Molecular Mediators of Estrogen Reduction-induced Otolith Shedding*

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[Abstract] Objective: Previous study suggested that estradiol (E2) plays an important role in otolith shedding by regulating the expression of otoconin 90 (OC90). The purpose of this article is to provide further data on the effect and mechanism of E2 on the morphology of otolith. **Methods:** The rats receiving bilateral ovariectomy (OVX) were used as animal models. Co-immunoprecipitation was used to observe the relationship between estrogen receptor (ER) and estrogen-related receptor α (ERRα) . The morphology of otolith was observed under the scanning electron microscopy. Western blotting and qPCR were used for quantitative analysis of the roles of ER and ERRα in regulating OC90 expression. **Results:** The looser otoliths were observed in rats receiving bilateral OVX, which could be reversed by supplementation with E2. The level of ERRα was decreased in bilateral OVX rats. ER and ERRα interacted with each other on the regulation of the expression of OC90. **Conclusion:** Our results suggest ER and ERRα are both important downstream receptors involved in regulating OC90 expression in utricles of rats, and ERRα probably functions by interacting with ER. This provides evidence for the mechanism of otolith shedding. And it may be significant for future studies of targeted prevention and therapies for benign paroxysmal positional vertigo.
Key words: estrogen-related receptor α; estrogen receptor; otoconin 90; otolith; benign paroxysmal positional vertigo

Benign paroxysmal positional vertigo (BPPV) is one of the most common peripheral vestibular disorders, which is caused by otolith shedding presenting with vertigo during changes in head position, and the cause of most cases is unclear. There is evidence suggesting that changes occur in otolith morphology after enucleation of ovaries[1]; however, the molecular mediators of estrogen reduction-induced otolith shedding require further investigation. In our previous study, we found that the serum level of estradiol (E2) in perimenopausal women with BPPV was lower than that in healthy perimenopausal women. In bilateral ovariectomy (OVX) rats, the expression of otoconin 90 (OC90) was largely decreased following a reduction in E2, and 17β-estradiol replacement reversed the decrease[2]. A previous study showed that in OVX rats, the number of otolith per unit area was decreased and their size was increased compared to a control group[1]. Using electron microscopy, we also found that the density of otolith was decreased in OVX rats with lower level of E2. OC90 was found to be largely synthesized by various nonsensory cell types in utricle[3, 4]. Identifying the key downstream receptor of E2 in regulation of OC90 expression will be a significant advance and may help in the search for new targets to prevent or reduce the risk of BPPV.

E2 is fat-soluble and can quickly bind to estrogen receptor (ER) α and ERβ, which are the nuclear receptors. And because E2 has the same affinity for each of the two receptors, which receptor is activated depends on their expression levels in specific tissues[5]. ERα and ERβ are extensively expressed in the inner ears of rodents, humans and fishes, and E2 is co-expressed with ERα and/or ERβ, in inner hair cells, outer hair cells, spiral ganglion cells, vestibular ganglion cells, vestibular dark cells and epithelial cells of the endolymphatic sac[6–8]. The downstream signaling of ERα/ERβ represents the "steroid signaling pathway", which can identify DNA sequences and ligand domains of estrogen responsive elements and then regulate the

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expression of target genes at the transcriptional level[9]. As a result, we propose that E2 also regulates the expression of OC90 protein through the ER-initiated steroid signaling pathway in the inner ear.

Estrogen-related receptor (ERR), which has three subtypes (ERRα, ERRβ and ERRγ), has strong homology with ER. ERR belongs to the third type of nuclear receptor, which combines with target gene hormone response elements in the form of dimer. For example, ERRα can be combined with the target gene DNA in homologous two dimers or ERRα-ER heterologous two dimers[10]. The difference between ERRα and ER is that ERRα directly regulates the target gene expression but not through combining with E2; thus, it is also called an orphan nuclear receptor. The expression of ERRα, ERRβ and ERRγ is tissue specific, with ERRα being expressed in the nervous system, glands and skeleton tissues, and ERRβ and ERRγ being mostly expressed in muscles and glands[11–13]. In our study, we only detected the expression of ERRα in inner ear of rats by Western blotting. ERR has been functionally implicated in the ER pathway, and has been associated with osteoporosis and breast cancer[14]. Recently, cholesterol has been found to bind to and activate the ERRα, and may be the endogenous ligand for the receptor. Moreover, the effects of cholesterol, statins, and bisphosphonates on osteoclastogenesis in bone tissue require ERRα; furthermore, cholesterol-induced bone loss or bisphosphonate osteoprotection is absent in ERRα knockout mice[15]. Because the formation of bone and otoconia shares similar mechanisms[16], it is reasonable to infer that ERRα may play an important subsidiary role in the process of otolith shedding in female patients with the idiopathic BPPV.

1 MATERIALS AND METHODS

1.1 Reagents and Animals

17β-estradiol (Abcam, ab120657), highly selective agonist of ERβ [diarylpropionitrile (DPN), H5915], ERRα inhibitor (inverse agonist) (XCT790, CAS No.: 725247-18-7) and ER inhibitor (Fulvestrant CAS No.: 129453-61-8) were purchased from Sigma Aldrich (USA). 17β-estradiol was dissolved in sesame oil. Primary and secondary antibodies against OC90 (SC-376855), ERα (SC-542), ERβ (SC-6822), ERRα (SC-32972), anti-ERα antibody (SC-7202), anti-ERβ antibody (SC-390243), anti-ERRα antibody (SC-32972) and β-actin (SC-8035) were purchased from Santa Cruz Technologies (USA). Selective agonist of ERα [1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), H6036] was purchased from Tocris Bioscience (USA). Animals in this study were purchased from Shanghai slac Laboratory Animal Company (China). Adult female Sprague Dawley rats (25 OVX and 5 sham-operated rats) with a body weight of 200–250 g (4-month-old) were housed in standard animal facilities. All experimental procedures conformed to Changzheng Hospital of Navy Medical University as well as international guidelines on the ethical use of animals. All efforts were made to minimize animal suffering and reduce the number of animals used. The rats were randomly divided into three groups. Rats in the OVX+E2 group were given a 17β-estradiol (0.25 mL/kg per day, 10 μg/0.1 mL) supplement (intraperitoneal injection) at 9:00 AM every day for 1 month after bilateral OVX. Rats in the OVX+progesterone group were given a progesterone (0.3 mL/kg per day, 10 mg/mL) supplement 1 month after bilateral OVX. The rats in the control (sham-operated) and (OVX+vehicle) groups received a daily injection of vehicle (sesame oil, 0.25 mL/kg). All supplement treatments continued for 30 days. All animals were sacrificed by decapitation under pentobarbital (30 mg/kg) anesthesia after 1 months of supplement treatments, and bilateral inner ears were carefully removed.

1.2 Scanning Electron Microscope

The temporal bone and labyrinth were carefully removed to expose the utricle. All samples were fixed in 2% glutaraldehyde for 1 day and osmic acid for 2 h, dehydrated using graded ethanol, dried using the critical point drying method and coated using silver. Utricle morphology was visualized by SU8000 electron microscope (Japan). The number of otoliths was counted by randomly selecting four regions under 3.0K UL, and the number of otoliths per unit area (100 μm^2) was finally calculated. Otoconial measurement was referred to the quantitative method of Dominique Vibert[1].

1.3 Western Blotting

Immunoblot analysis of OC90, ERα, ERβ and ERRα was performed on the tissue of the utricle. Samples were ground with liquid nitrogen and homogenized in a lysate buffer containing 100 mmol/L Tris-HCl, pH 6.7, 1% SDS, 143 mmol/L 2-mercaptoethanol and 1% protease inhibitor. The lysate was centrifuged at 12 000 r/min for 10 min at 4°C. Protein concentrations were determined using a BCA kit (Pierce Chemicals, USA). The samples were treated with the SDS sample buffer at 95°C for 5 min, loaded on a 10% SDS polyacrylamide gel and blotted to a PVDF membrane. Each blot was incubated with primary antibodies and secondary antibodies. Protein bands were detected by the ECL chemiluminescence system (Amersham, Buckinghamshire, UK). Immunoreactivity of the target protein was normalized to that of β-actin. Each experiment was repeated at least three times.

1.4 Quantitative RT-PCR for Assessment of mRNA Expression

Total RNA was extracted from tissue using the Rneasy MiNi Kit from Qiagen (USA). Quantitative

real-time PCR of the OC90 mRNA was performed using SYBR® Premix Ex Taq TM II (TaKaRa, China) through 40 PCR cycles (95°C for 10 s, 60°C for 25 s, and 72°C for 20 s). The primer sequences of OC90 were as follows: F: AGTGGTTTGGATGGTGCCAA and R: GCACCATCATTTCCACGAGC.

1.5 *In Vitro* Culture of Utricle Membrane

About 90 female pups were decapitated 3 days after birth. The 3 inner ears from pups were used for one sample for Western blotting and 2 inner ears for qPCR. Pups were used because they have similar and lower estrogen, and the otolith structure is rarely affected by various factors, such as the estrous cycle. The utricles were carefully removed to the 1.2 mL prepared cold growth media (Neurobasal-A medium containing 1× B27 supplement, 0.5 mmol/L L-glutamine). The utricle membrane was cultured in an incubator with carbon dioxide at a concentration of 5% at 37°C. After 24 h, the culture medium was replaced with fresh culture medium, with or without ERα inhibitor (XCT790 20 μmol/L), ER inhibitor (Fulvestrant, Ful), PPT+XCT (PPT 0.5 nmol/L+XCT790 20 μmol/L), DPN+XCT (DPN 0.3 nmol/L+XCT790 20 μmol/L), Ful (20 μmol/L), PPT (PPT 0.5 nmol/L), DPN (DPN 0.3 nmol/L) and DMSO (control group), and then cultured for 3 days.

1.6 Co-immunoprecipitation

Co-immunoprecipitation of ERα-ERRα and ERβ-ERRα was performed on utricle membranes of inner ear removed from female rats. Samples were homogenized in a RIPA lysis buffer (P0013B, Beyotime Biotechnology, China) containing 1% phenylmethanesulfonyl fluoride (PMSF, ST506, Beyotime Biotechnology, China) at 4°C after being removed. Protein concentrations were determined using an Enhanced BCA Protein Assay Kit. Each sample was incubated with either anti-ERα antibody (SC-7202), anti-ERβ antibody (SC-390243) or its control IgG for 1 h at 4°C. Pierce Protein A/G Magnetic Agarose Beads (78609, Thermo Fisher Scientific, USA) were added to each sample before incubating overnight at 4°C. The beads were collected by centrifugation and washed three times with lysis buffer. The immune complexes were released from the beads by boiling with 5× SDS-PAGE Sample Loading Buffer and analyzed by Western blotting using anti-ERRα antibody (SC-32972). The following antibodies were used in Western blotting analyses: IRDye® 800CW-conjugated secondary antibody (anti-goat, 926-32214, 1:1000, LI-COR, USA); IRDye® 800CW-conjugated secondary antibody (anti-rabbit, 926-32211, 1:1000, LI-COR, USA).

1.7 Statistical Analysis

Statistical analysis was performed using SPSS 16.0 for Windows (USA). For mRNA analysis by RT-qPCR, the data analysis was done by the comparative delta-delta Ct method, and the normalizer mRNA was β-actin. Quantitative data were presented as means±standard error (SE), and one way ANOVA was used for multiple comparisons. At the 0.05 level, the data of animal studies were normally distributed using K-S test, so the Student's *t*-test was used for data analysis. *P*-values <0.05 were considered statistically significant for all parameters.

2 RESULTS

2.1 Ultrastructural Manifestation of otolith in Rats

The morphology of the utricles in OVX group, OVX+E2 group, OVX+progesterone group and control group (sham-operated+sesame oil) was analyzed under the scanning electron microscopy. Most otoliths in OVX group couldn't be seen (having fallen off). The density of otolith was significantly decreased in OVX group as compared with that in the control group (fig. 1, $P<0.05$). There was no significant difference in the density of otolith between OVX+E2 group and control group, between OVX group and OVX+progesterone group and between OVX+progesterone and control group (fig. 1, $P>0.05$).

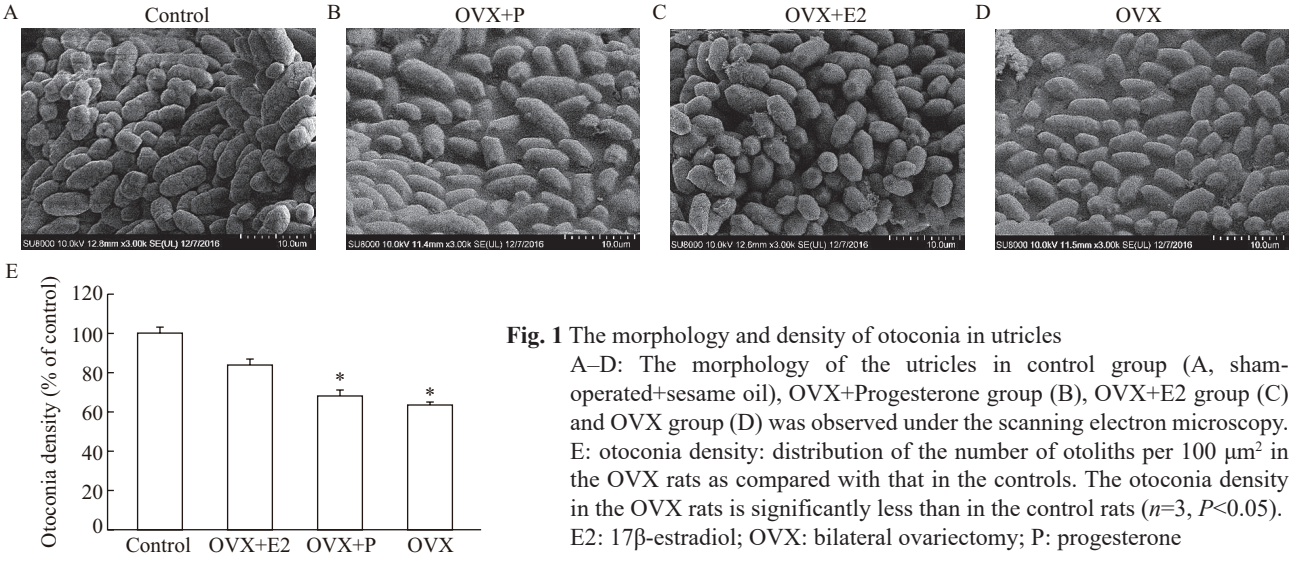

Fig. 1 The morphology and density of otoconia in utricles
A–D: The morphology of the utricles in control group (A, sham-operated+sesame oil), OVX+Progesterone group (B), OVX+E2 group (C) and OVX group (D) was observed under the scanning electron microscopy. E: otoconia density: distribution of the number of otoliths per 100 μm^2 in the OVX rats as compared with that in the controls. The otoconia density in the OVX rats is significantly less than in the control rats (n=3, $P<0.05$). E2: 17β-estradiol; OVX: bilateral ovariectomy; P: progesterone

2.2 OVX-induced Decreased Level of ERRα and Reversal by E2 Replacement Therapy

We examined the influence of E2 replacement therapy on the levels of E2-related proteins (ERα, ERβ and ERRα) in the inner ears of OVX rats. No significant differences were found in ERα or ERβ among sham-operated+vehicle group, OVX+E2 group, and OVX+ vehicle group. Only ERRα was reduced in OVX+vehicle group as compared with that in rest groups. The average percentages of ERRα protein were 100%±10.77%, 102.79%±14.59% and 70.35%±7.65% in sham-operated+vehicle group, OVX+E2 group and OVX+vehicle group, respectively (fig. 2, n=4).

2.3 Influence of ERRα Inhibitor (XCT790) and ER Inhibitor (Ful) on Expression of OC90 Protein and mRNA in Cultured Utricle Membranes from Neonatal Rats

We examined the expression of OC90 protein and mRNA in cultured utricle membranes from neonatal rats. The expression of OC90 protein and mRNA were both largely decreased after incubation with E2+XCT790 or E2+Ful as compared with incubation with E2+DMSO (fig. 3, n=4, P<0.05). However, there were no significant differences between E2+XCT790 group or E2+Ful group and control group. Also, there was no significant difference between E2+XCT790 group and E2+Ful group (fig. 3, n=4).

2.4 Influence of ERα Agonist (PPT), ERβ Agonist (DPN), ERRα Inhibitor (XCT790) and ER Inhibitor (Ful) on Expression of OC90 mRNA in Cultured Utricle Membranes from Neonatal Rats

To further explore the roles of ERα, ERβ and ERRα in regulation of OC90 expression, we examined the expression of OC90 mRNA in cultured utricle membranes from neonatal rats in XCT (XCT790: 20 μmol/L), PPT+XCT (PPT: 0.5 nmol/L+XCT790: 20 μmol/L), DPN+XCT (DPN: 0.3 nmol/L+XCT790: 20 μmol/L), Ful (20 μmol/L), PPT (PPT: 0.5 nmol/L), DPN (DPN: 0.3 nmol/L) and DMSO (control) groups. There were no significant differences in the OC90 mRNA expression among XCT, PPT+XCT, DPN+XCT and Ful groups, but the expression of OC90 mRNA in XCT,

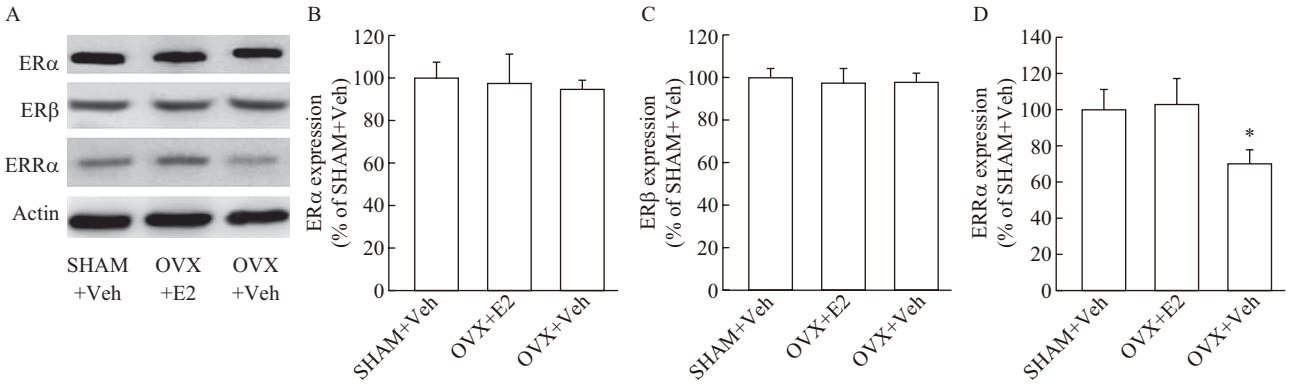

Fig. 2 Expression of ERα, ERβ and ERRα in the inner ears of SHAM+Veh group, OVX+E2 group and OVX+Veh group
A: immunoblots of ERα, ERβ and ERRα in the inner ears of rats in SHAM+Veh (sham-operated+sesame oil), OVX+E2 (17β-estradiol: 25 μg/kg·day, 10 mg/0.1 mL) and OVX+Veh groups. B–D: levels of ERα, ERβ and ERRα after normalization by β-actin in SHAM+Veh, OVX+E2 and OVX+Veh groups (n=4, $^*P$<0.05 *vs.* the SHAM+Veh group)

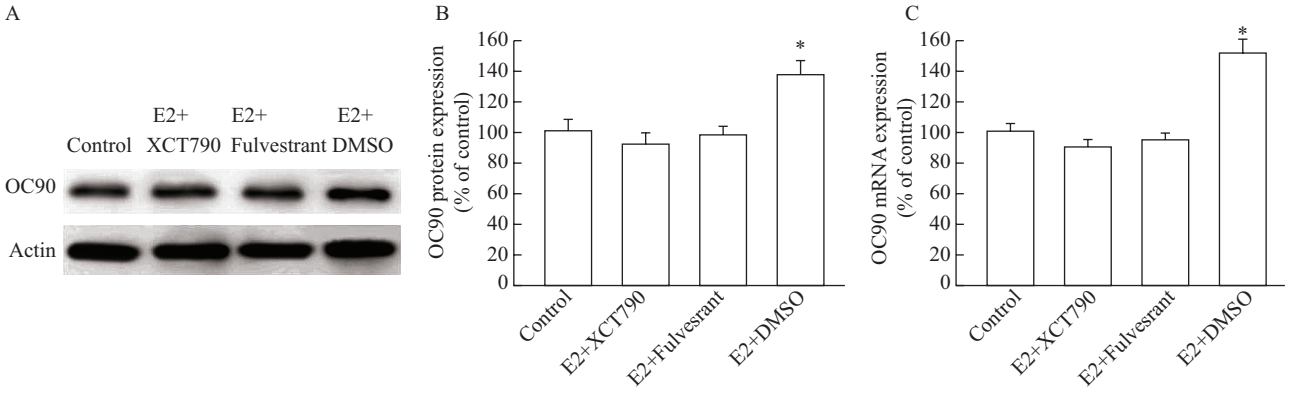

Fig. 3 Influence of ERRα inhibitor (XCT790) and ER inhibitor (Fulvestrant) on the expression of OC90 protein and mRNA in cultured utricle membranes from neonatal rats
A: immunoblots of OC90 in the inner ears of rats in the control (sesame oil+DMSO), E2+XCT790 (XCT790: 20 μmol/L), E2+Fulvestrant (Fulvestrant 20 μmol/L) and E2+DMSO groups. B: Average OC90 protein after normalization by β-actin in the above four groups (n=4, *P<0.05 *vs.* the control group). C: levels of OC90 mRNA in the above four groups by qPCR (n=4, $^*P$<0.05 *vs.* the control group).
OC90: otoconin 90; XCT: XCT790; E2: 17β-estradiol

PPT+XCT, DPN+XCT and Ful groups were lower than that in DMSO group. There was no significant difference in OC90 mRNA expression between PPT and DPN groups, and the expression of OC90 mRNA expression in PPT and DPN groups were significantly higher than that in DMSO group (fig. 4, n=3, $^*P$<0.05).

2.5 Relationship between ER and ERRα

We used co-immunoprecipitation assays to examine whether the interactions existed among ERα, ERβ and ERRαin the utricle of inner ear. In lysates of tissue samples incubated with anti-ERα and anti-ERβ antibody, ERRα was efficiently detected, indicating that ERα and ERRα, ERβ and ERRα interacted (fig. 5).

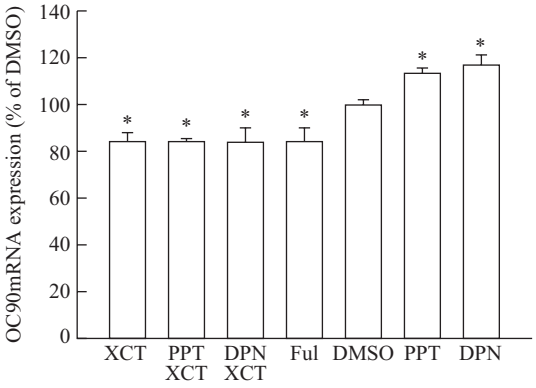

Fig. 4 Influence of ERα Agonist (PPT), ERβ Agonist (DPN), ERRα Inhibitor (XCT790) and ER Antagonist (Fulvestrant) on Expression of OC90 mRNA in Cultured Utricle Membranes from Neonatal Rats
Levels of OC90 mRNA in the XCT (XCT790 20 μmol/L), PPT+XCT (PPT 0.5 nmol/L+XCT790 20 μmol/L), DPN+XCT (DPN 0.3 nmol/L+XCT790 20 μmol/L), Ful (Fulvestrant 20 μmol/L), PPT (PPT 0.5 nmol/L), DPN (DPN 0.3 nmol/L) and DMSO groups in cultured utricle membranes from neonatal rats by qPCR (n=3, $^*P$<0.05 *vs.* the DMSO group)
XCT: XCT790; DPN: diarylpropionitrile; Ful: Fulvestrant; PPT: 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; qPCR: quantitative RT-PCR

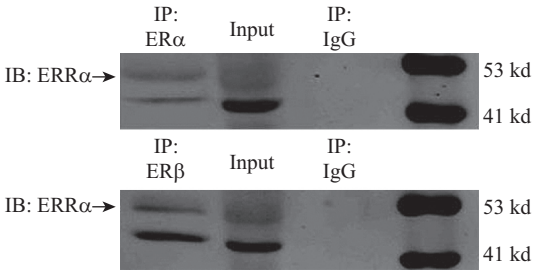

Fig. 5 Co-immunoprecipitation of ERα or ERβ with ERRα
The arrowed strip represents the band of ERRα (53 kDa) protein. Lysates were prepared from utricle membranes of adult female SD rats, and incubated either with anti-ERα antibodies and control IgG (negative control) or with anti-ERβ antibody and the IgG (negative control). The immunocomplexes were semiquantitatively analyzed by Western blotting using anti-ERRα antibody.

3 DISCUSSION

BPPV is commonly believed to be caused by dislocation of otolith into the semicircular canals[17]. Several reports have suggested OC90 deficiency would directly promote the dislocation of otolith[18, 19]. In our previous studies, we found statistically significant differences in E2 level between patients with BPPV and controls. And we not only found the level of OC90 was lower than the Sham group, but also found 17β-estradiol replacement could reverse the decrease of OC90 levels in bilateral OVX rats[2].

To further study the relationship between E2 and the dislocation of otolith, ultrastructural studies are necessary. Under the electron microscopy, we found that the density of otolith in OVX group was looser than that in the control group, which was similar to the result reported by Zhao *et al*[19]. This difference was mitigated by E2, but not progesterone replacement. We supposed that the reason why otolith couldn't maintain its normal morphology was the decrease of the OC90 induced by the decrease of E2. These results suggest that E2 is a key factor in the maintenance of normal morphology.

The peak age for incidence of BPPV is consistent with the age range of menopause. Hormone therapy will cause multitude of possible undesirable side effects in patients. So, it is supposed that if we can find the downstream pathway of E2 involved in regulating OC90 protein expression, we may find a target for BPPV treatment or prevention.

We examined the expression of ER and ERR in the utricle of OVX rats, finding that only the level of ERRα was reduced, suggesting that ERRα might play an important role in the effect of E2 on regulation of OC90 expression. To find the downstream signaling pathway of E2, we used ERRα inhibitor and ER inhibitor in cultured utricle membranes from neonatal rats. Surprisingly, the results showed that both ERRα inhibitor and ER inhibitor could lead to the decrease of the expression of OC90. There was no significant difference in the expression of ER between OVX rats and controls, and ERRα alone (E2+Ful group) couldn't activate OC90 expression. What this means is that both ER and ERRα may be important in regulating OC90 expression, and that ERRα may be an essential auxiliary factor for the ER-signaling-pathway involved in controlling the expression of OC90. This is supported by previous reports showing that the effect of ERRα is in the form of ERRα-ER heterologous two dimers in protein expression regulation[10]. To study the role of ERα, ERβ and ERRα, we further used synthetic modulators (inverse agonists, antagonists and agonists) to observe the expression of OC90 mRNA. The results indicated that both ERα and ERβ were involved in

the downstream pathway regulating the expression of OC90 mRNA and that ERRα may be an essential auxiliary factor for the ERα/β signaling pathway. We then assessed the relationship between ERRα and ER using co-immunoprecipitation, suggesting ERRα interacts with both ERα and ERβ.

This study explores the important role of ERRα in estrogen mediated otolith shedding. Next, we can use ERRα knockout mice (ERRαKO) for studying the pathogenesis of idiopathic BPPV, such as what was the endogenous nuclear receptor ligand of ERRα and how the receptor is regulated in inner ear, which was consistent with the Wei's study indicating that cholesterol is a functional endogenous ERRα agonist and further checks the physiological function of cholesterol and the pharmacological actions of cholesterol-lowering drugs require ERRα by using ERRαKO mice in vivo study[15].

In conclusion, E2 regulates OC90 protein expression through ER signaling in utricle of the inner ear and that ERRα may play an important subsidiary role. Although our study is most related to older women with idiopathic BPPV and cannot be fully extrapolated to human beings, it still has great significance for etiological study of positional vertigo related to otolith exfoliation, and for future studies of targeted preventions and therapies for idiopathic BPPV.

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Conflict of Interest Statement

All authors declare no conflicts of interest.

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