Biochemical and Biophysical Research Communications 433 (2013) 426–431

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Positive contribution of IRE1α–XBP1 pathway to the expression of placental cathepsins

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ARTICLE INFO

Article history:
Received 19 February 2013
Available online 26 March 2013

Keywords:
IRE1α
XBP1
Cts7
Cts8

ABSTRACT

IRE1α is an ER-located transmembrane RNase whose activation leads to the production of the transcriptional factor, XBP1. Recently, many studies report that IRE1α–XBP1 pathway has novel and significant roles in placenta. However, its molecular details have been still unknown. To address this point, we have focused on the molecular linkage between IRE1α–XBP1 pathway and Cts7 and Cts8, which are essential cathepsins for placenta formation. In cellular model, this pathway positively contributed to their expression at transcriptional level. In addition, the disruption of IRE1α or XBP1 in animal model significantly attenuated their transcripts in placenta. These results indicated that IRE1α–XBP1 pathway function as a specific program supporting the placenta formation by ensuring the moderate expression of specific subset of placental cathepsins.

1. Introduction

The endoplasmic reticulum (ER) plays a key role in the synthesis and modification of secretory and membrane proteins in all eukaryotic cells. Under normal conditions, these proteins are correctly folded and assembled in the ER [1,2]. However, when cells are exposed to environmental factors such as overproduction of ER proteins, viral infections, or glucose deprivation, the secretory and membrane proteins can accumulate in unfolded or misfolded forms in the lumen of the ER and, consequently, cause stress in the ER. To maintain cellular homeostasis, cells induce several responses to ER stress, that is totally called unfolded protein response (UPR) [3]. In mammalian cells, UPR is mediated by a diversity of signal pathways. There are three ER-located transmembrane proteins that play important roles in the mammalian UPR: activating transcription factor 6 (ATF6), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1 (IRE1).

IRE1, is an ER-located type I transmembrane protein with a kinase domain and RNase domain in the cytosolic region. When exposed to ER stress, via trans-autophosphorylation and activation of its RNase domain, IRE1 induces unconventional splicing of an mRNA encoding a specific transcription factor responsible for UPR activation [4–8]. IRE1 is highly conserved from yeast to humans, and two IRE1 paralogues have been reported in mammals: IRE1α and IRE1β [9–11]. Specifically, IRE1α induces the unconventional splicing of XBP1 mRNA under ER stress condition [12]. The spliced XBP1 mRNA is then translated into a functional transcription factor to induce UPR. Besides IRE1, two ER-located transmembrane proteins, PERK and ATF6, play important roles in mammalian UPR [13,14]. Upon ER stress, PERK induces the phosphorylation of eIF2α and the translational activation of ATF4 [15]. On the other hand, under ER stress condition, ATF6 is cleaved by Site-1 and Site-2 proteases, and its cytoplasmic domain is translocated to the nucleus [16,17]. The XBP1, ATF4, and the cleaved ATF6 work as transcription factors in UPR induction, as well as XBP1 which is activated by IRE1α.

Recently, many studies report that UPR has novel and significant function in placenta. The placenta is essential for sustaining the growth of the fetus during gestation, and defects in its function result in fetal growth restriction or, if more severe, fetal death and birth defects [18], and various kinds of genes governing placental development have been reported [19]. Previously, our reporter system identified the specific and significant placental activation of the IRE1α–XBP1 pathway, and serious morphological and birth defects were elicited by the deletion of IRE1α [20]. In line with this, the expression of several factors essential for placenta-formation was found to be mediated by the UPR pathways [19–22]. Also, it has been reported that specific kinds of cathepsins (PECs; placentally expressed cathepsins) are highly expressed in placenta [23–26], and mediates various function [27]. Importantly, Cts7 and

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http://dx.doi.org/10.1016/j.bbrc.2013.02.119

Cts8 that are essential for the placenta formation, were recently shown to have distinct roles in trophoblast function or vascular remodeling [28]. However, the molecular details how these cathepsins are induced in placenta have been still unknown. Here, we reported that IRE1α–XBP1 pathway positively contributes to the expression of these cathepsins, to ensure their moderate expression in placenta.

2. Materials and methods

2.1. RT-PCR, luciferase assay, and microarray analysis

For RT-PCR, Total RNAs from SM-10 cells (Fig. 1A and B) and placenta (Fig. 2C) were prepared from cells using Isogen reagent (Nippon Gene). A SuperScript® First-Strand Synthesis System (Invitrogen) was used to synthesize the cDNA, according to the manufacturer's instructions. The PCR experiments were performed with TaKaRa Ex-Taq (Takara) using the specific primers listed in Table 1.

For microarray analysis in Fig. 2B or Fig. S1, total RNAs were extracted from the placentas of wild type, IRE1α knockout, and XBP1 knockout mice (E11.5) [20], using Isogen reagent (Nippon Gene). RNAs from three littermates of each genotype were pooled. Microarray analysis was performed with the 3D-gene mouse Oligo chip 24 k (TORAY). Hybridization of the RNA samples to the microarray, detection and data analysis were carried out by TORAY.

Dual luciferase assay was performed using the dual luciferase assay system (Promega) and a luminometer (Berthold). As an internal control, phRL-TK (Promega) was used. The results are shown as mean ± s.e.m. from triplicate experiments. Each value is shown as a fold induction normalized to that of each non-treated (Fig. 1C), or non-treated without overexpression of XBP1, ATF6, and ATF4 (Fig. 2A), or non-treated without XBP1 overexpression (Fig. 3A and B), the value of which was set at 1.0.

2.2. Cell culture, transfection, and treatment

SM-10, a mouse placental trophoblastic cell line [29] kindly provided by Joan S. Hunt (University of Kansas Medical Center), was cultured in RPMI-1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum at 37 °C in 5% CO_2. The calcium phosphate-DNA precipitation method was used to introduce plasmid DNA into the SM-10 cells. To induce ER stress, cells were treated with tunicamycin (2.5 μg/ml) or thapsigargin (1 μM).

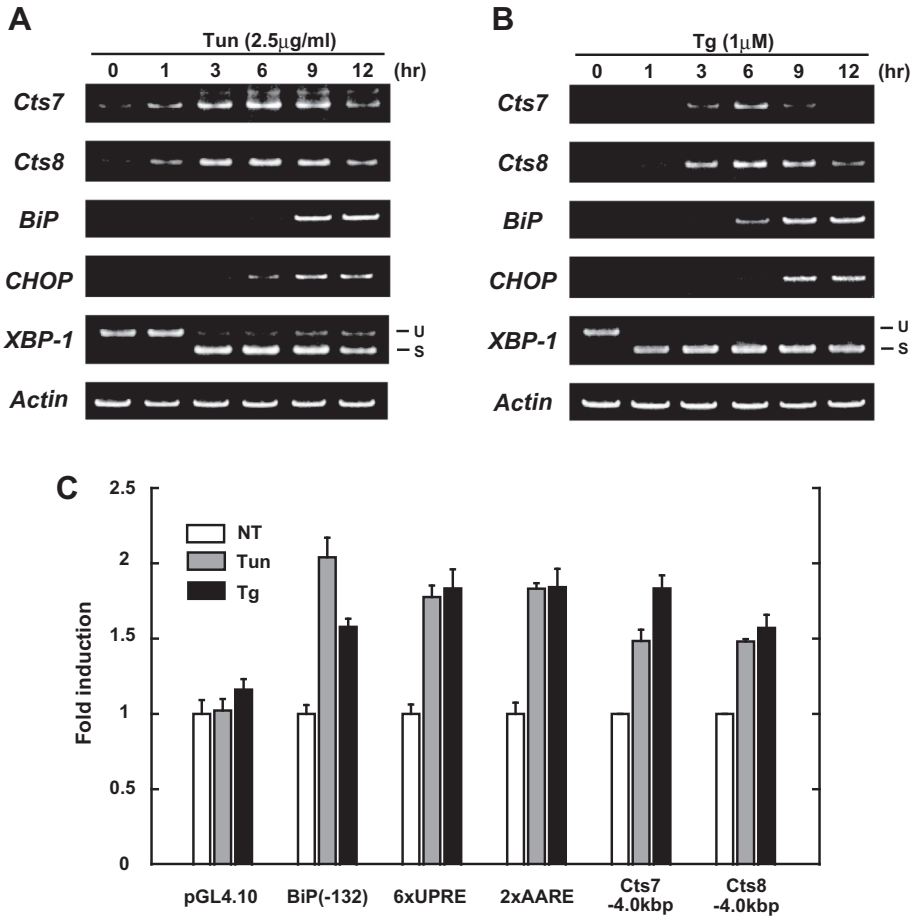
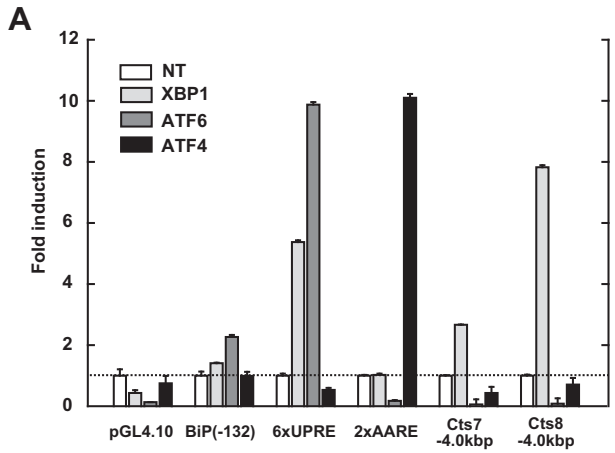


Fig. 1. Induction of Cts7 and Cts8 by ER stress in the placental cell line SM-10. (A and B) RT-PCR analysis of Cts7 and Cts8 in SM-10 cells. Total RNAs were obtained from SM-10 cells treated with tunicamycin (2.5 μg/ml) (A), or thapsigargin (1 μm) (B), for indicated time. Then the RNAs were subjected to RT-PCR using the primers listed in Table 1. XBP1 was used as a marker for ER stress-induced splicing. In (A) and (B), -u indicates the unspliced signal, and -s indicates the spliced signal of XBP1. BiP and CHOP was used as a marker for ER stress inducing gene. αActin was used as the internal standard. (C) Luciferase assay of the promoters for Cts7 or Cts8 in SM-10 cells. pGL4-Cts7(-4000) or pGL4-Cts8(-4000) was transfected into SM-10 cells and subjected to a luciferase assay. The assay was performed with or without treatment with tunicamycin (2.5 μg/ml), or thapsigargin (1 μm). BiP(-132) was used as a positive control for ATF6-inducible promoter, 6xUPRE was for XBP1-inducible promoter, and 2xAARE was for ATF4-inducible promoter. pGL4.10 was used as a negative control.



B

Gene	Accession No.	Fold change	
		IRE1α$^{-/-}$ / WT	XBP1$^{-/-}$ / WT
Cts7	NM_019539	0.2	0.43
Cts8	NM_019541	0.3	0.46

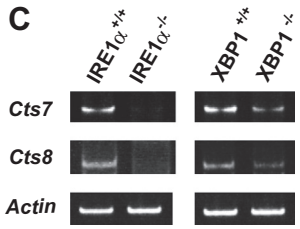


Fig. 2. Induction of Cts7 and Cts8 by IRE1α–XBP1 pathway. (A) Luciferase assay of the promoters for Cts7 or Cts8 in SM-10 cells. pGL4-Cts7(-4000) or pGL4-Cts8(-4000) was transfected into SM-10 cells and subjected to a luciferase assay. The assay was performed with or without overexpression of XBP1 (spliced), ATF6 (1-373), and ATF4. BiP(-132) was used as a positive control for ATF6-inducible promoter, 6xUPRE was for XBP1-inducible promoter, and 2xAARE was for ATF4-inducible promoter. pGL4.10 was used as a negative control. (B) Microarray analysis of placental RNAs from IRE1α- and XBP1-KO mice. Total RNAs from wild type, IRE1α-KO and XBP1-KO mice placentas (E11.5) were prepared as described in the Materials and methods, and subjected to microarray analysis. The relative abundance of Cts7 or Cts8 RNAs in IRE1α or XBP1-KO placentas were normalized against the wild type, which was set as 1.0. (C) RT-PCR analysis of placental RNAs from IRE1α or XBP1-KO mice.

2.3. Plasmids

For overexpression of transcription factors, pCAX-F-hXBP1(sp) [21], pCAX-hATF6(1-373)-F [30], and pCAX-F-mATF4 [31] were used. In the reporter assays, pGL4.10 (Promega) was used as a negative control without promoter. pGL3-BiP(-132) [30], pGL3-6xUPRE-cfos [21], and pGL3-2xAARE-TKbasal were used as positive controls with stress-inducible promoters. The pGL3-2xAARE-TKbasal, which has 2 repeat of the 19 bp AARE element from CHOP [32] and the TK basal promoter [33] on pGL3-basic (Promega), was constructed by PCR technique. To clone the promoters of Cts7 or Cts8, PCR was performed with mouse genomic DNA as the template. The resulting fragments were then inserted into the KpnI/XhoI sites (for Cts7) or SacI/XhoI sites (for Cts8) on pGL4.10 (Promega).

3. Results

3.1. Induction of Cts7 and Cts8 by ER stress in the placental cell line SM-10

As reported previously, specific kinds of cathepsin (PECs; placentally expressed cathepsins) are highly expressed in placenta, and mediate various functions, as typified by Cts7 or Cts8 in trophoblast function or vascular remodeling [28]. First, to check whether these cathepsins are regulated upon ER stress, the SM-10 cells, a mouse placental trophoblastic cell line [29], were subjected to RT-PCR analysis with primers listed in Table. 1. Among three UPR branches, ATF6 is known to induce BiP [16], activation of PERK leads to induction of CHOP [15], and IRE1α is known to mediate the unconventional splicing of XBP1 [8]. As shown in Fig. 1, all these ER stress-inducible actions were confirmed in SM-10 cells, by treatment with tunicamysin (Fig. 1A) or thapsigargin (Fig. 1B). Similarly, the transcripts of Cts7 and Cts8 were induced by ER stress with the peak of 6 h – treatment (Fig. 1A and B).

Then, the luciferase assay was performed with SM-10 cells, to investigate whether the induction of Cts7 and 8 depends on their promoters (Fig. 1C). In this experiment, BiP promoter was used as a control for ATF6-mediated activation [30], UPRE was for IRE1α -mediated activation [21], and AARE was for PERK activation [32]. The activities of all these controls were increased by 1.5 ∼ 2 times upon ER stress (Fig. 1C). Similarly, the activities of Ctss7 promoter (−4.0 kbp) and Cts8 promoter (−4.0 kbp) were significantly increased upon ER stress (Fig. 1C). These results indicated that the transcription of Cts7 and 8 are induced by ER stress.

Table 1
Primer sequences used for RT-PCR.

Gene	Accession No.	Primer name	Primer sequence	Size of PCR product (bp)
Cts7	NM_019539	mCts7–3F[a]	5′-CTTGGGAGTGGCCTTGGCTGC-3′	511
		mCts7–3R[a]	5′-AGGTCTGCCTCCATCACAGCCC-3′	
Cts7	NM_019539	mCts7–4F[b]	5′-GCTTGGGAGTGGCCTTGGCTG-3′	507
		mCts7–4R[b]	5′-TGCCTCCATCACAGCCCTTGG-3′	
Cts8	NM_019541	mCts8–2F	5′-CGGACACTGCAGGTACCACCC-3′	468
		mCts8–2R	5′-TGCAGCAGAGGCTGGTCATGGT-3′	
BiP	NM_001163434	mBiP-5F	5′-GGCTTCTGCGTGTGTGTGAGACC-3′	420
		mBiP-5R	5′-TGCGTCCGATGAGGCGCTTG-3′	
CHOP	NM_007837	mChop-2F	5′-GAGGTGGAGACCACACGGCG-3′	196
		mChop-2R	5′-AAGCCGAGCCCTCTCCTGGT-3′	
XBP1	NM_013842	m5x	5′-GAACCAGGAGTTAAGAACACG-3′	XBP1u; 205
		m3x	5′-AGGCAACAGTGTCAGAGTCC-3′	XBP1s; 179
β-actin	NM_007393	mBactin-F	5′-ATGGATGACGATATCGCT-3′	569
		mBactin-R	5′-ATGAGGTAGTCTGTCAGGT-3′	

[a] Were used only for the assays with cellular (SM-10) RNAs as substrates.
[b] Were used only for assays with placental RNAs as substrates.

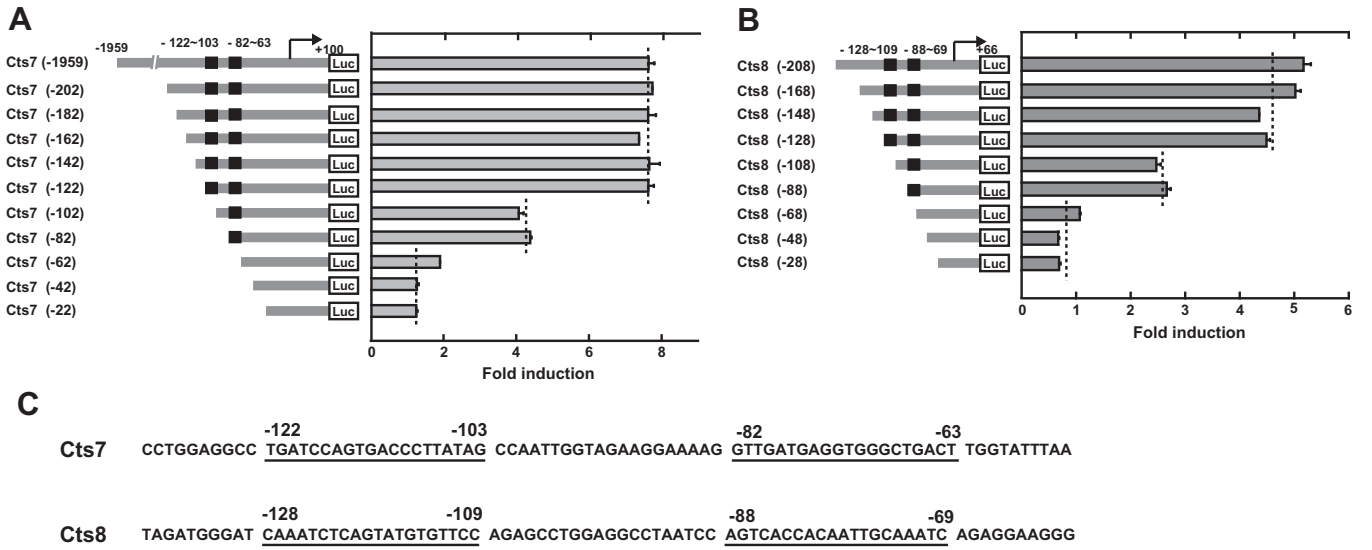


Fig. 3. Deletion scanning for the XBP1 responsive element in Cts7 and Cts8. (A) Luciferase assay of serial deletions of the Cts7 promoter. A plasmid expressing luciferase from each deletion on the Cts7 promoter was transfected into SM-10 cells and used for the luciferase assay. The assay was performed under overexpression of XBP1 (spliced). (B) Luciferase assay of serial deletions of the Cts8 promoter. The similar assay to (A) was performed with Cts8 promoter. (C) Sequence of the XBP1 responsive region on Cts7 and Cts8. The regions important for the XBP1 mediated induction were underlined.

3.2. Induction of Cts7 and Cts8 by IRE1α–XBP1 pathway

Next, to investigate which pathway in three UPR branches contribute to the induction of Cts7 and Cts8, luciferase assay was performed with the overexpression of each mediator (Fig. 2A). The truncated ATF6 (1-373) was used for case of ATF6 branch [16], XBP1 was for IRE1α branch [8], and ATF4 for PERK branch [15]. Corresponding with previous reports, the BiP promoter was best induced by the ATF6, and the UPRE element was induced by XBP1 or ATF6. Also, the AARE element was only induced by the overexpression with ATF4, not XBP1 or ATF6. Differently from these controls, the Cts7 promoter (−4.0 kbp) and Cts8 promoter (−4.0 kbp) were specifically and significantly induced only with XBP1, not with ATF6 or ATF4 (Fig. 2A).

The specific contribution of IRE1α–XBP1 pathway to the expression of Cts7 and Cts8 were further examined in the mice models. The placental RNAs from IRE1α-KO or XBP1-KO mice [20] were subjected to microarray analysis (Fig. S1 and Fig. 2B) and RT-PCR analysis (Fig. 2C). The former analysis revealed that among various kinds of cathepsins, the expression of Cts7 and Cts8 were importantly and significantly attenuated both in IRE1α-KO and XBP1-KO placenta (Fig. 2B). corresponding with this, the RT-PCR analysis confirmed the significant attenuation of Cts7 and Cts8 transcripts both in IRE1α-KO and XBP1-KO placenta (Fig. 2C). These results from cultured cells and mice models indicated that the moderate expression of Cts7 and Cts8 in placenta was ensured by the UPR pathways, especially by the IRE1α–XBP1 pathway.

3.3. Deletion scanning for the XBP1 responsive element in Cts7 and Cts8

Finally, serial deletion scanning analysis was performed on the Cts7 and Cts8 promoters to identify regions essential for the response to XBP1. This analysis identified two important regions whose individual deletion reduced promoter activity, respectively (Fig. 3A and B). In case of Cts7, the first was located in the 122–103 upstream region, and the second was positioned in the 82–63 upstream region, whose deletion drastically reduced the activation by XBP1 in a stepwise manner (Fig. 3A). In case of Cts8, these were in the 128–109 upstream region, and in the 88–69 upstream region (Fig. 3B). The sequences of these regions were shown in Fig. 3C. Interestingly, these did not contain any typical ER stress responsive element, including UPRE I or UPRE II. The details of this issue would be further discussed in the discussion section, including the possibility that XBP1 positively contributes to these promoters rather by indirect manner, than by direct binding or association.

4. Discussion

In this study, we showed that UPR, especially IRE1α–XBP1 pathway positively contributes to the expression of Cts7 and Cts8 in placenta. In the placental cell line SM-10, ER stress up-regulated the Cts7 and Cts8 at transcriptional level (Fig. 1), and this induction was mediated by the IRE1α–XBP1 pathway, among three branches in UPR (Fig. 2A). Correspondingly, the experiments with mice model confirmed that the disruption of IRE1α and XBP1 drastically attenuated the expression of Cts7 and Cts8 (Fig. 2B and C). These results indicated that placenta, which is constantly under ER stressed status possibly by high expression of secretory proteins like cytokines or hormones [20], utilizes IRE1α–XBP1 pathway to ensure the moderate expression of Cts7 and Cts8.

Previously, we have addressed the function of IRE1α–XBP1 pathway in the placenta formation, and revealed that the deletion of IRE1α or XBP1 in placenta disrupt placental morphology and its function leading to fetal growth defect, and that this pathway links UPR program to hypoxic program via the induction of VEGF [20]. Also, XBP1 positively contributes to the expression of PSGs in placenta, possibly to regulate the placental immuno-modulation to prevent rejection of the allotypic fetus as a foreign graft [21]. This study provides the novel possibility on the IRE1α–XBP1 pathway as a specific program supporting the normal placental development, and healthy fetal growth via the cathepsin expression.

This time, we focused on the Cts7 and Cts8 based on the recent study [28]. However, as show in Fig. S1, other cathepsins than Cts7 or Cts8 could be regulated by the IRE1α–XBP1 pathway. For example, CtsJ showed the similar attenuation pattern in IRE1α-KO or XBP1-KO placenta. Thus, this cathepsin might be regulated by the similar mechanism to Cts7 and Cts8. However, because we could not detect the induction of CtsJ transcript in SM-10 cell (data

not shown), we could not address this cathepsin in this study. In contrast, the expression of Cts K, CtsO, CtsF, CtsG, and CtsW were up-regulated in the KO placentas (Fig. S1). Thus, these cathepsins might be negatively controlled by the IRE1α–XBP1 pathway. In addition, as CtsC, CtsE, and CtsL were rather enriched in IRE1α-KO than in XBP1-KO, these cathepsins might be negatively regulated by some IRE1α-specific mechanisms, including RIDD (regulated IRE1-dependent decay [34]).

Another unsolved issue in this study is how XBP1 positively contribute to the expression of Cts7 and Cts8. As shown in Fig. 3C, these two regions did not contain any previously identified XBP1-responsive elements, including UPRE (TGACGTGG/A) or UPRE-II (ATTGG-N-CCGCGT) [35]. Nor did they have the known XBP1-binding sequences GCCACG, GACGTG, ACGT, or CGGAAG [36]. This is the similar case to PSG promoters [21], in which XBP1 did not associate with their promoters in chromatin- IP experiment. Therefore, it is likely that XBP1 activates the Cts7 or Cts8 promotes in an indirect manner, possibly through up-regulation or activation of intermediates, including placenta specific factors. The fact that these promoters were not activated in HeLa cells (data not shown) which is surely activated in placental cell line (Fig. 1C), corresponds with this hypothesis.

Nevertheless, this study provides the important information on the expression program of placental cathepsins, supporting the normal placental development, and healthy fetal growth. As mentioned above, it has been reported that specific kinds of cathepsins (PECs; placentally expressed cathepsins) are highly expressed in placenta [23–26], and mediates various function [27] including the case of Cts7 and Cts8 [28]. Also, it has been reported that placenta is significantly and constantly under ER stressed status [20], and that an increased ER stress is associated with the impaired placental development and fetal growth restriction [37–39]. Thus, information about the molecular mechanism how UPR pathways contribute to the placental development, or molecular linkage between ER stress and abnormal pregnancy would provide novel possibility on therapeutic methods of these disorders. Further studies are required.

Acknowledgments

We thank R. Akai, C. Asai, and Y. Takahashi for technical assistance. We are grateful for the support of Research Resources Center (BSI, RIKEN) and Biosignal Genome Resources Center (Gunma University) for DNA sequencing analysis. This work was supported by Program to Disseminate Tenure Tracking System of the MEXT granted to Gunma University, and supported by Grants from RIKEN and MEXT (Nos. 24390049 and 24113702).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.119.

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