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ORIGINAL RESEARCH COMMUNICATION

Wnt Signaling Activates TP53-Induced Glycolysis and Apoptosis Regulator and Protects Against Cisplatin-Induced Spiral Ganglion Neuron Damage in the Mouse Cochlea

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Abstract

Aims: Cisplatin can damage spiral ganglion neurons (SGNs) and cause sensorineural hearing loss. Wnt activation protects against neomycin-induced hair cell damage in the mouse cochlea, but the role of Wnt signaling in protecting SGNs from cisplatin treatment has not yet been elucidated. This study was designed to investigate the neuroprotective effects of Wnt signaling against cisplatin-induced SGN damage.

Results: First, we found that Wnt signaling was activated in SGNs after cisplatin treatment. Next, we discovered that overexpression (OE) of Wnt signaling in SGNs reduced cisplatin-induced SGN loss by inhibiting caspase-associated apoptosis, thus preventing the loss of SGN function after cisplatin treatment. In contrast, inhibition of Wnt signaling increased apoptosis, made SGNs more vulnerable to cisplatin treatment, and exacerbated hearing loss. TP53-induced glycolysis and apoptosis regulator (TIGAR), which scavenges intracellular reactive oxygen species (ROS), was upregulated in SGNs in response to cisplatin administration. Wnt/β-catenin activation increased TIGAR expression and reduced ROS level, while inhibition of Wnt/β-catenin in SGNs reduced TIGAR expression and increased the ROS level. Moreover, OE of TIGAR reduced ROS and decreased caspase 3 expression, as well as increased the survival of SGNs in Wnt-inhibited SGNs. Finally, antioxidant treatment rescued the more severe SGN loss induced by β-catenin deficiency after cisplatin treatment.

Innovation and Conclusion: This study is the first to indicate that Wnt signaling activates TIGAR and protects SGNs against cisplatin-induced damage through the inhibition of oxidative stress and apoptosis in SGNs, and this might offer novel therapeutic targets for the prevention of SGN injury. *Antioxid. Redox Signal.* 30, 1389–1410.

Keywords: spiral ganglion neuron, Wnt/β-catenin signaling, cisplatin, TIGAR, reactive oxygen species

Introduction

CISPLATIN IS AN EFFECTIVE antineoplastic agent that is widely used against a variety of tumor types (39). Unfortunately, serious toxic side effects after cisplatin treatment, such as ototoxicity, frequently occur (31), and audiometric studies show that 93% of patients will have a certain degree of progressive and irreversible sensorineural hearing loss after cisplatin chemotherapy (33). Ototoxicity induced by cisplatin occurs mainly in the inner ear with three major targets, including the outer hair cells (HCs) of the organ of Corti, spiral ganglion neurons (SGNs), and the stria

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Innovation

Wnt/β-catenin signaling is activated in spiral ganglion neurons (SGNs) after cisplatin treatment and overactivation of Wnt signaling protects against cisplatin-induced SGN loss both *in vitro* and *in vivo*. The expression of TP53-induced glycolysis and apoptosis regulator (TIGAR) is increased in SGNs after cisplatin treatment, and this is regulated by Wnt signaling. Wnt activation in SGNs upregulates TIGAR expression together with inhibition of reactive oxygen species accumulation and oxidative stress-induced apoptosis. This study demonstrates that Wnt signaling activates TIGAR and protects SGNs against cisplatin-induced damage through the inhibition of oxidative stress and apoptosis in SGNs, and this might offer novel therapeutic targets for the prevention of SGN injury.

vascularis (37). Notably, unlike some ototoxic drugs, the cytotoxic effects induced by cisplatin in both the organ of Corti and the SGNs are suggested to occur independently of each other as evidenced by animal models (37, 38). Furthermore, Harris *et al.* reported a unique case in which cisplatin-associated hearing loss occurred after some years of successful cochlear implant use, and the patient subsequently lost the benefit from the device following cisplatin therapy (15). This case has important implications if the benefits of cochlear implantation can be lost by the long-term destructive effect of cisplatin chemotherapy on SGNs. Unfortunately, there are no treatments for cisplatin-induced ototoxicity, thus strategies that can eliminate ototoxicity without interfering with the anticipated therapeutic effects of cisplatin are urgently needed.

One of the mechanisms behind the cochleotoxic effects induced by cisplatin is believed to be mediated through the generation of reactive oxygen species (ROS) and the consumption of antioxidant scavenger molecules such as glutathione peroxidase (7). Lipid peroxidation and the generation of malondialdehyde and other toxic agents subsequently induce calcium influx and apoptosis (17, 24). Cisplatin also activates apoptosis by triggering the release of cytochrome c, activating caspases 3, 8, and 9, lowering the expression of Bcl-2, and enhancing the expression of Bax (12, 16). However, all of the mechanisms behind cisplatin-induced ototoxicity have not yet been fully explained.

The Wnt signaling transduction cascade controls numerous biological activities throughout development and during adult life in animals (6). The Wnt signaling pathway is closely related to the generation and metabolism of ROS in cells, and it can play a protective role in many kinds of tissues and organs when they are subjected to oxidative stress injury (11, 23, 27, 36). Wnt signaling also plays an important role in the development of the inner ear, where it participates in otic specification, the formation of vestibular structures, and the development of the cochlea (25). Recent studies have shown that the overexpression of Wnt signaling protects against HC damage induced by neomycin in the mouse cochlea by inhibiting the accumulation of ROS in HCs (22). TP53-induced glycolysis and apoptosis regulator (TIGAR) is a metabolic enzyme that regulates ROS levels and apoptosis by maintaining the NADPH levels required to regenerate glutathione peroxidase, a pivotal intracellular antioxidant (2). TIGAR also regulates intracellular ROS in response to nutrient and metabolic stresses to prevent ROS-induced autophagy (1). Cheung *et al.* reported that activation of Wnt signaling in an intestinal adenoma model induces TIGAR expression through upregulation of the Wnt target gene Myc, which prevents the accumulation of ROS in the intestine (4). Because TIGAR has been shown to modulate cellular redox status, we felt it was relevant to investigate the expression and effect of TIGAR in cisplatin-induced SGN damage. Also, it remains unknown which regulatory network controls the expression of TIGAR in response to cisplatin exposure.

In this study, we conducted a complete investigation of the neuroprotective effects of the Wnt signaling pathway against SGN damage induced by cisplatin both *in vitro* and *in vivo* by taking advantage of Wnt agonist/antagonists and gain-of-function/loss-of-function mouse models. We found that Wnt signaling activates TIGAR and protects SGNs against cisplatin-induced damage through the suppression of oxidative stress and apoptosis in SGNs.

Results

Cisplatin treatment induces both necrosis and apoptosis in SGNs in vitro

To determine the neurotoxic effect of cisplatin on SGNs, cochlear explant cultures from P3 wild-type (WT) C57BL/6 mice were treated with 25, 50, or 150 μM of cisplatin for 48 h (Supplementary Fig. S1A). The somas of SGNs in normal control cultures were large and round or oval-shaped and had intense Tuj 1 labeling of the cytoplasm and weak labeling of the nucleus, while the peripheral auditory nerve fibers (ANFs), which project out radially from SGNs to HCs, were strongly labeled with Tuj 1 (green). The radial ANFs were organized into smooth and thick fascicles (Supplementary Fig. S1B). Treatment with 25 μM cisplatin induced minor SGN loss (Table 1), and the distal ends of some ANFs were slightly fragmented. When treated with 50 μM cisplatin, the number of SGNs was obviously decreased, and the somas of the remaining SGNs were shrunken and shriveled and their nuclei were rarely visible. Most ANFs were fragmented and pixelated with an almost complete loss of peripheral fiber ends approaching the HCs. When the concentration of cisplatin was increased to 150 μM, however, we found that the effect of cisplatin was weaker than in the 50 μM group, but stronger than in the 25 μM group, as the mean density of SGNs was greater compared with the 50 μM group but lower compared with the 25 μM group (Table 1). Because 50 μM cisplatin treatment for 48 h induced the most degenerative changes in SGNs and ANFs, these conditions were chosen for the SGN explant culture treatment in all subsequent experiments.

We used a terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay to measure cisplatin-induced apoptosis in SGNs. The cochlear explants were treated with 50 μM cisplatin for 48 h *in vitro*, and SGNs that were double labeled by Tuj 1 (green fluorescence) and TUNEL (red fluorescence) in the nuclei were considered to be apoptotic SGNs. No double-positive SGNs were detected in the control group, while significant numbers of apoptotic SGNs were observed in the cisplatin-treated groups *in vitro* ($p < 0.01$, $n = 9$) (Supplementary Fig. S1C, D, and Table 2).

Table 1. Spiral Ganglion Neuron Number Per 0.01 mm^2 in the Middle-Turn Cochlea of Wild-Type Mice *In Vitro* ($n=9$)

Cisplatin	*0* μM	*1* μM	*5* μM	*25* μM	*50* μM	*150* μM
SGN number/0.01 mm^2	55.3 ± 4.99	52.6 ± 6.92	50.3 ± 4.78	41.5 ± 7.02	28.8 ± 2.50	37.3 ± 3.61

SGN, spiral ganglion neuron.

The expression of apoptosis-related genes was used to assess the extent of cisplatin-mediated SGN apoptosis. We identified the expression changes of cleaved caspase 3 by immunostaining. After being treated with 50 μM cisplatin for 48 h, there were significantly more cleaved caspase 3/Tuj 1 double-positive SGNs in the cisplatin-treated group ($p<0.01$, $n=9$) (Supplementary Fig. S1E, F, and Table 2), suggesting that caspase 3 was activated in SGNs after cisplatin treatment *in vitro*. We further examined the apoptosis-related genes in SGNs by real-time polymerase chain reaction (RT-PCR). We found that SGNs treated with 50 μM cisplatin for 48 h had significantly higher expression of the proapoptotic genes *Caspase 3*, *Caspase 8*, *Caspase 9*, *Apaf1*, *Bax*, and *p53* compared with the control group and significantly lower mRNA expression of *Bcl-2*, which is an antiapoptotic gene (Supplementary Fig. S1G).

Cisplatin treatment induces apoptosis in SGNs and leads to hearing loss in vivo

Postnatal day (P) 30 WT mice were injected intraperitoneally (i.p.) with 3 mg/kg cisplatin daily for 7 days, and the impact of cisplatin-induced SGN death on auditory function was evaluated by measuring the auditory brainstem response (ABR) threshold and compound action potential (CAP) input/output functions in response to tone bursts (Supplementary Fig. S2A). The drug administration was chosen according to our previous report (40) that 3 mg/kg cisplatin i.p. injection for 7 days leads to significant hearing loss and SGN loss in mice. Before the cisplatin treatment, the ABR and CAP tests were performed to make sure that all of the mice had normal auditory function. After 7 days of drug treatment, the ABR thresholds of all frequencies were increased in the cisplatin-treated group compared with the control group ($p<0.05$, $n=6$) (Supplementary Fig. S2B). The CAP amplitudes measured at 90 dB sound pressure level (SPL) across all frequencies showed an ~35.3%–42.3% reduction in the cisplatin-treated group compared with the control group. The most reduced amplitude was at 16 kHz, and the amplitudes were 120.17 ± 7.51 μV and 69.3 ± 4.08 μV in the control and cisplatin groups, respectively ($p<0.05$ for 4, 8, 24, and 32 kHz; $p<0.01$ for 16 kHz, $n=6$).

The numbers of surviving SGNs in the middle-turn cochlear sections were counted in the two groups. The mean density of SGNs was reduced in the cisplatin-treated group to 33.8 ± 4.13 cells/0.01 mm^2 compared with the density of 56.1 ± 4.78 cells/0.01 mm^2 in the control group ($p<0.05$, $n=9$) (Supplementary Fig. S2D, E).

After the cisplatin treatment, immunostaining was used to measure the caspase 3 expression in SGNs *in vivo*. In the control group, no cleaved caspase 3/Tuj 1 double-positive SGNs could be observed. In the cisplatin-treated group, 21.5% ± 2.57% of the SGNs were cleaved caspase 3/Tuj 1 double positive ($p<0.001$, $n=9$) (Supplementary Fig. S2F, G).

Wnt signaling is activated in SGNs after cisplatin treatment both in vitro *and* in vivo

The Wnt/β-catenin signaling pathway has been shown to play a role in various cell processes, including cellular protection, and overexpression of Wnt signaling protects against neomycin-induced HC damage in the mouse cochlea by inhibiting the accumulation of ROS (22). To determine whether Wnt/β-catenin signaling also plays a role in SGN survival, we tested whether Wnt signaling is activated in SGNs after cisplatin treatment. β-catenin, the central player in the canonical Wnt pathway, migrates to the nucleus on Wnt activation to activate the expression of Wnt target genes. Therefore, the nuclear translocation of β-catenin and the regulation of Wnt-target gene expression represent the activation of the Wnt signaling pathway. Thus, we first measured the expression of β-catenin and Wnt target genes in the SGNs after cisplatin treatment. In *in vitro* experiments, the middle-turn cochlear and SGN explants from P3 WT mice were treated with 50 μM cisplatin for 48 h (Fig. 1A). We used an antibody against Neu N to label the SGN nuclei and the py489-β-catenin antibody to target phospho-Y489-β-catenin in the SGN nuclei (Fig. 1B). In WT control mice without cisplatin treatment, the expression of nuclear β-catenin in SGNs was almost undetectable, while the expression of nuclear β-catenin was significantly upregulated in the cisplatin-treated group (Fig. 1B). In addition, the activation level of Wnt signaling in SGNs was further confirmed by the mRNA expression of the Wnt downstream target

Table 2. Spiral Ganglion Neuron Number Per 0.01 mm^2, TUNEL-Positive SGN Percentage, and Caspase 3-Positive SGN Percentage in the Middle-Turn Cochlea of Wild-Type Mice *In Vitro* ($n=9$)

	Control	*Cisplatin*	*Cis + Wnt 3a + RS-1*	*Cis + IWP-2*
SGN number/0.01 mm^2	55.3 ± 4.99	28.8 ± 2.50	48.3 ± 4.16	17.8 ± 2.22
TUNEL+ SGN (%)	0.7 ± 0.58	20.9 ± 2.11	6.1 ± 1.43	50.9 ± 4.29
Caspase 3+ SGN (%)	1.3 ± 0.58	27.3 ± 5.53	9.8 ± 1.38	67.7 ± 7.14

RS-1, R-spondin1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling.

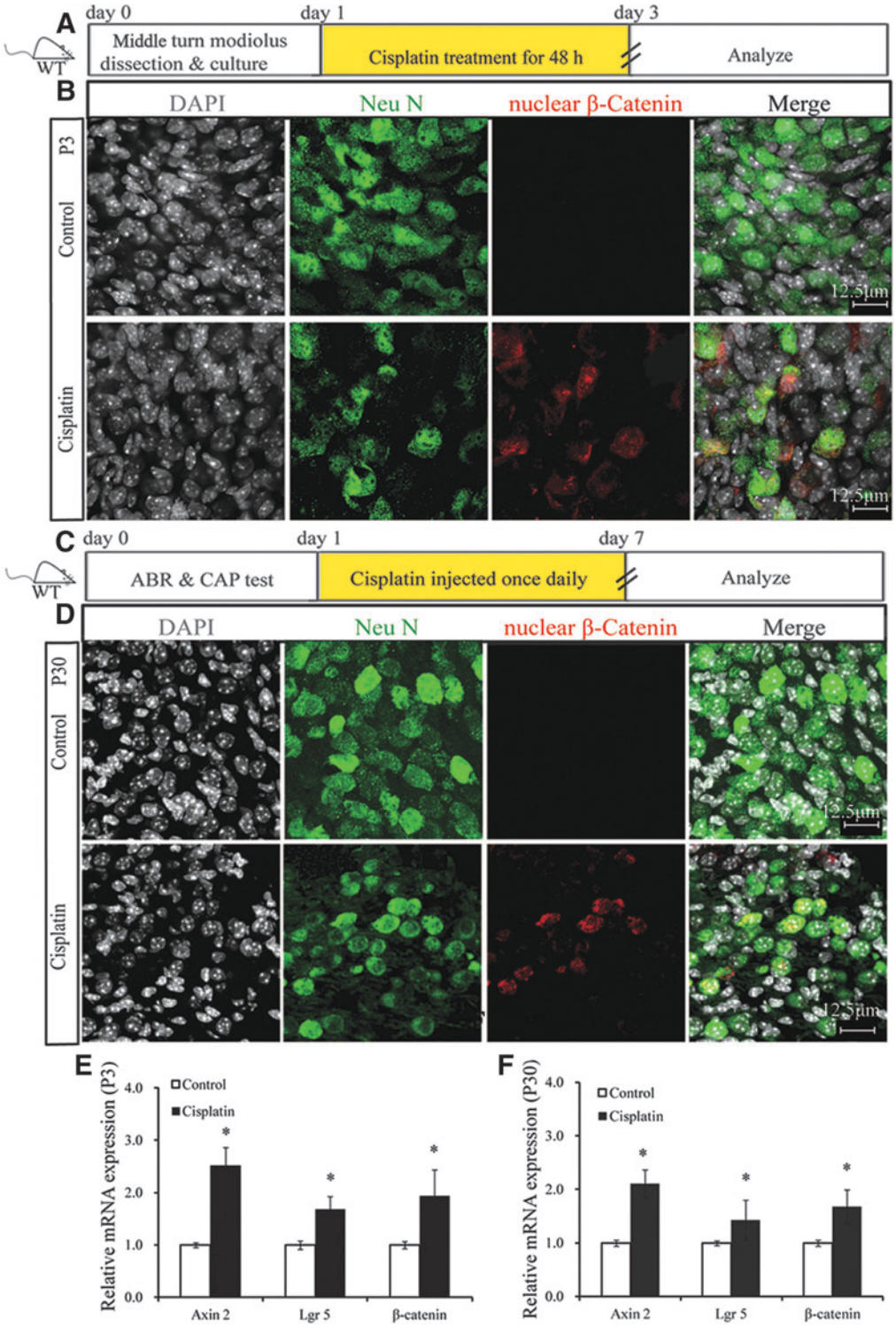


FIG. 1. The Wnt/*β*-catenin signaling pathway was activated in SGNs after cisplatin treatment both *in vitro* and *in vivo*. **(A)** The diagram of the assay for **(B**, **E)**. The middle-turn cochleae and SGNs from P3 C57BL/6 WT mice were cultured and incubated with cisplatin for 48 h and then used for immunostaining and RT-PCR. **(B)** Representative images of SGNs labeled with DAPI (*gray*), Neu N (*green*), and nuclear β-*catenin* (*red*). There was no double labeling of Neu N and nuclear *β*-catenin in SGNs in the WT control mice, whereas many of the SGNs were double labeled by Neu N and nuclear *β*-catenin in the cisplatin-treated group. **(E)** RT-PCR showed that the mRNA expression of β-*catenin*, *Axin2*, and *Lgr5* was significantly upregulated after cisplatin treatment. **(C)** The diagram of the assay for **(D**, **F)**. P30 WT C57 mice were injected with 3 mg/kg i.p. cisplatin daily for 7 days, the middle-turn cochlear section was immunostained with NeuN and py489-*β*-catenin antibody, and RT-PCR was performed to measure the mRNA expression of β-*catenin*, *Axin2*, and *Lgr5*. **(D–F)** Both immunofluorescence and RT-PCR results revealed that the expression of *β*-catenin was increased in the SGNs after cisplatin treatment *in vivo*, and the mRNA expression of *Axin2* and *Lgr5* was enhanced after cisplatin treatment. $*p < 0.05$ *versus* control group, each group, $n = 9$. ABR, auditory brainstem response; CAP, compound action potential; DAPI, 4′,6-diamidino-2-phenylindole; i.p., intraperitoneally; P, postnatal day; RT-PCR, real-time polymerase chain reaction; SGN, spiral ganglion neuron; WT, wild-type.

genes *Axin2* and *Lgr5*, both of which have been used as the reporter genes of Wnt signaling pathway in many studies (22). RT-PCR showed that the mRNA expressions of β-*catenin*, *Axin2*, and *Lgr5* were all significantly upregulated in SGNs after cisplatin treatment *in vitro* (Fig. 1E).

In *in vivo* experiments, P30 WT mice were injected with 3 mg/kg cisplatin daily for 7 days (Fig. 1C). The middle-turn cochleae of the mice were dissected out after they were sacrificed, we immunostained the cochleae with Neu N and py489-β-catenin antibodies, and we performed RT-PCR to assess the β-*catenin*, *Axin2*, and *Lgr5* mRNA expression. Both immunofluorescence and RT-PCR results revealed that the expression of β-catenin was significantly increased in the SGNs after cisplatin administration *in vivo* (Fig. 1D, F) and that the mRNA expression of *Axin2* and *Lgr5* was significantly enhanced after cisplatin treatment (Fig. 1F).

However, it is confusing why cisplatin produces hearing loss while it also activates the Wnt signaling pathway. One possible reason for this might be that lower doses of cisplatin activate the self-repair system of the cochleae by activating Wnt signaling, thus producing a self-healing injury. In testing this hypothesis, we measured the effects of low concentrations of cisplatin (1, 5 μM) on cultured SGNs. Cochlear explant cultures from P3 WT C57BL/6 mice were treated with 1 or 5 μM cisplatin for 48 h, and there was no significant difference of SGN number between the control group and cisplatin-treated groups (Supplementary Fig. S3A, B, and Table 1). Nevertheless, the expression of nuclear β-catenin was detected in both the 1 and 5 μM cisplatin-treated groups, indicating that Wnt signaling was also activated in cells that are spared apoptosis under the low-dose cisplatin treatment condition (Supplementary Fig. S3C). Together, these results suggest that Wnt signaling was activated in SGNs after cisplatin treatment and might play a role in protecting SGNs against cisplatin damage.

Wnt signaling protects against cisplatin-induced SGN loss both in vitro *and* in vivo

To explore how Wnt/β-catenin protects SGNs against damage induced by cisplatin, Wnt agonists (Wnt-3a and R-spondin1 [RS-1]), a Wnt antagonist (IWP-2), and gain-of-function/loss-of-function mouse models were used to activate or suppress Wnt signaling both *in vitro* and *in vivo*. First, we tested the efficiency of the β-catenin gain-of-function and loss-of-function mouse model with Bhlhb5-cre mice, β-catenin overexpression (OE), and β-catenin knockout (KO) mice (Supplementary Fig. S4A). Bhlhb5-cre mice are in a C57BL/6 background and express Cre recombinase under the Bhlhb5 promoter. When crossed with β-catenin-OE or β-catenin-KO transgenic mice, which have LoxP sites flanking exon 3 or exons 2–6 of β-catenin, respectively, Cre-induced recombination with these LoxP sites results in increased levels of β-catenin or β-catenin-null alleles, respectively, thus specifically upregulating or downregulating the expression of β-catenin in the SGNs of the mouse cochlea. Immunostaining showed that there was no nuclear β-catenin expression in Bhlhb5-cre control mice or Bhlhb5; β-catenin KO mice, while intense β-catenin expression was seen in the nuclei of SGNs in Bhlhb5; β-catenin OE mice (Supplementary Fig. S4B). The activation of Wnt signaling in SGNs was determined by measuring the mRNA levels of Wnt downstream target genes by RT-PCR. The mRNA expression levels of β-*catenin*, *Axin2*, and *Lgr5* were all significantly increased in SGNs from Bhlhb5; β-catenin OE mice and were all decreased in Bhlhb5; β-catenin KO mice compared with Bhlhb5-cre control mice without cisplatin treatment (Supplementary Fig. S4C). These results revealed that the Wnt signaling pathway was activated in SGNs in Bhlhb5; β-catenin OE mice and suppressed in Bhlhb5; β-catenin KO mice.

In *in vitro* experiments, the middle-turn cochlear explants from P3 WT mice were treated with 50 μM cisplatin, 50 μM cisplatin together with Wnt agonists (200 ng/mL Wnt-3a and 1 μg/mL RS-1), or 50 μM cisplatin together with a Wnt antagonist (1 μM IWP-2) for 48 h (Fig. 2A). Tuj 1 staining showed that the Cis + Wnt-3a + RS-1 group had significantly more surviving SGNs compared with the cisplatin-treated control group, while the Cis + IWP-2 group had significantly fewer surviving SGNs compared with the cisplatin-treated control group ($p < 0.05$, $n = 9$) (Fig. 2B; Table 2). Next, in a separate *in vitro* explant culture experiment, we cultured middle-turn cochlear explants from Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice in the presence or absence of 50 μM cisplatin for 48 h (Fig. 2C). Without cisplatin there was no decrease in SGN number in any of the groups (data not shown). With cisplatin treatment, the Bhlhb5; β-catenin OE cochlear explants had significantly more surviving SGNs compared with the Bhlhb5-cre group, and Bhlhb5; β-catenin KO explants had significantly fewer surviving SGNs ($p < 0.05$, $n = 9$ for all groups) (Fig. 2D; Table 3), suggesting that Wnt/β-catenin signaling could promote SGN survival after cisplatin exposure *in vitro*. However, when we added Wnt 3a+ RS-1 to SGNs 48 h after cisplatin treatment to test whether a Wnt agonist can promote SGN survival after cisplatin administration, there was no significant difference in surviving SGN numbers between the cisplatin group and the Wnt 3a+ RS-1-cis group (Supplementary Fig. S3D, E).

In *in vivo* experiments, transgenic Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice were injected daily with 3 mg/kg cisplatin for 7 days starting at P30. We then measured hearing function in the mice by ABR and CAP and then dissected out the middle-turn cochleae for immunostaining (Fig. 2E). In Bhlhb5; β-catenin OE mice, the ABR thresholds were only slightly lower than the Bhlhb5-cre control mice ($p = 0.061$), but the CAP amplitudes were higher than the Bhlhb5-cre control mice ($p < 0.05$, $n = 9$) (Fig. 2F, G). In the Bhlhb5; β-catenin KO mice, the ABR thresholds were significantly increased while the CAP amplitudes were significantly decreased compared with Bhlhb5-cre controls ($p < 0.05$, $n = 9$) (Fig. 2F, G). Immunostaining showed that Bhlhb5; β-catenin OE mice had significantly more surviving SGNs in the middle turn of the cochlea compared with the Bhlhb5-cre controls and that Bhlhb5; β-catenin KO mice had significantly fewer surviving SGNs ($p < 0.05$, $n = 9$) (Fig. 2H; Table 4). Therefore, these results demonstrated that activation of the Wnt signaling pathway contributes to the survival of SGNs after cisplatin treatment and reduces the level of hearing loss, while inhibition of Wnt signaling makes SGNs more vulnerable to the ototoxicity induced by cisplatin and leads to more severe hearing loss.

Wnt regulates caspase expression in SGNs as a result of cisplatin treatment both in vitro *and* in vivo

We further investigated the effect of Wnt/β-catenin signaling on the apoptosis of SGNs induced by cisplatin

in vitro. Cochlear explants treated with cisplatin together with Wnt agonists (200 ng/mL Wnt-3a and 1 μg/mL RS-1) had fewer TUNEL/Tuj 1 double-positive SGNs and caspase 3/Tuj 1 double-positive SGNs compared with the cisplatin-treated control group, while SGN explants treated with cisplatin together with Wnt antagonist (1 μM IWP-2) had significantly more TUNEL/Tuj 1 double-positive and caspase 3/Tuj 1 double-positive SGNs in the middle turns ($p < 0.05$, $n = 9$) (Fig. 3A–E; Table 2). Moreover, RT-PCR data indicated that the mRNA levels of the proapoptotic genes *Caspase 3*, *Caspase 8*, *Caspase 9*, *Bax*, *p53*, and *Apaf1* were significantly decreased in the Cis + Wnt-3a + RS-1 group and were significantly increased in the Cis + IWP-2 group compared with the cisplatin-only group (Fig. 3F). Next, we investigated the protective effect of β-catenin against cisplatin injury using cochlear explants obtained from Bhlhb5; β-catenin OE and Bhlhb5; β-catenin KO transgenic mice. In these experiments, we found that after cisplatin treatment *in vitro*, there were significantly fewer TUNEL/Tuj 1 double-positive SGNs and caspase 3/Tuj 1 double-positive SGNs in the Bhlhb5; β-catenin OE cochlear explants, while Bhlhb5; β-catenin KO cochlear explants had more TUNEL/Tuj 1 double-positive SGNs and caspase 3/Tuj 1 double-positive SGNs in the middle turns compared with the Bhlhb5-cre controls ($p < 0.05$, $n = 9$) (Fig. 3G–K; Table 3). RT-PCR data also showed that the mRNA expression of proapoptotic genes was decreased in Bhlhb5; β-catenin OE cochleae and increased in Bhlhb5; β-catenin KO cochleae compared with Bhlhb5-cre controls (Fig. 3L).

We then investigated the effect of Wnt signaling on the caspase-associated apoptosis that was induced by cisplatin injection *in vivo*. Transgenic Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice were given daily injections of 3 mg/kg cisplatin for 7 days starting at P30 (Fig. 4A). Immunostaining showed that Bhlhb5; β-catenin OE cochleae had very few caspase 3/Tuj 1 double-positive SGNs compared with the Bhlhb5-cre controls, while Bhlhb5; β-catenin KO cochleae had more caspase 3/Tuj 1 double-positive SGNs in cultured middle turns ($p < 0.05$, $n = 9$) (Fig. 4B, C; Table 4). We also performed RT-PCR and observed that the mRNA expression of proapoptotic genes was significantly reduced in the Bhlhb5; β-catenin OE cochleae and significantly enhanced in the Bhlhb5; β-catenin KO mice compared with Bhlhb5-cre controls (Fig. 4D) Therefore, these results demonstrated that activation of Wnt/β-catenin inhibits cisplatin-induced SGN apoptosis, while KO of β-catenin enhances cisplatin-induced SGN apoptosis both *in vitro* and *in vivo*.

Wnt increases TIGAR expression after cisplatin treatment

A previous report showed that in the mouse intestine, Wnt/β-catenin activates the antiapoptotic factor TIGAR (4), and TIGAR has been shown to protect against apoptosis by preventing the accumulation of ROS (4, 5, 18, 21). Here, we first investigated TIGAR expression in cisplatin-treated SGNs by *in vitro* and *in vivo* immunostaining. In *in vitro* experiments, we found that TIGAR was expressed in P3-cultured control SGNs, and after cisplatin treatment for 48 h the TIGAR expression was significantly enhanced, even though the SGN number decreased, which indicated that TIGAR signaling was activated in response to cisplatin-induced SGN damage *in vitro* (Fig. 5A, B; for original blots see Supplementary Fig. S9A). SGNs had much stronger TIGAR expression in the Cis + Wnt-3a + RS-1 group and in Bhlhb5; β-catenin OE cochleae, while TIGAR expression in the Cis + IWP-2 group and in Bhlhb5; β-catenin KO cochleae was significantly inhibited (Fig. 5A, B). In *in vivo* experiments, we performed immunostaining after 7 days of daily i.p. injections of 3 mg/kg cisplatin in P30 transgenic mice (Fig. 5C). Immunostaining showed that TIGAR was also expressed in P30 mouse SGNs. Consistent with the P3-cultured cochlea, the cisplatin treatment also strengthened the expression of TIGAR in the adult mouse SGNs, and TIGAR expression was significantly increased in the Bhlhb5; β-catenin OE mice and significantly decreased in the Bhlhb5; β-catenin KO mice after cisplatin treatment (Fig. 5D).

Western blot and RT-PCR were used to verify the *in vitro* changes in TIGAR expression in SGNs. We found that TIGAR expression was increased after cisplatin treatment and became much stronger when cotreated with Wnt-3a and RS-1

FIG. 2. Wnt/β-catenin signaling promoted SGN survival after cisplatin-induced damage *in vitro* and *in vivo*. (A) The diagram of the assay for **(B)**. The middle-turn cochlear and SGN explants from P3 WT mice were cultured *in vitro*, and the explants were treated with cisplatin (50 μM), cisplatin (50 μM) + Wnt-3a (200 ng/mL) + RS-1 (1 μg/mL), or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h. **(B)** Tuj 1 staining (*green*) showed that the Cis + Wnt-3a + RS-1 group had significantly more surviving SGNs than the cisplatin-only group, while in the Cis + IWP-2 group there were significantly fewer surviving SGNs compared with the cisplatin-only group. **(C)** The diagram of the assay for **(D)**. The middle-turn cochleae and SGNs were dissected out from P3 Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice and cultured *in vitro* with 50 μM cisplatin for 48 h. **(D)** After cisplatin treatment, Bhlhb5; β-catenin OE middle-turn cochlear explants had significantly more surviving SGNs compared with the Bhlhb5-cre group, while the Bhlhb5; β-catenin KO explants had significantly fewer surviving SGNs. **(E)** The diagram of the assay for **(F–H)**. P30 transgenic Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice first underwent ABR and CAP testing and then were given daily injections of cisplatin at 3 mg/kg i.p. for 7 days. Hearing function was again measured by ABR and CAP before mice were sacrificed and the middle-turn cochleae were dissected out for immunostaining. **(F, G)** In Bhlhb5; β-catenin OE mice, the ABR thresholds were slightly lower than the Bhlhb5-cre control mice, but the CAP amplitudes were significantly higher than the Bhlhb5-cre control mice. In Bhlhb5-cre; β-catenin KO mice, the ABR thresholds were significantly increased and the CAP amplitudes were significantly decreased compared with the Bhlhb5-cre controls. **(H)** Immunostaining showed that Bhlhb5; β-catenin OE mice had significantly more surviving SGNs in the middle turn of the cochleae, while the loss of SGNs was significantly greater in the Bhlhb5; β-catenin KO mice compared with the Bhlhb5-cre controls. $^*p < 0.05$ *versus* the cisplatin-treated group. Cis, cisplatin. Each group, $n = 9$. KO, knockout; OE, overexpression; RS-1, R-spondin1.

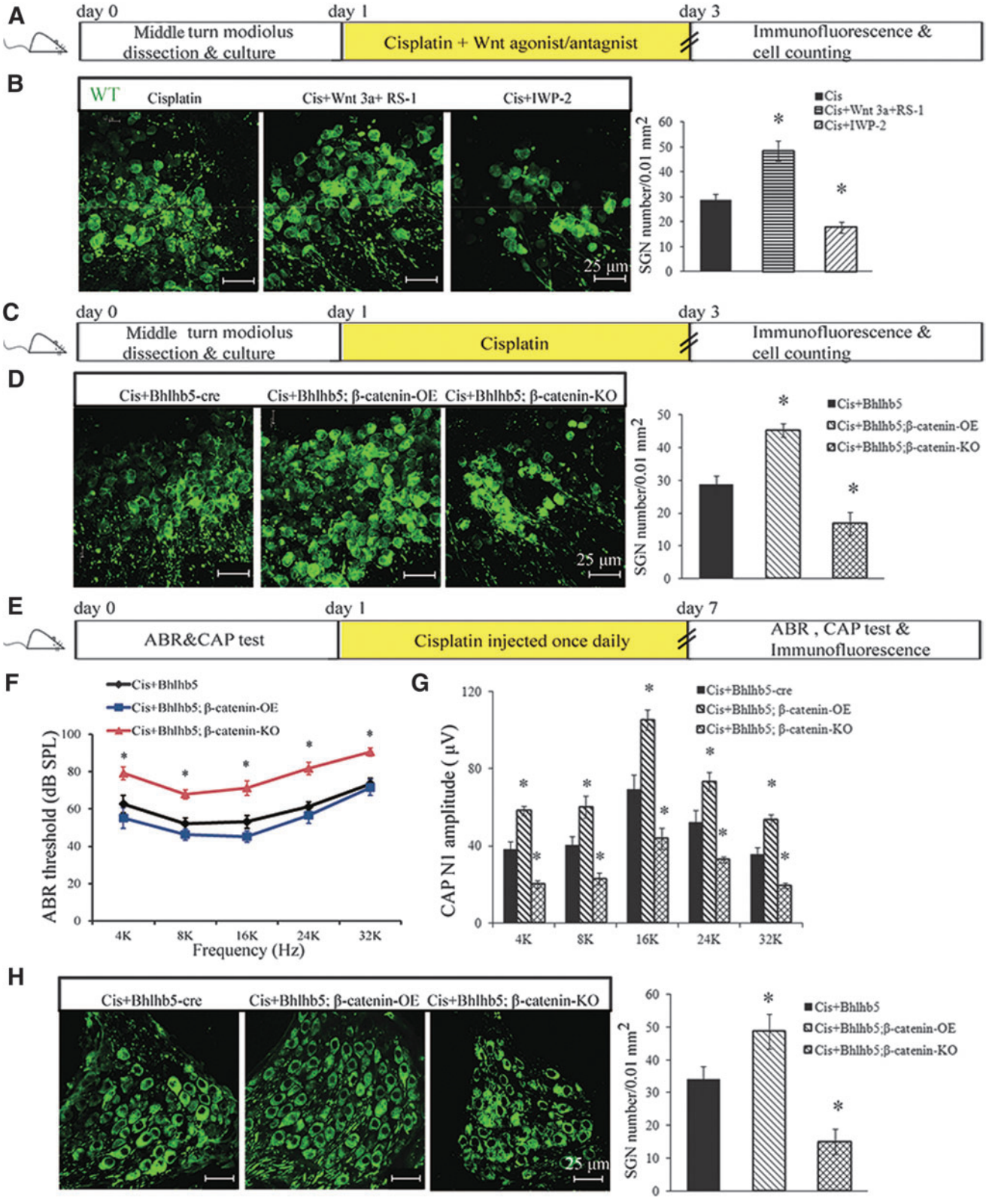


Table 3. Spiral Ganglion Neuron Number Per 0.01 mm^2, TUNEL-Positive SGN Percentage, and Caspase 3-Positive SGN Percentage in the Middle-Turn Cochlea of Transgenic Mice *In Vitro* ($n=9$)

	Bhlhb5-cre	*Cis + Bhlhb 5-cre*	*Cis + Bhlhb5; β-catenin-OE*	*Cis + Bhlhb5; β-catenin-KO*
SGN number/0.01 mm^2	57.2 ± 3.27	27.5 ± 4.15	45.3 ± 2.08	16.8 ± 3.59
TUNEL+ SGN (%)	0.6 ± 0.31	22.1 ± 1.91	5.0 ± 1.13	57.9 ± 3.73
Caspase 3+ SGN (%)	1.0 ± 0.43	25.4 ± 3.53	12.4 ± 1.81	55.7 ± 6.71

KO, knockout; OE, overexpression.

and in Bhlhb5; β-catenin OE cochleae and was significantly decreased when cotreated with IWP-2 and in Bhlhb5; β-catenin KO cochleae compared with the cisplatin-only controls (Fig. 5E, F and Supplementary Fig. S4).

Myc is a key transcriptional target of the Wnt signaling pathway and has been reported to mediate many cellular responses to Wnt signaling (32, 34). It has been reported that TIGAR expression is modulated by Wnt signaling through activation of Myc, but not as a direct response to β-catenin/T cell factor (TCF) activity. Thus, we further examined the changes of Myc expression in our experimental conditions to examine the possible mechanism underlying the regulation of Wnt/β-catenin on TIGAR expression. We investigated mRNA and protein expression levels of Myc by RT-PCR and Western blot, respectively, after the P3 middle-turn cochleae had been cultured and treated with cisplatin *in vitro*. We found that the expression of Myc was significantly upregulated after cisplatin treatment in SGNs, and the protein and mRNA levels of Myc were significantly increased in the Cis + Wnt-3a + RS-1 group and in Bhlhb5; β-catenin OE cochleae compared with the controls after cisplatin administration, while the expression of Myc was markedly decreased in the Cis + IWP-2 group and in Bhlhb5; β-catenin KO cochleae compared with the controls after cisplatin treatment (Fig. 5E, F). Taken together, all of these results suggested that the expression of TIGAR in SGNs is regulated by Wnt/β-catenin signaling after cisplatin treatment, and the induction of TIGAR might be a response to the activation of Myc.

Wnt signaling regulates reduced ROS levels in SGNs after cisplatin treatment

Cisplatin-induced formation of ROS is closely related to SGN apoptosis in the mouse cochlea (28), and previous studies reported that TIGAR can prevent ROS accumulation and thus protect against apoptosis induced by oxidative stress (4, 18, 21, 43). Also, Wnt/β-catenin has been shown to protect many organs, including the cochlea, against apoptosis that is induced by oxidative stress (11, 22, 23, 27, 36). In this study, to investigate the regulation of Wnt signaling on the ROS level in SGNs after cisplatin treatment, we performed the Wnt/β-catenin activation or inhibition experiments *in vitro* (Fig. 6A, D). MitoSOX Red, a redox fluorophore that signals the presence of mitochondrial superoxide, was used to evaluate the generation of mitochondrial ROS in SGNs after cisplatin treatment. Without cisplatin treatment, no MitoSOX/Tuj 1 double-positive SGNs were found in the control group, whereas a significant number of MitoSOX/Tuj 1 double-positive SGNs were observed after 48 h of cisplatin treatment *in vitro* ($p<0.05$, $n=9$) (Fig. 6A–C; Table 5). Moreover, treatment with Wnt3a and RS-1 significantly reduced the number of cisplatin-induced MitoSOX/Tuj 1 double-positive SGNs in the cultured middle-turn cochleae, while treatment with IWP-2 significantly increased the number of MitoSOX/Tuj 1 double-positive SGNs ($p<0.05$, $n=9$) (Fig. 6A–C; Table 5). The regulatory effects of Wnt signaling on cisplatin-induced ROS accumulation in SGNs using Bhlhb5; β-catenin OE and β-catenin KO transgenic mice have been investigated (Fig. 6D). Consistent with the above results, after cisplatin treatment *in vitro*, a significant number of MitoSOX/Tuj 1 double-positive SGNs were found in the Bhlhb5-cre cochleae. The number of MitoSOX/Tuj 1 double-positive SGNs in Bhlhb5; β-catenin OE cochleae was significantly lower, while Bhlhb5; β-catenin KO cochleae had more MitoSOX/Tuj 1 double-positive SGNs compared with the Bhlhb5-cre group after cisplatin administration ($p<0.05$, $n=9$) (Fig. 6E, F; Table 5). All of these results demonstrated that cisplatin-induced ROS accumulation in SGNs was significantly inhibited *in vitro* by the activation of Wnt signaling or upregulation of β-catenin, and markedly increased by Wnt inhibition.

Moreover, to test whether TIGAR is the effector of Wnt signaling and can protect SGNs from ROS accumulation and apoptosis after cisplatin treatment, we constructed an adenovirus (Ad) that overexpressed TIGAR (Ad-TIGAR) and used it to transfect SGNs exposed to cisplatin. First, the adenovirus vector Ad-GFP was used to evaluate the transfection efficiency, and after transfection with 5×10^7 PFU/mL Ad-GFP and culture for 72 h, the green fluorescence of GFP was found in SGNs (Fig. 7B), while no SGN death was found and the number of surviving SGNs was not significantly different from that in the normal control SGNs (Fig. 7C). Furthermore, the specific activity of TIGAR in SGNs was detected by Western blot, and the protein expression of TIGAR was increased 2.14 ± 0.08-folds in SGNs after transfection with Ad-TIGAR and culture for 72 h

Table 4. Spiral Ganglion Neuron Number Per 0.01 mm^2 and Caspase 3-Positive SGN Percentage in the Middle-Turn Cochlea of Transgenic Mice *In Vitro* ($n=9$)

	Bhlhb5-cre	*Cis + Bhlhb 5-cre*	*Cis + Bhlhb5; β-catenin-OE*	*Cis + Bhlhb5; β-catenin-KO*
SGN number/0.01 mm^2	54.3 ± 5.56	34.3 ± 3.77	48.8 ± 5.32	15.1 ± 3.82
Caspase 3+ SGN (%)	0.4 ± 0.08	21.5 ± 2.57	4.0 ± 1.62	41.2 ± 3.05

compared with normal control group (Fig. 7D; for original blots see Supplementary Fig. S9B). In the next experiment, 5×10^7 PFU/mL Ad-TIGAR was added to the cultured cochleae. The transfection medium was replaced 24 h later with normal medium, and the cochleae were then further treated with cisplatin (50 μM) or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h and harvested for immunostaining and counting analysis. As illustrated in Figure 7E–I, the numbers of MitoSOX/Tuj 1 double-positive SGNs (Fig. 7E, G) and caspase 3/Tuj 1 double-positive SGNs (Fig. 7F, H) were significantly decreased, and the number of surviving SGNs (Fig. 7I) was significantly increased in the Cis + Ad-TIGAR group compared with the cisplatin group ($p < 0.05$, $n = 9$), and more importantly in the Cis + IWP-2 + Ad-TIGAR group compared with the Cis + IWP-2 group ($p < 0.05$, $n = 9$) (Fig. 7; Table 6). This indicated that TIGAR OE could reduce ROS accumulation and decrease caspase 3 expression, as well as increase the survival of SGNs after cisplatin treatment, even when Wnt signaling was inhibited by IWP-2.

Antioxidant treatment rescues SGN loss induced by β-catenin deficiency after cisplatin treatment

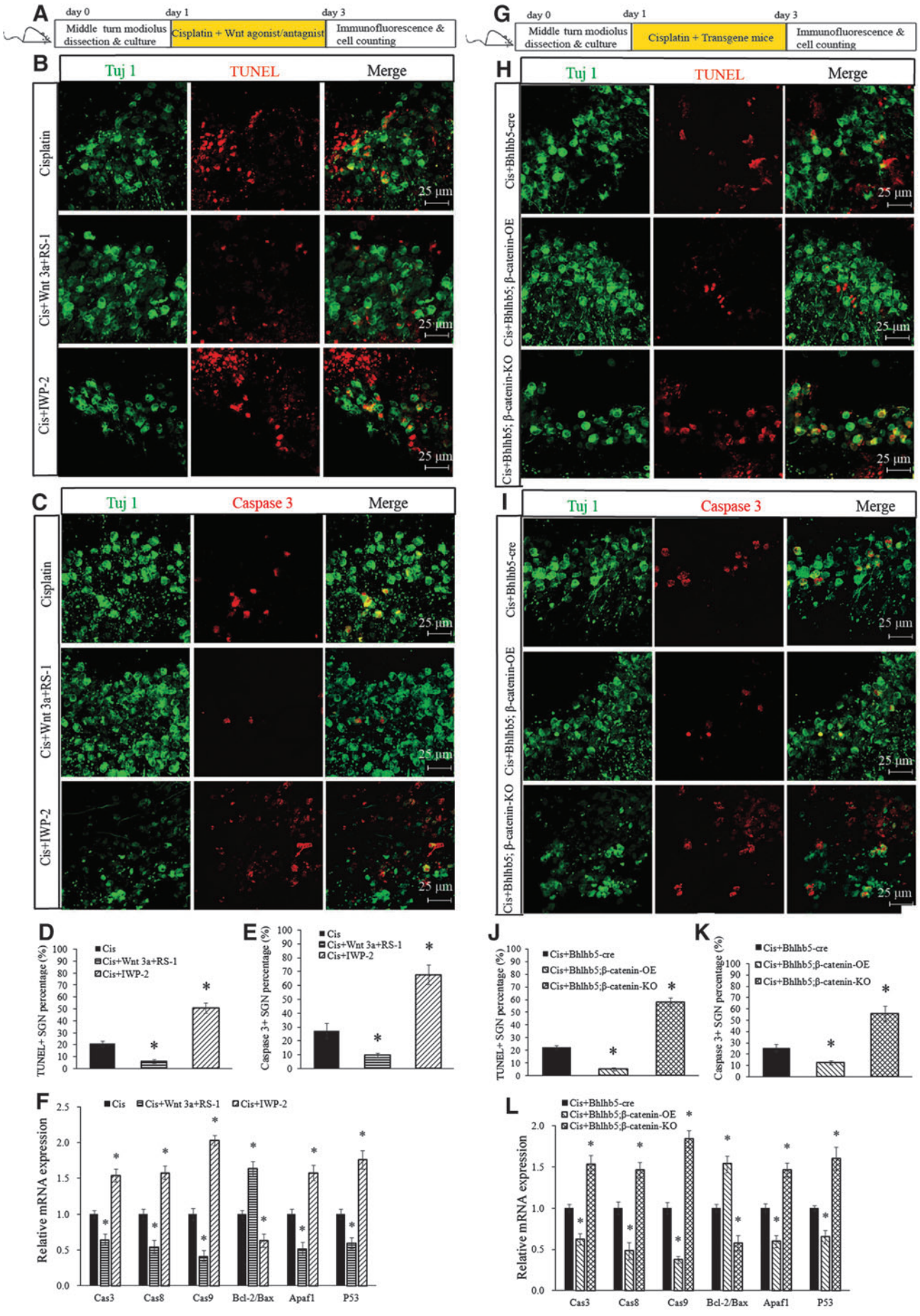
To further investigate whether increased ROS levels contribute to the severity of cisplatin-induced injury in β-catenin-deficient SGNs, the middle turns of the cochleae were cotreated with 2 mM of the antioxidant *N*-acetylcysteine (NAC), a direct scavenger of reactive oxygen intermediates and a provider of reduced glutathione, after cisplatin treatment and Wnt inhibition (Fig. 8A, C). After NAC treatment, MitoSOX Red immunohistochemistry showed significantly decreased numbers of MitoSOX/Tuj 1 double-positive SGNs in both the cisplatin-only group and the Cis + IWP-2 group ($p < 0.05$, $n = 9$) (Fig. 8B, E; Table 5). Moreover, after NAC treatment, the numbers of surviving SGNs were significantly greater in both the cisplatin-only group and the Cis + IWP-2 group ($p < 0.05$, $n = 9$) (Fig. 8B, F; Table 6). The number of surviving SGNs was not significantly different between the cisplatin-only group and the Cis + IWP-2 group when treated with NAC (Fig. 8B, F; Table 7), suggesting that NAC treatment successfully rescued SGN loss induced by Wnt inhibition after cisplatin administration. We used Bhlhb5; β-catenin KO transgenic mice to verify this finding in a separate *in vitro* cochlear explant culture experiment. Consistent with the results above, we found that NAC treatment significantly decreased the ROS level and increased the number of surviving SGNs in the cochleae of both Bhlhb5-cre and Bhlhb5; β-catenin KO mice after cisplatin treatment ($p < 0.05$, $n = 9$) (Fig. 8D–F; Tables 5 and 6), and there was only a slight difference in the number of surviving SGNs between the Bhlhb5-cre and Bhlhb5; β-catenin KO mice (Fig. 8D, F; Table 7).

Moreover, to test if NAC rescued the SGNs by inhibiting caspase-associated apoptosis, we further measured caspase 3 expression in NAC-treated SGNs after cisplatin exposure. Immunostaining results showed that NAC administration reduced the number of caspase 3/Tuj 1 double-positive SGNs after exposure to cisplatin even when Wnt signaling was inhibited by IWP-2 ($p < 0.05$, $n = 9$) (Fig. 9; Table 8). Together, these results indicated that Wnt signaling protects against cisplatin-induced SGN damage by inhibiting ROS accumulation and the subsequent oxidative stress-induced apoptosis in SGNs.

Wnt activation protects SGNs against cisplatin treatment in the basal turn of the cochlea

Because the greatest damage produced by cisplatin is reported to be localized to the basal region of the cochlea (40), we also performed experiments to investigate the protective effects of Wnt signaling in the basal turn of cochlea both *in vitro* and *in vivo*. The Wnt agonist treatment procedures were the same as described above. Immunostaining and cell counting showed that the basal turn of the cochlear explants treated with 50 μM cisplatin together with Wnt agonists (200 ng/mL Wnt-3a and 1 μg/mL RS-1) had significantly greater SGN survival compared with the cisplatin-treated control group ($p < 0.05$, $n = 9$), while the basal turn of cochlear explants treated with cisplatin together with Wnt antagonist (1 μM IWP-2) had significantly fewer surviving SGNs ($p < 0.05$, $n = 9$) (Fig. S5B, F). Moreover, there were significantly more SGNs in the basal turn of cultured Bhlhb5; β-catenin OE cochleae, and markedly fewer SGNs in the basal turn of Bhlhb5; β-catenin KO cochleae compared with that of control cochleae after treatment with cisplatin *in vitro*

FIG. 3. Wnt regulates caspase expression in SGNs as a result of cisplatin treatment both *in vitro* and *in vivo*. **(A)** The diagram of the assay for **(B–F)**. The middle-turn cochlear and SGN explants from P3 WT mice were cultured *in vitro* and incubated with cisplatin (50 μM), cisplatin (50 μM) + Wnt-3a (200 ng/mL) + RS-1 (1 μg/mL), or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h before immunostaining and RT-PCR. **(B–E)** After cisplatin treatment, the SGN explants treated with cisplatin and Wnt-3a and RS-1 had significantly fewer TUNEL/Tuj 1 and caspase 3/Tuj 1 double-positive SGNs compared with the cisplatin-only group, whereas the cisplatin and IWP-2 cotreated cochleae had significantly more TUNEL/Tuj 1 and caspase 3/Tuj 1 double-positive SGNs in the cultured middle turns. **(F)** RT-PCR showed that the expression of the proapoptotic genes *Caspase 3*, *Caspase 8*, *Caspase 9*, *Apaf1*, *p53*, and *Bax* was significantly reduced in the Cis + Wnt-3a + RS-1 explants compared with the cisplatin-only controls and was significantly increased in the Cis + IWP-2 SGN explants. **(G)** The diagram of the assay for **(H–L)**. *T*he middle-turn cochleae and SGN explants from P3 Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice were cultured *in vitro* and treated with 50 μM cisplatin for 48 h. **(H–K)** After cisplatin treatment *in vitro*, there were significantly fewer TUNEL/Tuj 1 and caspase 3/Tuj 1 double-positive SGNs in the Bhlhb5; β-catenin OE group compared with Bhlhb5-cre controls, while Bhlhb5; β-catenin KO cochlear explants had significantly more TUNEL/Tuj 1 and caspase 3/Tuj 1 double-positive SGNs. **(I)** RT-PCR showed that the mRNA expression of *Caspase 3*, *Caspase 8*, *Caspase 9*, *Apaf1*, *p53*, and *Bax* was significantly decreased in Bhlhb5; β-catenin OE cochleae compared with control groups and was significantly enhanced in Bhlhb5; β-catenin KO mice. *$p < 0.05$ *versus* the cisplatin-only controls. Cis, cisplatin; Cas 3, caspase 3; Cas 8, caspase 8; Cas 9, caspase 9. Each group, $n = 9$. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling.



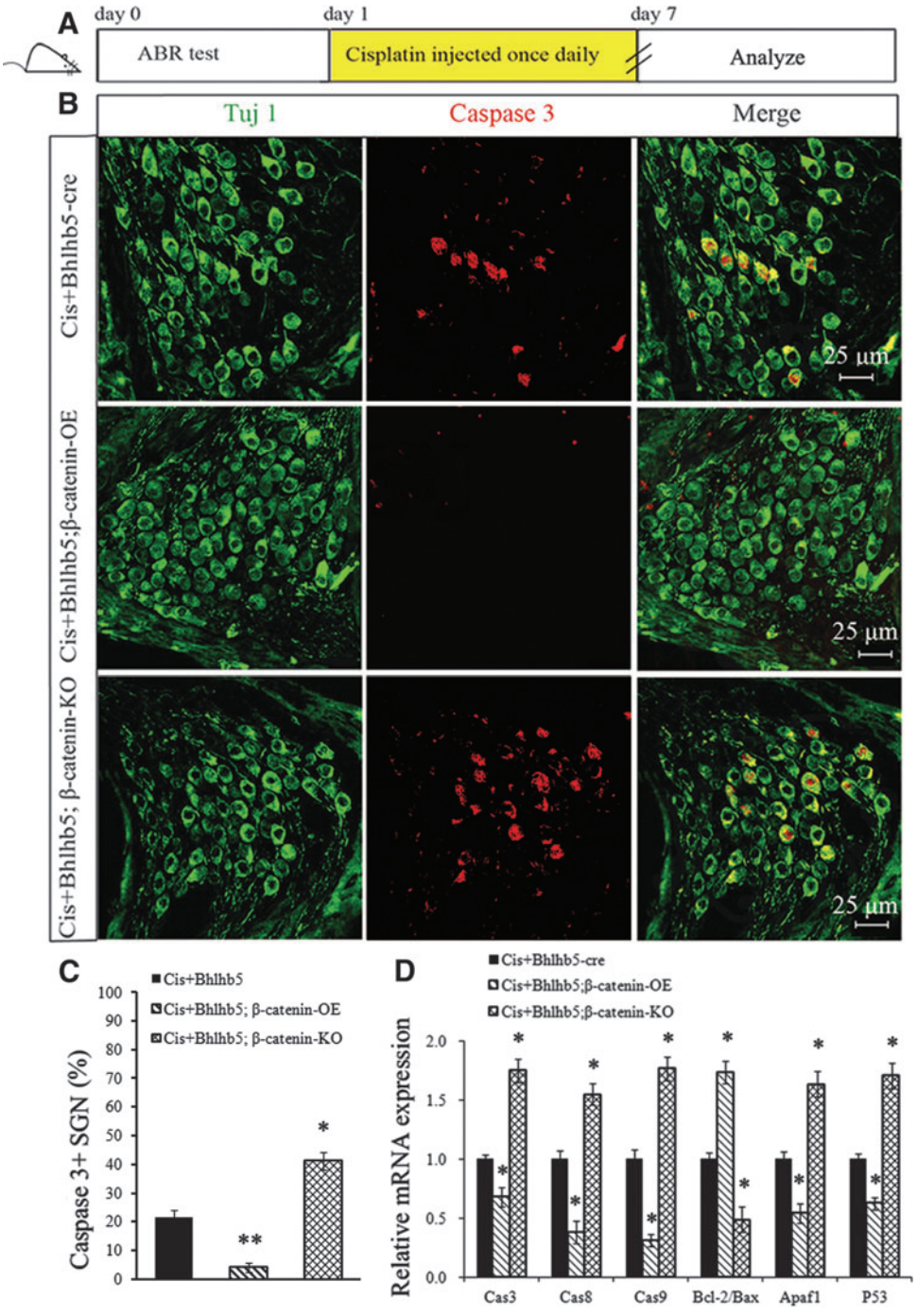


FIG. 4. The effect of Wnt/β-catenin signaling on the SGN apoptosis induced by cisplatin administration *in vivo*. Transgenic Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice were injected i.p. with 3 mg/kg cisplatin daily for 7 days starting at P30. **(A)** The diagram of the assay for **(B–D)**. **(B, C)** Immunostaining and statistical analysis showed that Bhlhb5; β-catenin OE cochleae had very few caspase 3/Tuj 1 double-positive SGNs in the middle turn of the cochlea compared with the Bhlhb5-cre control mice, while Bhlhb5; β-catenin KO cochleae had observably more caspase 3/Tuj 1 double-positive SGNs in the middle turn. **(D)** RT-PCR showed that the mRNA expression of Caspase 3, Caspsase 8, Caspase 9, Apaf1, p53, and Bax was significantly decreased in Bhlhb5; b-catenin OE mice cochleae compared with control groups and was significantly enhanced in Bhlhb5;b-catenin KO mice. $*p<0.05$, $**p<0.01$ *versus* cisplatin-treated Bhlhb5-cre mice. Each group, $n=9$.

($p<0.05$, $n=9$) (Supplementary Fig. S5C, G). The *in vivo* procedures were also the same as those described above; transgenic mice were injected daily with 3 mg/kg cisplatin for 7 days starting at P30. We also found significantly more SGNs in the basal turn of Bhlhb5; β-catenin OE cochleae, and markedly fewer SGNs in the basal turn of Bhlhb5; β-catenin KO cochleae compared with that of the control Bhlhb5-cre cochleae *in vivo* ($p<0.05$, $n=9$ for cell counting) (Supplementary Fig. S5E, H).

Furthermore, consistent with the results observed in the SGNs of the middle turn of the cochlea, the Cis + Wnt-3a + RS-1 group had significantly fewer TUNEL/Tuj 1 double-positive SGNs (Supplementary Fig. S6B, D) and caspase 3/Tuj 1 double-positive SGNs (Supplementary Fig. S6C, E), greater TIGAR expression (Supplementary Fig. S7), and fewer MitoSOX Red/Tuj 1 double-positive SGNs (Supplementary Fig. S8) in the basal turn of the cochlea compared with the cisplatin-treated control group ($p<0.05$, $n=9$). The Cis + IWP-2 group had significantly more TUNEL/Tuj 1 double-positive SGNs (Supplementary Fig. S6B, D) and caspase 3/Tuj 1 double-positive SGNs (Supplementary Fig. S6C, E), less TIGAR expression (Supplementary Fig. S7), and more MitoSOX Red/Tuj 1 double-positive SGNs (Supplementary Fig. S8) compared with the cisplatin-treated control group ($p<0.05$, $n=9$). The results together indicated that Wnt signaling also has protective effects on basal-turn SGNs against cisplatin treatment both *in vitro* and *in vivo*.

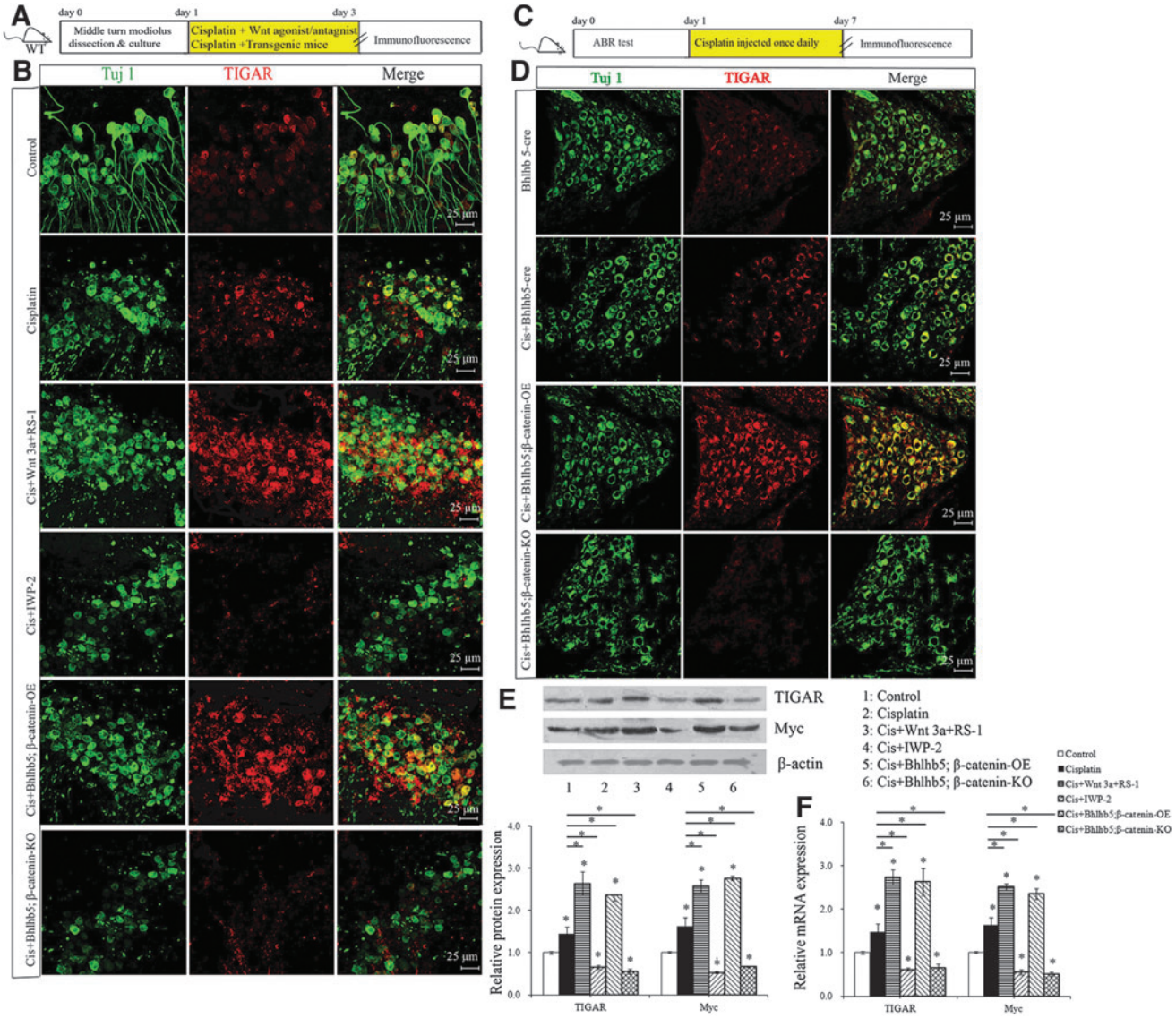


FIG. 5. Wnt signaling increases TIGAR expression after cisplatin treatment. **(A)** The diagram of the assay for **(B**, **E**, **F)**. The middle-turn cochleae and SGN explants from P3 WT mice or transgenic mice were cultured *in vitro*. The explants from WT mice were treated with cisplatin (50 μM), cisplatin (50 μM) + Wnt-3a (200 ng/mL) + RS-1 (1 μg/mL), or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h, and the explants from the Bhlhb5; β-catenin OE and Bhlhb5; β-catenin KO mice were treated with cisplatin (50 μM) for 48 h. **(B)** TIGAR staining was observed in P3-cultured control SGNs, and TIGAR staining was enhanced after cisplatin treatment for 48 h. In the WT cochleae cotreated with Wnt 3a and RS-1 and in the Bhlhb5; β-catenin OE cochleae, the SGNs had much stronger TIGAR staining intensity, whereas in the WT cochleae cotreated with IWP-2 and in Bhlhb5; β-catenin KO cochleae the TIGAR staining in SGNs was very faint. **(C)** The diagram of the assay for **(D)**. P30 Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice were injected with 3 mg/kg cisplatin daily for 7 days, and then, the middle-turn cochlear sections were dissected out for immunostaining. **(D)** The fluorescence staining of TIGAR was positive in P30 mouse SGNs, and cisplatin increased the expression of TIGAR in the adult mouse SGNs. The TIGAR staining was much stronger in the Bhlhb5; β-catenin OE cochleae and much weaker in the Bhlhb5; β-catenin KO cochleae after cisplatin treatment. **(E, F)** Western blot and RT-PCR showing that the protein and mRNA expressions of both TIGAR and Myc were significantly increased after cisplatin treatment and became much stronger in WT cochleae cotreated with Wnt-3a + RS-1 and in Bhlhb5; β-catenin OE cochleae but significantly decreased in cochleae from IWP-2-treated WT mice and Bhlhb5; β-catenin KO mice compared with the cisplatin-only groups. $*p < 0.05$. Each group, $n = 9$. TIGAR, TP53-induced glycolysis and apoptosis regulator.

Discussion

Finding ways to protect SGNs against cisplatin-associated injury is a very important clinical problem. Here, consistent with previous observations (8, 12), we also found that SGNs underwent massive degeneration in response to cisplatin treatment *in vitro* and *in vivo*, as demonstrated by reduced cell numbers, increased apoptosis, increased expression of apoptosis-related factors in SGNs, and impaired auditory function (Supplementary Figs. S1 and S2). It is worth noting that when we tested three

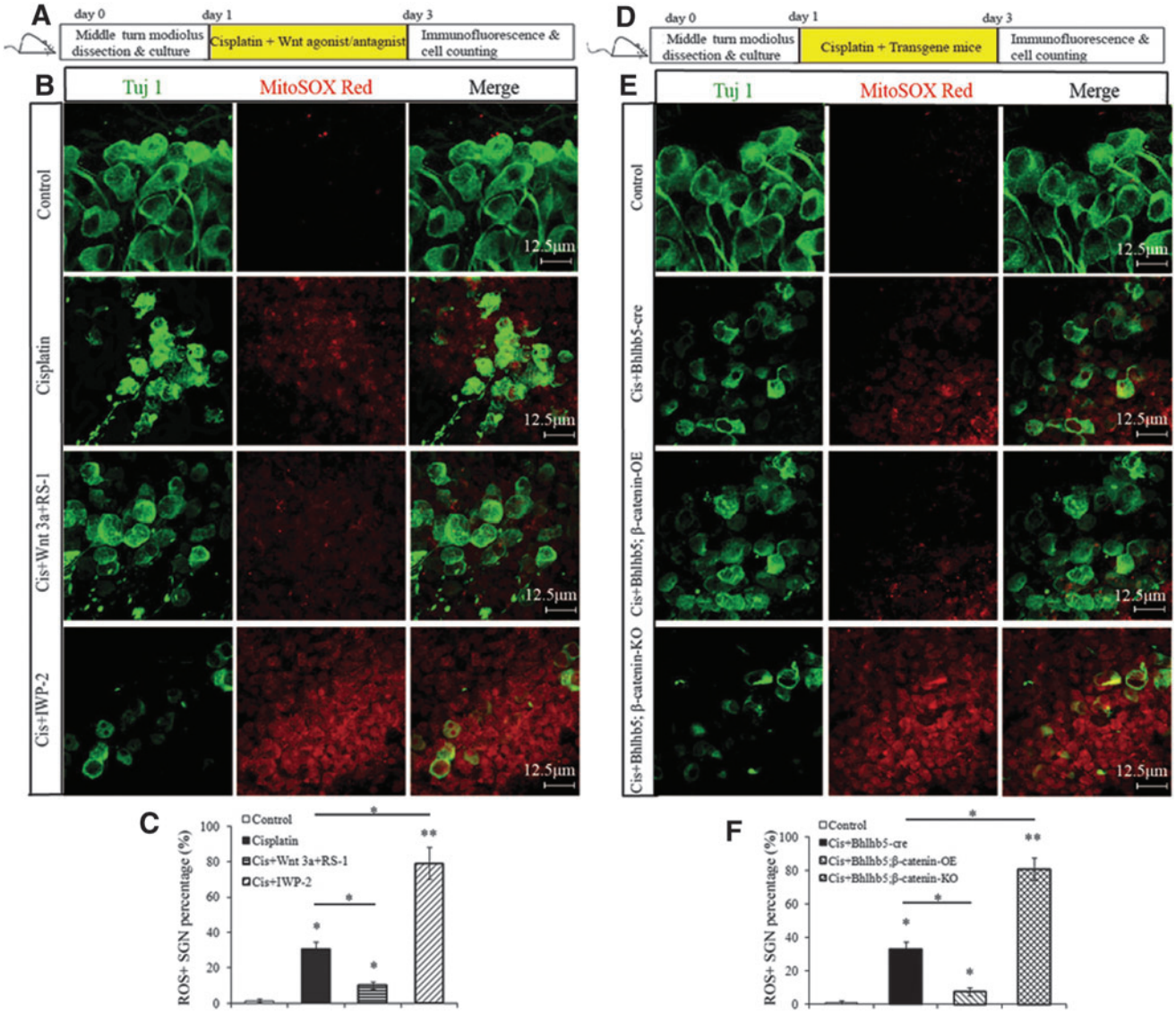


FIG. 6. Wnt signaling reduces ROS levels in SGNs after cisplatin treatment. **(A)** The diagram of the assay for **(B, C)**. The middle-turn cochleae and SGN explants from P3 WT mice were cultured *in vitro*, and incubated with cisplatin (50 μM), cisplatin (50 μM) + Wnt-3a (200 ng/mL) + RS-1 (1 μg/mL), or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h. **(B, C)** Representative images showing that no MitoSOX (*red*)/Tuj 1 (*green*) double-positive SGNs were found in the normal control group, whereas significant numbers of MitoSOX/Tuj 1 double-positive SGNs were observed in the cisplatin-treated groups. Moreover, cotreatment with Wnt-3a and RS-1 reduced the number of cisplatin-induced MitoSOX/Tuj 1 double-positive SGNs in the middle-turn cochlea, while cotreatment with IWP-2 significantly increased the number of MitoSOX/Tuj 1 double-positive SGNs. **(D)** The diagram of the assay for **(E, F)**. The middle-turn cochleae of P3 Bhlhb5-cre, Bhlhb5; β-catenin OE, and Bhlhb5; β-catenin KO mice were cultured *in vitro* and incubated with 50 μM cisplatin for 48 h. **(E, F)** Bhlhb5; β-catenin OE cochleae had remarkably fewer MitoSOX/Tuj 1 double-positive SGNs compared with the Bhlhb5-cre group after cisplatin administration, and Bhlhb5; β-catenin KO cochleae had significantly more MitoSOX/Tuj 1 double-positive SGNs. $^*p < 0.05$, $^{**}p < 0.01$. Each group, $n = 9$. ROS, reactive oxygen species.

concentrations of cisplatin (25, 50, 150 μM) in the *in vitro* experiments, the 150 μM cisplatin had a milder effect compared with 50 μM (Supplementary Fig. S1B and Table 1), which corresponded with a previous study showing that the cytotoxic efficacy of high concentrations of cisplatin is reduced in SGNs and HCs compared with low concentrations (9). That work showed that the minimal HC loss observed at high concentrations coincided with limited intracellular accumulation of cisplatin, but the exact mechanism responsible for this resistance is still unclear. We speculate that it might be because of the intrinsic resistance of SGNs to high levels of extracellular cisplatin, and thus, high concentrations of cisplatin do not accumulate intracellularly to sufficient concentrations to cause SGN injury (9, 20), and this hypothesis should be tested in future studies.

In this study, we show that Wnt signaling is activated in SGNs after cisplatin exposure both *in vitro* and *in vivo* (Fig. 1) and even under lower concentrations of cisplatin (1, 5 μM) treatment *in vitro*, in which there is no significant SGN loss (Supplementary Fig. S3). We speculate that the low-dose cisplatin might produce a self-healing injury in the cochlea by

Table 5. MitoSOX/Tuj 1 Double-Positive Spiral Ganglion Neuron Percentage in the Middle-Turn Cochlea *In Vitro* ($n=9$)

Group	*MitoSOX+ /Tuj 1+ SGN (%)*
Control	1.5 ± 1.00
Cisplatin	30.9 ± 3.78
Cis + Wnt 3a + RS-1	10.1 ± 1.90
Cis + IWP-2	78.9 ± 9.01
Bhlhb5-cre	1.3 ± 0.81
Cis + Bhlhb 5-cre	33.2 ± 4.14
Cis + Bhlhb5; β-catenin-OE	7. 6 ± 2.49
Cis + Bhlhb5; β-catenin-KO	81.1 ± 6.52
Cis + NAC	8.1 ± 2.93
Cis + IWP-2 + NAC	10.6 ± 3.18
Cis + Bhlhb5; β-catenin-KO + NAC	11.3 ± 3.59

NAC, *N*-acetylcysteine.

activating Wnt signaling, but with the high-dose cisplatin (25, 50, or 150 μM) treatment, this self-protection effect is not sufficient to resist the cisplatin-induced injury, and it might need to be augmented in advance of the insult to be effective. A large body of work has demonstrated that Wnt signaling participates in cell survival, proliferation, apoptosis inhibition, and cellular protection (6, 11, 19), and the canonical Wnt/β-catenin signaling pathway has been shown to be important for the development of the mouse cochlea, including HC regeneration and cell differentiation (25). In addition, Liu *et al.* reported that Wnt signaling protects HCs from apoptosis after neomycin damage by regulating forkhead box O3 transcription factor expression, antioxidant enzyme expression, and ROS levels in HCs (22). However, whether Wnt/β-catenin signaling is essential for SGN survival is not known. In the present study, we used both Wnt agonist/antagonists and transgenic mice with either OE or KO of β-catenin, and thus, Wnt signaling in SGNs (Supplementary Fig. S4) to thoroughly examine the effect of Wnt/β-catenin signaling on SGNs after exposure to cisplatin. We found that Wnt signaling overactivation protects against cisplatin-induced SGN loss and that inhibition of Wnt/β-catenin makes the SGNs more sensitive to cisplatin-induced ototoxicity both *in vitro* and *in vivo* (Fig. 2). Moreover, in our adult transgenic mouse experiment, the ABR thresholds of all frequencies were not significantly changed in the cisplatin-treated Bhlhb5; β-catenin OE mice, which corresponded to the reduced SGN loss in these mice, but the CAP amplitudes were significantly reduced compared with the Bhlhb5-cre mice. In the Bhlhb5; β-catenin KO mice, cisplatin caused not only an increase in ABR thresholds but also a decrease in CAP amplitudes compared with the Bhlhb5-cre mice (Fig. 2). This difference is due to the fact that ABR tests the whole auditory transduction pathway, including the HCs, the auditory neurons, and the auditory cortex, but the auditory CAP amplitudes mainly test the function of the auditory nerve, and thus, Wnt activation only significantly increased the CAP amplitude and did not significantly decrease the ABR threshold.

In attempting to determine the mechanisms underlying the protection provided by Wnt signaling against cisplatin-induced SGN damage, we found that Wnt/β-catenin prevented the SGN apoptosis induced by cisplatin injury both *in vitro* and *in vivo* (Figs. 3 and 4). Previous studies have demonstrated that cisplatin causes the death of auditory cells through activation of the mitochondrial apoptotic pathway (8, 12, 40). The proapoptotic protein Bax changes the permeability of mitochondrial membranes and causes the release of cytochrome *c* and activated caspase 9, in turn activating the downstream caspase 3 by forming cleaved caspase 3, which induces apoptosis. The Bcl-2 family of proteins prevents apoptosis by binding to Bax protein. Our TUNEL and immunostaining experiments showed that activation of Wnt/β-catenin signaling significantly reduced the number of TUNEL-positive SGNs and reduced caspase 3-mediated apoptosis in SGNs after cisplatin exposure. Furthermore, Wnt/β-catenin signaling downregulated the expression of *Caspase 8*, *Apaf 1*, *Caspase 9*, *Caspase 3*, and *Bax* and increased the expression of *Bcl-2*, suggesting that Wnt/β-catenin intervenes in the mitochondria-initiated cell death-signaling pathway to protect SGNs against cisplatin ototoxicity.

Another major finding of this study was that TIGAR, an antiapoptotic factor, was expressed both in the neonatal and adult mouse SGNs and that this expression was significantly enhanced after cisplatin exposure, thus limiting its injury both *in vitro* and *in vivo* (Fig. 5) and indicating that TIGAR might be involved in cisplatin injury. More importantly, we found that TIGAR expression in SGNs was upregulated by Wnt/β-catenin activation after cisplatin treatment (Fig. 5), suggesting that TIGAR might be regulated by Wnt signaling

FIG. 7. TIGAR overexpression protects SGNs from cisplatin treatment when Wnt signaling is inhibited. (A) The diagram of the assays. The middle-turn cochleae and SGN explants from P3 WT mice were cultured *in vitro.* Ad-TIGAR was diluted 1:1000 in EIS to a final concentration of 5×10^7 PFU/mL and added to the cochleae. The transfection medium was replaced 24 h later with normal medium and the cochleae were further cultured for 48 h for transfection efficiency test and TIGAR protein expression test (**B**–**D**). Or the cochleae were then treated with cisplatin (50 μM) or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h after being replaced with normal media for the cisplatin treatment test (**E**–**I**). **(B)** The transfection efficiency test with adenovirus vector Ad-GFP transfection showed that after 5×10^7 PFU/mL Ad-GFP transfection, the *green* fluorescence of GFP was found in SGNs, **(C)** while no SGN death was found, and the number of surviving SGNs was not significantly different from that in the normal control SGNs. **(D)** The specific activity of TIGAR in SGNs was detected by Western blot, and the protein expression of TIGAR was increased 2.14 ± 0.08-folds in SGNs after transfection with Ad-TIGAR and culture for 72 h compared with normal control group. (**E**–**I**) The 5×10^7 PFU/mL adenovirus Ad-TIGAR was added to the cultured cochleae, the transfection medium was replaced 24 h later with normal medium, and cochleae were further treated with cisplatin (50 μM) or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h. **(E, G)** The numbers of MitoSOX/Tuj 1 double-positive SGNs and **(F, H) c**aspase 3/Tuj 1 double-positive SGNs were significantly decreased. **(I)** The number of surviving SGNs was significantly increased in the Cis + Ad-TIGAR group compared with the cisplatin group, and more importantly in the Cis + IWP-2 + Ad-TIGAR group compared with the Cis + IWP-2 group. $^*p<0.05$. Each group, $n=9$. Ad, adenovirus; EIS, enhanced infection solution; PFU, plaque forming unit

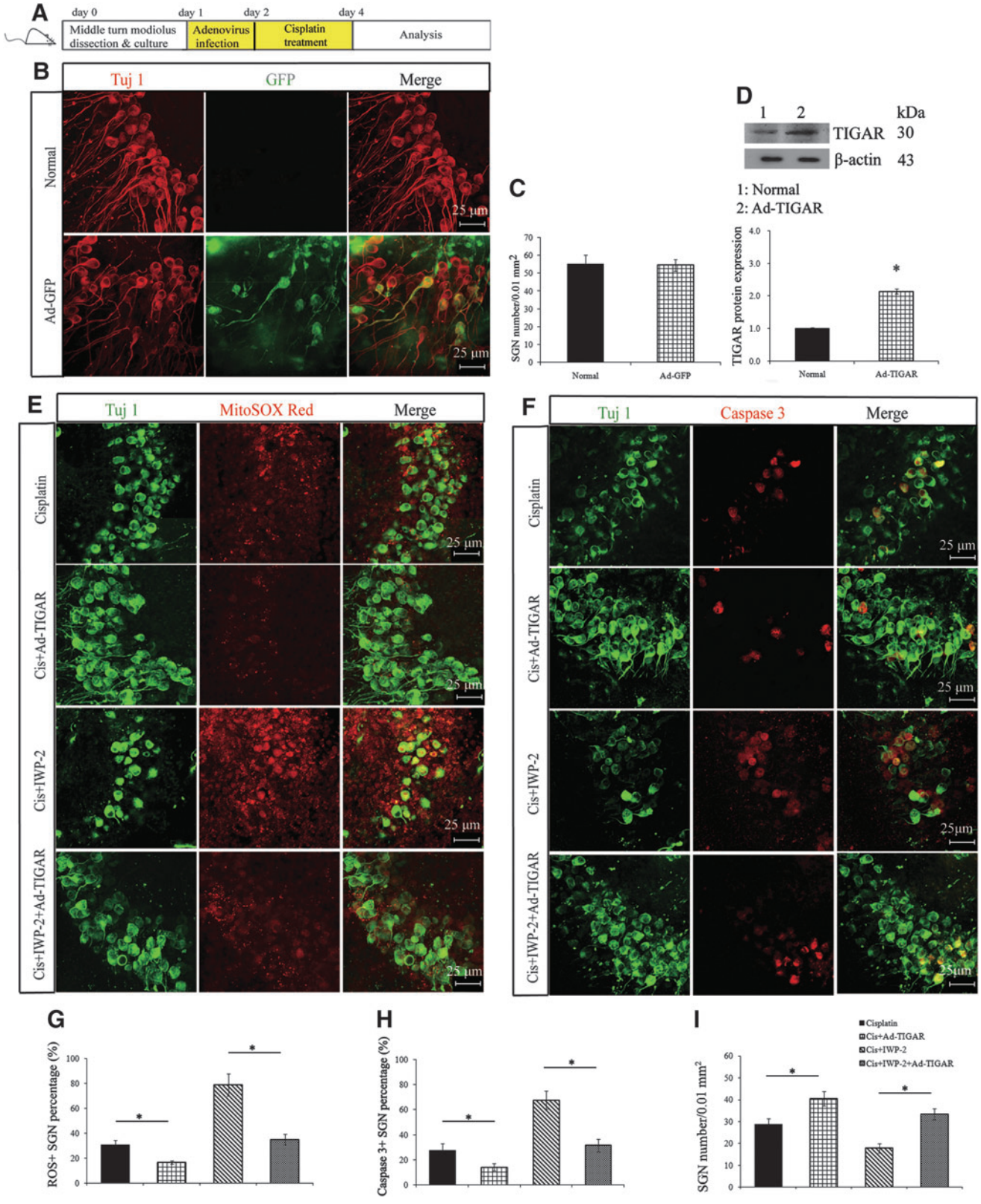


Table 6. Spiral Ganglion Neuron Number Per 0.01 mm^2, MitoSOX/Tuj 1 Double-Positive SGN Percentage, and Caspase 3-Positive SGN Percentage in the Middle-Turn Cochlea of Wild-Type Mice *In Vitro* ($n=9$)

	Cisplatin	*Cis + Ad-TIGAR*	*Cis + IWP-2*	*Cis + IWP-2 + Ad-TIGAR*
SGN number/0.01 mm^2	28.8 ± 2.50	40.5 ± 3.28	17.8 ± 2.22	33.4 ± 2.59
MitoSOX+ SGN (%)	30.9 ± 3.78	16.8 ± 1.43	78.9 ± 9.01	55.2 ± 4.18
Caspase 3+ SGN (%)	27.3 ± 5.53	14.2 ± 2.85	67.7 ± 7.14	41.5 ± 5.20

Ad, adenovirus; TIGAR, TP53-induced glycolysis and apoptosis regulator.

to play a role in cisplatin-treated SGNs. However, the mechanism for how Wnt signaling regulates the expression of TIGAR in SGNs is still unclear. According to Cheung *et al.*, TIGAR expression is regulated by Wnt signaling as a direct response to the activation of Myc, which is a known Wnt/β-catenin/TCF pathway target gene (4, 21). The absence of Myc in their experiments did not prevent the activation of Wnt, but it did prevent the induction of TIGAR in crypts (21). In another lung cancer model, Myc activation and substantial upregulation of TIGAR were observed in the premalignant lesions compared with the adjacent normal lung tissue (26). In our present work, Western blot and RT-PCR data also showed that changes in Myc expression went together with changes in TIGAR expression in SGNs after cisplatin treatment and in response to Wnt signaling regulation (Fig. 5), suggesting that Myc might also regulate the expression of TIGAR. However, further investigation of the role of Myc in the regulation of TIGAR expression still needs to be performed, preferably in transgenic mice in which Myc expression in SGNs is regulated.

TIGAR was first reported as a P53-inducible regulator of glycolysis and apoptosis (2, 13, 41). The expression of TIGAR reduces fructose-2,6-bisphosphate levels in cells, leading to the inhibition of glycolysis and an overall decline in intracellular ROS levels and the maintenance of cell survival (5, 21, 41), and it protects against neuron injury by preserving mitochondrial function (21). These functions of TIGAR protect cells from ROS-associated apoptosis (18, 21, 43).Therefore, we speculated that the activation of TIGAR might, in part, reflect the enhanced levels of ROS in SGNs after cisplatin exposure and that TIGAR might play a neuroprotective role because accumulation of cisplatin in the tissues of the cochlea can cause ROS production and damage to inner ear cells through the oxidative stress pathway (28, 29). To verify this speculation directly, we examined the ROS levels in SGNs after cisplatin injury and found that cisplatin significantly increased mitochondrial ROS levels in SGNs and that cisplatin-induced ROS accumulation in SGNs was significantly inhibited by the activation of Wnt signaling and significantly increased by Wnt inhibition (Fig. 6), which indicated that Wnt/β-catenin controls ROS levels in SGNs and therefore protects SGNs against cisplatin treatment. To further explore whether the increased ROS levels contribute to the aggravated cisplatin-induced damage in β-catenin-deficient SGNs, we tested the effect of antioxidant treatment. We showed that NAC successfully reduced ROS accumulation, decreased caspase 3 expression, and reduced the SGN loss in β-catenin-deficient mice (Figs. 8 and 9), suggesting that Wnt signaling might protect against cisplatin-induced SGN injury by inhibiting ROS accumulation and the subsequent oxidative stress-induced cell apoptosis in SGNs. Moreover, OE of TIGAR by adenovirus could reduce ROS accumulation, decrease caspase 3 expression, and increase the survival of SGN after cisplatin treatment, even when Wnt signaling was inhibited by IWP-2 (Fig. 7). Therefore, we conclude that Wnt signaling regulates the susceptibility of SGNs to cisplatin treatment *via* the regulation of TIGAR expression and accumulated ROS. However, the specific role and mechanism of TIGAR in regulating oxidative stress in SGNs need to be further investigated in the future.

In summary, we show in this study that Wnt/β-catenin signaling is activated in SGNs after cisplatin treatment, and the overactivation of Wnt signaling pathway protects against SGN loss after exposure to cisplatin, whereas the suppression of Wnt signaling in SGNs makes them more vulnerable to cisplatin treatment. We demonstrated that activation of Wnt/β-catenin signaling in SGNs upregulates TIGAR expression together with inhibition of ROS accumulation and oxidative stress-induced apoptosis, thereby protecting SGNs against caspase-associated apoptosis after cisplatin exposure. Our findings indicate that Wnt signaling is requisite in protecting SGNs from cisplatin-induced damage and thus might offer novel therapeutic targets for the prevention of cisplatin-induced SGN death.

Materials and Methods

Mouse models and genotyping

C57BL/6 WT mice and transgenic mice in the C57BL/6 background were used. Bhlhb5-cre mice (10) were generously provided by Dr. Lin Gan (University of Rochester, New York), β-catenin-OE mice (14) were the kind gift of Mark Taketo (Kyoto University, Kyoto, Japan), and β-catenin-KO mice (3) were bought from The Jackson Laboratory (Stock # 004152). The Bhlhb5 gene is only expressed in the SGNs and not in HCs or glia cells in the cochleae of Bhlhb5-cre mice (30), as shown in our previous report (42). The genotyping for all transgenic mice with PCR was performed using genomic DNA from tail tips incubated in 70 μL 50 m*M* NaOH at 98°C for 1 h, followed by neutralization in 7 μL 1 *M* HCl. The genotyping primers are listed in Table 9. All animal procedures were performed according to protocols approved by the Animal Care Committee of Shandong University, China (No. ECAESDUSM 20123011), and were consistent with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to prevent their suffering.

Organotypic culture of neonatal mouse cochleae and drug treatment

C57BL/6 WT mice or transgenic mice were decapitated at P3, and we collected only the segment isolated from the middle cochlear turn to ensure consistency. Tissue dissection

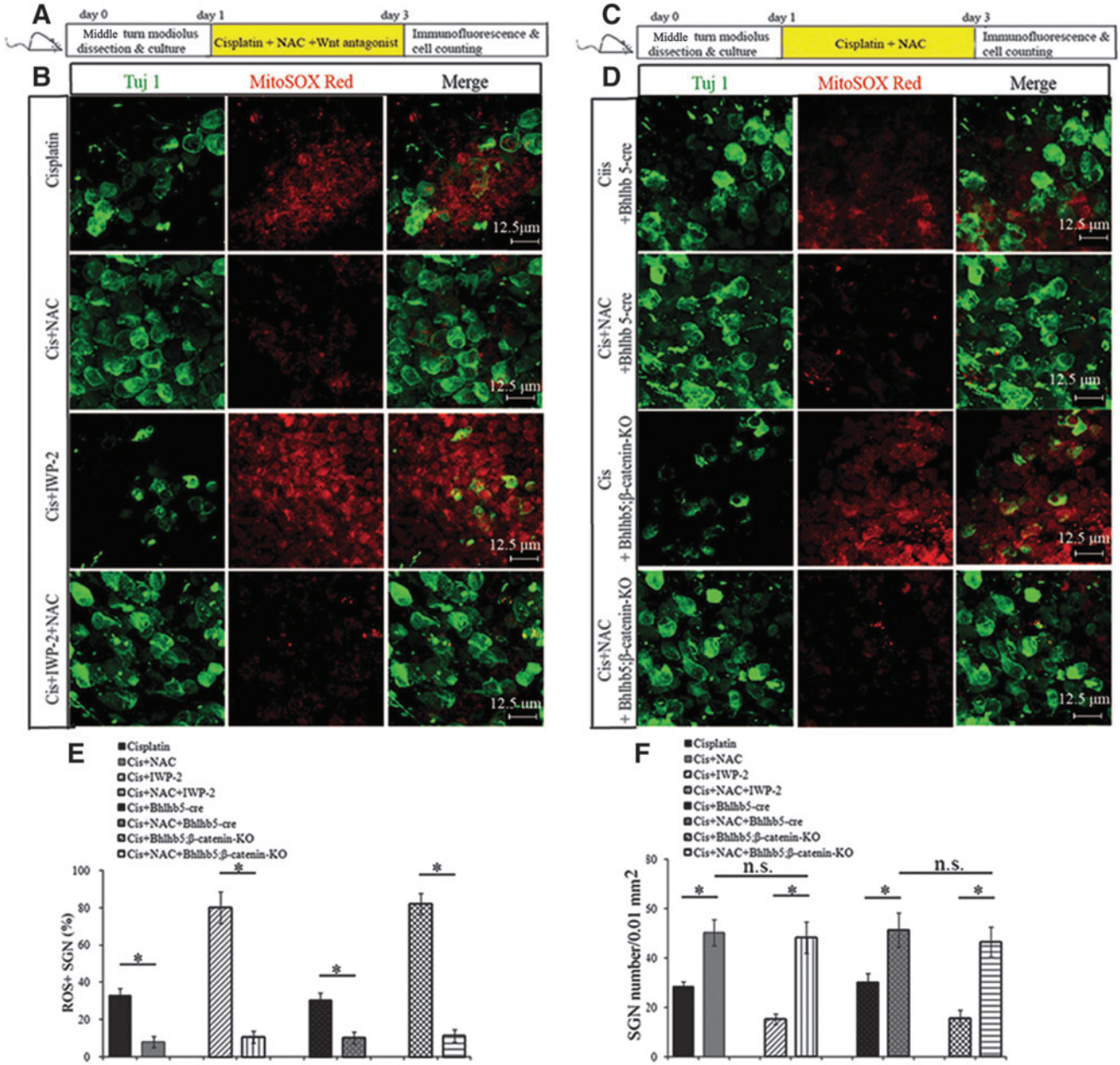


FIG. 8. Antioxidant treatment rescued the severe SGN loss induced by *β*-catenin deficiency after cisplatin treatment. (A) The diagram of the assay for **(B**, **E**, **F)**. The middle-turn cochleae from P3 WT mice were dissected out and cultured *in vitro* with cisplatin (50 μM), cisplatin (50 μM) + NAC (2 mM), or cisplatin (50 μM) + IWP-2 (1 μM) + NAC (2 mM) for 48 h. **(B)** MitoSOX Red immunohistochemistry showed that the *red* fluorescence staining of MitoSOX Red was greatly reduced in both the Cis + NAC group and the Cis + NAC + IWP-2 group compared with the cisplatin-only group and the Cis + IWP-2 group. **(C)** The diagram of the assay for **(D–F)**. The middle-turn cochleae from P3 Bhlhb5-cre and Bhlhb5; *β*-catenin KO mice were dissected out and cultured *in vitro* with cisplatin (50 μM) or cisplatin (50 μM) + NAC (2 mM) for 48 h. **(D)** NAC treatment significantly inhibited the ROS level in both Bhlhb5-cre and Bhlhb5; *β*-catenin KO mice after cisplatin administration. **(E**, **F)** Statistical analysis showed that NAC administration significantly decreased MitoSOX/Tuj 1 double-positive SGN numbers and significantly reduced SGN loss in the cisplatin-only cochlea, Cis + IWP-2 cochlea, Bhlhb5-cre cochlea, and Bhlhb5; *β*-catenin KO cochlea compared with the groups without NAC treatment after cisplatin administration. The numbers of surviving SGNs were not significantly different between the Cis + NAC group and Cis + NAC-IWP-2 group or between the Cis + NAC + Bhlhb5-cre group and Cis + NAC + Bhlhb5; *β*-catenin KO group $*p < 0.05$; n.s., no significant difference. Each group, $n = 9$. Cis, cisplatin; NAC, *N*-acetylcysteine.

Table 7. Spiral Ganglion Neuron Number Per 0.01 mm^2 in the Middle-Turn Cochlea *In Vitro* ($N=9$)

Group	*SGN number/ 0.01 mm^2*
Cisplatin	28.4 ± 2.15
Cis + NAC	50.2 ± 5.36
Cis + IWP-2	15.3 ± 2.22
Cis + IWP-2 + NAC	48.2 ± 6.32
Cis + Bhlhb5; β-catenin-KO	15.6 ± 3.16
Cis + Bhlhb5; β-catenin-KO + NAC	46.6 ± 5.91

procedures were performed as described in previous studies with some slight modifications (35, 42). Briefly, the cochlear capsule was removed from the temporal bones to expose the membranous labyrinth under a dissecting microscope, and then, the stria vascularis was removed. The middle-turn cochlear explants containing the SGNs were then placed onto 10 mm coverslips (Fisher Scientific, PA) precoated with Cell-TaK (BD Biosciences) and incubated in Dulbecco's modified Eagle's medium/F12 (Invitrogen) supplemented with ampicillin (50 mg/mL; Sigma), N2 (1:100 dilution; Invitrogen), B27 (1:50 dilution; Invitrogen), epidermal growth factor (20 ng/mL; Sigma), fibroblast growth factor-basic (10 ng/mL; Sigma), insulin-like growth factor-1 (50 ng/mL; Sigma), and heparan sulfate (50 ng/mL; Sigma) at 37°C in a 5% CO_2 atmosphere.

The next day, samples were changed into fresh media containing cisplatin (Sigma-Aldrich, St. Louis, MO) alone, cisplatin and Wnt-3a (200 ng/mL; R&D Systems) and RS-1 (1 μg/mL; R&D Systems), or cisplatin and IWP-2 (1 μ*M*; Stemgent) as indicated in the text. Samples were cultured for 48 h and then used in the immunostaining and other assays.

In vivo *drug treatments*

Experiments were performed on age- and sex-matched P30C57BL/6 WT or transgenic mice. All animals were bred and housed in a climate-controlled room with an ambient temperature of 23 ± 2°C and a 12-h light/12-h dark cycle. Control mice were administered 0.9% physiological saline (0.6 mL/100 g body weight by i.p. injection) daily for seven consecutive days, and experimental mice were administered cisplatin (3 mg/kg i.p.) daily for seven consecutive days.

Auditory functional tests

The ABR and CAP were measured before and after 7 days of injection of cisplatin in a sound proof chamber. Before the recording, the animals were anesthetized using chloral hydrate (400 mg/kg). During the recording, the body temperature of the animals was maintained at 38°C with a thermal static heating device (FHC, MA). We used three subdermal needle electrodes to record the ABRs. The noninverting electrode, the reference electrode, and the grounding electrode were inserted at the vertex and at the neck posterior to the auditory bullas of both sides, respectively. To record the CAPs, a silver ball electrode was placed at the round window membrane *via* a small hole drilled in the auditory bulla posterior-inferior to the external ear canal. The other end of the electrode was attached to the noninverting channel of the preamplifier.

Tucker-Davis Technologies (TDT System III, Alachua, FL) system hardware and software were used to generate stimuli and record the responses. The acoustic stimuli were 10 ms tone bursts for ABR and CAP with cos^2 gating and a 0.5 ms rise/fall time. The stimuli were played through a broadband speaker (MF1; TDT) that was placed 10 cm in front of the head of the animal. The evoked responses were amplified 20-fold and then filtered between 100 and 3000 Hz using a preamplifier (RA16PA; TDT), which digitized the signal at a sampling rate of 25 kHz. The responses were averaged 1000 times for ABR and 100 times for CAP. The ABR thresholds and the tone-burst

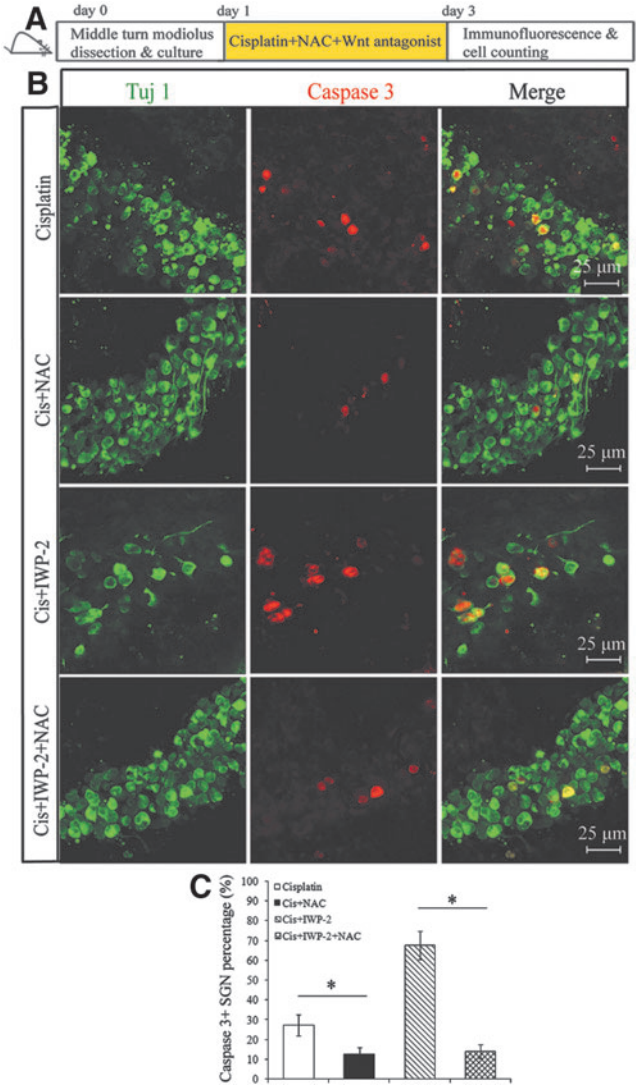


FIG. 9. NAC treatment reduced the caspase 3 expression in SGNs induced by cisplatin treatment. (A) The diagram of the assay. The middle-turn cochleae from P3 WT mice were dissected out and cultured *in vitro* with cisplatin (50 μ*M*), cisplatin (50 μ*M*) + NAC (2 m*M*), or cisplatin (50 μ*M*) + IWP-2 (1 μ*M*) + NAC (2 m*M*) for 48 h. **(B, C)** Immunostaining showed that the caspase 3 expression was significantly reduced in both the Cis + NAC group and the Cis + NAC + IWP-2 group compared with the cisplatin-only group and the Cis + IWP-2 group separately, and statistical analysis revealed that NAC administration significantly decreased the numbers of caspase 3/Tuj 1 double-positive SGNs in the Cis + NAC group and Cis + NAC + IWP-2 group compared with the cisplatin and Cis + IWP-2 groups, respectively. *$p < 0.05$. Each group, $n = 9$.

Table 8. Caspase 3-Positive Spiral Ganglion Neuron Percentage in the Middle-Turn Cochlea of Wild-Type Mice *In Vitro* ($n=9$)

	Cisplatin	*Cis + NAC*	*Cis + IWP-2*	*Cis + IWP-2 + NAC*
Caspase 3+ SGN (%)	27.4 ± 5.26	12.8 ± 3.30	67.7 ± 7.14	14.19 ± 3.51

CAP input/output functions were tested at 4, 8, 16, 24, and 32 kHz. At each frequency, the test was started at 90 dB SPL and tracked in 5 dB steps before the response disappeared. In the ABR test, the focus was the thresholds, which were defined as the lowest level at which a repeatable wave III response could be seen. In the CAP test with tone bursts, the focus was the peak-to-peak amplitude, which was measured at different sound levels to generate input/output functions.

Immunostaining

After culture or cryosection, the cochlear explants or tissue sections were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS), and the samples were immersed in blocking solution (0.1% Triton X-100, 8% donkey serum, 1% bovine serum albumin [BSA], and 0.02% sodium azide in PBS) at room temperature for 1 h. The samples were then incubated with primary antibodies against Tuj 1 (1:1000 dilution; Neuromics), Neu N (1:1000 dilution; CST), cleaved-caspase 3 (1:400 dilution; CST), PY489-β-catenin (1:400 dilution; DSHB), or TIGAR (1:800 dilution; Abcam) diluted in blocking solution at 4°C overnight. The next day, cells were incubated with fluorescein isothiocyanate-conjugated, tetramethylrhodamine-conjugated, or Cy5-conjugated (Invitrogen) secondary antibody along with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in 0.1% Triton X-100 and 1% BSA in PBS at room temperature for 1 h. Coverslips were then mounted and the samples were observed under a laser scanning confocal microscope (Leica, Germany).

Cryosectioning

Cochleae from P30 mice after cisplatin injection were removed and fixed with 4% paraformaldehyde in PBS at 4°C overnight. Tissues were then incubated in 10%, 20%, and 30% sucrose in 1×PBS, embedded in O.C.T compound (Tissue-Tek; Sakura Finetek), snap-frozen on dry ice, and then stored frozen at −80°C. Frozen sections were then cut into 7 μm sections using a cryostat (Leica CM 1850, Nussloch, Germany).

TUNEL assay

Cell apoptosis was measured by DNA fragmentation with a TUNEL staining kit (Click-iT Plus TUNEL Assay for *In Situ* Apoptosis Detection; Invitrogen) according to the manufacturer's instructions. Each section was stained with DAPI solution (1:1000 dilution) for 15 min at room temperature and protected from light. After washing with PBS, samples were examined using a laser scanning confocal microscope (Leica, Germany).

RNA extraction and RT-PCR

TRIzol (Life Technologies) was used to obtain total RNA following the manufacturer's instructions. For P30 adult cochleae, a grinding machine (Jingxin JXFSTPRP-48, Shanghai, China) was used to triturate the cochlea with bone, and then, RNA was extracted by TRIzol. The cDNA was synthesized by reverse transcription using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocol. Quantitative RT-PCR was performed using SYBR Premix Ex Taq (TaKaRa, Japan). *GAPDH* was used as the housekeeping gene. All data were analyzed using an Eppendorf Realplex 2. PCR primers for the genes are listed in Table 10.

Western blot

After being treated with cisplatin for 48 h, the proteins from the cultured middle-turn cochleae were extracted, and the expressions of TIGAR and Myc were examined by Western blot. Briefly, total protein was obtained from SGNs of the cochleae using radioimmunoprecipitation assay lysis buffer. A total of 30 μg of each protein sample was separated on 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gels. The primary antibodies were anti-TIGAR (1:1000 dilution), anti-Myc (1:1000 dilution), and anti-β-actin (1:2000 dilution) (Santa Cruz Biotechnology). The relative optical density ratio of TIGAR and Myc to β-actin was calculated with the ImageJ software.

Adenovirus administration

E1/E3/polymerase/terminal protein-deleted adenoviral (Ad) type 5 vectors with a mouse TIGAR gene insert (Ad-TIGAR), driven by the cytomegalovirus promoter, were generated using the AdMax system (Microbix Bioscience, Ontario, Canada). Crude virus was amplified through several rounds to produce a high titer, purified using the Adeno-X Virus Purification Kit (BD Biosciences, Clontech), and stored at a concentration of 5×10^{10} PFU/mL in Ad storage buffer at −80°C. The transfection efficiency was evaluated by adenovirus vector Ad-GFP transfection. Briefly, cochlear samples were treated with

Table 9. Genotyping Primers Used in the Experiments

Gene	*Forward sequence*	*Reverse sequence*
Bhlhb5-cre	GGGATTGGACTCAGAGGCGGTAGC	GCCCAA ATGTTGCTGGATAT
β-Catenin-OE-Mutant	AACTGGCTTTTGGTGTCGGG	TCGGTGGCTTGCTGATTATTTC
β-Catenin-KO-Mutant	AAGGTAGAGTGATGAAAGTTG	CACCATGTCCTCTGTCTATTC

Table 10. Polymerase Chain Reaction Primer Sequences Used in the Experiments

Gene	*Forward sequence*	*Reverse sequence*
Casp3	GGAGCAGCTTTGTGTGTGTG	CTTTCCAGTCAGACTCCGGC
Casp8	GCTGTATCCTATCCCACG	TCATCAGGCACTCCTTT
Casp9	GGACCGTGACAAACTTGAGC	TCTCCATCAAAGCCGTGACC
Apaf1	TGTGTGAAGGTGGAGTCAAGG	CCTCTGGGGTTTCTGCTGAA
Bcl-2	TGACTTCTCTCGTCGCTACCG	GTGAAGGGCGTCAGGTGCAG
Bax	CGTGGTTGCCCTCTTCTACT	TTGGATCCAGACAAGCAGCC
p53	GGTGCTGACGAAGAAGAGGA	AGCCCAACTGTGATGAAGCA
TIGAR	GTCACTCCGTTCAGGTGCTC	TTTCCTCCATCACTCCCAAC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Axin 2	TGACTCTCCTTCCAGATCCCA	TGCCCACACTAGGCTGACA
Lgr 5	CCTACTCGAAGACTTACCCAGT	GCATTGGGGTGAATGATAGCA
β-Catenin	ATGCGCTCCCCTCAGATGGTGTC	TCGCGGTGGTGAGAAAGGTTGTGC

Ad-GFP vector at different dilutions (1:10, 1:100, 1:1000, 1:10,000) in enhanced infection solution (EIS). After incubation for 24 h, the medium was replaced with normal medium, and cells were further cultured for 48 h at 37°C and 5% CO_2. Samples were then prepared and analyzed. According to the transfection efficiency, the 1:1000 dilution of adenovirus was shown to be the best concentration for SGN transfection.

The specific TIGAR expression was then tested with 1:1000 diluted Ad-TIGAR adenovirus transfected SGNs. Briefly, Ad-TIGAR was diluted 1:1000 in EIS to a final concentration of 5×10^7 PFU/mL and added to the cochleae. The transfection medium was replaced 24 h later with normal medium and cultured for 48 h, then samples were harvested for Western blot.

To test the effect of TIGAR OE in SGNs after cisplatin treatment and Wnt signaling inhibition, cochleae were treated with 5×10^7 PFU/mL Ad-TIGAR diluted in EIS for 24 h before the transfection medium was replaced with normal medium, and then, the cochleae were further treated with cisplatin (50 μM) or cisplatin (50 μM) + IWP-2 (1 μM) for 48 h and harvested for immunostaining and counting analysis.

SGN counting

The cultured middle-turn cochlear explants were imaged using a confocal microscope (400×) with a 10 μm distance between each optical section. SGNs were immunolabeled with the Tuj 1 antibody that specifically labels both SGN bodies and neurites. SGNs in which the nucleus comprised 40% of the soma area were counted in each optical section using the NIH ImageJ software. The total number of SGNs in each spiral ganglion explant was obtained by summing the SGN counts in all consecutive sections, and the SGN density per unit area (0.01 mm^2) was calculated.

The *in vivo* SGN density was quantified from midmodiolar sections of cochleae dissected out of P30 mice. Using NIH ImageJ software, the number of SGNs in each section was divided by the area of the Rosenthal's canal, and the total number of SGNs was counted. The density of SGNs was then calculated within the unit area (0.01 mm^2) from the middle-turn sections of each cochlea.

Statistical analyses

Statistical analyses were conducted using Microsoft Excel and GraphPad Prism software. For each experiment, at least three individual samples were used. Data are presented as mean ± standard error of the mean. Two-tailed, unpaired Student's *t*-tests were used to determine statistical significance when comparing two groups. A one-way analysis of variance followed by a Dunnett's multiple comparisons test was used when comparing more than two groups. A value of $p<0.05$ was considered to be statistically significant.

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Author Disclosure Statement

No competing financial interests exist. The data contained in this article have not been previously published.

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Abbreviations Used

Ad = adenovirus
ABR = auditory brainstem response
ANF = auditory nerve fiber
BSA = bovine serum albumin
CAP = compound action potential
DAPI = 4′,6-diamidino-2-phenylindole
EIS = enhanced infection solution
HC = hair cell
i.p. = intraperitoneally
KO = knockout
NAC = *N*-acetylcysteine
OE = overexpression
P = postnatal day
PBS = phosphate-buffered saline
PFU = plaque forming unit
ROS = reactive oxygen species
RS-1 = R-spondin1
RT-PCR = real-time polymerase chain reaction
SGN = spiral ganglion neuron
SPL = sound pressure level
TCF = T cell factor
TIGAR = TP53-induced glycolysis and apoptosis regulator
TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling
WT = wild type