generator and amplifier set (custom made; EM-Tech Co., Ltd.; Fig. 1A). Output power and frequency were 24 Vrms and 30 kHz, respectively. Inaudible sound was produced using the Piezo speaker 300ST18M (Pro-Wave Electronics Corporation). The sound pressure level produced was 144 dB at 1 cm distance. Cells were exposed to inaudible sound at 37˚C in a humidified incubator with 95% O_2 and 5% CO_2 for 4 h in serum free low glucose DMEM supplemented with L-glutamine and sodium pyruvate media (cat. no. SH30021.01; HyClone). Cells were irradiated with inaudible sound from above the media.

Reagents. MNX and DMSO were purchased from Sigma-Aldrich (Merck KGaA). The human vascular endothelial growth factor (VEGF), Dickkopf-1 (DKK-1) and TGF-β1 levels were evaluated using Human VEGF Quantikine ELISA Kit (cat. no. DVE00), Human DKK-1 Quantikine ELISA kit (DKK100B) and Human TGF-β1 Quantikine ELISA Kit (cat. no. DB100B), purchased from R&D Systems, Inc.

Isolation and culture of hDPCs and human outer root sheath (ORS) keratinocytes. During the hair transplant procedure, performed between October 2020 and May 2021 at Dankook University Hospital (Cheonan, South Korea), hair biopsy specimens were obtained from the non-balding occipital scalp region of three male patients (aged 32, 34 and 37) with androgenic alopecia during a hair transplant procedure. Patients had received 1 mg/day oral finasteride treatment for at least a year before the procedure. The Medical Ethical Committee of Dankook Medical College (Cheonan, South Korea) approved all the described protocols and informed written consent was obtained from all patients (approval no. DKUH 2013-08-012-001). HFs were isolated and cultured as described previously, with minor modifications to the procedure (28-30). Briefly, a subcutaneous fat of scalp skin including lower hair

follicles was dissected from the epidermis and dermis. The follicles were then separated using a binocular microscope with forceps and maintained at 6 follicle units/well in 24-well plates, and grown in Williams E medium (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified incubator with 5% CO_2. Cultured hDPCs and ORS keratinocytes of, no later than passage 3, were used for the subsequent experiments and were maintained at 37˚C in a humidified incubator with 5% CO_2. hDPCs were grown in low glucose DMEM supplemented with L-glutamine and sodium pyruvate, which was supplemented with 10% FBS (Gibco; thermo Fisher Scientific, Inc.). ORS keratinocytes were grown in Epilife medium with 60 μM calcium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with EpiLife Defined Growth Supplement (EDGS; Gibco; Thermo Fisher Scientific, Inc.).

hDPC viability assay. Cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay. Briefly, hDPCs were seeded at a density of 1.5×10^5 cells/well into 6-well plates and cultured for 24 h at 37˚C in a humidified incubator with 5% CO_2. The cells were subsequently treated with an inaudible sound frequency of 20 and 30 kHz for 4 h in FBS-free medium at 37˚C in a humidified incubator with 5% CO_2. After 48 h, cell viability was assessed using a CCK-8 assay (Dojindo Molecular Technologies, Inc.), and the absorbance at 450 nm was analyzed using a plate reader after the CCK-8 solution was added and cells were incubated at 37˚C in a humidified incubator with 5% CO_2 for 2 h. The cell viability rates were determined and are represented as percentages of the control value (untreated cells).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmbH) and 2 μg RNA was reverse-transcribed into complementary DNA using SuperScript® III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using the ABI 7500HT Fast System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the Taqman™ Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) to determine the expression of the following genes: Bcl-2 (assay ID: Hs00608023_m1), Bax (Hs00180269_m1), VEGFA (Hs00900055_m1) and ribosomal protein lateral stalk subunit P0 (RPLP0, Hs00420895_gH). The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min; followed by 40 cycles of amplification at 95˚C for 15 sec, annealing at 65˚C for 30 sec, and extension at 72˚C for 30 sec. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (31). RPLP0 was used as a housekeeping gene to normalize data.

Secretion factor analysis. VEGF, DKK-1 and TGF-β1 secretion was quantified using commercial ELISA kits, according to the manufacturer's protocol. For assessing the factors secreted by hDPCs treated with inaudible sound, hDPCs (passage 3) were plated overnight at a density of 1.5×10^5 cells/well into 6-well plates at 37˚C in a humidified incubator with 5% CO_2. The cells were washed twice with PBS and then added to FBS-free media. For quantification of VEGF, the cells were then incubated for 48 h following treatment with inaudible sound of

30 kHz at 37°C in a humidified incubator with 5% CO_2 for 4 h. In order to quantify the involvement of DKK-1 and TGF-β1, cells were treated with 100 nM DHT following the inaudible sound treatment. The concentrations of VEGF, DKK-1 and TGF-β1 in the medium were subsequently analyzed. Briefly, a capture antibody coated 96-well microplate (included in the kit) was blocked with 1% BSA (Thermo Fisher Scientific, Inc.) in PBS and incubated at room temperature for 1 h. After washing three times with wash buffer, the conditioned media, obtained from cells treated with inaudible sound or the untreated control was added, and cells were further incubated for 2 h at room temperature. The plate was then washed three times with wash buffer and detection antibody was added and cells were incubated for 2 h at room temperature. After washing three times, streptavidin-HRP solution was added and samples were incubated for 20 min at room temperature. The plate was washed three times with wash buffer followed by the addition of substrate solution and the plate was incubated for 20 min at room temperature. Stop solution was added to stop the enzyme reaction. Optical density was assessed using the Synergy™2 ELISA reader (BioTek Instruments, Inc.) at 450 nm.

Antiapoptotic effect assay. DKK-1 and TGF-β1, known as hair loss inducers, are known to induce apoptosis in ORS keratinocytes and to suppress cell viability (32). To analyze the antiapoptotic effect of ultrasound, ORS keratinocytes were seeded at a density of $3x10^5$ cells/well into 6-well plates and cultured at 37°C in a humidified incubator with 5% CO_2 for 24 h. Prior to treatment, the culture medium was replaced with a EDGS-free medium. After the cells were subsequently treated with inaudible sound at 37°C in a humidified incubator with 5% CO_2 for 4 h, 50 ng/ml DKK-1 and 50 ng/ml TGF-β1 were added for 48 h. The efficacy of any antiapoptotic effects was determined using the CCK-8 assay. The absorbance at 450 nm was assessed using a plate reader after treating the cells with CCK-8 solution at 37°C in a humidified incubator with 5% CO_2 for 2 h. The cell viability rates were determined using the optical density readings and are represented as percentages of the control value (untreated cells).

TUNEL assay. A TUNEL kit (*In Situ* Cell Death Detection Kit, Fluorescein; Roche Diagnostics GmbH) was used according to the manufacturer's protocol to evaluate apoptotic cells. Briefly, ORS keratinocytes at $2x10^4$ cells/200 μl were seeded into 8-chamber slides (Thermo Fisher Scientific, Inc.). After adherence, the cell medium was replaced with a growth supplement-free medium. Subsequently, the cells were treated with inaudible sound at 37°C in a humidified incubator with 5% CO_2 for 4 h and treated with 50 ng/ml DKK-1 and 50 ng/ml TGF-β1 at 37°C in a humidified incubator with 5% CO_2 for 24 h. These cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were incubated with 0.1% Triton X-100 in 0.1% sodium citrate for 1 h at room temperature. After washing with PBS, the cells were treated with TUNEL reagent and incubated in a humidified incubator for 1 h at 37°C in the dark. After washing with PBS, the cells were stained with DAPI and mounted using Fluoroshield with DAPI (cat. no. F6057; Sigma-Aldrich, USA), which was applied to the solution by addition of 3-4 drops, after which, the cover glass was mounted and the samples were left at room temperature for 5 min. DAPI staining was used to visualize the nuclei. Representative images were captured using a fluorescence microscope (Olympus Corporation; magnification, x100).

HF organ culture and assessment of hair elongation. Anagen HFs were obtained from human scalp skin specimens. Six HFs per well in 24-well plates were cultured in 37°C in a humidified incubator with 5% CO_2 with 500 μl of Williams E medium supplemented with 10 μg/ml insulin (Sigma-Aldrich; Merck KGaA), 10 ng/ml hydrocortisone (Sigma-Aldrich; Merck KGaA), 2 mM L-glutamine (Sigma-Aldrich; Merck KGaA), 0.1% fungizone (Gibco; Thermo Fisher Scientific, Inc.), 10 μg/ml streptomycin and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) according to Philpott's method (33). Each experimental group contained at least 30 anagen HFs derived from three different patients. HFs were treated with 30 kHz inaudible sound at 37°C in a humidified incubator with 5% CO_2 for 4 h, once a day. The incubation medium was renewed every two days. HF elongation was analyzed directly at 2 and 5 days of culture using a stereomicroscope (Olympus Corporation).

Ki-67 immunohistochemistry. Ki-67 staining was performed as previously described (34,35). To confirm the effect of ultrasound treatment on hair, HFs were treated with 30 kHz inaudible sound at 37°C in a humidified incubator with 5% CO_2 for 4 h, once a day for 3 days. After 3 days, HFs were embedded in optimal cutting temperature (OCT) freezing compound (Thermo Fisher Scientific, Inc.) and frozen 6-μm sections were prepared using a CM1850 cryostat (Leica Microsystems GmbH). The cryostat temperature was maintained between-15 and -23°C. The follicles were then cryo-sectioned and fixed in 10% paraformaldehyde for 10 min at room temperature. The samples were blocked using 4% BSA in TBS with 0.1% Tween-20 (TBST) for 30 min at room temperature, followed by washing with TBST. The samples were then incubated with Ki-67 monoclonal antibody (1:50; cat. no. 14-5698-82, Invitrogen; Thermo Fisher Scientific, Inc.) at 4°C overnight. After washing, Alexa Fluor™ 488 anti-rat secondary antibody (1:50; cat. no. A-11006, Invitrogen; Thermo Fisher Scientific, Inc.) was added and incubated for 1 h at room temperature. The samples were subsequently mounted and counterstained with DAPI as above. The samples were imaged using a fluorescence microscope (IX-73 with U-HGLGPS; Olympus Corporation; magnification, x100).

Statistical analysis. Data are presented as the mean ± SD. Comparisons among three or more groups were analyzed using a one-way ANOVA followed by the Tukey's honestly significant difference test. Comparisons between two groups were analyzed using a paired two-tailed Student's t-test. Results were processed using SPSS for Windows, version 22.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inaudible sound stimulates cell proliferation and the expression of hair growth related factors in hDPCs. To
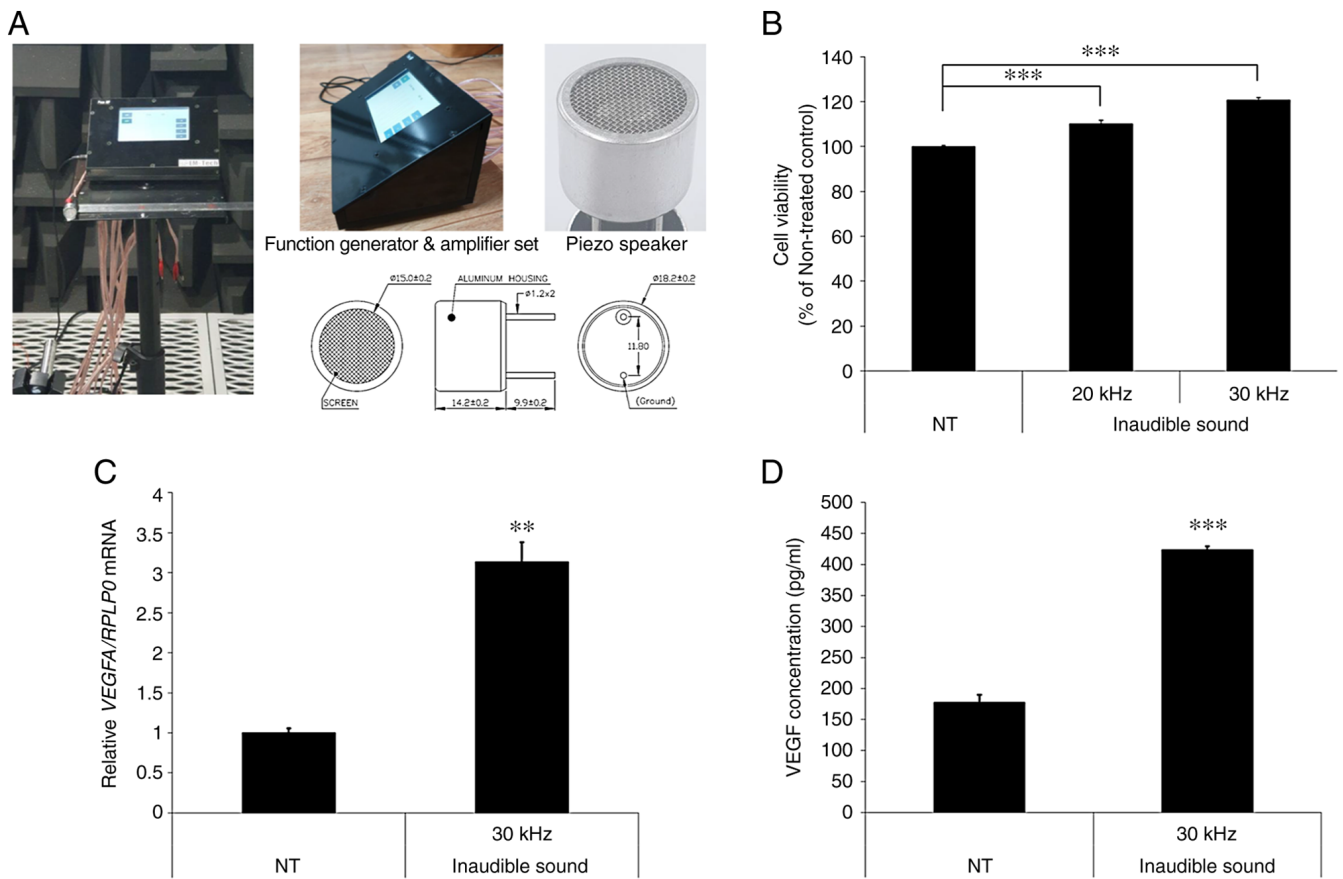
Figure 1. Inaudible sound stimulates proliferation and expression of hair growth-related factors in human dermal papilla cells. (A) Electrical sine waves were generated using a custom made function generator and amplifier set. (B) Cells were treated with inaudible sound (30 kHz) for 4 h and the Cell Counting Kit-8 assay was performed on day 2. (C) VEGF mRNA expression levels were assessed using reverse transcription-quantitative PCR. The mRNA expression levels of VEGF were normalized against the mRNA expression levels of RPLP0, a house-keeping gene. (D) VEGF concentration was determined using ELISA. Data are presented as the mean ± SD of three independent experiments. Statistically significant differences were determined using a one-way ANOVA followed by Tukey's honestly significant difference test or a paired Student's t-test. $^{**}P<0.01$ and $^{***}P<0.001$. VEGF, vascular endothelial growth factor; RPLP0, ribosomal protein lateral stalk subunit P0; NT, non-treated control.

determine the potential effect of ultrasound on the proliferation of hDPCs, the CCK-8 assay was performed 2 days after treatment with 20 and 30 kHz inaudible sound for 4 h. The results demonstrated that ultrasound significantly enhanced hDPC proliferation in a frequency-dependent manner compared with the non-treated control (NT) (Fig. 1B). A frequency 30 kHz was chosen for subsequent experiments because it elevated hDPC proliferation more than 20 kHz. To confirm the effect of inaudible sound on hDPC proliferation, RT-qPCR and ELISA were performed. VEGF is a typical growth factor that enhances hair growth and the effect of MNX on VEGF expression has previously been investigated (36). The results showed that treatment with inaudible sound significantly increased the mRNA expression levels and concentration of VEGF in hDPCs compared with the NT (Fig. 1C and D).

Inaudible sound abrogates DHT-induced secretion of catagen-related factors in hDPCs. To investigate the potential role of inaudible sound frequencies on the inhibition of catagen-related secretion factors, such as DKK-1 and TGF-β1, in hDPCs, ELISAs were performed 2 days following treatment in the presence or absence of DHT and 30 kHz inaudible sound. The results demonstrated that DHT significantly increased the concentrations of catagen-related secretion factors, DKK-1

and TGFβ1, in hDPCs, whereas inaudible sound significantly reduced the concentrations of DKK-1 and TGFβ1 compared with the NT group (Fig. 2A and B). These results suggested that the inhibition of catagen-related secretion factors may be likely mechanism responsible for increased hair loss following treatment with inaudible sound in DHT-treated hDPCs, as opposed to increased hair growth.

Inaudible sound inhibits apoptosis mediated by DKK-1 and TGF-β1 and regulates the expression of apoptosis-related genes in ORS keratinocytes. To investigate the potential role of inaudible sound on the inhibition of apoptosis in ORS keratinocytes, the CCK-8 assay was performed 1 day after treatment in the presence or absence of DKK-1, TGF-β1 and inaudible sound. The DKK-1 and TGF-β1 concentrations used were determined in preliminary tests (Fig. S1). Treatment with DKK-1 (50 ng/ml) and TGF-β1 (50 ng/ml) significantly inhibited the viability of ORS keratinocytes compared with the untreated NT group. The inhibitory effect of apoptosis by DKK-1 and TGFβ-1 on ORS keratinocyte viability was significantly reversed by 30 kHz inaudible sound compared with the NT and DKK1 + TGFβ-1 group (Fig. 3A).

To confirm the inhibitory effect of inaudible sound on apoptosis in ORS keratinocytes, the TUNEL assay was
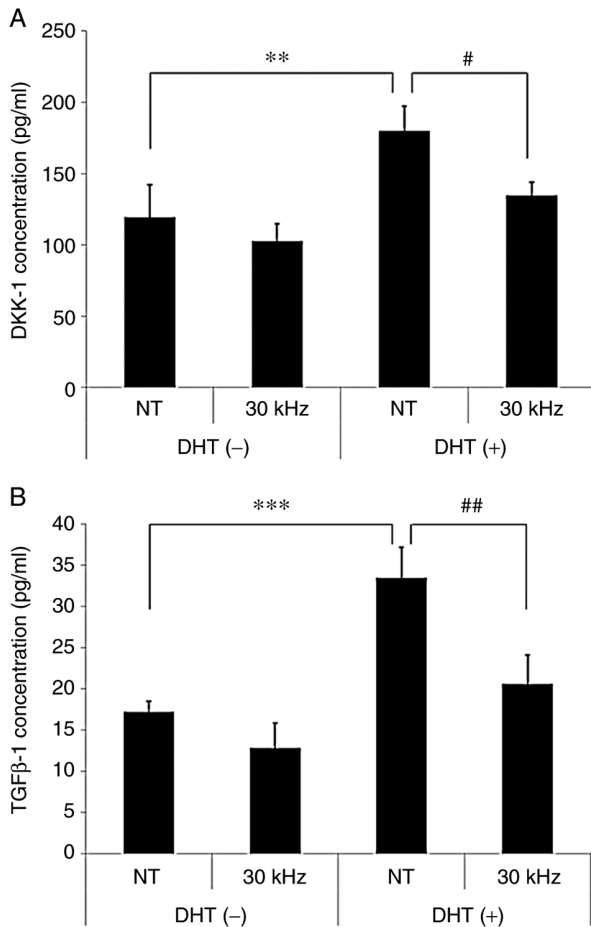
Figure 2. Inaudible sound abrogates DHT-induced catagen-related secretion factors in human dermal papilla cells. Cells were treated with inaudible sound at 30 kHz for 4 h and DHT for 48 h, or with both 30 kHz inaudible sound and DHT. (A) DKK-1 and (B) TGF-β1 concentrations were determined using ELISA on day 2. Data are presented as the mean ± SD of three independent experiments. Statistically significant differences were determined using a one-way ANOVA followed by the Tukey's honestly significant difference test. $^{**}P<0.01$, $^{***}P<0.001$, $^{#}P<0.05$ and $^{##}P<0.01$. DHT, dihydrotestosterone; DKK-1, Dickkopf-1; NT, non-treated control.

performed. The results demonstrated that TUNEL-positive cells undergoing apoptosis were significantly increased when ORS keratinocytes were treated with DKK-1 and TGFβ-1 compared with the NT group (Fig. 3B). TUNEL-positive cells were significantly decreased by 30 kHz inaudible sound despite co-treatment with DKK-1 and TGFβ1, compared with the NT DKK1 + TGFβ-1 group.

To further investigate the antiapoptotic effects of inaudible sound on DKK-1 and TGF-β1, changes in mRNA expression levels of apoptosis-related genes were investigated via RT-qPCR. Bcl-2 and Bax genes, which are related to DKK-1 and TGF-β1 have previously been demonstrated to induce apoptosis in numerous cell types, including HF cells (30,37,38) and were therefore used to measure apoptosis. ORS keratinocytes were treated with 30 kHz and/or a combination of DKK-1 and TGF-β1. In ORS keratinocytes, combination treatment with DKK-1 and TGFβ-1 significantly decreased the mRNA expression levels of antiapoptotic factor, Bcl-2, compared with the NT group without treatment. Furthermore, treatment with 30 kHz significantly reversed the inhibition of Bcl-2 mRNA expression induced by DKK-1

and TGFβ-1, compared with the NT DKK1 + TGFβ-1 group. DKK-1 and TGF-β1 also significantly induced the mRNA expression of the proapoptotic factor Bax compared with the untreated NT group. Moreover, treatment with 30 kHz significantly inhibited Bax mRNA expression levels in ORS keratinocytes compared with the NT DKK1 + TGFβ-1 group (Fig. 3C), despite the presence of DKK-1 and TGFβ-1. These results therefore indicated t that inaudible sound may promote the survival of ORS keratinocytes and increase the ratio of Bcl-2/Bax to inhibit cell death.

Inaudible sound promotes HF elongation and hair matrix keratinocyte proliferation in human HF organ culture. To examine the hair growth effect of inaudible sound at the organ level, *ex vivo* cultures of whole human scalp HFs were investigated. MNX and vehicle served as the positive and negative controls, respectively. It was observed that HFs treated with 30 kHz inaudible sound grew significantly longer compared with the NT HFs at 5 days, which was similar to the growth of HFs treated with MNX (Fig. 4A and B).

To confirm the cell proliferation promoting effect of inaudible sound in HF matrix keratinocytes, immunofluorescence staining for Ki-67 was performed after human HFs were exposed to 30 kHz inaudible sound for 4 h every day, over 3 days. HF sections derived from three different individuals were analyzed for the proliferation of follicular matrix keratinocytes in the hair bulb (green fluorescence; Fig. 4C). Nuclei were counterstained with DAPI (blue fluorescence). For quantitative analyses, the number of Ki-67$^+$ cells were counted and normalized to the number of DAPI-stained cells (Fig. 4D). The Ki-67$^+$ signal was especially strong in the upper part of the matrix above the hDPCs. After 3 days of culture, numerous NT HFs switched to the catagen phase, thus losing proliferative follicular matrix keratinocytes in the process. However, 30 kHz inaudible sound-treated cells displayed significantly enhanced cell proliferation levels in HFs compared with the NT and maintained the anagen phase.

Discussion

In the present study, the effects of inaudible sound on hair growth in hDPCs and HFs, and whether it can inhibit hair loss induced by DHT at the cellular level, were investigated. Although the molecular pathogenic mechanism of DHT is unclear, its role in male-pattern baldness has been well-documented (1,39). Circulating androgens such as DHT enter the follicle via capillaries, bind with the androgen receptors (ARs) on hDPCs and subsequently activate target genes, including DKK-1 and TGFβ1 (40). Therefore, in the present study hair loss signals were induced by treating hDPCs with DHT and it was then observed whether inaudible sound inhibited the secretion level of the hair loss-inducing factors, DKK1 and TGF-β1, which are induced by DHT. Furthermore, the results demonstrated that inaudible sound could significantly inhibit apoptosis induced by DKK1 and TGF-β1 in ORS keratinocytes, which was determined using a cell viability assay, TUNEL assay and the mRNA expression levels of apoptosis-related genes, Bcl-2 and Bax. Moreover, the results demonstrated that the direct treatment of human HFs with inaudible sound accelerated the growth rate of hair.
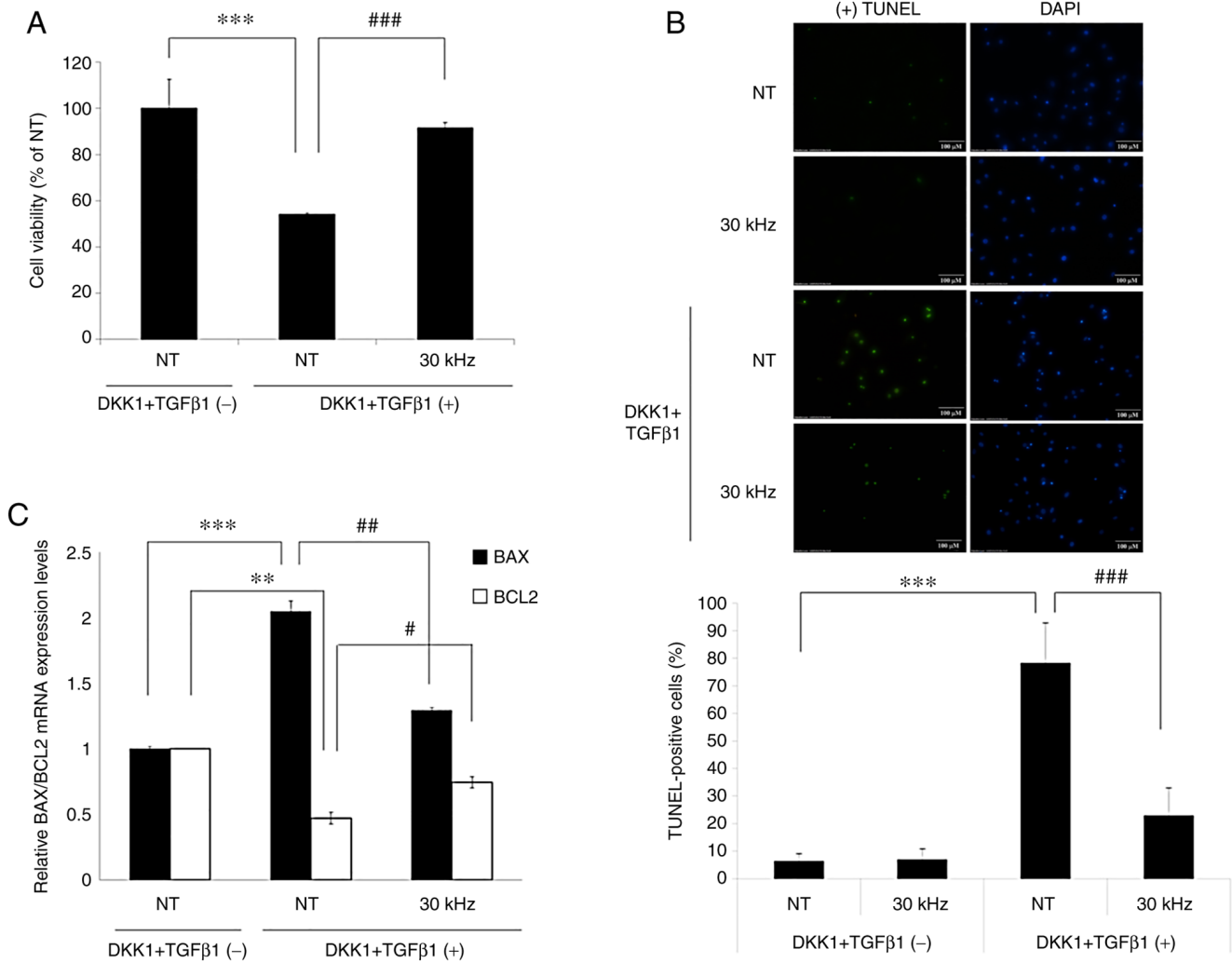
Figure 3. Inaudible sound inhibits DKK-1 and TGF-β1-mediated apoptosis and regulates the expression of apoptosis-related genes in outer root sheath keratinocytes. Cells were treated with a combination of apoptosis stimulator (DKK-1 and TGF-β1) or inaudible sound at 30 kHz or with a combination of both. The (A) Cell Counting Kit-8 and (B) TUNEL assays were subsequently performed on day 2. (C) Bax and Bcl-2 mRNA expression levels were analyzed using reverse transcription-quantitative PCR. mRNA expression levels were normalized against the mRNA expression levels of RPLP0, a housekeeping gene. Data are presented as the mean ± SD of three independent experiments. Statistically significant differences were determined using a one-way ANOVA followed by the Tukey's honestly significant difference test. $^{**}P<0.01$, $^{***}P<0.001$, $^{#}P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$. DKK-1, Dickkopf-1; RPLP0, ribosomal protein lateral stalk subunit P0; NT, non-treated control.

The protein expression of Ki-67, a proliferation marker of matrix cells, was also significantly increased in the hair bulb in response to inaudible sound.

To the best of our knowledge, the present study is the first to report this phenomenon, which can be explained by two hypotheses. The first hypothesis in the present study was that promotion of hair growth occurred due to activation of the Wnt/β-catenin signaling pathway caused by inaudible sound vibrations. Our lab observed that inaudible sound altered Wnt related genes expression in hDPCs (data not shown). Canonical Wnt signaling is transmitted via allosteric modulation induced by a structural change in the Wnt receptor when it binds to Wnt ligands (41). Weak vibrations such as inaudible sound, may lead to structural changes in membrane receptors (42). Structural changes caused by inaudible sound in the Wnt receptor may mimic the structural change caused by Wnt ligand binding, resulting in allosteric modulation. The present study provides the first report of hair growth promotion by inaudible sound; however, it is well known that signal changes

caused by vibrations exert effects such as stem cell activity, wound healing and bone fracture healing in a number of cell types (27,43,44). Therefore`, in future studies it will be necessary to verify if inaudible sound exhibits hair growth effects via allosteric modulation of the Wnt receptor.

The second hypothesis is that promotion of hair growth occurs as a result of mechanotransduction from the cell membrane of HF cells, which is modified by inaudible sound. Organs and cells routinely face mechanical stress such as muscle contraction, blood flow, or stretching. Within the cell, forces such as those generated by the actin-myosin cytoskeleton or surface tension generated by the membrane are present. These forces themselves act as signals to cells and are known to affect cell fate or organ formation (45-47). Recently, Thompson *et al* (48) reported the activation of mesenchymal stem cells (MSC) by low-intensity vibration (LIV). LIV is a micro-vibration that occurs upon light exercise or blood flow. This study reported that when LIV is applied to bone marrow, Yes-associated protein (YAP), a
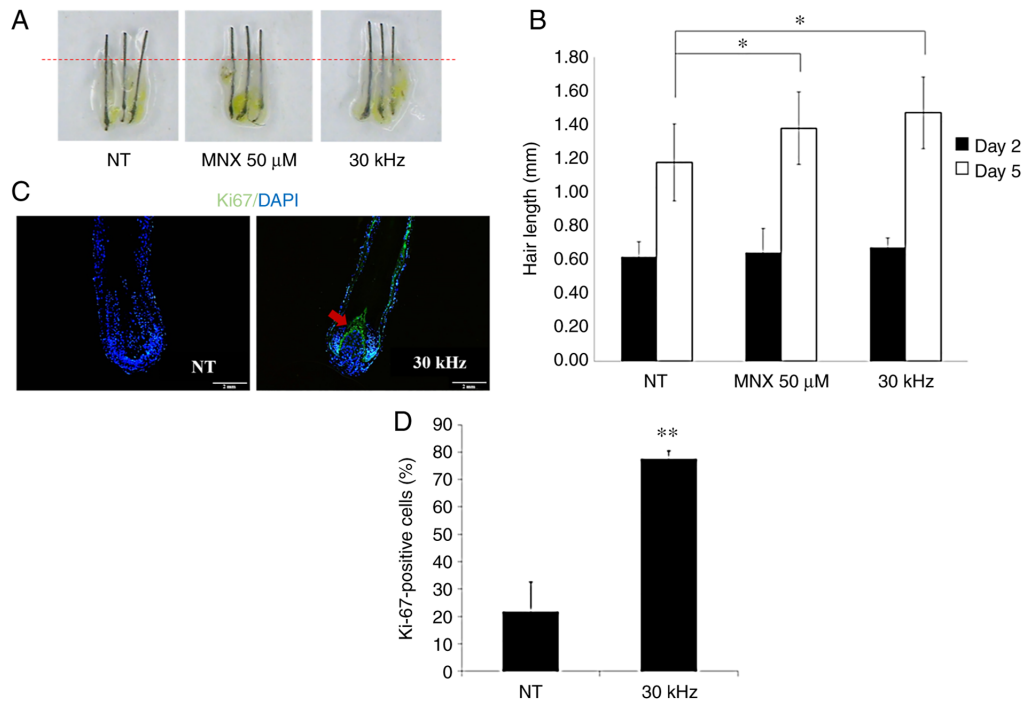
Figure 4. Inaudible sound promotes HF elongation and hair matrix keratinocyte proliferation in human HF organ culture. In total, 180 human scalp HFs with intact dermal papillae were obtained and treated for 5 days with inaudible sound at 30 kHz or with MNX. (A) HF length was analyzed under a stereomicroscope on day 2 and 5. (B) Relative length of each hair shaft. MNX (50 μM) served as the positive control for stimulating HF growth. Red dotted line, hair starting point. (C) Immunofluorescence staining of Ki-67 (green fluorescence) was performed after treating human HFs with inaudible sound at 30 kHz for 4 h every day, for 3 days. Nuclei were counterstained with DAPI (blue fluorescence). (D) For quantitative analysis, the number of Ki-67$^+$ cells were counted and normalized to the number of DAPI-stained cells. Data are presented as the mean ± SD of three independent experiments. Statistically significant differences were determined using a one-way ANOVA followed by Tukey's honestly significant difference test or a paired Student's t-test. $^*P<0.05$ and $^{**}P<0.01$. HF, hair follicle; MNX, minoxidil; NT, non-treated control.
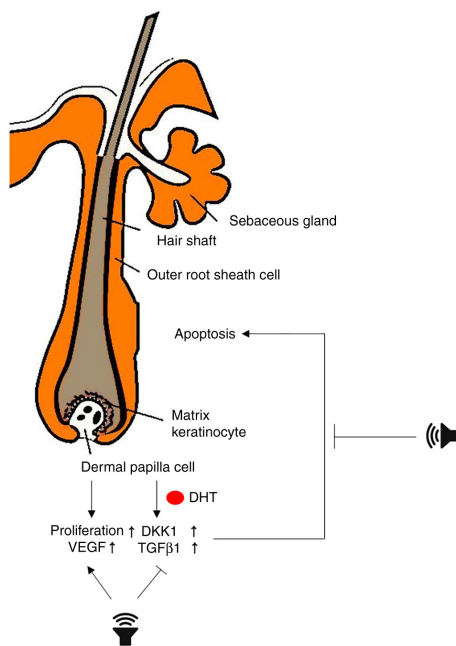

Figure 5. Potential mechanism for the stimulation of hair growth by inaudible sound. A schematic summarizing the proposed mechanism of action of inaudible sound. The present study demonstrated that inaudible sound potentially induced cell proliferation and the release of VEGF, a hair growth-related growth factor and abrogated DHT-induced catagen-related secretion factors, such as DKK-1 and TGF-β1 in human dermal papilla cells. Furthermore, inaudible sound was demonstrated to prevent apoptosis induced in HFs by catagen-related secretion factors and induced the proliferation of HF keratinocytes. VEGF, vascular endothelial growth factor; DHT, dihydrotestosterone; DKK-1, Dickkopf-1; HF, hair follicle.

mechanotransducer, is translocated into the nucleus from the cytosol of MSCs. YAP enters the MSC nucleus where it acts as a transcriptional co-activator and transcribes various genes associated with bone production. It has been hypothesized that this is the mechanism by which bone stiffness increases upon exercise (48). In the present study, inaudible sound was used as a scalp stimulator, which was transmitted to the scalp via the air. Moreover, the inaudible sound transmitted to the scalp may transmit LIV to the HF. Transferred LIV has been reported to affect HF stem cells (HFSC) of the HF bulge, which may promote the expression of various genes to promote hair growth by translocating YAP from the HFSC cytosol into the nucleus. In the present study it has been demonstrated that inaudible sound may inhibit hair loss by blocking DHT-induced apoptotic signaling and promoting follicle cell proliferation; however, further studies are needed to verify the aforementioned hypotheses for determining the mechanisms underlying this phenomenon.

In conclusion, the present study demonstrated that inaudible sound promoted the proliferation of HF cells and that this effect inhibited hair loss signals induced by DHT (Fig. 5). Although the mechanism of action remains to be elucidated, the present study has provided the basis for a new therapeutic approach that can replace or complement existing hair loss treatments.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HC and BJK were responsible for the conceptualization of the present study. HC, YL and SHS designed the methodology and HC, JN, WSP and BJK validated the data. Data was collected by HC, YL and SHS and the investigation and interpretation of the data was performed by HC, YL, SHS, BCP and BJK. Statistical analysis was performed by HC. HC prepared the original draft manuscript and HC, YL and BJK edited and wrote the manuscript. HC, WSP and BJK supervised the project. HC, JN, WSP and BJK edited the manuscript. WSP and BJK confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Medical Ethical Committee of Dankook Medical College (Cheonan, South Korea) approved all the described protocols and informed written consent was obtained from all patients (IRB approval no. DKUH 2020-11-004).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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