Transmembrane BAX Inhibitor Motif-6 (TMBIM6) protects against cisplatin-induced testicular toxicity

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STUDY QUESTION: Is the Transmembrane BAX Inhibitor Motif-6 (TMBIM6) involved in the molecular mechanism by which cisplatin causes reproductive toxicity?

SUMMARY ANSWER: TMBIM6 protects against cisplatin-induced testicular toxicity through up-regulation of heme oxygenase-1 (HO-1), which maintains the levels of steroidogenic enzymes by decreasing oxidative stress in the endoplasmic reticulum (ER).

WHAT IS KNOWN ALREADY: Testosterone production is highly suppressed as a main complication of cisplatin (cis-diamminedichloro-platinum) anticancer therapy.

STUDY DESIGN, SIZE, DURATION: Groups of seven wild type or Tmbim6 KO C57BL/6J mice were given a single i.p., injection of cisplatin (30 mg/kg body wt) and testis and serum were collected 3 days later. Tmbim6-lentivirus-mediated testicular expression-rescued KO mice were analyzed to confirm function was restored. Tmbim6-over expressing TM3 mouse Leydig cells were exposed to cisplatin in vitro.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After collection of the specimens serum testosterone level and testicular weight and structure were compared between the groups. Quantitative PCR, immunoblot, and assays for ROS, HO-1 activity and protein disulfide isomerase (PDI) carbonylation were performed.

MAIN RESULTS AND THE ROLE OF CHANCE: Phospho protein kinase B (p-Akt), nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), and its downstream gene product HO-1 and the levels of testosterone synthesis-associated enzymes, including steroidogenic acute regulatory protein (StAR), a rate limiting enzyme for testosterone production, were significantly expressed in the presence of Tmbim6 and maintained after cisplatin treatment. Excessive post-translational oxidation of protein disulfide isomerase (PDI), altered folding capacitance and ROS accumulation, and ER stress were also decreased in the presence of Tmbim6. Higher levels of ER stress and protein hypercarbonylation were consistently observed in KO tests, compared with WT tests. In the Tmbim6 KO mice, lentivirus-mediated testicular expression of Tmbim6 rescued the above phenotypes. Furthermore, the protective role of Tmbim6 against testicular toxicity was consistently shown in Tmbim6-overexpressing TM3 Leydig cells (testosterone producing cells). We conclude that TMBIM6 protects against cisplatin-induced testicular toxicity by inducing HO-1 and enhancing ER folding capacitance.

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: This study was performed using a short, 3-day cisplatin treatment condition. Therefore, the results need to be cautiously interpreted with regard to cisplatin-associated chronic toxicity. Moreover, to determine the clinical relevance of the role of TMBIM6, further studies in testicular cancer are needed.

WIDER IMPLICATIONS OF THE FINDINGS: Cisplatin-associated ER stress and redox imbalance might be implicated as toxicity mechanisms associated with anticancer therapy.
Introduction

Platinum derivative drugs play an important chemotherapeutic role in the treatment of solid tumors. Among them, cisplatin (cis-diaminedichloroplatinum) is the most commonly used (Pabla and Dong, 2008). However, despite its curative effects on solid tumors, cisplatin shows nephrotoxicity, anaphylaxis, ototoxicity, and bone marrow suppression (Hartmann et al., 2000; Hartmann and Lipp, 2003). Recently, side effects of cisplatin have been observed in testis (Ciftci et al., 2011). Testosterone production capacity is highly decreased after exposure to cisplatin due to the generation of reactive oxygen species (ROS) as a result of impaired mitochondrial oxidative phosphorylation and through the generation of DNA adducts (Marullo et al., 2013).

The steroidalogenic activity of cells is regulated by a protein called steroidalogenic acute regulatory protein (StAR). StAR mediates the rate-limiting step in the transfer of cholesterol from the outer mitochondria to the inner mitochondrion (Rone et al., 2009). Increased activity of StAR protein transports cholesterol into mitochondria, where it is further metabolized to pregnenolone by cytochrome P450 11A1 (CYP11A1). The pregnenolone is transferred from mitochondria to the endoplasmic reticulum (ER) and is converted to progesterone by the action of 3β-hydroxysteroid dehydrogenase Δ5–Δ4-isomerase (3β-HSD). The progesterone is finally converted to testosterone by 17β-hydroxysteroid dehydrogenase (HSD17β6) (Stocco, 2001). An increase in ROS induced by cisplatin is one cause of a decrease in StAR, whereas ER-localized heme oxygenase-1 (HO-1) inhibits ROS-induced apoptosis (Diemer et al., 2003). HO-1 activity has been also reported to be linked to the StAR protein (Shiraishi and Naito, 2005).

ER promotes the synthesis of new proteins and protein folding and post-translational modification. Disturbance in the usual activities of the ER leads to ER stress (Yadav et al., 2014). ER stress can be generated by oxidative stress due to chemicals, changes in calcium storage, glycosylation inhibition, nutrient deprivation and pathogen infection (Wang and Kaufman, 2012). Increased ER stress leads to defects in the synthesis of steroidalogenic enzymes (Park et al., 2013). To control ER stress, the unfolded protein response (UPR) is activated, which in turn induces IRE1, PERK and ATF6α signaling. Chaperone proteins such as glucose-regulated protein Grp78, calreticulin, calnexin and protein disulfide isomerase (PDI) also contribute to the UPR process. Among them, PDI, an important ER catalyst, is involved in disulfide bond formation (Bulleid, 2012) and the regulation of thiol/disulfide exchange. A decrease in PDI activity and binding to client proteins leads to an inability of PDI to form disulfide bonds in the ER, resulting in increased ER stress (Bhandary et al., 2012). ROS have been suggested to induce excessive post-translational oxidation through post-translational modification of PDI and carbonylation in the ER (Kenche et al., 2013; Lee et al., 2016). Cisplatin-induced ROS production might be linked to the alteration of the redox state of PDI and its hypercarboxylation impairing ER protein folding, leading to ER stress.

The antiapoptotic protein transmembrane BAX inhibitor motif-6/Bax inhibitor-1 (TMBIM-6/BI-1) is an ER stress inhibitor (Chae et al., 2004). Cisplatin-induced ER stress (Mandic et al., 2003) leads to defects in folding and maturation of ER-localized steroidalogenic enzymes. A defect in the maturation of ER-localized steroidalogenic enzymes leads to a decrease in testosterone formation. As HO-1 activity is high in the presence of TMBIM6 (Lee et al., 2007), we hypothesized that TMBIM6 might have protective activity against ROS-induced testicular toxicity and allow the production of more testosterone in the presence of ER stress. To investigate the protective role of TMBIM6 against ER stress and the associated alteration of steroidogenesis and the underlying mechanism, we examined cisplatin-induced testicular toxicity in a Tmbim6 knockout mouse model and Tmbim6-overexpressing Leydig cells.

Materials and Methods

Reagents

Cisplatin (Cis), N-ethylmaleimide (NEM), Rapamycin and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against HO-1, and VDAC1 were obtained from Abcam. Antibodies against Nrf2, β-actin, StAR, 3β-HSD, GRP78, and eIF2α were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against CYP11A1, p-AKT, AKT, CHOP, histone H3 and p-eIF2α were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against PDI was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Pregnenolone measurement kit (Immuno-biological Laboratories, MN, USA) and testosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) were used in this study.

Cell culture

TM3 Leydig cells from an immortalized mouse testis cell line were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea).

TM3 Leydig cells were stably transfected with pcDNA™3.1/Hygro(+) and pcDNA™3.1/Hygro(+)–Tmbim6 plasmids using the lipofectamine3000 transfection reagent (Invitrogen). The cells were cultured for 2 weeks in 100 μg/mL hygromycin (Invitrogen) and then grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% v/v fetal bovine serum (FBS) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO2.

Animal experiments

Mice (C57BL/6J) were housed in groups of five animals at 21 ± 1°C and 55 ± 5% humidity under a 12 h/12 h light/dark cycle. Mice were maintained in specific pathogen-free housing and were cared for in accordance with the regulations of the Care and Use of Laboratory Animals guide of Chonbuk National University with approval from the Institutional Animal Care and Use Committee of Chonbuk National University laboratory animal center (CBNU 2016-70). The TMBIM6+/+ wild-type (WT) and TMBIM6−/− knockout (KO) mice used in this study were described previously (Chae et al., 2004). In
animal experiments, to evaluate the effect of cisplatin, mice were divided into four groups (n = 7 each): TMBIM6+/+ and TMBIM6−/− cisplatin treatment groups which received a single intraperitoneal injection of 30 mg/kg body weight of cisplatin on the first day as acute models, and TMBIM6+/+ and TMBIM6−/− vehicle control groups received a saline. The cisplatin dose used was based on single-dose intravenous cisplatin therapy frequently applied to cancer patients. The cisplatin dose given (100 mg/m²) is based on body surface area but is equivalent to 28–30 mg/kg (Chang et al., 2013; Mitzaki et al., 2013). For tests experiments, the left testis (n = 7, each) was used for immunoblotting, RNA expression, PDI redox and DNP experiments, and the other testis was used for the other experiments (n = 7, each). Animals were anesthetized with ketamine on the third day, and blood and testis tissue samples were collected.

**Lentivirus-mediated gene transfer in vivo**

For lentivirus-mediated gene transfer in vivo, Tmbim6 KO mice were infected via a single injection of purified 1.0 × 10⁹ virus particle (Genennmed Inc., Seoul, Korea) in a volume of 50 μl (diluted in saline) into the testis, as previously described (Ogawa et al., 1997). After 2 days of infection with the virus, mice were treated with cisplatin or saline for the subsequent 3 days. Mice were sacrificed, and testis samples were collected and stored at −80°C for subsequent analysis.

**Immunohistochemistry and immunofluorescence**

We used formalin-fixed paraffin and Tissue-Tek OCT compound embedded testis tissues for immunohistochemistry and immunofluorescence staining, respectively. The tissue samples were stained with antibodies as previously described (Chae et al., 2004). Serial 5-μm thick sections were prepared, blocked with 5% serum after antigen retrieval, and incubated at 4°C overnight with one of the following primary antibodies. Immunostaining was visualized with 3,3′-diaminobenzidine (DAB) and sections were counterstained with hematoxylin and eosin.

**Measurement of testosterone**

Blood samples were centrifuged for 15 min at 2000g using a bench centrifuge, and plasma was stored at 4°C for testosterone assay using a commercially available Testosterone ELISA kit (Enzo, USA) in accordance with the manufacturer’s instructions.

**Quantitative real-time PCR**

Total RNA was extracted using TRizol reagent (Invitrogen) and reverse transcription was performed using a superscript III first strand synthesis kit (Invitrogen) according to the manufacturer’s instructions, which were determined by qPCR. The primer sequences and reaction conditions are listed in Supplementary Table SIV.

**Immunoblot analysis**

Cells or tissue samples were lysed with RIPA buffer (GenDEPOT, USA) containing a protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich) for 30 min on ice, followed by centrifugation at 10 000g for 20 min at 4°C. The resulting supernatants were collected and used as protein extracts. Protein extracts (30 μg) were separated on polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Bio-Rad, USA) for 1 h. After 1 h in blocking buffer containing 5% w/v non-fat dry milk (Bio-Rad, USA) and 0.05% v/v Tween 20 in Tris-buffered saline (TBS-T), the membranes were probed overnight at 4°C with the relevant primary antibodies. The membranes were washed three times with TBS-T for 30 min, and incubated with secondary antibodies at room temperature for 1 h, and washed three times with TBS-T for 30 min. The protein bands were detected using an enhanced electrochemiluminescence (ECL) Detection System (Thermo Fisher Scientific, USA), and immunoreactive bands were visualized using an LAS-3000 system (Fuj Film, Japan). Band intensities were measured and quantified using ImageJ software (National Institutes for Health).

**Electron microscopy**

Testis tissues were fixed by a conventional method (2% glutaraldehyde and 2% formaldehyde in 0.05 M sodium cacodylate buffer, pH 7.2) (Nishida et al., 2009; Park et al., 2011a). Fixed samples were embedded in Spurr’s resin, and thin sections (80 nm) were cut and stained with uranyl acetate and lead citrate for observation under a transmission electron microscope (H7650, accelerating voltage 100 kV, Hitachi) at the Center for University-wide Research Facilities (CBNU).

**PDI redox state and high-molecular-weight protein complex formation in the testis samples**

Procedures were performed as described previously (Molteni et al., 2004). Briefly, testis tissues were washed twice with ice-cold PBS supplemented with 20 mM N-ethylmaleimide (NEM) to protect the reduced disulfide bonds from further oxidation during lysis, lysed in lysis buffer (20 mM Tris, pH 7.4; 150 mM NaCl; 0.5% v/v Triton X-100) for 30 min on ice, and cleared by centrifugation. The 50 μg of the total protein was separated on non-reducing polyacrylamide gels (no SDS, not boiled) at 50 V to distinguish between redox forms of PDI.

**Detection of carbonylated PDI**

For detection of carbonylated PDI, proteins (100 μg) were derivatized for 5 min using the OxyBlot kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. PDI was immunoprecipitated as described previously (Lee et al., 2016), resolved in non-reducing gels, and subjected to Western blot analysis with anti-DNP rabbit antibodies according to the OxyBlot kit instructions.

**Heme oxygenase-1 activity**

Heme oxygenase-1 activity was measured in isolated microsomes as previously described (Nath et al., 1992). The samples were homogenized in phosphate buffer (pH 7.4), sonicated on ice, and centrifuged at 10000g for 10 min at 4°C. The resulting supernatants were centrifuged at 100 000g for 60 min at 4°C. The pellet was suspended in phosphate buffer (pH 7.0) containing 2 mM MgCl₂ and designated the microsome fraction. An aliquot of the microsomal fraction was added to a reaction mixture containing cytosol of the cells (2 mg of cytosolic protein), hemein (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate-dehydrogenase (0.2 units), and NADPH (0.8 mM) and incubated at 37°C for 2 h in the dark. The bilirubin formed during incubation was extracted using chloroform. The change in optical density was measured at 464–530 nm. HO-1 activity was expressed as picomoles of bilirubin formed per h/mg of protein.

**Detection of intracellular ROS levels**

For the detection of ROS in cells, cells were incubated with 100 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) at 37°C for 30 min after treatment with cisplatin. The fluorescence intensity of 2′,7′-dichlorofluorescein formed by a reaction between DCF-DA and intracellular ROS was analyzed by PAS flow cytometry (Partec, Münster, Germany) at excitation and emission wavelengths of 488 and 525 nm, respectively. For the detection of ROS in testis tissues, cryosection tissues slides were incubated with dihydroethidium (DHE) as biomarker of oxidative stress.
In situ hybridization

In situ detection of Tmbim6 transcripts in WT and tmbim6−/− mouse testis samples was performed using the RNAscope assay kit (Advanced Cell Diagnostics, 310035, Newark, CA, USA), as described previously (Jang et al., 2013). Briefly, 5-μm-thick paraffin-embedded tissue sections were deparaffinized, pretreated with heat and proteases and then hybridized with a target probe for the Tmbim6 gene. Next, an HRP-based signal amplification system was hybridized to the target probe before color development with 3,3′-diaminobenzidine tetrahydrochloride (Advanced Cell Diagnostics, 310035, Newark, CA, USA). Positive staining was defined as the presence of brown punctate dots in the nucleus and/or cytoplasm. The housekeeping gene Ubc (ubiquitin C) served as a positive control. The presence of brown punctate dots in the nucleus and/or cytoplasm. The housekeeping gene Ubc (ubiquitin C) served as a positive control. The DapB gene, which is derived from a bacterial gene sequence, was used as a negative control.

Statistical analysis

All data are presented as the mean ± SEM of several independent experiments, as specified in the figure legends. Differences among the four groups in the animal and cell culture studies (in animal study: saline-treated WT, saline-treated KO, cisplatin-treated WT, and cisplatin-treated KO mice; in cell culture study: saline-treated vector TM3, cisplatin-treated vector TM3, cisplatin-treated Tmbim6 TM3 and cisplatin-treated Tmbim6 TM3) were analyzed using one-way analysis of variance (ANOVA). Origin 8.0 software from OriginLab® Corp (Northampton, MA, USA) and GraphPad Prism 5.01 were used for statistical calculations. A P-value <0.05 was considered statistically significant.

Results

Tmbim6 protects testis against cisplatin toxicity

Expression of Tmbim6 was clearly detectable in the spermatogonia, Sertoli cells, Leydig cells, primary spermatocytes and spermatids in mouse testis, as examined by in situ hybridization (Fig. 1a). After cisplatin treatment, the testicular weight in cisplatin-treated Tmbim6 KO mice was decreased to a significantly greater extent than in Tmbim6 wild type (WT) (Fig. 1b), as was the serum testosterone concentration (Fig. 1c). Consistent with these findings, testosterone production was not significantly changed after cisplatin treatment in Tmbim6-overexpressing TM3 cells whilst it showed a dose dependent decrease in vector-only transfected TM3 cells (Supplementary Fig. S1a and b). Histopathologic analysis of seminiferous epithelium showed that cisplatin-treated Tmbim6 KO mice had greater testicular damage, vacuole formation, and decrease in germ cells with wide lumen compared with cisplatin-treated Tmbim6 WT mice (Fig. 1d, Supplementary Table S1). After cisplatin treatment, TUNEL-positive staining was increased significantly more in the Tmbim6 KO testis compared with the treated Tmbim6 WT testis (Fig. 1e). The Tmbim6-overexpressing TM3 cells also showed more resistance to cisplatin-induced toxicity compared with only vector-transfected TM3 cells (Supplementary Fig. S1c–e). After cisplatin treatment the ROS level was increased significantly more in the testis of Tmbim6 KO mice, compared with WT mice (Fig. 1f). Ultrastructure analysis of sperm revealed a well-organized structure in both non-treated Tmbim6 WT and KO mice (Fig. 2). After cisplatin treatment, Tmbim6 KO spermatozoa showed more abnormal mitochondria structure, more pronounced loss of microtubules, greater vacuolation in the sperm plasma membrane and greater degradation of the acrosome compared with Tmbim6 WT (Yao et al., 2002; Zhou et al., 2011). The Golgi bodies, which are important for the formation of the acrosome, were more affected by cisplatin in the Tmbim6 KO condition compared with the WT. Consistent with these findings, the weights of seminal vesicles and epididymis plus vas deferens, and sperm count in the cisplatin-treated Tmbim6 KO testis were significantly more decreased than in Tmbim6 WT testis (Supplementary Table SII). Together, these findings suggest that Tmbim6 has a protective role against cisplatin-induced testicular toxicity.

Tmbim6 protects against cisplatin-induced testicular toxicity through pAKT–Nrf2–HO-1 signaling

To understand the Tmbim6-induced protective mechanism against cisplatin, we examined Akt–Nrf2–HO-1 signaling. First, after cisplatin treatment the level of pAkt was increased significantly more in Tmbim6 WT testis compared with KO testis (Fig. 3a and b). The downstream expression of HO-1 protein and mRNA, and nuclear localization of Nrf2 were also more highly increased in the cisplatin-treated WT testis compared with treated KO testis (Fig. 3a–c). In immunostaining analysis, HO-1 expression was also highly increased in Leydig cells, and slightly increased in Sertoli cells after cisplatin treatment (Fig. 3d). Moreover, the level of p-Akt, HO-1, and nuclear expression of Nrf2 were more highly increased in Tmbim6-overexpressing TM3 Leydig cells (Tmbim6 TM3 cells) than vector-transfected TM3 cells (vector TM3 cells) (Fig. 3e, Supplementary Fig. S2). HO-1 activity was also increased to a greater extent in cisplatin-treated Tmbim6 TM3 cells than in the treated vector TM3 cells (Fig. 3f). The testosterone level was relatively highly maintained in Tmbim6 WT mice compared to Tmbim6 KO mice under the constitutive activation of p-AKT by rapamycin (Supplementary Fig. S3a and b). Consistent with these observations, cisplatin-induced ROS accumulation was also significantly inhibited in the presence of Tmbim6 (Fig. 3g). These data indicate that cisplatin-associated p-Akt, Nrf2 and HO-1 signaling is highly enhanced in the presence of Tmbim6.

Tmbim6 maintains expression of testosterone synthesis-associated proteins in cisplatin-treated conditions

The expression of StAR was decreased significantly more in the cisplatin-treated Tmbim6 KO mice compared with the treated Tmbim6 WT mice (Fig. 4a and b) (Diemer et al., 2003). Cyp1Ia1 was not affected in Tmbim6 WT testis after cisplatin treatment, whereas it was significantly decreased in the treated KO testis. Moreover, expression of StAR and Cyp1Ia1 was significantly decreased in the treated vector TM3 cells, while that expression was maintained at a high level in cisplatin-treated Tmbim6 TM3 cells (Fig. 4c and d). However, in a quantitative RT-PCR analysis, the mRNA expressions of StAR and Cyp1Ia1 were not significantly different between the treated Tmbim6 WT and the KO testis (Supplementary Fig. S4a and b). Markedly, the expression of StAR was rapidly decreased after cisplatin treatment, a representative phenomenon for protein degradation such as ubiquitination (Li et al., 2004; Zhang et al., 2015). Expectedly, the ubiquitination of StAR was increased in cisplatin-treated control TM3 cells...
compared to the treated Tmbim6 TM3 cells (Fig. 4e). The other steroidogenic enzyme, Cyp11a1 was also confirmed to be ubiquitinated. Treatment with cisplatin inhibited mitochondrial localization of StAR, whereas the mitochondrial localization was relatively stable in the cisplatin-treated TMBIM6-overexpressing cells, compared with the treated vector expressing cells (Supplementary Fig. S5). Consistent with these findings, although cisplatin did not affect serum cholesterol it decreased serum pregnenolone concentration but to a greater extent in Tmbim6 KO mice than in WT (Fig. 4f and g), suggesting that TMBIM6 protects steroidogenic activities in the presence of cisplatin.
and that StAR signaling contributes to the TMBIM6-associated steroidogenic activities.

**TMBIM6 controls ER folding capacitance, thus regulating ER stress in cisplatin-treated condition**

Expression of 3β-HSD was significantly more decreased in the cisplatin-treated Tmbim6 KO mice than the treated Tmbim6 WT mice (Fig. 5a and b). In the presence of cisplatin, 3β-HSD mRNA expression was obviously decreased, although the difference between Tmbim6 WT and KO tests were not significant (Supplementary Fig. S4c). Expectedly, levels of the ER stress marker proteins Grp78, Chop and p-EIF2 were prominently increased in cisplatin-treated Tmbim6 KO tests, compared with the treated Tmbim6 WT tests (Fig. 5c, Supplementary Fig. S6). The formation of high-molecular weight complexes (HMWCs) by PDI was also significantly increased in Tmbim6 KO, compared with Tmbim6 WT tests (Fig. 5d). These data indicate that PDI binds to the high-molecular weight proteins, leading to a decrease in disulfide bond formation, especially in the Tmbim6 knockout condition. In TMBIM6-overexpressing TM3 cells, PDI-bound HMWC formation was reduced compared with control TM3 cells (Fig. 5e). PDI was highly carbonylated in cisplatin-treated Tmbim6 KO relatively to Tmbim6 WT tests (Fig. 5f), indicating that the presence of TMBIM6 decreases the level of ROS and protein oxidation in cisplatin-treated conditions.

**Tmbim6 maintains steroidogenic enzyme activity in a cisplatin-treated condition through a pAKT-Nrf2-HO-1 axis**

The role of Tmbim6 in the testis was further examined through lentiviral Tmbim6 testicular seminiferous infection into Tmbim6 KO mice. Compared with the GFP-infected control, testicular Tmbim6 expression had little effect on testosterone in the absence of cisplatin but conferred significant resistance to the testosterone lowering effect of the drug (Fig. 6a). Expression of Tmbim6 increased the level of phosphorylated Akt and expression of HO-1 before and after cisplatin treatment (Fig. 6b and c). Under control conditions infection of Tmbim6 had little effect on the expression of StAR, 3β-HSD and Cyp11a1 but conferred significant resistance against cisplatin (Fig. 6b and c). In immunostaining analysis, HO-1 expression was also increased in the cisplatin-treated or non-treated Tmbim6 overexpression system (Fig. 6d). These data suggest that Tmbim6-protective signaling against cisplatin includes Akt–HO-1 signaling.

**Discussion**

In this study, we explored the protective activities of TMBIM6 in the testis against cisplatin treatment. TMBIM6 protects against cisplatin-induced testicular toxicity through the regulation of ROS metabolism through Nrf2 and related heme oxygenase-1. The presence of TMBIM6 also modulates cisplatin-induced ER folding disturbance and ER stress, maintaining a relatively high level of testosterone after cisplatin treatment compared with the Tmbim6 KO condition. Cisplatin treatment significantly decreased the steroidogenic enzymes StAR and 3β-HSD, whereas the presence of TMBIM6 recovered the decreased endocrine mechanism through the control of intra-ER resident HO-1 expression and ER redox balance.

We showed that cisplatin-induced signaling pathways including p-AKT, Nrf2 and HO-1 were down-regulated in the absence of TMBIM6. Cisplatin-induced ROS are compensated with endogenous antioxidant mechanisms including HO-1 (Jiang et al., 2006; Jung et al., 2014). In this study, the absence of TMBIM6 seems to disturb the compensatory antioxidant system of HO-1 and the Akt–Nrf2–HO-1 axis signaling (Fig. 3a), showing ROS accumulation with decreased testosterone (Fig. 1f and c). PI3K-Akt serves as an important signaling pathway in the induction of HO-1 (Park et al., 2011b). The increase in HO-1 promoter activity is also preceded by an increase in Akt phosphorylation, whereas Akt knockdown with siRNA almost completely suppressed HO-1 expression in response to H2O2 (Brunt et al., 2006). This study also suggests the involvement of ROS in the cisplatin-associated testicular structure alteration, showing greater testicular damage, vacuole formation and decrease in germ cells with a wide lumen in the tmbim6-KO compared with WT condition (Figs 1d
and 2e). In the cisplatin-induced ROS accumulation and its testicular toxicity model, the HO-1-linked mechanism is strongly implicated to explain the protective effect of TMBIM6 against cisplatin as summarized in Fig. 6e. With regard to the endogenous expression of HO-1 in the presence of TMBIM6, there has been some debate (Lisbona et al., 2009). To better clarify the endogenous expression of HO-1, immunoblotting was performed in MEF cells and Tmbim6-overexpressing cells, showing more significant expression of HO-1 in non-treated Tmbim6 mouse embryo fibroblasts and in TMBIM6-overexpressing TM3 cells compared with each counter group (Supplementary Fig. S7a and b). The AKT–Nrf2–HO-1 axis might contribute to the preservation of testicular structure, which is disrupted in the cisplatin-treated Tmbim6 knockout condition.

The expression of StaAR was significantly decreased after cisplatin treatment, especially in Tmbim6 KO tests (Fig. 4a). Similarly, the expression of CYP11A1 and 3β-HSD was also decreased in Tmbim6...

Figure 3 TMBIM6 enhances cisplatin-induced pAKT–Nrf2–HO-1 signaling. Tmbim6 WT and KO mice were injected with 30 mg/kg cisplatin or normal saline (n = 7/group). Three days later, immunoblotting with antibodies against p-AKT, AKT, HO-1 and β-actin for whole cell lysates; and Nrf2 and histone H3 for the nuclear fraction (a), quantification of the indicated proteins (b), real-time PCR analysis for HO-1 mRNA (c), and immunostaining with anti-HO-1 antibody (d) were performed. Scale bars = 100 μm. Vector TM3 and TMBIM6 TM3 cells were treated with 20 μM cisplatin for 0, 2, 4, 8, 12 or 24 h. Immunoblotting with antibodies against p-AKT, AKT, HO-1 and β-actin for whole cell lysates and against Nrf2 or histone for nuclear fraction (e) was performed (n = 3). Vector and TMBIM6-stable TM3 cells were treated with 20 μM cisplatin for 24 h, and HO-1 activity was measured as described in Materials and Methods (n = 3) (f). Vector and TMBIM6 TM3 cells were treated with 20 μM cisplatin for 12 h and incubated with 2',7'-dichlorofluorescein diacetate (DCF-DA) for 30 min following DCF-DA analysis by flow cytometry (n = 3) (g). Data normalized to the saline-treated Tmbim6 WT group. One-way ANOVA, *P < 0.05, **P < 0.001 and ***P < 0.0001.
KO, indicating relatively low production of testosterone and pregnenolone in the absence of TMBIM6 (Figs 1c and 4e). The mRNA levels of StAR, CYP11A1 and 3β-HSD showed no difference between cisplatin-treated Tmbim6 WT and Tmbim6 KO (Fig. S4). It seems likely that translational modification contributes to TMBIM6-associated maintenance of testosterone production even in the presence of cisplatin.

Figure 4 TMBIM6 maintains steroidogenic protein expression. Tmbim6 WT and KO mice were injected with 30 mg/kg cisplatin or normal saline (n = 7/group). Three days later, immunoblotting of testis tissues and mitochondrial (Mito) fraction with the indicated antibodies (a) and the quantification analysis of the indicated proteins were performed (b). Vector and TMBIM6-stably transfected TM3 cells were treated with 20 μM cisplatin for the indicated times. Immunoblotting with antibodies against StAR or Cyp11a1 (c) and its quantification analysis (n = 3) (d) and immunoprecipitation (IP) with the StAR or Cyp11a1 antibody and the immunoblotting with Ubiquitin antibody (e) were performed in the cell lysates (n = 3). Pregnenolone (f) and cholesterol analysis (g) were performed from Tmbim6 WT and KO mice as described in Materials and Methods. Data normalized to the saline-treated Tmbim6 WT group. One-way ANOVA, *P < 0.05, **P < 0.001 and ***P < 0.0001.
Figure 5 TMBIM6 regulates cisplatin-induced ER stress. Tmbim6 WT and KO mice were injected with 30 mg/kg cisplatin or normal saline (n = 7/group). Three days later, immunoblotting with antibodies against 3β-HSD (a) and quantification analysis (b) were performed. Immunoblotting with antibodies against Grp78, Chop, p-eIF2α, eIF2α or β-actin was performed (c). Whole testis lysates were analyzed for the presence of PDI in HMWCs using non-reducing (upper) and reducing (lower) gels (d). Vector TM3 cells and Tmbim6 TM3 cells were treated with 20 μM cisplatin for 0, 2, 4, 8, 12 or 24 h. Whole cell lysates were analyzed for PDI in HMWCs using non-reducing gels, and immunoblotting with anti-PDI antibody was performed (n = 3) (e). Whole testis lysates were derivatized with DNPH. Immunoprecipitated PDI was run on a non-reducing gel and analyzed by immunoblotting using anti-DNP antibody (n = 3) (f). PDI, protein disulfide isomerase; HMWCs, high molecular weight complexes. Data normalized to the saline-treated Tmbim6 WT group. one-way ANOVA, *P < 0.05, **P < 0.001 and ***P < 0.0001.
For folding of these steroidogenic proteins, ER folding capacitance represented by PDI and its related redox-coupled cycles are essential (Gidalevitz et al., 2013). This study suggests that the ER stress-mediated reduction in steroidogenic protein expression is related to dysfunctional PDI chaperone activity, a fundamental system in assisting protein folding (Lee et al., 2016). The unspecified folding proteins associated with the chaperone protein PDI were clearly accumulated as an aggregated form, especially in the Tmbim6 KO mice (Fig. 5d), indicating that the Tmbim6 KO condition disrupts function of the chaperones and isomerases such as PDI. Carbonylated proteins were also highly associated with PDI, especially in the Tmbim6 KO mice (Fig. 5f), suggesting that the majority of oxidized PDI resides within the HMWC fraction in association with client proteins, rather than releasing these proteins to their destinations. This study shows reduced/altered function of protein folding capacity, especially in the cisplatin-treated Tmbim6 KO condition. Throughout this study, cisplatin-induced ER stress is shown to be linked with PDI alterations and the stress-associated ROS accumulation during the steroidogenic protein folding/secretion processes, which are regulated by TMBIM6.

TMBIM6 is highly expressed in several cancers including prostate, breast and lung cancer (Grzmil et al., 2003, 2006; Lu et al., 2015), but has not yet been studied in testicular cancer. To identify whether

**Figure 6** TMBIM6 restored stereogenic enzyme activity through pAKT–Nrf2–HO-1 signaling. Tmbim6 KO mice were infected with lentiviral-GFP and lentiviral-Tmbim6 in testes. After 1 day of viral infection, mice were intraperitoneally injected with 30 mg/kg cisplatin or saline (n = 7/group). Three days later, testosterone concentration in serum was measured (a). Immunoblotting with indicated antibodies (b) and quantification analysis (c) were performed. Immunostaining with anti-HO-1 antibody was performed (d). Schematic diagram of the protective mechanism of Tmbim6 against cisplatin-induced testicular toxicity (e). Data normalized to saline-treated Tmbim6 WT group. one-way ANOVA, *P < 0.05, **P < 0.001 and ***P < 0.0001.
testicular cancer expresses TMBIM6, we analyzed GSE8607 data from the open source GEO database of NCBI, in which TMBIM6 expression was significantly increased in testicular cancer (n = 40) compared with normal samples (n = 3) (Supplementary Fig. 5B). We also confirmed the expression of TMBIM6 in various cancer types through the Human Protein Atlas database. In RNA-seq data analysis from 17 cancer types, we found the expression of TMBIM6 in testicular cancer to be relatively lower than other tumor types, for example, ovarian and lung cancers, which show resistance against cisplatin chemotherapy (Supplementary Table SIII); however, we also found the expression of TMBIM6 in various cancer types through the Human Protein Atlas database.

Cisplatin resistance also has implications for ongoing treatment since chemotherapy is associated with a poor long-term survival rate. Cisplatin-induced ROS are linked to endoplasmic reticulum stress. Further studies are necessary to determine the role of TMBIM6 in testicular cancer. In summary, cisplatin-induced ROS are linked to ER redox imbalance and ER stress, affecting steroidogenic protein maturation and ultimately decreasing testosterone levels. These responses are regulated by TMBIM6, which allows recovery of ER function through effective induction of HO-1 and finally contributes to the protection against cisplatin-induced testicular toxicity.

Supplementary data
Supplementary data are available at Human Reproduction online.

Authors’ roles
H.K.K.: experiment, data analysis, data interpretation, drafting of the article; R.K.Y.: experiment, drafting of the article; K.R.B.: experiment of IHC analysis and data interpretation; H.W.J.: experiment of animal and data interpretation; H.R.K. and H.J.C.: conception and design, data interpretation, revision of the article. All authors read and approved the article.

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Conflict of interest
None declared.

References
Abuzeid WM, Davis S, Tang AL, Saunders L, Brenner JC, Lin J, Fuchs JR, Light E, Bradford CR, Prince ME et al. Sensitization of head and neck cancer to cisplatin through the use of a novel curcumin analog. Arch Oncolaryngol Head Neck Surg 2011; 137: 499–507.

Bagrodia A, Lee BH, Lee W, Cha EK, Sfakianos JP, Iyer G, Pietzak EJ, Gao SP, Zabor EC, Ostrovnya I et al. Genetic determinants of cisplatin resistance in patients with advanced germ cell tumors. J Clin Oncol 2016; 34: 4000–4007.

Bhandary B, Marahatta A, Kim HR, Chae HJ. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. Int J Mol Sci 2012; 14: 434–456.

Brunt KR, Fennrich KK, Kiani G, Tse MY, Pang SC, Ward CA, Melo LG. Protection of human vascular smooth muscle cells from H2O2-induced apoptosis through functional co-codendence between HO-1 and AKT. Arterioscler Thromb Vasc Biol 2006; 26: 2027–2034.

Bulleid NJ. Disulfide bond formation in the mammalian endoplasmic reticulum. Cold Spring Harb Perspect Biol 2012; 4: a013219.

Chae HJ, Kim HR, Xu C, Bailly-Maire B, Krajewska M, Krajewski S, Banares S, Cui J, Dicicaglio M, Ke N et al. Bl-1 regulates an apoptosis pathway linked to endoplasmic reticulum stress. Mol Cell 2004; 15: 355–366.

Chang WW, Liu JJ, Liu CF, Liu WS, Lim YP, Cheng YJ, Lee CH. An extract of Rhodobacter sphaeroides reduces cisplatin-induced nephrotoxicity in mice. Toxins (Basel) 2013; 5: 2353–2365.

Ciftci O, Beytuer O, Cakir O, Gurbuz N, Vardi N, Vardi N. Comparison of reproductive toxicity caused by cisplatin and novel platinum-N-heterocyclic carbene complex in male rats. Basic Clin Pharmacol Toxicol 2011; 109: 328–333.

Diemer T, Allen JA, Hales KH, Hales DB. Reactive oxygen disrupts mitochondrial MA-10 tumor Leydig cells and inhibits steroidogenic acute regulatory (StAR) protein and steroidogenesis. Endocrinology 2003; 144: 2882–2891.

Gidalevitz T, Stevens F, Argon Y. Orchestra of secretory protein folding by ER chaperones. Biochim Biophys Acta 2013; 1833: 2410–2424.

Grzmił M, Kaulfuss S, Thelen P, Hemmerlein B, Schwayer S, Obenauer S, Kang TW, Burfeind P. Expression and functional analysis of Bax inhibitor-1 in human breast cancer cells. J Pathol 2006; 208: 340–349.

Grzmił M, Thelen P, Hemmerlein B, Schwayer S, Voigt S, Mury D, Burfeind P. Bax inhibitor-1 is overexpressed in prostate cancer and its specific down-regulation by RNA interference leads to cell death in human prostate carcinoma cells. Am J Pathol 2003; 163: 543–552.

Hartmann JT, Fels LM, Knop S, Stott H, Kanz L, Bokemeyer C. A randomized trial comparing the nephrotoxicity of cisplatin/ifosfamide-based combination chemotherapy with or without amifostine in patients with solid tumors. Invest New Drugs 2000; 18: 281–289.

Hartmann JT, Lipp HP. Toxicity of platinum compounds. Expert Opin Pharmacother 2003; 4: 889–901.

Jang BG, Lee BL, Kim WH. Distribution of LGR5+ cells and associated implications during the early stage of gastric tumorigenesis. PLoS One 2013; 8:e82390.

Jiang F, Roberts SJ, Data Sr, Dusting GJ. NO modulates NADPH oxidase function via heme oxygenase-1 in human endothelial cells. Hypertension 2006; 48: 950–957.

Jung SH, Kim HJ, Oh GS, Shen A, Lee S, Choe SK, Park R, So HS. Capsaicin ameliorates cisplatin-induced renal injury through induction of heme oxygenase-1. Mol Cells 2014; 37: 234–240.
Kenche H, Baty CJ, Vedagiri K, Shapiro SD, Blumental-Perry A. Cigarette smoking affects oxidative protein folding in endoplasmic reticulum by modifying protein disulfide isomerase. FASEB J 2013;27:965–977.

Lee GH, Kim HK, Chae SW, Kim DS, Ha KC, Cuddy M, Kress C, Reed JC, Kim HR, Chae HJ. Bax inhibitor-1 regulates endoplasmic reticulum stress-associated reactive oxygen species and heme oxygenase-1 expression. J Biol Chem 2007;282:21618–21628.

Lee HY, Lee GH, Bhattarai KR, Park BH, Koo SH, Kim HR, Chae HJ. Bax inhibitor-1 regulates hepatic lipid accumulation via ApoB secretion. Sci Rep 2016;6:27799.

Li D, Ueta E, Kimura T, Yamamoto T, Osaki T. Reactive oxygen species (ROS) control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination. Cancer Sci 2004;95:644–650.

Lisbona F, Rojas-Rivera D, Thielen P, Zamorano S, Todd D, Martinon F, Glavic A, Kress C, Lin JH, Walter P et al. BAX inhibitor-1 is a negative regulator of the ER stress sensor IRE1alpha. Mol Cell 2009;33:679–691.

Lu B, Li Y, Li H, Zhang Y, Xu J, Ren L, Fu S, Zhou Y. Bax inhibitor-1 is over-expressed in small cell lung cancer and promotes its progression and metastasis. Int J Clin Exp Pathol 2015;8:1411–1418.

Mandic A, Hansson J, Linder S, Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. J Biol Chem 2003;278:9100–9106.

Marullo R, Werner E, Degtjareva N, Moore B, Altavilla G, Mayer F, Stoop H, Scheffer GL, Scheper R, Oosterhuis JW, Looijenga LH, Lisbona F, Rojas-Rivera D, Thielen P, Zamorano S, Todd D, Martinon F, Glavic A, Kress C, Lin JH, Walter P et al. BAX inhibitor-1 is a negative regulator of the ER stress sensor IRE1alpha. Mol Cell 2009;33:679–691.

Lu B, Li Y, Li H, Zhang Y, Xu J, Ren L, Fu S, Zhou Y. Bax inhibitor-1 is over-expressed in small cell lung cancer and promotes its progression and metastasis. Int J Clin Exp Pathol 2015;8:1411–1418.

Mayer F, Stoop H, Schefer GL, Scheper R, Oosterhuis JW, Looijenga LH, Bokemeyer C. Molecular determinants of treatment response in human germ cell tumors. Clin Cancer Res 2003;9:767–773.

Mitsuzaki S, Hashimoto M, Matsushashi Y, Homma S, Suto M, Kato N, Nakagawasai Q, Tan-No K, Hiraika W, Yoshida M et al. Interleukin-6 modulates oxidative stress produced during the development of cisplatin nephrotoxicity. Life Sci 2013;92:694–700.

Molteni SN, Fassio A, Cirolino MR, Filomeni G, Pasqualetto E, Fagioli C, Sitia R. Glutathione limits Ero1-dependent oxidation in the endoplasmic reticulum. J Biol Chem 2004;279:32667–32673.

Natih KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, Rosenberg ME. Induction of heme oxygenase is a rapid, protective response in rhabdomyosarcoma in the rat. J Clin Invest 1992;90:267–270.

Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S. Discovery of A5G/A7G-independent alternative macroautophagy. Nature 2009;461:654–658.

Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 1997;41:111–122.

Pabla N, Dzug. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int 2008;73:994–1007.

Park KH, Kim BJ, Kang J, Nam TS, Lim JM, Kim HT, Park JK, Kim YG, Chae SW, Kim JW. Ca2+ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. Sci Signal 2011a;4:ra31.

Park SJ, Kim TS, Park CK, Lee SH, Kim JM, Lee KS, Lee IK, Park JW, Lawson MA, Lee DS. hCG-induced endoplasmic reticulum stress triggers apoptosis and reduces steroidogenic enzyme expression through activating transcription factor 6 in Leydig cells of the testis. J Mol Endocrinol 2013;50:151–166.

Parks SY, Park DJ, Kim YH, Kim Y, Kim SG, Shon KJ, Choi YY, Lee SJ. Upregulation of heme oxygenase-1 via PI3K/Akt and Nrf2 signaling pathways mediates the anti-inflammatory activity of Schisandrin in Porphyromonas gingivalis LPS-stimulated macrophages. ImmunoLett 2011b;139:93–101.

Rone MB, Fan J, Papadopoulos V. Cholesterol transport in steroid biosynthesis: role of protein-protein interactions and implications in disease states. Biochim Biophys Acta 2009;1791:646–658.

Shiraishi K, Naito K. Increased expression of Leydig cell haem oxygenase-1 preserves spermatogenesis in varicocele. Hum Reprod 2005;20:2608–2613.

Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. Oncogene 2003;22:1318–3151.

Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 2001;63:193–213.

Wang S, Kaufman RJ. The impact of the unfolded protein response on human disease. J Cell Biol 2012;197:857–867.

Yadav RK, Chae SW, Kim HR, Chae HJ. Endoplasmic reticulum stress and cancer. J Cancer Prev 2014;19:75–88.

Yao R, Ito C, Natsume Y, Sugitani Y, Yamanaka H, Kuretake S, Yanagida K, Sato A, Toshimori K, Noda T. Lack of acrosome formation in mice lacking a Golgi protein, GOPC. Proc Nat Acad Sci USA 2002;99:11211–11216.

Zhang J, Tripath DN, Jing J, Alexander A, Kim J, Powell RT, Dere R, Tait-Mulder J, Lee JH, Paul TT et al. ATM functions at the peroxisome to induce pexophagy and reduces steroidogenic enzyme expression through activating transcription factor 6 in Leydig cells of the testis. J Mol Endocrinol 2013;50:151–166.

Zhao X, Fabian L, Bayraktar JL, Ding HM, Brill JA, Chang HC. Auxillin is required for formation of Golgi-derived clathrin-coated vesicles during Drosophila spermatogenesis. Development 2011;138:1111–1120.