



## Decreased Expression of TRPV4 Channels in HEI-OC1 Cells Induced by High Glucose Is Associated with Hearing Impairment

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**Purpose:** Previous reports have shown that hyperglycemia-induced inhibition of transient receptor potential vanilloid sub type 4 (TRPV4), a transient receptor potential ion channel, affects the severity of hearing impairment (HI). In this study, we explored the role of TRPV4 in HI using HEI-OC1 cells exposed to high glucose (HG).

**Materials and Methods:** HEI-OC1 cells were cultured in a HG environment (25 mM D-glucose) for 48 hours, and qRT-PCR and Western blotting were used to analyze the expression of TRPV4 at the mRNA and protein level. TRPV4 agonist (GSK1016790A) or antagonist (HC-067047) in cultured HEI-OC1 cells was used to obtain abnormal TRPV4 expression. Functional TRPV4 activity was assessed in cultured HEI-OC1 cells using the MTT assay and a cell death detection ELISA.

**Results:** TRPV4 agonists exerted protective effects against HG-induced HI, as evidenced by increased MTT levels and inhibition of apoptosis in HEI-OC1 cells. TRPV4 overexpression significantly increased protein levels of phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK), while TRPV4 antagonists had the opposite effect. Our results indicated that TRPV4 is a hyperglycemia-related factor that can inhibit cell proliferation and promote cell apoptosis by activating the MAPK signaling pathway in HEI-OC1 cells.

**Conclusion:** Our results show that the overexpression of TRPV4 can attenuate cell death in HEI-OC1 cells exposed to HG.

**Key Words:** TRPV4, high glucose, hearing impairment, HEI-OC1 cells

### INTRODUCTION

Hearing impairment (HI) is one of the most highly prevalent

chronic diseases<sup>1</sup> and represents a huge burden on society and on individuals in terms of reduced emotional, social, mental, and physical health.<sup>2-4</sup> HI has many causes, the most common of which is presbycusis, followed by noise exposure, ototoxic drugs, and viral infections.<sup>5,6</sup> In addition to these factors, accumulating evidence suggests that HI is yet another disabling complication of diabetes.<sup>7</sup> We believe that, as time goes on, hyperglycemia may impair cochlear microvascular circulation and affect the steady state of hearing cells, thereby reducing patient hearing.<sup>8</sup>

Hearing is a complex system that depends on various tissue and cell types in the inner ear. Damage to cochlear hair cells is the main reason for threshold elevation in hearing loss.<sup>9</sup> Previous studies have shown that abnormalities of homeostasis and apoptosis of cochlear hair cells are important contributing factors leading to HI.<sup>10</sup> The way that auditory cell apoptosis affects HI has been studied for a long time.<sup>11</sup> However, the mechanisms of cochlear hair cell apoptosis during the pro-

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gression of HI remain poorly understood.

As a member of the TRP super family, transient receptor potential vanilloid sub type 4 (TRPV4) is a receptor-activated nonselective cation channel that is endogenously expressed in many tissue and cell types, such as skin,<sup>12</sup> cortical astrocytes,<sup>13</sup> keratinocyte cell lines,<sup>14</sup> and tracheal epithelial cells,<sup>15</sup> including cochlear hair cells.<sup>16</sup> Previous studies have found that TRPV4 is linked to the development of diabetes.<sup>17</sup> In addition, multiple studies have found that damage to TRPV4 can cause hearing delay, which makes the cochlea vulnerable to noise injury. It has been suggested that TRPV4 is associated with hearing loss.<sup>18,19</sup> TRPV4 expression was down-regulated in high glucose (HG)-cultured retinal microvascular endothelial cells<sup>20</sup>; however, the potential role of TRPV4 expression in HG-cultured cochlear hair cells has not been investigated. In this study, we sought to detect the expression of TRPV4 and discover its role in HG-mediated HI.

In mammals, mitogen-activated protein kinase (MAPK) signaling cascades regulate important cellular processes, including gene expression, cell proliferation, cell survival and death, and cell motility.<sup>21</sup> Recently, research has shown that TRPV4 channels, as TRP cation channel subfamily V members, are involved in MAPK activation in dorsal root ganglion neuropathic pain.<sup>22</sup> Research by Jie, et al.<sup>21</sup> has shown that the TRPV4 gene contains two conserved MAPK-binding sites that confer responsiveness to calcineurin-MAPK signaling, thus making MAPK and its downstream signals promising targets for therapeutic intervention. However, the specific mechanism whereby MAPK signaling pathways affect HI has not been fully understood.

Therefore, in this study, our aim was to confirm the expression levels of TRPV4 in HG-induced cochlear hair cells. Our experimental results show that the ectopic expression of TRPV4 in cochlear hair cells inhibits cell proliferation and promotes cell apoptosis *in vitro*. In addition, we also demonstrate that the MAPK signaling pathway is a direct target of TRPV4 and show that TRPV4 activates the MAPK signaling pathway. These findings show that TRPV4 could have potential implications for therapeutic approaches to HI as a complication of diabetes.

## MATERIALS AND METHODS

### Cell culture and transfection

The cochlear auditory cell line HEI-OC1 was purchased from the House Ear Institute (Los Angeles, CA, USA). HEI-OC1 cells were cultured in DMEM medium containing 10% fetal bovine serum at 33°C under 10% CO<sub>2</sub> (permissive conditions). Glucose was supplemented in the culture medium to induce a diabetes cell model; an appropriate concentration of mannitol was supplemented in the control culture medium. After 72 hours of culture, cells were divided into three groups: a euglycemic control group (5 mM D-glucose), a hyperglycemia group (25 mM D-

glucose), and an osmotic control group (5 mM D-glucose+20 mM mannitol). HEI-OC1 cells were transfected with pcDNA3.1 empty vector, pcDNA3.1-TRPV4 vector, TRPV4-siRNA, and scrambled siRNA control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### Drug treatment

The TRPV4 antagonist HC-067047 and agonist GSK1016790A were purchased from Calbiochem (Merck, Darmstadt, Germany). Following pre-culture, HEI-OC1 cells were stimulated either with GSK1016790A (100 nM<sup>23</sup>; Sigma-Aldrich, St. Louis, MO, USA) or HC067047 (1 μM<sup>24</sup>; Maybridge Ltd., Cambridge, UK) for 24 hours. The p38 inhibitor SB203580 (10 μM; Tocris Bioscience, Bristol, UK) was given to the experimental groups at the recommended concentrations.<sup>25</sup>

### RNA extraction and RT-PCR

Total RNA was isolated from HEI-OC1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For reverse transcription (RT), the oligonucleotide sequences of primers specific for TRPV4 were 5'-GATGGGCGAC CAAATCTGC-3' and 5'-GAGGACTCATATAGGGTGGACTC-3'. Aliquots of the reaction mixture were used as templates under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 45 s. PCR experiments were performed in triplicate. As a control, β-actin expression was analyzed (forward, 5'-ACCGAGCGCG GCTACA-3' and reverse, 5'-CAGCCGIGGCCATCTCTT-3').

### Western blot assays

Cultured cells were washed twice with PBS and lysed on ice using ice-cold RIPA buffer for 20 minutes. The proteins were then subjected to SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was blocked in a 5% ECL priming blocker for 1 hour and incubated overnight at 4°C with primary antibody: rabbit anti-TRPV4 (1:800, Abcam, Cambridge, UK), rabbit anti-p38 polyclonal antibody (1:200, CST, Beverly, MA, USA), rabbit anti-Pp38 polyclonal antibody (1:1000, CST), and anti-β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times with TBST, the membrane was placed in 1:5000 diluted secondary antibody (sc-2054, Santa Cruz Biotechnology) and incubated for 1 hour at room temperature. The immunoreaction products were visualized using the chemiluminescence-emanating ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

### Cell proliferation assay

HEI-OC1 cells were cultured in a 96-well plate at a concentration of 2×10<sup>5</sup> cells/well. 20 μL of a 5 mg/mL MTT solution was added to each well, and the plate was incubated at 33°C for 4 hours under 10% CO<sub>2</sub> and 95% air. The supernatants were removed and 250 μL of DMSO added. An automatic microplate reader was used to read the absorbance of each well at 540 nm.

### Apoptosis assay

To measure the influence of ectopic expression of TRPV4 protein on cell apoptosis, we used the cell death detection ELISA-Plus (Roche, Indianapolis, IN, USA) to analyze the percentage of apoptotic cells, according to the manufacturer's protocol. The assay system quantifies the amount of histone-coupled DNA fragments by measuring absorbance at 405 nm.

### ELISA analysis

An ELISA was used to analyze p38 concentrations in the culture medium according to the manufacturer's protocol. Absorbance was measured at 450 nm using a 680XR Microplate reader (Bio-Rad). All of the samples were analyzed in duplicate.

### Statistical analysis

All experiments were independently repeated at least three times. Data are presented as means  $\pm$  SEM. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was tested with a one-way ANOVA test or Student's *t*-test. *p* values  $< 0.05$  were considered to indicate statistically significant differences.

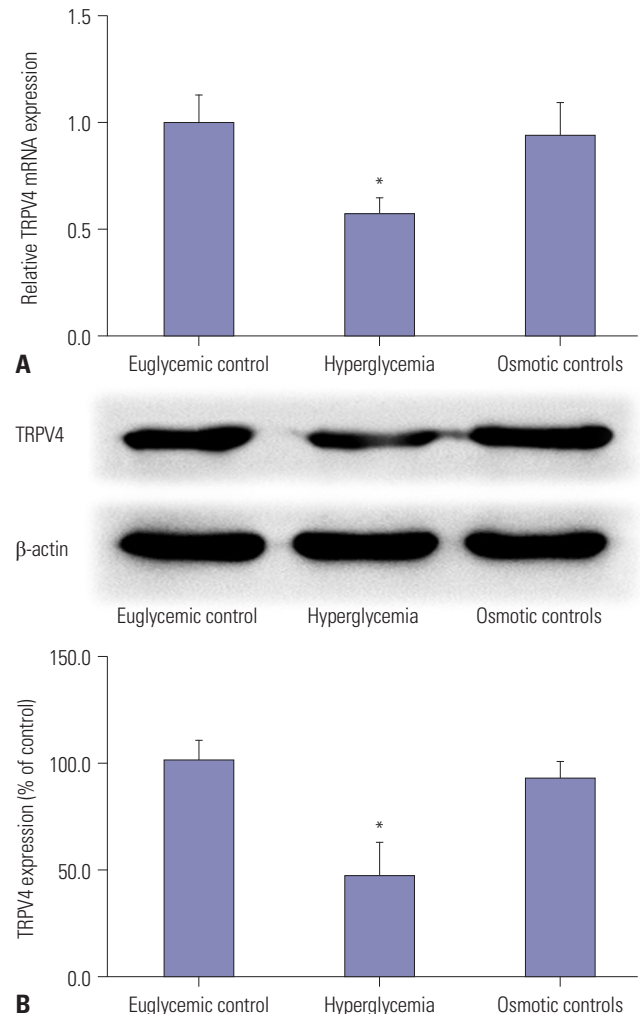
## RESULTS

### HGs reduce the expression of TRPV4 in HEI-OC1 cells

To investigate the role of TRPV4 in HEI-OC1 cells cultured in HG, qRT-PCR and Western blotting were used to detect the expression of TRPV4 at the mRNA level and the protein level, respectively. As shown in Fig. 1A, TRPV4 expression was significantly reduced in HEI-OC1 cells following culture in HG for 48 h, compared with the euglycemic and osmotic controls. Western blotting shows that the expression of the TRPV4 protein was decreased compared with the controls (Fig. 1B).

### Expression of TRPV4 in agonist and inhibitor stimulated HEI-OC1 cells

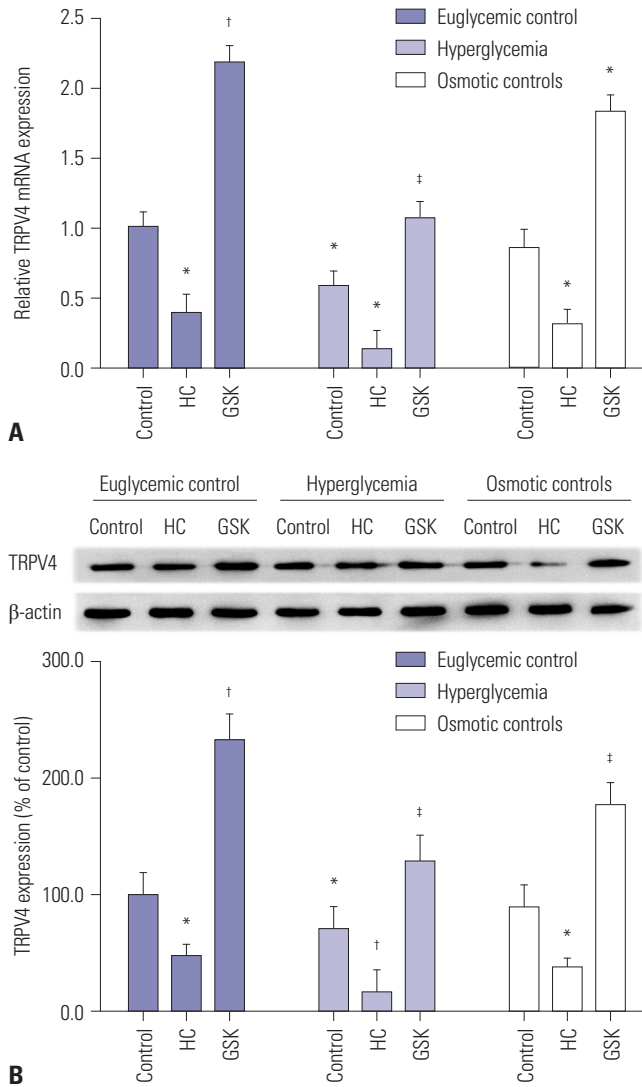
To investigate whether TRPV4 expression is affected by pharmacological agonists or inhibitors, HEI-OC1 cells were treated with 100 nM of a TRPV4 agonist or 1  $\mu$ M of a TRPV4 antagonist. The control group was given normal saline in an equal quantity. After stimulation of HEI-OC1 cells with the TRPV4 antagonist HC-067047, mRNA expression was significantly decreased. Conversely, stimulation with the TRPV4 agonist GSK1016790A significantly promoted mRNA expression (Fig. 2A). Western blotting showed that the expression of TRPV4 protein was similarly affected compared with the controls (Fig. 2B). Moreover, our results suggested that TRPV4 expression significantly decreased in HEI-OC1 cells treated with HG, compared with HEI-OC1 cells treated with TRPV4 agonist in the euglycemic condition. While the HG condition induced HEI-OC1 cell death, we believe that TRPV4 agonist may rescue HEI-OC1 cell death from HG status.



**Fig. 1.** HG reduces the expression of TRPV4 in HEI-OC1 cells. HEI-OC1 cells were cultured under euglycemic, hyperglycemia, and osmotic control conditions for 48 h, and qRT-PCR (A) and Western blot (B) were used to analyze the expression of TRPV4 mRNA and protein. Results are representative of three independent experiments. \*Indicates differences from controls at  $p < 0.05$ . TRPV4, transient receptor potential vanilloid sub type 4; HG, high glucose.

### Effects of HG on cell proliferation and apoptosis in HEI-OC1 cells

To identify the mechanisms of TRPV4 action on cellular behavior, we first examined the effects of HG on cell proliferation in HEI-OC1 cells. Cell proliferation was measured using the MTT-8 assay in HEI-OC1 cells. Overexpression of TRPV4 significantly increased cell proliferation following 48 h of exposure to HG. Conversely, in the HG condition, down-regulated TRPV4 inhibited cell proliferation in comparison with the control group (Fig. 3A). The cell death detection ELISA-Plus cell apoptosis assay showed that overexpression of TRPV4 inhibits cell apoptosis. Conversely, down-regulated TRPV4 promoted cell apoptosis in comparison with the corresponding control group (Fig. 3B). To further confirm the effects of TRPV4 on HEI-OC1 cell proliferation and apoptosis, TRPV4

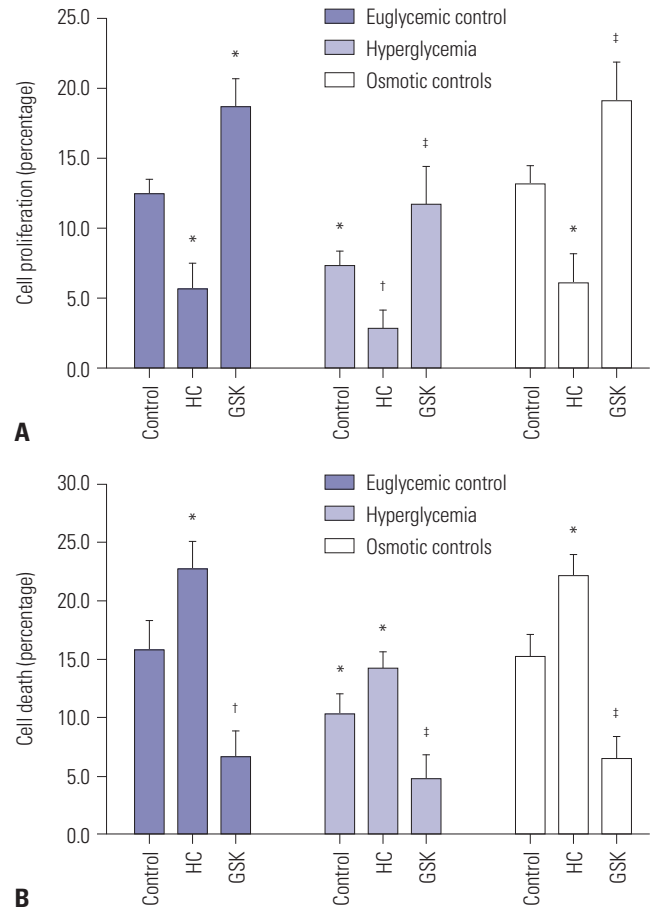


**Fig. 2.** The effect of reagents on the expression of TRPV4. (A) The relative expression of TRPV4 mRNA in high glucose cultured HEI-OC1 cells after treatment with HC-067047 (HC) or GSK1016790A (GSK), compared with the untreated control, was determined by qRT-PCR. (B) Western blotting was used to analyze the expression of TRPV4 protein in HEI-OC1 cells after treatment with HC-067047 or GSK1016790A. Data are representative of three experiments. \*Indicates differences from euglycemic controls at  $p < 0.05$ , <sup>†</sup>Indicates differences from euglycemic controls at  $p < 0.01$ , <sup>‡</sup>Indicates differences from untreated controls at  $p < 0.05$ , respectively. TRPV4, transient receptor potential vanilloid sub type 4.

overexpression and silence HEI-OC1 cell were constructed (Supplementary Fig. 1, only online). Moreover, we found that TRPV4 overexpression induced HEI-OC1 cell proliferation and impeded cell apoptosis, whereas TRPV4 depletion had the opposite effects (Supplementary Fig. 1, only online).

**Overexpression of TRPV4 promotes activation of MAPK pathways**

In order to verify the relationships between TRPV4 and MAPK pathways, we studied the effect of HG on the phosphorylation of p38 MAPK in HEI-OC1 cells. HEI-OC1 cells were incubated

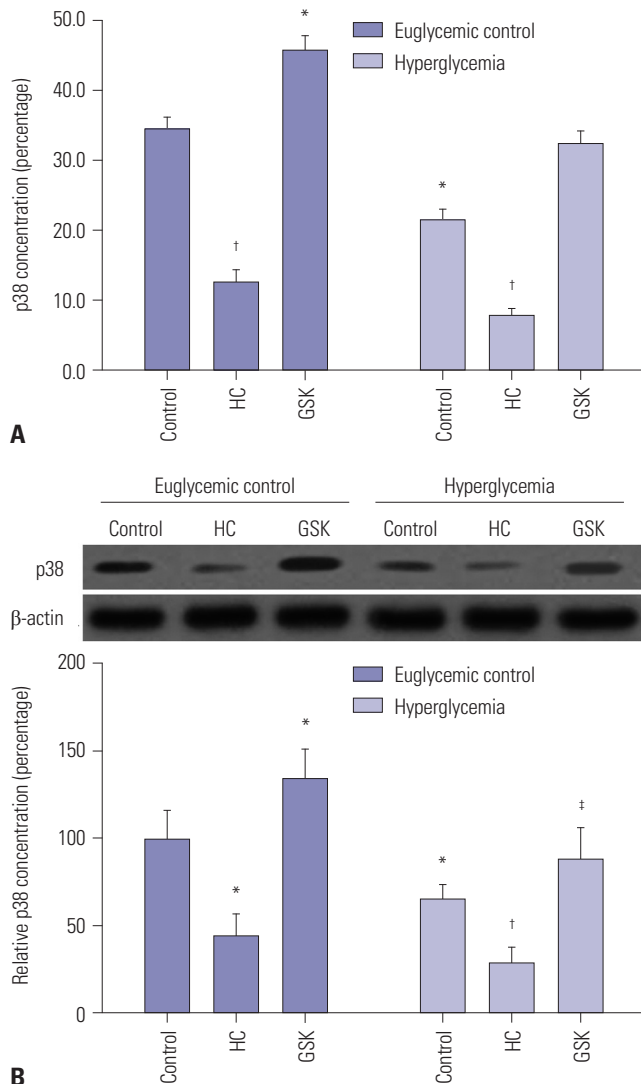


**Fig. 3.** TRPV4 directly affects proliferation and apoptosis in HEI-OC1 cells. (A) The effect of TRPV4 on cell proliferation of HEI-OC1 cells was detected using the MTT assay. (B) The effect of TRPV4 on cell apoptosis of HEI-OC1 cells was detected using the cell death detection ELISA-Plus apoptosis assay. Data are representative of three experiments. \*Indicates differences from euglycemic controls at  $p < 0.05$ , <sup>†</sup>Indicates differences from euglycemic controls at  $p < 0.01$ , <sup>‡</sup>Indicates differences from untreated controls at  $p < 0.05$ , respectively. TRPV4, transient receptor potential vanilloid sub type 4; HC, TRPV4 antagonist HC-067047; GSK, TRPV4 agonist GSK1016790A.

in a HG environment for 24 hours, and an anti-p-p38 polyclonal antibody was used to quantify the phosphorylated form of the p38 protein. The results showed that overexpression of TRPV4 significantly increased the concentrations of p-p38 in the HEI-OC1 cell culture medium (Fig. 4A). In addition, we also found that the expression of TRPV4 could positively regulate the protein expression of p-p38 (Fig. 4B). This result indicated that TRPV4 is involved in the activation of the MAPK pathway.

**TRPV4 modulates HEI-OC1 cell proliferation and apoptosis by targeting MAPK signaling pathways**

To further clarify the effect of MAPK on TRPV4-modulated HEI-OC1 cell growth and apoptosis, the MAPK signaling inhibitor SB203580 was used. After a series of functional restoration assays, we found that the decrease in HEI-OC1 cell proliferation (Fig. 5A) induced by TRPV4 up-regulation was

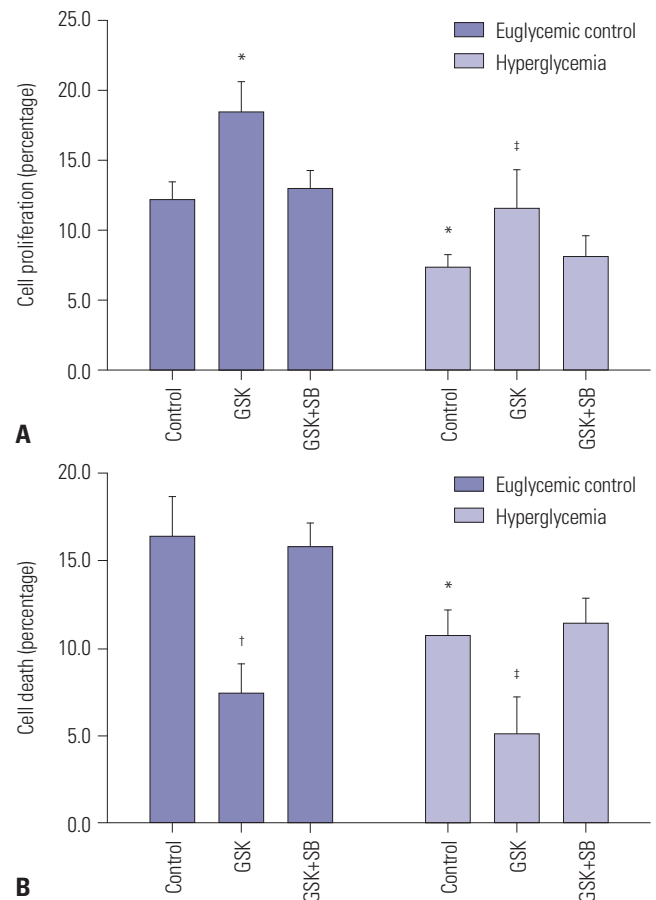


**Fig. 4.** Overexpression of TRPV4 triggered activation of the MAPK signaling pathway. (A) After pretreatment with the TRPV4 agonist GSK1016790A (GSK) or the TRPV4 antagonist HC067047 (HC), the p38 concentration in the culture medium of HEI-OC1 cells was assessed by ELISA. (B) The protein expression of p38 was analyzed by Western blotting. Data represent means $\pm$ SD from three independent experiments. \*Indicates differences from euglycemic controls at  $p < 0.05$ , †Indicates differences from euglycemic controls at  $p < 0.01$ , ‡Indicates differences from untreated controls at  $p < 0.05$ , respectively. TRPV4, transient receptor potential vanilloid sub type 4; MSPK, mitogen-activated protein kinase.

dramatically attenuated after stimulation with SB203580. Moreover, disruption of MAPK signaling by SB203580 treatment also significantly increased the apoptosis rate (Fig. 5B). These results indicate that TRPV4 may promote cochlear hair cell proliferation and repress apoptosis by modulating MAPK signaling.

## DISCUSSION

The calcium-permeable cation channel protein TRPV4 is



**Fig. 5.** TRPV4 regulates cochlear hair cell development by targeting MAPK signaling pathways. After pretreatment with the TRPV4 agonist GSK1016790A (GSK) or a combination of GSK1016790A and the p38 inhibitor SB203580 (GSK+SB), the effect of TRPV4 on cell proliferation of HEI-OC1 cells was detected using the MTT assay, and the effect of TRPV4 on cell apoptosis of HEI-OC1 cells was detected using the cell death detection ELISA-Plus apoptosis assay. (A) Inhibition of MAPK signaling rescued cell proliferation induced by TRPV4 in HEI-OC1 cells. (B) Disruption of MAPK signaling promoted cell apoptosis induced by TRPV4 in HEI-OC1 cells. Data are represented as means $\pm$ SD from three independent experiments. \*Indicates differences from euglycemic controls at  $p < 0.05$ , †Indicates differences from euglycemic controls at  $p < 0.01$ , ‡Indicates differences from untreated controls at  $p < 0.05$ , respectively. TRPV4, transient receptor potential vanilloid sub type 4; MSPK, mitogen-activated protein kinase.

known to function as a  $Ca^{2+}$ -permeable TRP channel in cells and is involved in a variety of different physiological processes. TRPV4 has been reported to be expressed in the inner-ear hair cells.<sup>26</sup> However, previous published reports on TRPV4 expression in hearing tissues/cells were discordant. TRPV4 family member expression has been reported in cochlear hair cells.<sup>27-29</sup> A study by Zanini and Göpfert<sup>30</sup> indicated that TRP family members are associated with hearing impairment; however, no TRPV4 transcripts were detected in HEI-OC1 cells. Therefore, in this study, the expression of TRPV4 in HEI-OC1 cells was investigated. Our results showed that TRPV4 was expressed in HEI-OC1 cells.

As an intrinsic constituent of receptor-operated cation entry, TRPV4 may participate in numerous physiological functions.<sup>20,31,32</sup> Increasing evidence suggests that TRPV4 may be involved in the development of HG-induced HI.<sup>27</sup> Therefore, in our study, we demonstrated that HG influences the expression of TRPV4 in HEI-OC1 cells, inducing a significant down-regulation thereof, consistent with the results of previous *in vivo* studies, which indicated significantly decreased expression of TRPV4 following diabetes.<sup>33,34</sup> However, the exact functions and mechanisms of TRPV4 in cochlear hair cells are largely unknown. We found that the mRNA and protein levels of TRPV4 were much lower in HG-cultured HEI-OC1 cells, compared with controls. Our findings are consistent with previous reports, and allow us to speculate that the high-expression of TRPV4 may be conducive to improving the hearing of diabetic patients.

Studies have indicated that TRPV4 promotes activation of MAPK signaling. MAPK is an important mediator of signal transduction from the cell surface to the nucleus. Recently, many studies have confirmed that the MAPK signaling pathway plays a crucial role in cell development, including effects on proliferation and survival.<sup>11</sup> The significance of MAPK and its therapeutic value as a target for hearing loss therapy have been investigated in several reports,<sup>35,36</sup> in which MAPK status was found to be positively correlated with oxidative stress, cell injury, and inflammation.<sup>37,38</sup> Our work broadens our understanding of the roles that TRPV4 plays in HEI-OC1, showing that it targets MAPK signaling pathways and that it can be a target for therapy for hearing damage caused by diabetes.

In conclusion, this study showed that TRPV4 expression is lower in a diabetic hearing cell model, consistent with previous studies. We also found that, in the diabetic cell model, overexpression of TRPV4 induces cell proliferation and inhibits apoptosis by activation of MAPK signaling pathways associated with cochlear hair cell growth. Thus, increased TRPV4 expression in diabetic patients may decrease cochlear hair cell amplification. Therefore, our study may provide a therapeutic strategy for improving HI in diabetic patients.

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