IRE1α is essential for Xenopus pancreas development

Li Yuan*a, Xinxin Li*, Jiaojiao Feng*, Chenyang Yin*, Fang Yuan*, Xinru Wang†b

*aDepartment of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing, Jiangsu 210029, China;
†Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing, Jiangsu 210029, China.

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Abstract

Inositol requiring enzyme-1 (IRE1) is highly conserved from yeasts to humans. Upon the endoplasmic reticulum (ER) stress, IRE1 activates X-box-binding protein 1 (XBP1) by unconventionally splicing XBP1 mRNA, which activates the unfolded protein response (UPR) to restore ER homeostasis. In mice, IRE1α inactivity leads to embryonic death and IRE1α plays an essential role in extraembryonic tissues and the placenta. However, its precise action in the embryo proper is still unknown. In this study, the loss of function analysis was performed to investigate the function of Xenopus IRE1α (xIRE1α) during pancreas development. Firstly, the complete open reading frame of xIRE1α was amplified and the expression pattern was detected. The effects of Xenopus IRE1α and XBP1 during embryo development were detected with whole-mount in situ hybridization. The results demonstrated that xIRE1α was much closer to human IRE1α when compared with their sequence alignment. xIRE1α was expressed strongly in developing pancreas and the knockdown of xIRE1α inhibited the differentiation and specification of the pancreas. xIRE1α, which was required for cytoplasmic splicing of XBP1 pre-mRNA and XB–P1MO, also showed inhibitory effects on pancreas development. These results suggest that xIRE1α is essential for pancreas development during embryogenesis and functions via the XBP1 dependent pathway.

Keywords: IRE1α, Xenopus laevis, pancreas, XBP1

INTRODUCTION

The endoplasmic reticulum (ER) plays an important role in the synthesis and modification of secretory and membrane proteins in all eukaryotic cells. The accumulation of the unfolded/ misfolded proteins in the ER could cause ER stress and affect the overall integrity of the cell. A series of adaptive responses, called the unfolded protein response (UPR), is the transcriptional/translational regulatory pathway that mitigates such impairment of cellular integrity upon the detection of ER stress by the sensor proteins. The UPR is transduced through 3 forms of ER-resident transmembrane sensors, including inositol requiring enzyme-1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Each sensor protein senses the ER stress in its own fashion and induces the expression of its target genes, which facilitate the protein-folding capacity in the ER[1-3].

The IRE1-dependent branch is highly conserved from yeasts to humans[4]. IRE1 is an ER-located type I transmembrane protein with a kinase domain and an
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 endoribonu-
clease[5-7]. The specific activity of the endoribonuclease is responsible for the unconventional cytosolic splicing of HAC1 in yeasts and the excision of the 26-nucleo-
ite intron of the X-box-binding protein 1 (XBP-1) transcription factor in metazoan organisms. The re-
moval of intron causes a frame shift and the produc-
tion of a spliced XBP1 (XBP1s) mRNA, and encodes the active transcription factor XBP1s from unspliced XBP1 mRNA (XBP1U)[8,9]. 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.

Although PERK, ATF6 and IRE1 have common features as UPR inducers, there are several differ-
ences among their functions in vivo. PERK is highly expressed in mouse pancreas and is indispensable in pancreas development, while PERK−/− mice postnatally exhibit a phenotype of diabetes mellitus and exo-
crine pancreatic dysfunction[10]. ATF6α and ATF6β are ubiquitously expressed, and double knockout of ATF6α and ATF6β in mammals causes embryonic lethality in the early developmental stage (by 8.5 days of gestation), although a single knockout of each gene does not cause developmental abnormality[11,12]. IRE1α is also known to be ubiquitously expressed in fetal and adult mice[13,14], especially in the pancreas and the placenta. IRE1α inactivation results in wide-
spread developmental defects, leading to embryonic death after 12.5 days of gestation in mice[15]. Embryo proper-restricted IRE1α conditional KO mice, which specifically express IRE1α in the extra-embryonic tissues, can avoid embryonic lethality. It indicates that a defective IRE1α−/− placenta may be one of the reasons for embryonic lethality. However, it has been hitherto unclear in which tissues of the embryo proper IRE1α functions during embryogenesis.

In IRE1α conditional KO mice, embryonic viability disruption of IRE1α caused histological abnormality of the pancreatic acinar and increased blood glucose level that started occurring four weeks after birth. In Xenopus, IRE1α was found to be expressed in the do-
main that probably represents the dorsal pancreas an-
lagen[16]. These lines of evidence suggested that IRE1α plays a role during pancreas organogenesis.

Pancreas development is conserved and early pan-
creas development in Xenopus closely resembles that

of mice and humans, and is applicable to mammalian cells[17]. In fact, it is becoming clear that the same genes used in mammalian pancreas development are involved in Xenopus pancreas development[18]. In this study, the complete open reading frame (ORF) of Xenopus IRE1α was cloned, and the knockdown of IRE1α was performed to study the role of IRE1α in pancreas formation.

**MATERIALS AND METHODS**

**Embryo manipulation**

Xenopus laevis eggs were obtained from in vitro fertilization, dejellied in 2% cysteine hydrochloride (pH 7.8-8.0) and cultured in 0.1 × MBSH (8.8 mmol/L NaCl, 0.24 mmol/L NaHCO3, 0.1 mmol/L KCl, 0.082 mmol/L MgSO4, 0.041 mmol/L CaCl2, 0.033 mmol/ L Ca(NO3)2, and 1 mmol/L HEPES, pH 7.4). Embryo-
one stages were determined according to Nieuwkoop and Faber[18].

**Plasmids and constructs**

To complete the Xenopus IRE1α ORF, we used rapid amplification of cDNA ends (RACE) tech-
nique to extend the known partial cDNA to its 5’ and 3’ ends. For the 5’ RACE of xIRE1α, the following primers were used: 5′-TGTTCTCAGCA GTCCAC-
CAG-3′ and 5′-GGTTCTGTGACGTGTGTTGG-3’; for the 3’ RACE of xIRE1α, 5′-GT TTTTCGACAGC-
GGAGGTG-3′ and 5′-GCTATTTCTGCACCCGAG-
GAGG-3′ were used. The ORF of xIRE1α encoding 969 amino acids was established by joining the 1963 bp cDNA sequence, the 5’ RACE and the 3’ RACE sequences. To make the pCS2+-xIRE1α expression plasmid, IRE1α ORF was amplified from a cDNA pool consisting of st.1, st.8, st.10, st.15, st.20 and st.28 cDNAs and was subcloned to pCS2

**In vitro transcription of RNA, antisense mor-
pholino oligonucleotide (MO) and microin-
jection**

Plasmids of pCS2+-xIRE1α were linearized with NorI. Capped mRNA for microinjection was synthe-
sized with SP6 mMessage mMachine™ kit (Ambion, Thebarton, SA, Australia). The sequence of antisense MO (Gene Tools, Philomath, OR, USA) used for xIRE1α’s functional knockdown (IRE1α MO) was
5′-AAGAGAAACCCGAGGGCCCATGT-3′; the sequence of an antisense MO named XBP(C) MO that was used to inhibit the cytoplasmic splicing of xXBP1 was 5′-GACATCTGGCCGCTGCTCTCCT-GCTGCA-3′. Standard control MO (CoMO) was 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Fifty ng IRE1α MO or XBP(C)MO was injected into 4 blastomeres at the 4-cell stage for scoring the phenotype and marker gene analysis.

In vivo assay for morpholino function

To detect the specificity of IRE1α MO, the N-terminus coding region of xIRE1α containing the IRE1α MO binding site was fused to green fluorescent protein (GFP) (xIRE1α/GFP). For control, the N-terminus of xIRE1α was mutated at 6 bases and fused to GFP (xIRE1αmut/GFP). The mRNAs were transcribed and injected either alone or with 50 ng IRE1αMO or CoMO, respectively. At the desired stage, the embryos were analyzed by fluorescence microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from embryos was extracted and digested with DNaseI, and purified by RNase kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized with RevertAid™ first strand cDNA synthesis kit (Fermentas, Ontario, Canada). Semi-quantitative RT-PCR was performed and primers for xXBP1 splicing were detected as previous described. In parallel, ODC was amplified to confirm equal amounts and integrity of different RNA preparations.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to standard procedures. The probes were

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**Fig. 1 Xenopus IRE1α sequence analysis.** A: Alignment of Xenopus xIRE1α, xIRE1β, hIRE1α and hIRE1β amino acid sequences. Identical residues are marked by asterisks. Gaps are introduced to achieve optimum alignment. B: Percentage of identity between IRE1 proteins. C: Phylogenetic tree of IRE1 proteins of different species created by ClustalW (h, Homo sapiens; x, Xenopus laevis).
prepared as follows: pDrive-IRE1α was cut with HindIII and transcribed with T7 RNA polymerase. pdx1, ptf1a, insulin and amylase antisense probes were prepared as previously described[20].

RESULTS

Isolation of Xenopus IRE1α

In a previous study, we obtained a piece of 1,963 bp cDNA containing partial xIRE1α ORF[16]. Now, the ORF of xIRE1α coding for 969 amino acids was completed by using RACE. A phylogenetic analysis of xIRE1α with other vertebrate homologues by using ClustalW (MacVector, Cary, NC, USA) showed that this peptide shared 57% identity to the Xenopus IRE1β (xIRE1β) and 49% identity to the human IRE1β (hIRE1β). However, xIRE1α exhibited 78% identity to human IRE1α (hIRE1α) (Fig. 1A and B), which indicated that the isolated xIRE1α sequence and hIRE1α were genetically close (Fig. 1C).

IRE1α expression in the developing pancreas

In tail bud embryos, IRE1α was detected in a domain that is probably representing the dorsal pancreas anlagen[16]. To further explore the spatial expression patterns of IRE1α in Xenopus embryos at later stages, we carried out whole-mount in situ hybridization. During the tadpole stages, high expression of IRE1α was observed in the pancreas (Fig. 2), suggesting a potential role of IRE1α in the Xenopus pancreas development.

Xenopus IRE1α knockdown inhibits the expression of pancreatic differentiation marker genes

To perform loss-of-function studies, we designed morpholino antisense oligos for IRE1α, which cover the ATG initiation codon. To test whether IRE1α MO could efficiently block IRE1α translation in vivo, a 300 bp 5′-coding sequence of xIRE1α was fused which contained the putative MO binding site to GFP.
Xenopus IRE1α and pancreas formation

Since cleavage of XBP1 pre-mRNA by IRE1 is a well conserved mechanism throughout all organisms examined\textsuperscript{[8,9,16,21]}, we tested whether the IRE1 homologue xIRE1α could also cleave Xenopus XBP1 pre-mRNA and xXBP1(U). Non-injected control and xIRE1α-injected embryos were collected at stages 11 and 18 and subjected to RT-PCR. As previously reported, for non-injected embryos at stage 11, only the unspliced (xXBP1(U)) and the nuclear splice form of xXBP1 (xXBP1(N)) were detected; at later stages, the conventional cytoplasmic splice form of xXBP1 (xXBP1 (C)) was detected\textsuperscript{[16]}. In embryos injected with xIRE1α mRNA, the band representing xXBP1(C) at stage 11 was detected, and xXBP1(C) was significantly increased concomitant with a decrease of xXBP1(U) in comparison to the controls at stages 18. In embryos injected with xIRE1αMO, the band for xXBP1 (C) at stage 18 disappeared (Fig. 6A).

We further monitored the effects of xIRE1α on xXBP1 splicing in vivo using a fluorescence sensor, by fusing the C terminal coding region of a 121-254 of unspliced xXBP1 with the stop codon to the 5’-end of the coding region of the fluorescent protein, EosFP\textsuperscript{[22]}. In the xXBP1(U)-EosFP mRNA injected embryos, green fluorescence was not detected (Fig. 6B). However, the xXBP1(U)-EosFP mRNA co-injected with xIRE1α mRNA resulted in the appearance of green fluorescence in embryos (Fig. 6C). In the embryos injected with xXBP1(U)-EosFP, xIRE1α mRNA and XBP1(C)MO, no more fluorescence was detected (Fig. 6D). These results suggest that xIRE1α is required for cytoplasmic splicing of xXBP1 pre-mRNA.

Xenopus XBP1 knockdown inhibits pancreas formation

IRE1α is the most evolutionarily conserved branch of the UPR. Upon activation, it initiates the unconventional splicing of mRNA encoding the transcriptional factor XBP1 to attenuate ER stress by mediating UPR. To investigate whether knockdown of XBP1 affects pancreas formation, we injected 50 ng of spliced form of XBP1 (C) MO that bind to the splice site to repress the splice of XBP1 into 4 blastomeres at the 4-cell stage for scoring the phenotype. As shown in Fig. 7, the expression of pancreas specific marker insulin and amylase was significantly reduced compared to the control embryos. Injection of XBP1(C) MO also caused gut-coiling defect.

DISCUSSION

Previous studies have established the essential role of IRE1α during embryogenesis\textsuperscript{[13,23,24]}; however, it has been hitherto unclear in which tissues in the embryo proper it functions and how IRE1α functions during embryogenesis. Here, we demonstrated that Xenopus IRE1α is essential for pancreas organogenesis. We initially cloned the full length of Xenopus IRE1α and found that it was predominantly expressed in the developing pancreas during Xenopus embryogenesis. Then, we demonstrated that knockdown of IRE1α led to the suppressed expression of pancreas differentia-
**Fig. 4** IRE1α knockdown specifically inhibits the expression of differentiation marker genes. Whole mount in situ hybridization analyses revealed that the expression of insulin (B) and amylase (D) was not detected in IRE1α MO injected embryos at stage 43 compared with control MO injected embryos (A, C). The white arrows point to the positive staining of insulin and amylase.

**Fig. 5** IRE1α knockdown inhibits the expression of specification marker genes. Whole mount in situ hybridization analyses revealed that the expression of pdx1 (B) and ptf1α (D) was significantly suppressed in IRE1α MO injected embryos at stage 30 compared with control MO injected embryos (A, C). The white arrows indicate positive staining.
**Xenopus IRE1α and pancreas formation**

Fig. 6 Effects of xIRE1α on cytoplasmic splicing of xXBP1. RT-PCR (A) detected an increase of cytoplasmic variant xXBP1 (C) in embryos injected with xIRE1α mRNA and a decrease of the xXBP1(C) in embryos injected with IRE1α MO at stage 11 and 18. ODC (ornithine decarboxylase) served as a loading control. Monitoring the xXBP1 splicing by xIRE1α in vivo (B-D). Embryos injected with 500 pg xXBP1(U)-EosFP RNA individually or in combination with 1 ng xIRE1α RNA and/or 50 ng XBP (C) MO. xXBP1 (U), unspliced xXBP1; xXBP1(N), nuclear spliced xXBP1. RT- no reverse transcriptase control.

Fig. 7 IRE1α knockdown inhibits pancreas marker gene expression. Whole mount in situ hybridization analyses revealed that the expression of insulin (B) and amylase (D) was not detected in 50 ng XBP1MO injected embryos at stage 43 compared with control MO injected embryos (A,C). The white arrows indicate positive staining.

Finally, we demonstrated that IRE1α functions via the XBP1 dependent pathway.

Only IRE1 is conserved in all eukaryotes of the ER stress sensors, including fungi, plants and animals. Yeasts and nematodes have only one *IRE1* gene in
their genome, and the inactivation of this gene is not lethal to these organisms under normal conditions\textsuperscript{[25,26]}, while knockout of IRE\textsubscript{1}α causes embryonic lethality in mice\textsuperscript{[15]}. This evidence suggests that IRE\textsubscript{1}α has a unique function in the developmental processes. Sequence alignment showed that Xenopus IRE\textsubscript{1}α is much more similar to human IRE\textsubscript{1}α. xIRE\textsubscript{1}α, like mammalian IRE\textsubscript{1}α\textsuperscript{[27]}, cleaves XBP1 pre-mRNA in vivo.

Abundant expression of IRE\textsubscript{1}α has been reported in the mammalian pancreas\textsuperscript{[13]}. A previous report showed that xIRE\textsubscript{1}α is expressed in a domain that probably represents the dorsal pancreas anlagen\textsuperscript{[16]}. This study showed that xIRE\textsubscript{1}α was expressed during the development of the pancreas during Xenopus embryogenesis. The developmental expression of a number of pancreatic markers has been reported in Xenopus including nuclear factors (Pax6, NeuroD, Islet1, Pdx1 and XpabPiII), hormones (insulin, glucagon and somatostatin) and digestive enzymes (amylase, elastase, trypsinogen and carboxypeptidase A\textsubscript{\textalpha})\textsuperscript{[17]}. Sox9 and Pdx1 are expressed around stage 25 in the prospective pancreatic rudiments, and most of the other markers are not detected until the pancreatic buds become discernible\textsuperscript{[17]}. Therefore, IRE\textsubscript{1}α is one of the earliest genes expressed in the developing pancreatic tissue and has the potential role in specification and differentiation of the pancreas.

Pancreas morphogenesis begins with the evagination of the embryonic endoderm for the formation of dorsal or ventral buds whose development is guided by distinct transcription programs\textsuperscript{[28]}. To investigate whether IRE\textsubscript{1}α plays a role in pancreas development, IRE\textsubscript{1}α was knocked down and pancreas developmental marker genes were detected with the whole-mount in situ hybridization. Knockdown of IRE\textsubscript{1}α resulted in dramatic gut defects after stage 40. The expression of the endocrine and exocrine differentiation markers, insulin and amylase at stage 43 was almost completely abolished, which suggested that the pancreas structure was destroyed in IRE\textsubscript{1}α deficient embryo and the final differentiation of endocrine and exocrine cells was affected in IRE\textsubscript{1}α knockdown embryos. However, it does not address whether this effect is seen earlier in development when the pancreatic domain is first specified. This is especially important as IRE\textsubscript{1}α is expressed early in the domain representing the pancreas anlagen.

Pancratic progenitor cells first express the homeodomain transcription factor Pdx1, then expressing the basic helix-loop-helix (bHLH) factor Ptf1α\textsuperscript{[29]}. The whole-mount in situ hybridization showed that knockdown of IRE\textsubscript{1}α caused reduced expression of pancreas specification markers, including pdx1 and ptf1α. Both genes are expressed in pancreatic progenitors, and are necessary and sufficient for pancreas development\textsuperscript{[17]}. The defect seen in Pdx1 knockdown Xenopus is similar to that observed in mice; although loss of Pdx1 leads to pancreatic agenesis, there is a small dorsal bud present that produces insulin and glucagons\textsuperscript{[17]}. Knockdown of Ptf1α resulted in a complete loss of acinar cells, and both insulin and glucagons were lost at late stage\textsuperscript{[30]}. Based on the previous report, knockdown of IRE\textsubscript{1}α has no effect on germ layer formation\textsuperscript{[31]}, which suggest that knockdown of IRE\textsubscript{1}α firstly inhibited the progenitor genes of endocrine and exocrine, and then repressed the differentiation of the pancreas.

In ER stress response, IRE\textsubscript{1}α and XBP1 function in the same signal transduction pathway\textsuperscript{[32]}. However, some other studies showed that not only a known IRE\textsubscript{1}α-dependent XBP1 function but also an XBP1-independent IRE\textsubscript{1}α function exists\textsuperscript{[21,22,33]}. We found that the knockdown of IRE\textsubscript{1}α and XBP1 led to a similar phenotype, which indicated that XBP1 functions downstream of IRE\textsubscript{1}α. XBP1 is a transcription factor and was reported to physically interact with and negatively regulate the levels of forkhead box O1 (FoxO1)\textsuperscript{[34]}. FoxO1 may play a role on beta cell differentiation in the human fetal pancreas by controlling critical transcription factors, including ngn3 and Nkx6.1\textsuperscript{[35]}. These findings suggest that during pancreas development, IRE\textsubscript{1}α may function via the XBP1-dependent pathway, and then XBP1 regulates the downstream transcription factors, which needs to be further confirmed.

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