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Research Paper

IRE1α knockdown rescues tunicamycin-induced developmental defects and apoptosis in *Xenopus laevis*

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Abstract

Inositol requiring enzyme-1 (IRE1) is highly conserved from yeasts to humans. Upon endoplasmic reticulum (ER) stress, IRE1 activates X-box-binding protein 1 (XBP1) by unconventional splicing of *XBP1* mRNA, which activates unfolded protein response (UPR) to restore ER homeostasis. In mice, IRE1α plays an essential role in extraembryonic tissues. However, its precise action during the early stage of development is unknown. In this study, the gain and loss-of-function analyses were used to investigate the function of *Xenopus* IRE1α (xIRE1α). The effects of xIRE1α during embryo development were detected with RT-PCR and whole mount *in situ* hybridization. ER stress was induced by tunicamycin. The apoptotic cells were measured by TUNNEL assays. Although both gain and loss of xIRE1α function had no significant effect on *Xenopus* embryogenesis, knockdown of xIRE1α could rescue tunicamycin-induced developmental defects and apoptosis. The finding indicates that xIRE1α is not required for embryogenesis but is required for tunicamycin-induced developmental defects and apoptosis in *Xenopus laevis*.

Keywords: IRE1α, *Xenopus laevis*, tunicamycin, developmental defects

INTRODUCTION

The endoplasmic reticulum (ER) plays an important role in the synthesis and modification of secretary and membrane proteins in all eukaryotic cells. However, the function of the ER is disrupted when the inflow of unfolded polypeptide chains exceeds the folding or processing capacity of the ER, which is called ER stress. A series of adaptive responses, called the unfolded protein response (UPR), can up-regulate the transcription of various genes to increase the activity of protein-folding and protein-degradation for maintaining ER homeostasis. However, the persistent activation of UPR will trigger the cell death pathway because of prolonged ER dysfunction.

The UPR is transduced through three forms of ER-resident transmembrane sensors: IRE1, PKR-like ER Kinase (PERK), and activating transcription factor 6 (ATF6). The IRE1-dependent branch is highly evolutionarily conserved from yeasts to humans[1–4]. IRE1 is an ER-located type I transmembrane protein with a kinase domain and RNase domain in the cytosolic region. It plays a central role in the ER stress response. Upon ER stress, IRE1 is activated and the signal is transduced to the cytosol by the sequential dimerization/multimerization, trans-autophosphorylation, and activation of its endoribonuclease[5–7]. The specific activity of the endoribonuclease is responsible for the unconventional cytosolic splicing of HAC1 in yeast or excision of the 26-nucleotide intron of the X-box-

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binding protein 1 (XBP-1) transcription factor in metazoan organisms. The removal of the intron causes a frame shift and results in production of a spliced XBP1 mRNA, and encodes the active transcription factor XBP1s from the unspliced XBP1 mRNA (XBP1U)[8,9]. In *Xenopus*, there is another form of frame shift generated by the complete removal of exon 4[10]. The active form of XBP1 up-regulates chaperones to enhance protein folding and genes that mediate ER-associated degradation (ERAD) to target degradation of misfolded proteins in ER stress response[4]. Therefore, the splicing of XBP1 mRNA is a major event to mediate the UPR. Tunicamycin (TM) is an inducer of UPR and it was reported that TM induced severe ER stress, apparently with increased XBP1 splicing, and caused serious developmental defects in *Xenopus*[11].

In mammals, two IRE1 paralogues (IRE1α and IRE1β) have been reported. IRE1α is expressed ubiquitously and IRE1β primarily in intestinal epithelial cells. The two isoforms appear to have the same *in vitro* activities; subcellular localizations, and downstream target (XBP1 mRNA)[5–7]. However, they play a different role in embryo development. IRE1β deletion does not lead to significant developmental defects in mammals[12,13]. IRE1α is confirmed to be expressed ubiquitously in fetal and adult mice and to be essential for mammalian developmental processes[5]. Knockout of *IRE1α* caused widespread developmental defects, leading to embryonic death after 12.5 days of gestation in mice[14], which suggested an important role of IRE1α during development. To date, it has been reported that, during development, IRE1α is required for B-cell differentiation[14], exocrine tissues[15] and placental development and embryonic viability[16]. However, the data could not explain why *IRE1$\alpha^{-/-}$* embryos died at early stage.

Recently, two IRE1 homologues in *Xenopus* (xIRE1α and xIRE1β) were identified, and differentially expressed during embryogenesis. It was reported that xIRE1β is not only required for cytoplasmic splicing of xXBP1 pre-mRNA but also for mesoderm formation[17]. Although the expression pattern of IRE1α during embryogenesis was reported, the function of IRE1α during embryogenesis was unknown until now. In this study, knockdown and over-expression of IRE1α analyses were used to study the role of IRE1α during *Xenopus* embryogenesis. The results showed that knockdown of IRE1α had no significant effects on germ layer formation but it rescued TM induced developmental defects in *Xenopus* (*X.*) *laevis*.

MATERIALS AND METHODS

Ethics statement

The care of *X. laevis* (Nasco), the in vitro fertilization procedure, and embryo study were performed according to protocols approved by the Ethics Committee of Nanjing Medical University (permit number: 20080205).

Embryo manipulation and TM treatment

X. laevis eggs were obtained via in vitro fertilization, dejellied with 2% cysteine hydrochloride (pH 7.8–8.0) and cultured in 0.1 × MBSH (8.8 mmol/L NaCl, 0.24 mmol/L $NaHCO_3$, 0.1 mmol/L KCl, 0.082 mmol/L $MgSO_4$, 0.041 mmol/L $CaCl_2$, 0. 033 mmol/L $Ca(NO_3)_2$, and 1 mmol/L HEPES, pH 7.4). Embryonic stages were determined according to Nieuwkoop and Faber[18]. TM (Sigma, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) to obtain a stock solution (5 mg/mL). Four-cell stage embryos were cultured in 0.1 × MBSH containing TM (2 μg/μL). Embryos were grown in 0.1 × MBSH without TM were used as control. When control sibling embryos reached mid-blastula stage, treated embryos were transferred to fresh culture media without TM.

In vitro transcription of RNA, antisense morpholino oligonucleotides and microinjection

Plasmids pCS2$^+$-xIRE1α were linearized with *Not*I. Capped mRNA for microinjection was synthesized with SP6 mMessage mMachine™ kit (Ambion, USA) and cleaned-up with RNeasy kit (Qiagen, Germany). The antisense morpholino oligonucleotide (Gene Tools, USA) used for *xIRE1α* functional knockdown (IRE1α MO) was 5′-AAGAGAACCGCCAGAGGCGCC-ATGT-3′; an antisense morpholino oligonucleotide XBP(C)MO designed to inhibit the cytoplasmic splicing of xXBP1 was 5′-GACATCTGGGCCTGCTC-CTGCTGCA-3′; a standard control mopholino oligonucleotide, CoMO) was 5′-CCTCTTACCTCA-GTTACAATTTATA-3′. One nanogram of xIRE1α and 50 ng of IRE1α MO were injected into four blastomeres at 4-cell stage for scoring the phenotype and marker gene analysis.

Gene expression analysis

Total RNA from embryos was extracted, digested by DNase I and purified by RNeasy kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized with RevertAid™ first strand cDNA synthesis kit (Fermentas, Barlington, ON, Canada). Semi-quantitative RT-PCR was performed and primers for xXBP1 splicing and

Xbra, Xsox17a, α-actin, XMyoD, and Xnot were described previously[10]. In parallel, ODC was amplified to confirm equal amounts and integrity of different RNA preparations. Real time RT-PCR was performed and primers for chop were as described previously[19].

Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was performed according to standard procedures[20].

TUNNEL assay

TUNNEL assay on whole embryos was done using the protocol previously described[21].

Statistical analysis

Statistical analyses were performed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). Student's *t*-test was used to compare the differences among groups. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

IRE1*α* is not required for early stage development of *X. laevis*

The xIRE1α expression pattern during early stage of development was reported[17], but the role of xIRE1α is still unknown. To elucidate the function of xIRE1α during early embryonic development, the role of xIRE1α was focused on germ layer formation by gain of function (GOF) and loss of function (LOF) experiments. Different doses (from 0.5 to 1.5 ng/embryo) of xIRE1α mRNA were injected into all blastomeres at 4-cell stage, respectively. All the injected embryos at the tailbud stage were nearly normal (***Fig. 1C***). Whole mount *in situ* hybridization of embryos injected with 1.5 ng xIRE1α revealed that the expression of the pan-mesodermal marker gene *Xbra* and the endodermal gene *Xsox17a* at stage 10.5 was not significantly changed, compared with control MO injected embryos (***Fig. 1F*** and ***I***).

Next, a LOF analysis was performed by using an antisense morpholino oligonucleotide (IRE1α MO) directed against xIRE1α[22]. To examine the embryonic phenotype in response to *xIRE1α* knockdown, 50 ng IRE1α MO was injected into 4 blastomeres of 4-cell stage embryos. Interestingly, the injected embryos showed completely different phenotype from that of the *xIRE1β* knockdown embryos. Even until the tailbud stage, the xIRE1α knockdown embryos still looked normal (***Fig. 1B***). Further detection of the germ layer marker expression with whole mount *in situ* hybridization also showed no change (***Fig. 1E*** and ***H***), compared with control MO injected embryos. Therefore, GOF and LOF of IRE1α could not cause an apparent change of phenotype. These results show that xIRE1α is not required for development of *X. laevis* at early stage.

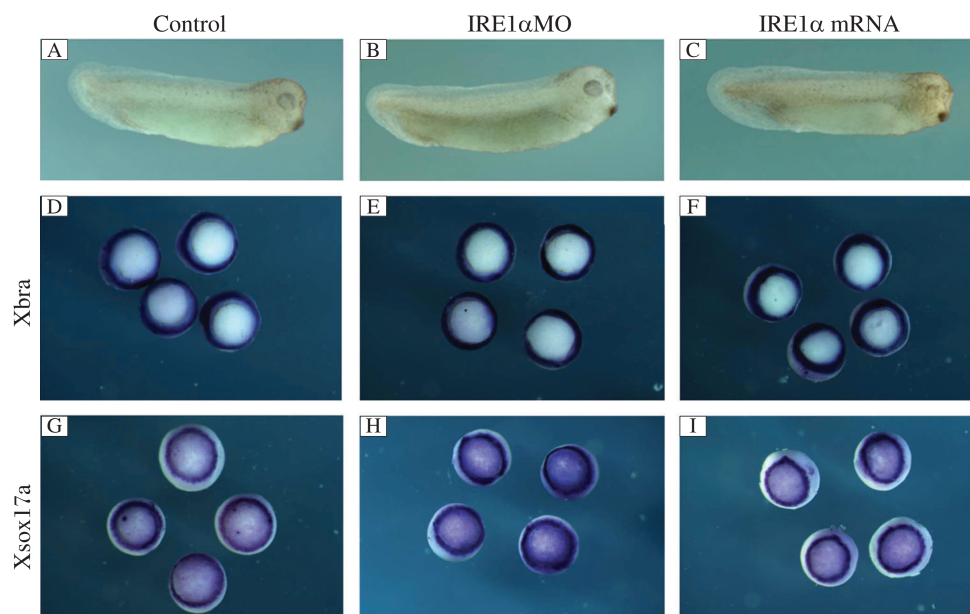

Fig. 1 **xIRE1*α* does not affect mesoderm and endoderm formation.** A: control MO injected embryo at tailbud stage. B and C: embryo injected with 50 ng xIRE1α MO or 1.5 ng *xIRE1α* mRNA. Overexpression or knockdown of *xIRE1α* did not change the expression of Xbra (D–F) and Xsox17a

Knockdown of *xIRE1α* rescued the developmental defects induced by TM

TM is the inducer of UPR, and it was reported that TM induced severe ER stress with an apparent increase in XBP1 splicing and caused serious developmental defects in *Xenopus*[19]. We speculated that, if XBP1 splicing is repressed, the developmental defects could be rescued. We injected 50 ng XBP1(C) MO into the 4 blastomeres at 4 cell stage embryos treated with 2 μg/mL TM. As shown in ***Fig. 2***, when control MO injected embryos reached the tailbud stage (***Fig. 2F***), TM-treated embryos still exhibited open neural folds and developed severe inhibition of anterior-posterior axis elongation and loss of head structure (***Fig. 2G***), while the developmental abnormality caused by TM was rescued in XBP1(C) MO injected embyos. The rescued embryos only showed a shorter axis, compared with the control sibling embryos (***Fig. 2I***).

Since both IRE1*α* and IRE1*β* have the same effect on XBP1 splicing, we detected if the rescue effect is dependent on the xIRE1*α*/XBP1 or xIRE1*β*/XBP1 pathway. At the neurula stage, untreated embryos developed normal blastopore closure and neural fold formation (***Fig. 2A***), while TM-treated embryos showed no blastopore closure and no clearly visible neural fold formation (***Fig. 2B***). We tried to do the rescue with injection of 50 ng of IRE1*β* MO into the embryos with the same treatment, but the rescue failed and the embryos died before or during gastrulation (***Fig. 2D***). Then, 50 ng of IRE1α MO was injected, as described above, and successful rescue was observed. The embryos looked nearly normal at the neurula stage (***Fig. 2C***) and showed a slightly shorter axis at the tailbud stage (***Fig. 2H***).

We also detected the rescue effect on XBP1 splicing after IRE1α MO injection with RT-PCR. It was shown that at stage 11, in control MO injected embryos, the cytoplasmic splicing was undetectable while the cytoplasmic splice form of xXBP1 (xXBP1(C)) was present in TM-treated embryos (***Fig. 2E***). After IRE1α MO injection, xXBP1(C) was undetectable.

Since TM treatment destroyed germ layer formation, we tested the rescue effect of IRE1α MO on the expression of germ layer markers. As shown in ***Fig. 3***, endodermal gene *sox17a*, mesodermal gene *xbra, XMyoD,* and *α-actin* and neuroectodermal marker *Xnot* were significantly inhibited in TM-treated embryos, while after knockdown of *xIRE1α*, the expression of these genes was recovered to nearly normal. These results suggested that knockdown of *IRE1α* could rescue the developmental defects through the xIRE1α /XBP1 pathway.

Knockdown of *xIRE1α* rescued apoptosis induced by TM

Increased ER stress triggers the apoptosis pathway, which was confirmed in TM treated embryos. The apoptotic cells were detected in most of the treated embryos (75%, n = 40, ***Fig. 4B***), which was one of

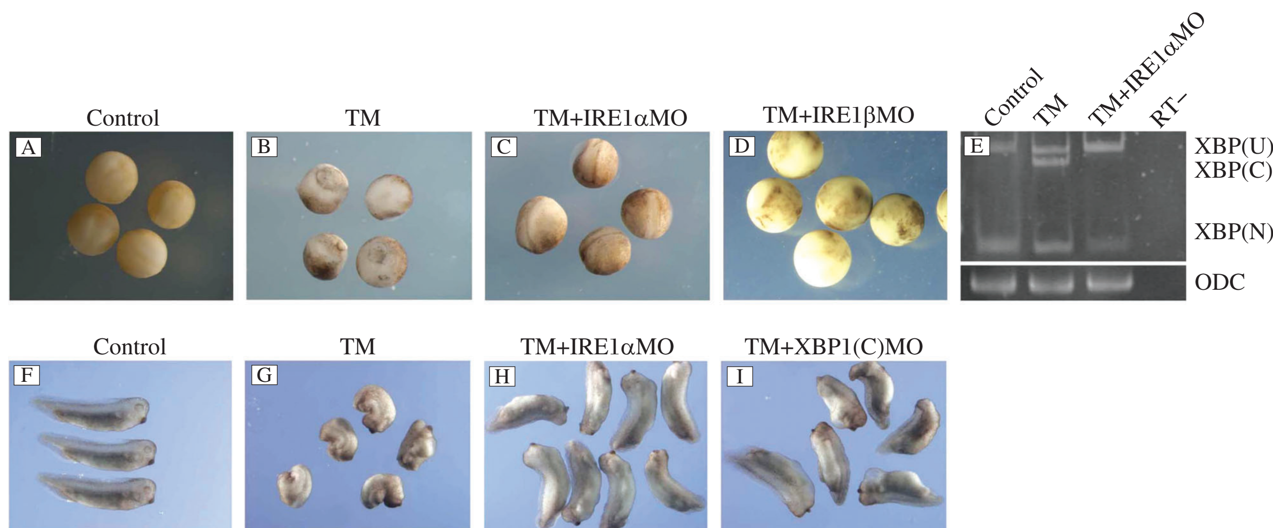

Fig. 2 **Developmental defects caused by tunicamycin (TM) were rescued with the injection of IRE1αMO and xXBP1(C) MO.** A: control MO injected embryos at stage 18. B: embryos treated with 2 μg/mL TM at stage 18. C: embryos treated with 2 μg/mL TM and injected with 50 ng IRE1αMO at stage 18. D: embryos treated with 2 μg/mL TM and injected with 50 ng IRE1*β*MO at stage 18. E: rescue effect on XBP1 splicing. TM treatment led to an increase of xXBP1(C) while TM treatment together with xIRE1α MO injection rescued the change in embryos at stage 11. F: control MO injected embryos at stage 32. G: embryos treated with 2 μg/mL TM at stage 32. H: embryos treated with 2 μg/mL TM and injected with 50 ng IRE1αMO at stage 32. I: embryos treated with 2 μg/mL TM and injected with 50 ng XBP1(C) MO at stage 32. RT-: no-reverse transcriptase control.

the possibilities that caused the developmental defects and even death of treated embryos. Then, we detected the rescue effects of *xIRE1α* knockdown on apoptosis with TUNNEL assay. As shown in ***Fig. 4C***, compared with TM-treated embryos, embryos injected with IRE1α MO and TM treatment showed dramatically decreased apoptotic signal.

CHOP codes for a bZIP transcription factor involved in the apoptosis pathway mediated by ER stress or DNA damage[23]. The higher increase in *CHOP* expression was observed in embryos treated with TM, while in xIRE1α knockdown embryos, the expression of *CHOP* decreased nearly to the normal level.These results clearly showed that knockdown of xIRE1α protected embryos from ER stress-induced cell death in *X. laevis*.

DISCUSSION

It has been established that ER stress is associated with various diseases[24–27] and ER stress-related mole–cules play an important role during embryo develop–ment[28–30]. IRE1α is the most evolutionarily conserved branch of the UPR and, upon activation, initiates the unconventional splicing of the mRNA encoding the transcriptional factor XBP1 to attenuate ER stress by mediating UPR. In the mammal, IRE1α and IRE1β have the same effect in mediating UPR, but they play different role in embryo develop–ment[11,12]. Recently, both IRE1α and IRE1β were iden–tified in *X. lavies*. Unexpectedly, besides mediating XBP1splicing, IRE1β played an essential role in germ layer formation[17], which has not been previously reported in mammals. The findings from the mammal model show that the function of IRE1α appears to be more important than that of IRE1β. Thus, we focused our study on the function of IRE1α in germ layer for–mation during *X. lavies* embryo development. However, we found that IRE1α is not required for germ layer formation during the early stage develop–ment of *X. lavies*.

Although knockdown of *xIRE1α* and *xIRE1β* can inhibit the splicing of xXBP1[17,21], their phenotype at the early stage of embryo development is different. These data also suggested that XBP1 may be not the only downstream molecule and other pathways med–iate the IRE1 function. On the other hand, XBP1 may have functions independent of xIRE1α and xIRE1β. In the studies of *XBP1* or *IRE1α* knock–out mice, their different phenotypes also support the possibility.[15,31]

Here we showed that *xIRE1α* knockdown did not show effects as strong as the effects caused by knock–down of *xIRE1β*, which suggests that xIRE1α and xIRE1β play different roles during the development. Furthermore, xIRE1β is essential in germ layer for–mation, while xIRE1α could be important in organo–genesis. In the mammal, IRE1α plays an essential function in extraembryonic tissues and IRE1a disrup–tion causes histological abnormality of the pancreatic acinar and exhibits mild hypoinsulinemia, hypergly–cemia, and a low-weight trend[15]. The previous study reported an expression pattern which showed that

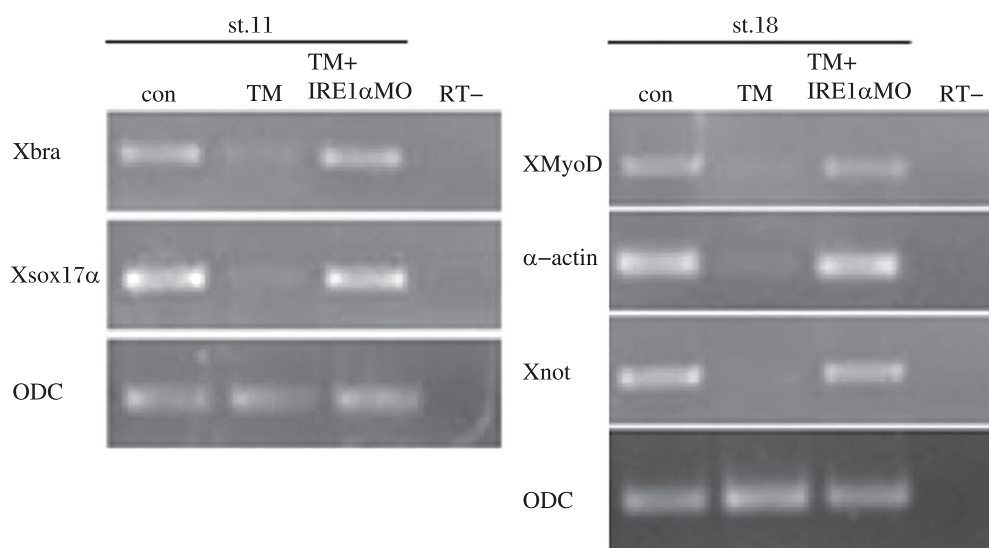

***Fig. 3* Rescue effects of *xIRE1α* knockdown on gene expression.** Control, TM treated and rescued embryos were collected at stage 11 or 18 and subjected to RT-PCR. Expression of mesodermal, endodermal, and neuroectodermal genes were inhibited in TM-treated embryos and rescued by *xIRE1α* knockdown. RT-: no-reverse transcriptase control.

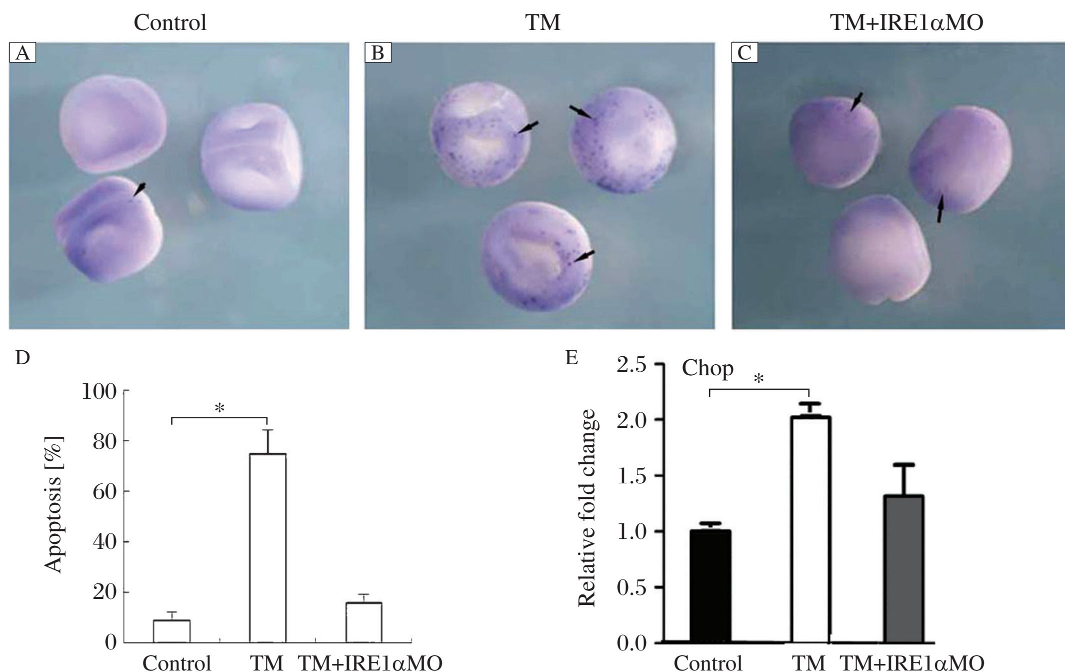

***Fig. 4 xIRE1α* knockdown rescued the apoptosis in embryos of stage 18 with TM treatment by TUNNEL.** A: The control embryos without TM treatment showed few staining indicating apoptosis (arrow). B: The TM-treated embryos showed significant signal for apoptosis (arrows). C: The embryos were injected with 50 ng of IRE1αMO at 4 cell stage, then treated with TM, and the apoptotic signal was decreased (arrows). D: A quantitative presentation is given. E: Expression of *CHOP* is inhibited in TM-treated embryos and rescued by xIRE1α knockdown. $^{*}P < 0.05$ compared with control embryos.

xIRE1α is expressed in the pancreas[17]. This suggested that xIRE1α could play a role in pancreas formation. The function of hIRE1α and the correlation between pancreas and hIRE1α will be investigated in future study.

Since the endogenous xIRE1α showed no effect on germ layer formation, we then detected the role of xIRE1α activated by ER stress. In a previous report[21], we found that embryos treated with TM showed significant abnormality during development and germ layer formation was obviously inhibited. In those embryos, xXBP1 splicing was enhanced, which suggested that the xIRE1/xXBP1 pathway was involved. We wonder that, if the xIRE1/xXBP1 pathway is inhibited, whether the developmental defects can be rescued. There are two ways to inhibit the xIRE1/xXBP1 pathway, one is to block the splicing of XBP1 to decrease the active form of XBP1; the other is to block the upstream gene *IRE1*. We tried to knockdown XBP1splicing and xIRE1α expression, and observed that both xXBP1 MO and xIRE1α MO had the rescue effects. However, when we tried to knockdown *xIRE1β* in TM treated embryos, the embryos died at early stage. These data indicated that it was not the xIRE1β/xXBP1 pathway, but the xIRE1α/xXBP1 pathway that was essential in TM caused developmental defects.

The splicing of XBP1 is increased in the case of overexpression of xXBP1 and TM treatment, but the following phenotype is different, which indicates that XBP1 could induce different kinds of downstream target genes and be multifunctional. In addition to the induction of UPR-related genes by ER stress, recent studies demonstrated that XBP1 also induces genes in cell type- and condition-specific manner[32]. In TM treated embryos xXBP1 showed UPR-related function mainly. Thus, *xIRE1α* knockdown can get rescue effect in TM treated embryos. Moreover, knockdown of *xIRE1α* showed better rescue effect than that showed in *XBP1* knockdown embryos indicated that xIRE1α has still other functions independent of XBP1.

In this study, the function of xIRE1α was compared during development, with TM treated embryos. The results showed that the phenotypes in TM treated embryos were significantly different from those during development, and indicated that IRE1α, in normal and ER stressed embryos, play different roles during embryogenesis. In summary, we showed that xIRE1α was not required for germ layer formation but was required for XBP1 splicing during embryogenesis, and knockdown of *xIRE1α* could rescue TM induced developmental defects and apoptosis in *X. laevis.*

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