Accumulation of type IV collagen in dilated ER leads to apoptosis in *Hsp47*-knockout mouse embryos via induction of CHOP

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Summary

Hsp47 is an endoplasmic reticulum (ER)-resident molecular chaperone that is specific for collagen. In $Hsp47^{-/-}$ mouse embryos at 9.5 days postcoitus (dpc), immunostaining indicated the absence of type IV collagen, but not of laminin and nidogen-1, in the basement membrane (BM). Electron immunomicroscopy revealed accumulation of type IV collagen in dilated ERs, but not in the BM of $Hsp47^{-/-}$ embryos, whereas it was only present in the BM in $Hsp47^{+/+}$ embryos. The BM structures stained with anti-laminin and anti-nidogen-1 antibody became disrupted in $Hsp47^{-/-}$ embryos at 10.5 dpc. Thus, in the absence of type IV collagen in the BM owing to the lack of Hsp47, the structure of the BM cannot be maintained during the dramatic morphological changes that take place

around 10.5 dpc. Type IV collagen is therefore indispensable for the maintenance of BM structures during the late-stage development of mouse embryos, although not essential for the initial formation of the BM. Just before the death of $Hsp47^{-/-}$ embryos, DNA fragmentation typical of apoptosis was observed at 10.5 dpc together with significantly upregulated CHOP mRNA expression. ER stress caused by the accumulation of misfolded collagen may have induced apoptosis in *Hsp47*-knockout embryos through the upregulation of CHOP.

Key words: Apoptosis, Basement membrane, CHOP, Hsp47, Molecular chaperone, Type IV collagen

Introduction

Collagen is the most abundant protein in mammals. It plays an essential role in the function of the extracellular matrix (ECM) and the lack of its expression or function has serious consequences (Kadler, 1995; Kadler et al., 1996). Twenty-seven types of collagen have been identified so far. Of these, the disruption of collagen types I, II and III is known to generate serious defects (Schnieke et al., 1983; Lohler et al., 1984; Li et al., 1995; Liu et al., 1997). The 47-kDa heat-shock protein (Hsp47) is a collagen-specific molecular chaperone (Nagata and Hosokawa, 1996; Nagata, 2003) that recognizes the Pro-Arg-Gly sequence in the Gly-X-Y repeats that constitute the triple helices of collagen (Koide et al., 2000; Koide et al., 2002). Hsp47 shows weak but significant amino acid sequence similarity with the serine proteinase inhibitor (serpin) family of proteins (Hirayoshi et al., 1991). Hsp47 is the only heat-inducible protein in the endoplasmic reticulum (ER) in mammalian cells, and it transiently binds to pro-collagen in the ER (Tasab et al., 2000; Saga et al., 1987). In vitro binding analyses have shown that Hsp47 can bind collagen types I-V (Nagata and Yamada, 1996; Natsume, 1994). In addition, yeast two-hybrid screening assays revealed that Hsp47 can bind to other collagenous proteins through their triple helical regions (Sato et al., 2002).

In the initial steps of collagen maturation, three alpha chains of newly synthesized pro-collagen assemble at the C-terminus and they fold into a triple helix from the C- to N-termini in a zipper-like fashion (Engel and Prockop, 1991; McLaughlin and Bulleid, 1998). After binding to pro-collagen in the ER, Hsp47 is co-transported with pro-collagen from the ER to the cis-Golgi or ER-Golgi intermediate compartment, where it dissociates from pro-collagen in a pH-dependent manner (Saga et al., 1987; Satoh et al., 1996). Hsp47 is then thought to return to the ER through the effect of the Arg-Asp-Glu-Leu (RDEL) sequence at its C-terminal end. This sequence is similar to Lys-Asp-Glu-Leu (KDEL), which is the ER retention signal (Sauk et al., 1998). Following or during secretion into the extracellular matrix, the N- and C-propeptides of the pro-collagens are cleaved off, which generates mature collagens in the extracellular matrix (Prockop et al., 1998; Cal et al., 2001). Consistent with the role of Hsp47 in collagen maturation, the expression level of Hsp47 is closely correlated with that of collagen in normal tissues (Levio et al., 1980; Masuda et al., 1994; Masuda et al., 1998; Moriyama et al., 1998). It is also known that Hsp47 is upregulated in the fibrotic legions of liver cirrhosis, glomerulosclerosis and keloids (Masuda et al., 1994; Moriyama et al., 1998; Naitoh et al., 2001), and that inhibition of Hsp47 expression by administering antisense *Hsp47* RNA can suppress the accumulation of collagen in glomerulonephritis (Sunamoto et al., 1998).

We previously produced *Hsp47*-knockout (*Hsp47*⁻/⁻) mice and found by silver staining that their basement membrane (BM) at 9.5 days postcoitus (dpc) exhibit no or very weak staining (Nagai et al., 2000). Moreover, electron microscopy revealed abnormal discontinuous morphology in these BMs (Nagai et al., 2000). Various abnormalities that are probably due to the lack of BMs were also observed in tissues and organs of the knockout embryos at 10.5 dpc, including the rupture of endothelial cell layers of blood vessels. Consequently, *Hsp47*⁻/⁻ embryos cannot survive beyond 11.5 dpc. The *Hsp47*⁻/⁻ fibroblastic cell lines derived from the knockout embryos were found to produce incorrectly folded type I collagen. These observations indicate that Hsp47 is essential for the production of type I collagen, and that the abnormal BM in the *Hsp47*⁻/⁻ embryos may be caused by the defective molecular maturation of type IV collagen, as this is a major component of the BM. However, there is no direct evidence of the relationship between the abnormal BM in *Hsp47*⁻/⁻ embryos and the function of Hsp47 in type IV collagen maturation.

Here, we examined the distribution of type IV collagen and other components that localize in the BM, including laminin and nidogen, in *Hsp47*⁻/⁻ embryos. These analyses revealed that unlike the other BM components, type IV collagen does not localize in the BM of the knockout embryos: it accumulates in the ER. We also report here that immediately prior to the death of the *Hsp47*⁻/⁻ embryos, they undergo apoptosis that occurs concomitantly with the upregulation of CHOP. We discuss the role of Hsp47 in type IV collagen maturation in vivo, and the role of type IV collagen in the maintenance of BM structures during development. We also discuss the biological significance of the induction of CHOP during the apoptosis of *Hsp47*⁻/⁻ embryos.

Materials and Methods
Antibodies
The following antibodies were used: mouse monoclonal antibodies specific for Hsp47 (Stressgen Biotechnologies, Victoria, Canada) and entactin/nidogen-1 (Neomarkers, CA, USA) and rabbit polyclonal antibodies specific for mouse collagen type IV (Chemicon, Temecula, CA, USA) and laminin (LSL, Tokyo, Japan). The anti-laminin antibody recognizing alpha 1, beta 1 and gamma 1 chains was affinity-purified by using mouse engelbreth-holm-swarm sarcoma laminin (Sigma, St. Louis, MO, USA) that was immobilized on CNBr-Sepharose (Amersham Biosciences, Buckinghamshire, England). Goat anti-rabbit Ig (Biosource International, Camarillo, CA, USA), anti-mouse Ig (Biosource International) and anti-rat IgG (Cappel, Darham, UK) conjugated with FITC or rhodamine were used as secondary antibodies.

Immunofluorescent staining
Mutant embryos and littermate controls at 9.5 and 10.5 dpc were fixed in 0.1 M phosphate buffer, pH 7.4 (PB) containing 4% paraformaldehyde at room temperature for 2 hours. The fixed embryos were then embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), snap frozen, and stored at –80°C. 4 µm cryosections were prepared using a freezing microtome, mounted on poly-L-lysine-coated cover glass and dried. After nonspecific binding was blocked with 1.5% horse normal serum in PBS for 30 minutes, the sections were incubated with the appropriate primary antibodies in PBS containing 1.5% normal horse serum for 1 hour. Following

incubation with secondary antibodies conjugated with FITC or rhodamine, the localization of the antibodies was visualized by using a fluorescent microscope.

Immunoelectron microscopy
The pre-embedding silver enhancement immunogold method was performed for immunoelectron microscopy as described (Mandai et al., 1997), with a slight modification. Briefly, mouse embryos were fixed in 4% paraformaldehyde in PB for 1 hour. 6 µm cryosections were prepared and incubated in blocking solution (PB containing 0.005% saponin, 10% bovine serum albumin, 10% normal goat serum and 0.1% coldwater fish skin gelatin) for 30 minutes. The sections were then treated with rabbit antibodies against type IV collagen or laminin in blocking solution overnight and then washed five times in PB containing 0.005% saponin for 10 minutes. After incubation with goat anti-rabbit IgG conjugated with colloidal gold (1.4 nm diameter) in the blocking solution for 2 hours, the sections were washed five times with PB for 10 minutes and fixed with 1% glutaraldehyde in PB for 10 minutes. Following several washes, the gold labeling was intensified by using a silver enhancement kit for 6 minutes at 20°C in the dark. After washing in distilled water, the sections were post-fixed in 0.5% OsO₄ for 90 minutes at 4°C, washed in distilled water, incubated with 50% ethanol for 10 minutes and stained with 2% uranyl acetate in 70% ethanol for 2 hours. The sections were further dehydrated with a graded series of ethanol and embedded in epoxy resin. Ultra-thin sections were prepared and doubly stained with uranyl acetate and lead citrate.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)
The in situ visualization of DNA fragmentation was carried out with embryo sections by using In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Agarose gel electrophoresis of embryonic DNA
The embryos were lysed in lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA and 0.1% SDS) supplemented with 0.5mg/ml proteinase K and incubated at 55°C overnight. Following centrifugation, the supernatant was recovered and DNA was isolated by phenol:chloroform extraction followed by ethanol precipitation. The precipitated DNA was dissolved in TE and DNA fragmentation was analysed by 1.8% agarose gel electrophoresis followed by ethidium bromide staining.

RT-PCR analysis
Embryos were lysed by shearing with syringes and their total RNA was isolated by acid phenol extraction using TRIzol (Invitrogen, Carlsbad, CA, USA). Equal amounts of total RNA (1 µg) were converted to first-strand cDNA by using reverse transcriptase and specific cDNA was amplified by PCR with Taq DNA polymerase and the following oligonucleotide primers: Hsp47, 5′-AAGACCAGGCG-GTGGAGAACATCC-3′ and 5′-TCTCGCATCTTGTCTCCCTTG-GGC-3′; CHOP, 5′-TCATACACCACCACACCTGAAAGC-3′ and 5′-AATGTACCGTCTATGTGDAAGCCG-3′; β-actin, 5′-GTTCGC-CATGGATGACGATATCGC-3′ and 5′-AGGTGGACAGTGAGGC-CAGGATGG-3′.

PCR consisting of 24 cycles for β-actin, 30 cycles for CHOP and 37 cycles for Hsp47 was then performed. Each cycle consisted of 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 1.5 minutes. The PCR products were analysed by 1.8% agarose gel electrophoresis followed by ethidium bromide staining.

Quantitative RT-PCR was carried out as follows. Total RNAs (1 µg)

Fig. 1. Type IV collagen does not localize in the basement membrane (BM) regions of *Hsp47^-/-^* mutant mouse embryos at 9.5 days post coitus (dpc). (A-F) Wild-type (WT) and *Hsp47^-/-^* embryos were fixed with paraformaldehyde. Neighboring cryosections of the embryos were then analysed by staining with haematoxylin and eosin (A,B) and by immunostaining with anti-Hsp47 antibody and FITC-conjugated second antibody (C,D) or anti-type IV collagen antibody and rhodamine-conjugated second antibody (E,F). Arrows indicate BM regions. ME, mesenchyme; EP, epithelium. Bar, 50 μm.

were converted to first-strand cDNA by First-strand cDNA Synthesis System for Quantitative RT-PCR (Marligen Biosciences, Nunc, Denmark) and amplified by PCR reaction in SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the specific primer sets of CHOP and β-actin. Fluorescence from DNA-SYBER Green complex was recorded by ABI PRISM 7700 Sequence Detection System (Applied Biosystems) throughout the PCR reaction, and the level of CHOP mRNA relative to β-actin mRNA was determined according to the manufacturer's instructions.

Results

Type IV collagen is not found in the BM of *Hsp47*-knockout embryos

Type IV collagen and laminin are the major components of the BM and are linked together with nidogen (Mayer et al., 1998). Previously, we reported that the BM of *Hsp47^-/-^* embryos exhibits a discontinuously disrupted abnormal morphology (Nagai et al., 2000). To investigate the molecular reason for these morphological abnormalities, we first examined sections of *Hsp47*-knockout embryos and littermate wild-type controls at 9.5 dpc by immunohistochemistry (Fig. 1). Hsp47 was detected in the epithelial cells and fibroblasts of normal embryos (Fig. 1C), which is consistent with the known expression patterns of collagen types IV and I in those cells, respectively. In contrast, expression of Hsp47 was undetectable in the *Hsp47^-/-^* embryos (Fig. 1D), which confirms the complete disruption of Hsp47 in these mice. Type IV collagen, a putative substrate of the Hsp47 chaperone, was not detectable in the BM regions of any tissues of the *Hsp47^-/-^* embryos (Fig. 1F), whereas the same antibody clearly stained the BM of wild-type embryos (Fig. 1E).

Fig. 2. Laminin and nidogen-1 localize normally in the BM regions of *Hsp47^-/-^* mutant mouse embryos at 9.5 dpc. (A-F) Neighboring sections of wild-type and *Hsp47^-/-^* embryos were analysed by immunostaining with antibodies against laminin (A,B), nidogen-1 (C,D) and fibronectin (E,F). Arrows indicate BM regions. Bar, 50 μm.

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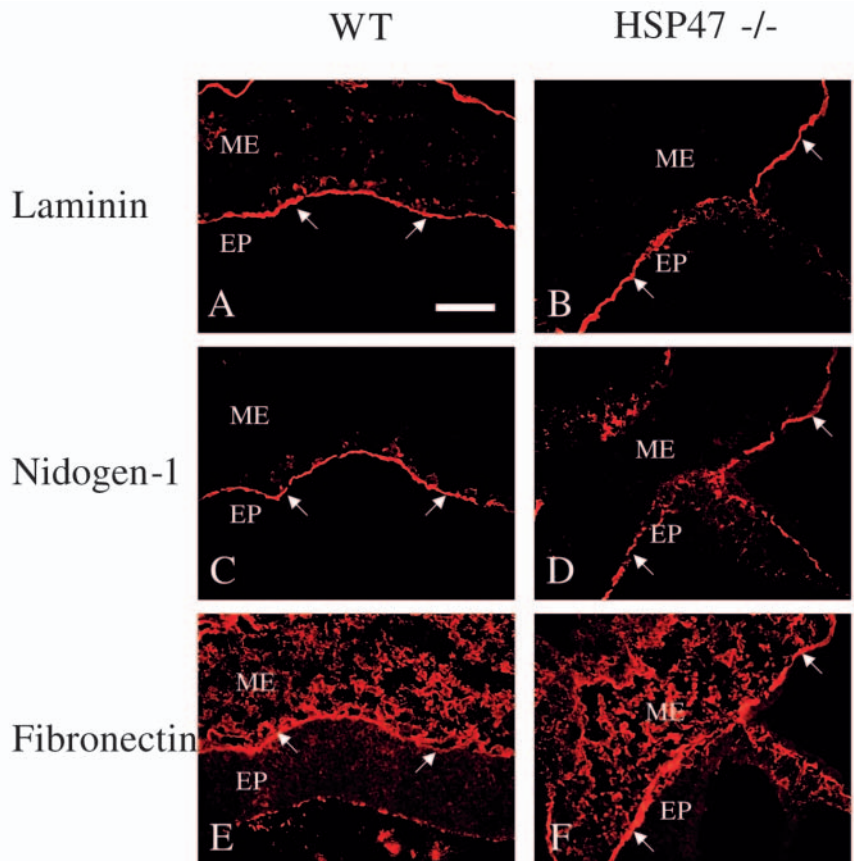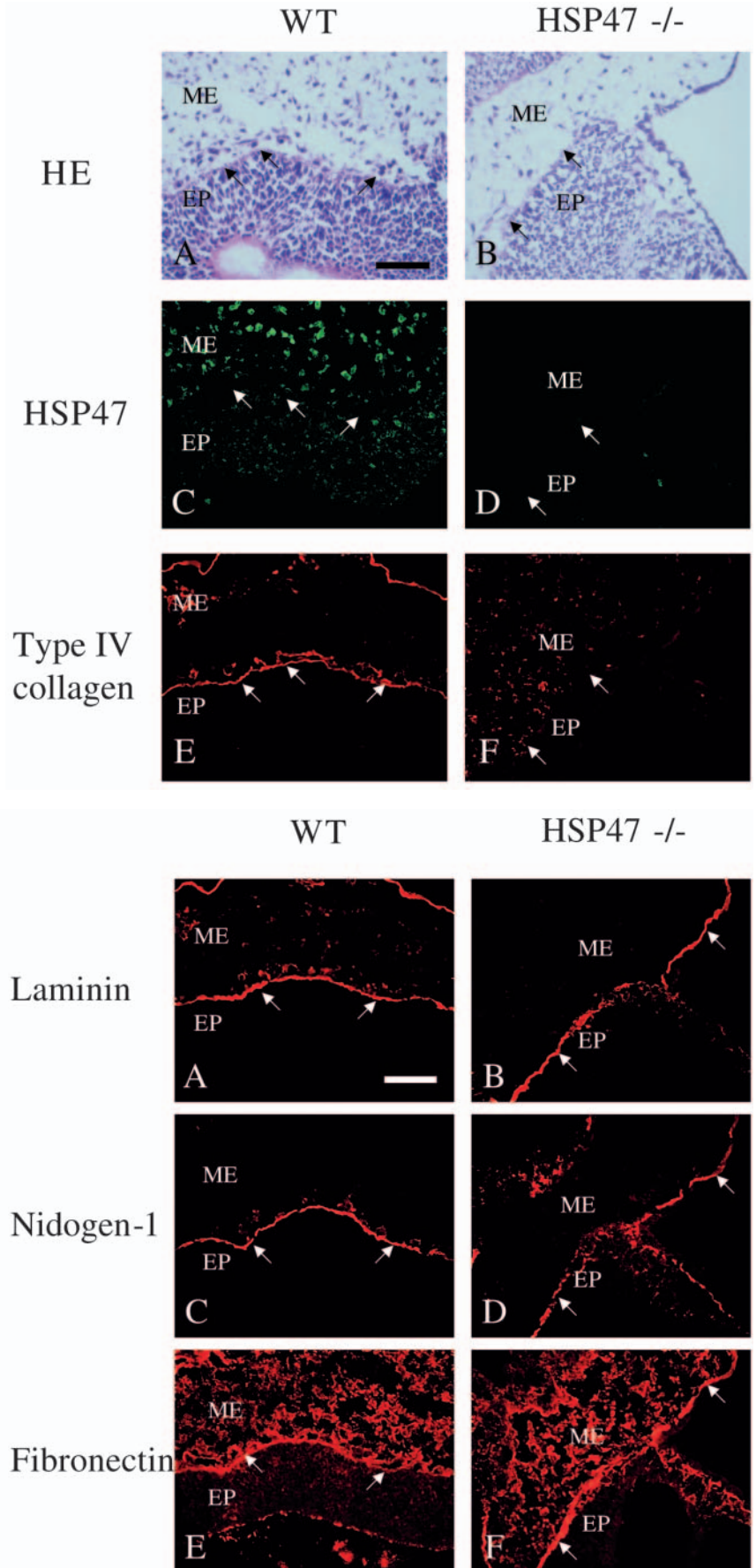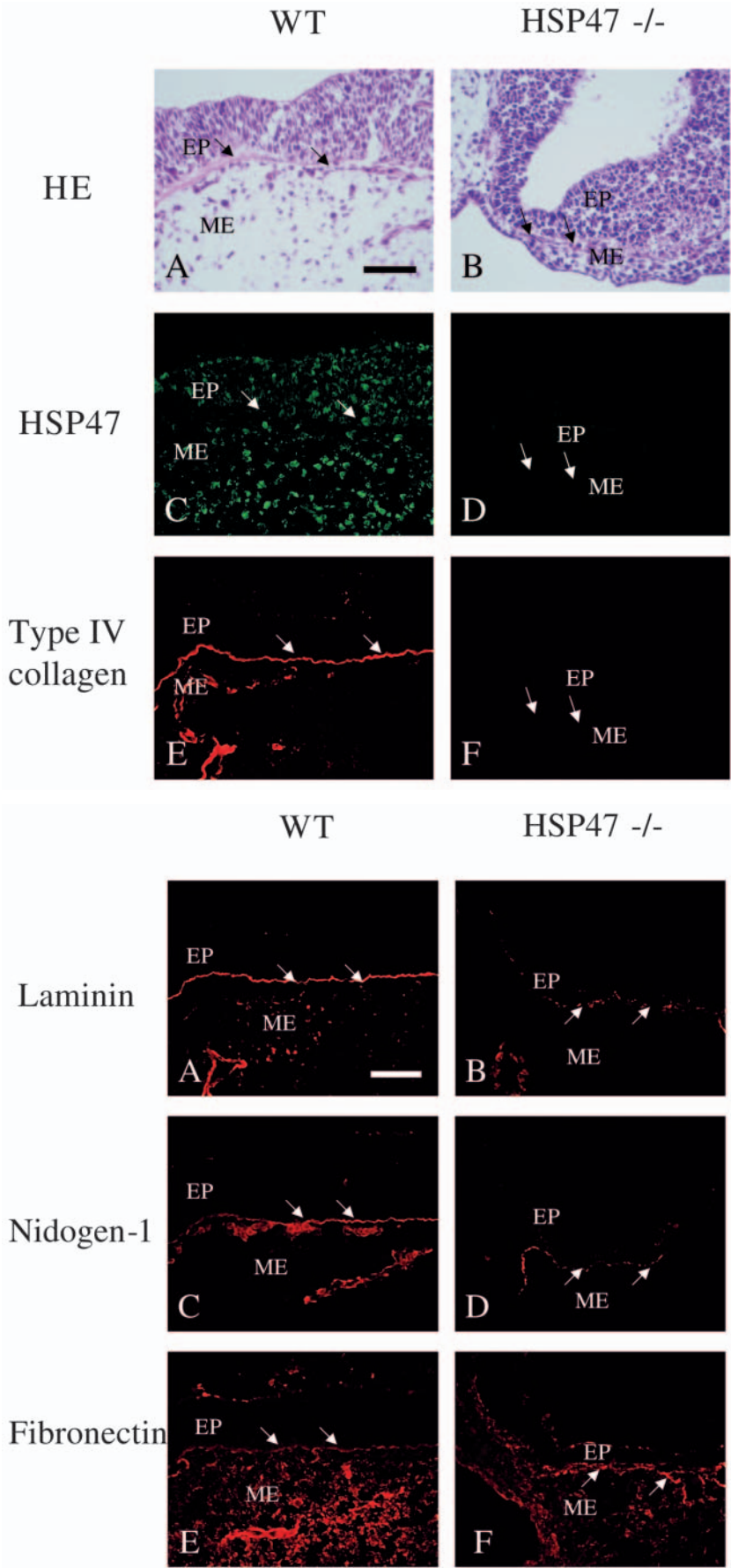
Fig. 3. Type IV collagen does not localize in the BM regions of *Hsp47*^{−/−} mutant mouse embryos at 10.5 dpc. (A-E) Neighboring sections of wild-type and *Hsp47*^{−/−} embryos that included the neural tube region were stained with haematoxylin and eosin (A,B) or with antibodies against Hsp47 (C,D) or type IV collagen (E,F). Arrows indicate BM regions. Bar, 50 μm.

Unlike type IV collagen, laminin, another major component of the BM, was present in the BM of *Hsp47*^{−/−} embryos at 9.5 dpc (Fig. 2B) indicating that the localization of laminin is not affected by the disruption of the *Hsp47* gene. Nidogen-1, a supporting component of the BM, was also shown to localize normally in *Hsp47*^{−/−} embryos (Fig. 2C,D). The distribution of fibronectin, a major component of the ECM, was also not affected by the absence of the *Hsp47* gene (Fig. 2E,F), which further confirms that the effect of *Hsp47* disruption is collagen specific. Thus, Hsp47 is required for the accumulation of type IV collagen in the BM, and the localization of other BM components, including laminin and nidogen-1, is not affected by the absence of type IV collagen.

The BM in *Hsp47*^{−/−} embryos becomes fractured at 10.5 dpc

As the Hsp47-deficient embryos die just after 10.5 dpc (Nagai et al., 2000), we next examined the distribution of the BM components in 10.5 dpc *Hsp47*^{−/−} embryos. In the wild-type embryos, the epithelial cells and fibroblasts of the neural tube epithelium and their supporting mesenchymal tissues, respectively, were clearly stained with the anti-Hsp47 antibody (Fig. 3C). In contrast, no staining was observed in *Hsp47*^{−/−} embryos (Fig. 3D). As with the 9.5 dpc embryos, the BM in 10.5 dpc *Hsp47*^{−/−} embryos was not stained with the anti-collagen IV antibody (Fig. 3F), which is in contrast to the strong staining of the BM in the wild-type embryos (Fig. 3E). Intriguingly, the anti-laminin antibody staining patterns of the BM in the 10.5 dpc *Hsp47*^{−/−} embryos now exhibited fragmented patterns (Fig. 4B), whereas normal continuous staining of the BM was observed in the 10.5 dpc wild-type embryos (Fig. 4A). Similar staining patterns were obtained with the antibody against nidogen-1 (Fig. 4C,D). These staining patterns were in marked contrast to

Fig. 4. Staining with anti-laminin and anti-nidogen antibodies reveals that the BM of *Hsp47*^{−/−} embryos at 10.5 dpc is fractured. (A-E) Neural tube regions of wild-type and *Hsp47*^{−/−} embryos were analysed for the immunolocalization of laminin (A,B), nidogen-1 (C,D) and fibronectin (E,F). Arrows indicate BM regions. Bar, 50 μm.

WT   *hsp47 -/-*

Type IV
collagen
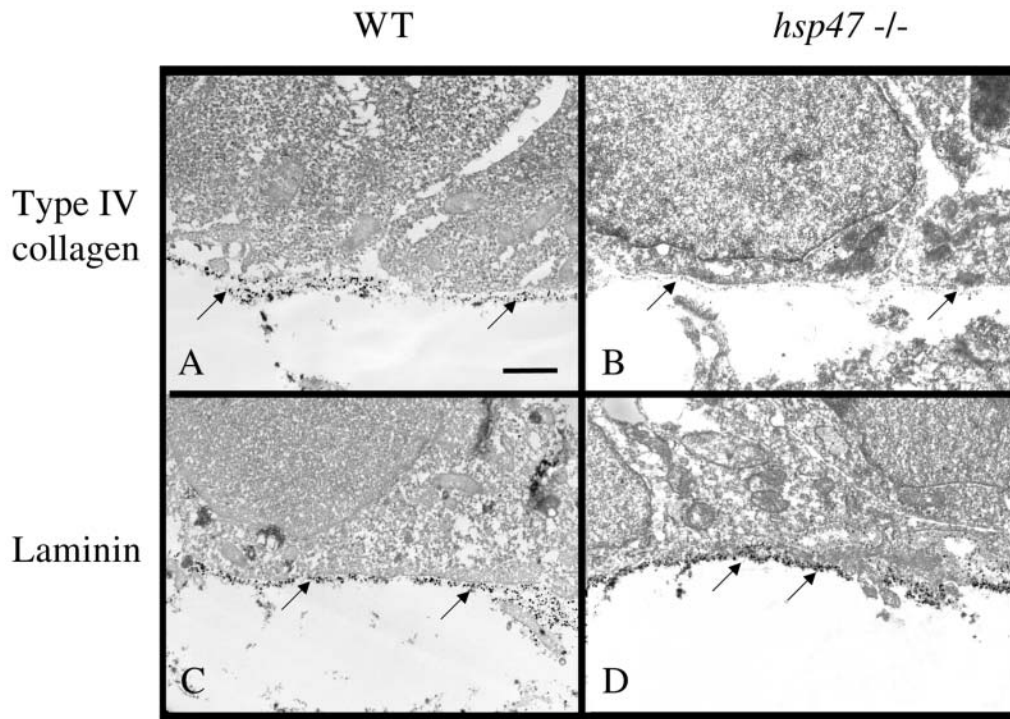
Laminin

Fig. 5. Type IV collagen, but not laminin, is absent from the BM of $Hsp47^{-/-}$ embryos at 9.5 dpc, as determined by immunoelectron microscopy. (A-D) Cryosections of wild-type and $Hsp47^{-/-}$ embryos that include the BM and neighbouring epithelial cells were first incubated with antibodies against type IV collagen (A,B) or laminin (C,D) and then with secondary antibodies conjugated with gold particles. Ultrathin sections were analysed by electron microscopy. Arrows indicate BM regions. Note that the staining of the BM with gold particles is observed in panels A, C and D but not in panel B. EP, epithelial cells; ME, mesenchyme. Bar, 0.1 µm.
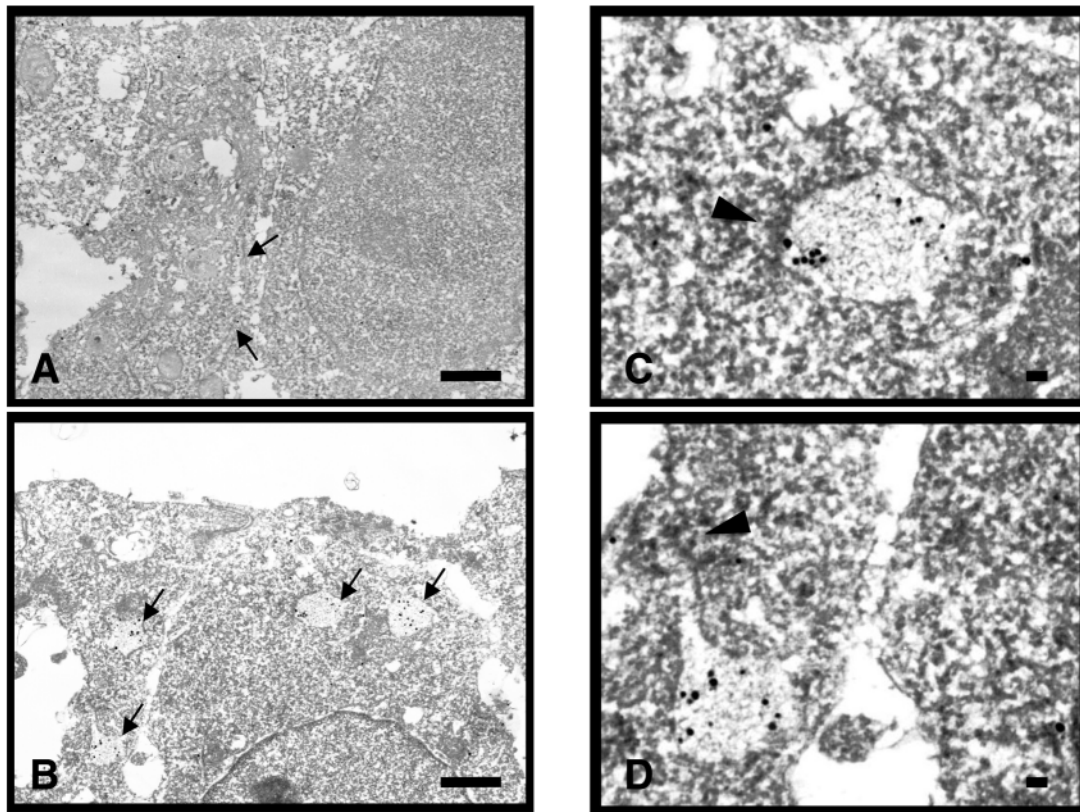
Fig. 6. Type IV collagen accumulates in dilated ERs in epithelial cells of $Hsp47^{-/-}$ embryos at 9.5 dpc. (A,B) Sections of epithelial cells of wild-type (A) or $Hsp47^{-/-}$ (B) embryos were analysed by immunoelectron microscopy using an antibody against type IV collagen. Arrows indicate ERs that contain type IV collagen. Bar, 0.1 µm. (C,D) Large magnification view of the dilated ER in $Hsp47^{-/-}$ embryos. Arrowheads indicate ribosomes associated with the dilated ER membranes. Note that the ERs of the $Hsp47^{-/-}$ cells are highly dilated and exhibit large accumulations of type IV collagen. Bar, 10 nm.

those of the 9.5 dpc embryos (compare Figs 2 and 4). The distribution of fibronectin was again not affected by the absence of Hsp47 in 10.5 dpc embryos (Fig. 4E,F). Thus, the continuous sheet-like structure of the BM cannot be maintained in an intact form in the absence of type IV collagen at 10.5 dpc, although the initial formation of the BM itself does not always require the presence of type IV collagen.
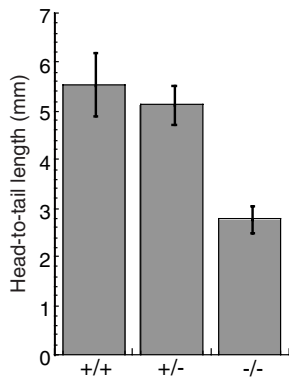
Type IV collagen accumulates in dilated ER in epithelial cells of *Hsp47*^{–/–} embryos

Electron microscopy with immunogold labeling confirmed that type IV collagen is not present in the BM of 9.5 dpc *Hsp47*^{–/–} embryos (Fig. 5B), unlike the BM of wild-type embryos (Fig. 5A). Immunoelectron microscopy also confirmed that laminin is present in the BM of *Hsp47*^{–/–} and wild-type embryos at 9.5 dpc (Fig. 5C,D). Thus, no type IV collagen is accumulated in the BM in the absence of Hsp47, despite the fact that laminin is present.

Hsp47^{–/–} embryos at 9.5 dpc have been reported to synthesize type IV collagen at the same level as 10.5 dpc wild-type embryos (Nagai et al., 2000). Thus, the absence of type IV collagen in the BM of *Hsp47*^{–/–} embryos is due either to the accumulation of the type IV collagen within the collagen-producing cell through the lack of its secretion, or it is degraded during or after its secretion. To test these possibilities, we analysed the epithelial cells that lie adjacent to the BM by immunoelectron microscopy. Most of the ERs of the epithelial cells of *Hsp47*^{–/–} embryos were found to be dilated compared to those in wild-type embryos, and intriguingly, a significant amount of type IV collagen was localized in these dilated ERs (Fig. 6A,B). In contrast, type IV collagen was rarely detected in the ERs of wild-type embryos. Thus, in *Hsp47*^{–/–} cells, type IV collagen is not secreted into the ECM, but it accumulates in the ER.

Apoptotic cell death in the *Hsp47*-knockout embryos

The disruption of *Hsp47* severely retards the growth of the embryo as determined by measuring the head-to-tail length (Fig. 7) and the number of somites (Nagai et al., 2000) and *Hsp47*^{–/–} emb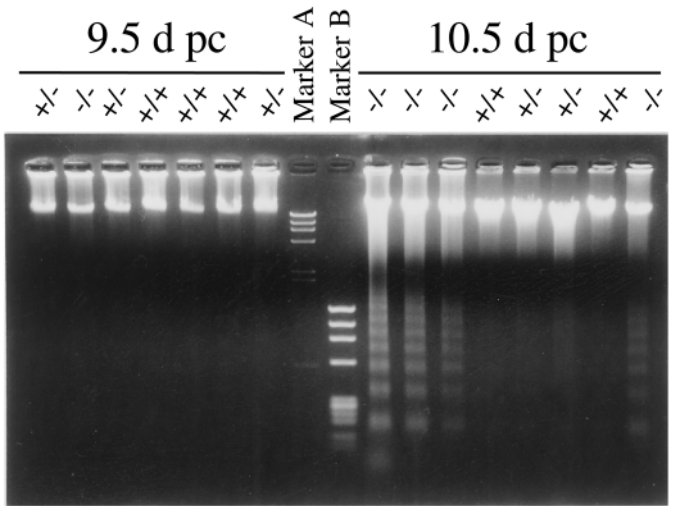ryos never survive beyond 11.5 dpc (Nagai et al., 2000). To investigate the mechanisms underlying the embryonic lethality of *Hsp47* disruption, we analysed the DNA fragmentation, which served as an indicator of apoptotic cell death, in the wild-type and mutant embryos. DNA extracted from *Hsp47*^{–/–}, *Hsp47*^{+/–} and *Hsp47*^{+/+} embryos from the same litter were analysed by agarose gel electrophoresis (Fig. 8). Although no DNA fragmentation was observed in the embryos of any genotype at 9.5 dpc, the DNA ladders that are typical of apoptosis were detected in *Hsp47*^{–/–} embryos at 10.5 dpc. In contrast, these ladders were not observed in heterozygotic or wild-type embryos. TUNEL staining also revealed that DNA fragmentation only occurred in 10.5 dpc *Hsp47*^{–/–} embryos (Fig. 9). Thus, apoptotic cell death occurred in *Hsp47*^{–/–} embryos prior to their death.

Stimulation of CHOP expression in *Hsp47*^{–/–} embryos

The accumulation of unfolded proteins in the ER is known to cause ER stress that results in the cells activating the so-called unfolded protein response (UPR) pathway. Moreover, it is known that the ER can become dilated when secretion-incompetent materials accumulate within it (Umebayashi et al., 2001). In addition, strong ER stress induces the expression of CHOP (Barone et al., 1994; Fleming et al., 1997; Zinszner, 1998; Jousse et al., 1999), which triggers a pathway that leads to apoptosis. As the ER of *Hsp47*^{–/–} mouse embryos exhibited a dilated morphology and an accumulation of type IV collagen, we examined the levels of CHOP mRNA in wild-type and *Hsp47*^{–/–} mouse embryos by RT-PCR (Fig. 10). At 10.5-11.0 dpc, the *Hsp47*^{–/–} embryos showed significantly higher CHOP mRNA levels compared to wild-type embryos, although at 9.5 dpc the CHOP mRNAs of the two genotypes did not significantly differ. The strong upregulation of CHOP

Fig. 7. Growth retardation of *Hsp47*^{–/–} embryos. The head-to-tail lengths of wild-type (*Hsp47*^{+/+}, *n*=3; *Hsp47*^{+/–}, *n*=11) and *Hsp47*^{–/–} mutant (*n*=5) mouse embryos at 10.5 dpc were measured, and the means and standard deviations are shown.

Fig. 8. DNA isolated from *Hsp47*^{–/–} embryos at 10.5 dpc, but not at 9.5 dpc, shows the ladder pattern that is typical of apoptosis. DNA extracted from wild-type and *Hsp47*^{–/–} embryos at 9.5 and 10.5 dpc was analysed by agarose gel electrophoresis. The DNA ladder was observed only in *Hsp47*^{–/–} embryos at 10.5 dpc (right side), although none of the embryos displayed DNA fragmentation at 9.5 dpc (left side). Size markers: A, lambda phage DNA digested with *Hind*III; B, φx174 DNA digested with *Hae*III.
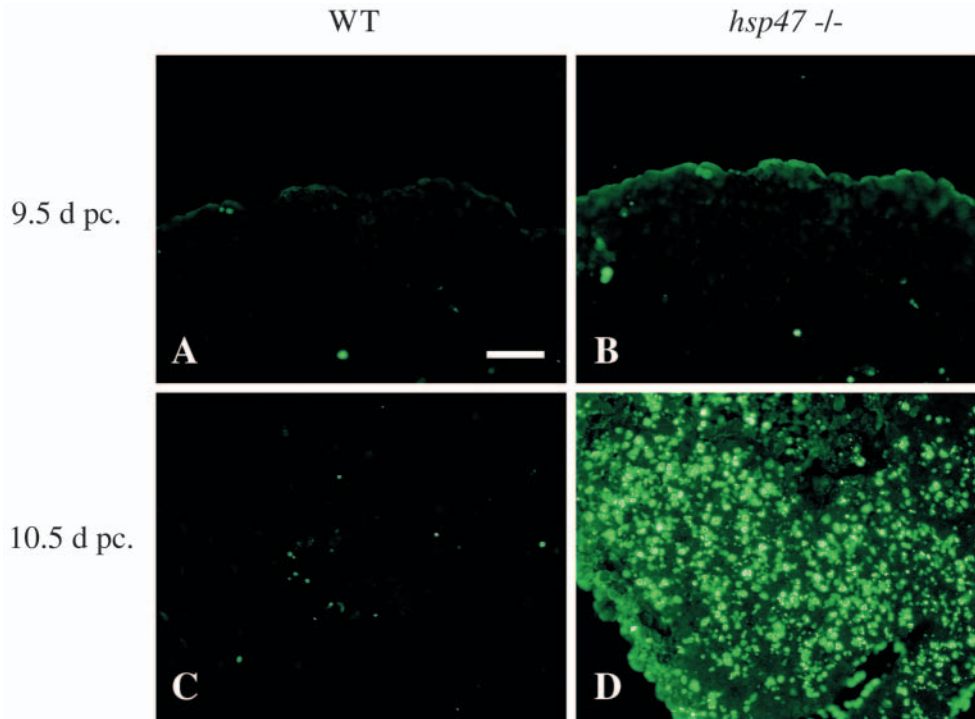
Fig. 9. DNA fragmentation was detected by TUNEL staining of sections of *Hsp47*⁻/⁻ mutant mouse embryos at 10.5 dpc. (A-D) Sections of wild-type and *Hsp47*⁻/⁻ embryos at 9.5 dpc (A,B) and 10.5 dpc (C,D) were analysed for DNA fragmentation by TUNEL staining. A strong signal was detected in *Hsp47*⁻/⁻ embryos at 10.5 dpc (D) but not at 9.5 dpc (B), whereas no significant signals were detected in the wild-type embryos at either time point (A,C). Bar, 50 μm.
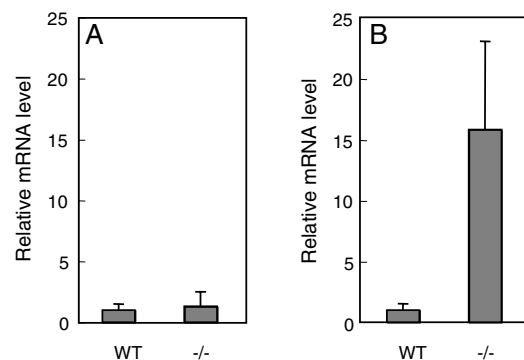
mRNA in the *Hsp47*⁻/⁻ embryos at 10.5 dpc, but not at 9.5 dpc, was confirmed by quantitative real-time PCR (Fig. 11). The elevated level of CHOP mRNA is probably responsible for the high level of apoptosis observed in the *Hsp47*⁻/⁻ mouse embryos.

Discussion

We previously demonstrated that Hsp47 plays an essential role in the production of mature type I collagen by mouse embryos, and that the type I collagen secreted by cultured embryonic fibroblasts is not correctly folded into the triple helices in the absence of Hsp47 (Nagai et al., 2000). In the present study, type IV collagen was also shown to require Hsp47 for its maturation because type IV collagen was not detected in the BM of *Hsp47*⁻/⁻ embryos (Figs 1, 3 and 5). Considered together with the fact that Hsp47 recognizes type IV collagen in vitro (Natsume et al., 1994), these observations clearly demonstrate the indispensable

Fig. 10. CHOP is upregulated in *Hsp47*⁻/⁻ mutant mouse embryos at 10.5-11.0 dpc. The levels of CHOP (A), Hsp47 (B) and β-actin (C) mRNAs in wild-type and *Hsp47*⁻/⁻ embryos at 9.5, 10.5 and 11.0 dpc were analysed by RT-PCR using specific primers. CHOP mRNA levels were elevated in *Hsp47*⁻/⁻ embryos at 10.5 and 11.0 dpc, but not at 9.5 dpc.

Fig. 11. Quantitative RT-PCR analysis measuring CHOP mRNA levels in wild-type and *Hsp47*⁻/⁻ embryos. Total RNA isolated from embryos at 9.5 dpc (A) and 10.5 dpc (B) were analysed by real time PCR using primers specific to CHOP and β-actin in the presence of SYBER Green. Levels of amplified products were recorded throughout the PCR reaction, and the level of CHOP cDNA was normalized against that of β-actin cDNA. The calculated CHOP mRNA levels of wild-type embryos were set at 1.0. Mean with standard deviations for 11-14 embryos are shown.

role of Hsp47 as a molecular chaperone of not just collagen type I but also type IV. This view is consistent with the previous observation that Hsp47 preferably binds the Pro-Arg-Gly sequence in Pro-Pro-Gly repeats in vitro (Koide et al., 2002) because type IV collagen has these sequences in abundance.

BMs play important roles in the morphogenesis of tissues during embryonic development. BMs separate epithelial (or endothelial) cells from mesenchymal tissues and maintain the function and morphology of epithelial cells. In the BM, laminin and type IV collagen form meshes that are linked by nidogen (Carlin et al., 1981; Timpl et al., 1983). We found here that laminin and nidogen-1 accumulate normally in the BM of 9.5 dpc $Hsp47^{-/-}$ embryos, despite the fact that the normal accumulation of type IV collagen in the BM does not occur (Figs 2 and 5). Thus, the accumulation of laminin and nidogen-1 in the BM does not seem to require type IV collagen. This is consistent with the previous observation that type IV collagen-free BM exists in *Caenorhabditis elegans* (Gupta et al., 1997). However, laminin is necessary for BM formation, as *LAMC1*-knockout mouse embryos cannot produce BM structures and die earlier than 5.5 dpc (Smyth et al., 1999).

More importantly, we demonstrated in the present study that the BM becomes fractured at 10.5 dpc in the absence of type IV collagen (Fig. 4). These observations indicate that type IV collagen is essential for the maintenance of the continuous sheet-like structures of the BM. Type IV collagen therefore seems to be important for providing the BM with the strength that is needed to cope with the growing tensile stresses imposed on the BM during the rapid morphological changes that take place during development.

Immunoelectron microscopic analysis of epithelial cells also revealed that type IV collagen accumulates within dilated ERs in the absence of Hsp47 (Fig. 6). Type I collagen secreted from $Hsp47^{-/-}$ cultured fibroblasts has been reported to be sensitive to protease digestion (Nagai et al., 2000), which indicates that the triple helix structure of the secreted type I collagen molecules is incompletely formed. Recently, we observed that type IV collagen secreted from $Hsp47^{-/-}$ embryonic stem cells also consists of an incomplete triple helix that is sensitive to protease digestion (Matsuoka et al., 2004). Thus, the type IV collagen that accumulates in the ER in the *Hsp47*-knockout embryos is probably misfolded and/or aggregated. Even if some of these proteins were secreted from the cells, the incompletely folded type IV collagen may be degraded by proteinases in the ECM. The accumulation of unfolded or misfolded proteins in the ER is known to activate the ER stress response that induces the expression of molecular chaperones and folding enzymes in the ER (Kaufman, 1999; Mori, 2000; Urano et al., 2000). ER stress also induces apoptosis that serves to remove severely damaged cells (Zinszner, 1998; Nakagawa et al., 2000; Iwawaki et al., 2001). In the present study, laddered DNA fragmentation typical of apoptotic cell death was observed in $Hsp47^{-/-}$ embryos at 10.5 dpc but not at 9.5 dpc (Fig. 8). This was also detected by TUNEL staining (Fig. 9). These observations indicate that apoptotic cell death occurs in $Hsp47^{-/-}$ embryos at 10.5 dpc just prior to the death of the embryos.

We also demonstrated that the mRNA levels of CHOP are elevated in $Hsp47^{-/-}$ embryos at 10.5-11.0 dpc (Figs 10 and 11). CHOP is known to be a transcription factor that is strongly upregulated by ER stress (Barone et al., 1994; Fleming et al.,

1997; Jousse et al., 1999) and that plays a crucial role in ER stress-induced apoptosis (Zinszner, 1998; Nakagawa et al., 2000; Iwawaki et al., 2001; Oyadomari, 2001). Thus, our present observations suggest that the accumulation of unfolded collagen in the ER induces apoptosis through the stimulation of CHOP expression. This is similar to the observation of the pancreatic β cells of mice that bear a C96Y mutation in their insulin gene: these cells die by apoptosis through the induction of CHOP (Oyadomari et al., 2002) after the accumulation of misfolded insulin in the ER stimulates the UPR pathway (Nozaki et al., 2004). However, the cell types that are affected in the insulin mutant are much more restricted than those in the $Hsp47^{-/-}$ embryos. It is worthwhile to note that we cannot exclude the possibility that CHOP might be induced by hypoxia or by impairment of nutrient exchange from the placenta, because of the disruption of the vasculature in the Hsp47-disrupted mouse (Nagai et al., 2000) or the disruption of the BM in the placenta, respectively. Further studies could be carried out by crossing knockout $Hsp47$ and knockout $CHOP$ mice (Oyadomari et al., 2001) to determine the role of CHOP in the death of $Hsp47^{-/-}$ embryos.

While this manuscript was in preparation, it was reported that the knockout mouse of type IV collagen α1 and α2 chains exhibits lethality at 10.5-11.5 dpc (Poschl et al., 2004). These authors also reported that although BM-like structures were present in the $Col4\alpha1/2$-knockout embryos, their ultrastructure was abnormal. The authors proposed that type IV collagen is important for the maintenance of the integrity of the BM but is dispensable for the formation of the BM. These observations are entirely consistent with our observations of the $Hsp47^{-/-}$ embryo, and strongly support the idea that the phenotype of the $Hsp47^{-/-}$ embryos is primarily due to the absence of functional type IV collagen. It is noteworthy that the $Hsp47^{-/-}$ embryos die slightly earlier than the $Col4\alpha1/2$-knockout embryos, as this suggests that the defects in BM structure and related functions are more severe in the $Hsp47^{-/-}$ embryos. This difference may be due to absence of other collagens in the BM and/or supporting connective tissues in the $Hsp47^{-/-}$ embryos, as Hsp47 recognizes many different types of collagen in vitro (Natsume et al., 1994). It is also conceivable that in addition to the defects in BM structures, the accumulation of misfolded type IV collagen in the ER may induce the earlier apoptosis-driven death of the $Hsp47^{-/-}$ embryos, because the ER stress due to the accumulation of misfolded collagen would not be expected in the case of the $Col4\alpha1/2$-knockout mice. Thus, the observations of Poschl and colleagues (Poschl et al., 2004) are consistent with our conclusion that Hsp47 plays an essential role in type IV collagen maturation and that type IV collagen is important for the maintenance of BM structures.

References

Barone, M. V., Crozat, A., Tabaee, A., Philipson, L. and Ron, D. (1994). CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. *Genes Dev*. **8**, 453-464.

Cal, S., Arguelles, J. M., Fernandez, P. L. and Lopez-Otin, C. (2001). Identification, characterization, and intracellular processing of ADAM-

TS12, a novel human disintegrin with a complex structural organization involving multiple thrombospondin-1 repeats. *J. Biol. Chem.* **276**, 17932-17940.

Carlin, B., Jaffe, R., Bender, B. and Chung, A. E. (1981). Entactin, a novel basal lamina-associated sulfated glycoprotein. *J. Biol. Chem.* **256**, 5209-5214.

Engel, J. and Prockop, D. J. (1991). The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. *Annu. Rev. Biophys. Biophys. Chem.* **20**, 137-152.

Fleming, J. V., Fontanier, N., Harries, D. N. and Rees, W. D. (1997). The growth arrest genes gas5, gas6, and CHOP-10 (gadd153) are expressed in the mouse preimplantation embryo. *Mol. Reprod. Dev.* **48**, 310-316.

Gupta, M. C., Graham, P. L. and Kramer, J. M. (1997). Characterization of alpha1(IV) collagen mutations in Caenorhabditis elegans and the effects of alpha1 and alpha2(IV) mutations on type IV collagen distribution. *J. Cell Biol.* **137**, 1185-1196.

Hirayoshi, K., Kudo, H., Takechi, H., Nakai, A., Iwamatsu, A., Yamada, K. M. and Nagata, K. (1991). HSP47: a tissue-specific, transformation-sensitive, collagen-binding heat shock protein of chicken embryo fibroblasts. *Mol. Cell. Biol.* **11**, 4036-4044.

Iwawaki, T., Hosoda, A., Okuda, T., Kamigori, Y., Nomura-Furuwatari, C., Kimata, Y., Tsuru, A. and Kohno, K. (2001). Translational control by the ER transmembrane kinase/ribonuclease IRE1 under ER stress. *Nat. Cell Biol.* **3**, 158-164.

Jousse, C., Bruhat, A., Harding, H. P., Ferrara, M., Ron, D. and Fafournoux, P. (1999). Amino acid limitation regulates CHOP expression through a specific pathway independent of the unfolded protein response. *FEBS Lett.* **448**, 211-216.

Kadler, K. (1995). Extracellular matrix 1: fibril-forming collagens. *Protein Profile* **2**, 491-619.

Kadler, K. E., Holmes, D. F., Trotter, J. A. and Chapman, J. A. (1996). Collagen fibril formation. *Biochem. J.* **316**, 1-11.

Kaufman, R. J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev.* **13**, 1211-1233.

Koide, T., Aso, A., Yorihuzi, T. and Nagata, K. (2000). Conformational requirements of collagenous peptides for recognition by the chaperone protein HSP47. *J. Biol. Chem.* **275**, 27957-27963.

Koide, T., Takahara, Y., Asada, S. and Nagata, K. (2002). Xaa-Arg-Gly triplets in the collagen triple helix are dominant binding sites for the molecular chaperone HSP47. *J. Biol. Chem.* **277**, 6178-6182.

Leivo, I., Vaheri, A., Timpl, R. and Wartiovaara, J. (1980). Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* **76**, 100-114.

Li, S. W., Prockop, D. J., Helminen, H., Fassler, R., Lapvetelainen, T., Kiraly, K., Peltarri, A., Arokoski, J., Lui, H., Arita, M. et al. (1995). Transgenic mice with targeted inactivation of the Col2 alpha 1 gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. *Genes Dev.* **9**, 2821-2830.

Liu, X., Wu, H., Byrne, M., Krane, S. and Jaenisch, R. (1997). Type III collagen is crucial for collagen I fibrillogenesis and fornormal cardiovascular development. *Proc. Natl. Acad. Sci. USA* **94**, 1852-1856.

Lohler, J., Timpl, R. and Jaenisch, R. (1984). Embryonic lethal mutation in mouse collagen I gene causes rupture of blood vessels and is associated with erythropoietic and mesenchymal cell death. *Cell* **38**, 597-607.

Mandai, K., Nakanishi, H., Satoh, A., Obaishi, H., Wada, M., Nishioka, H., Itoh, M., Mizoguchi, A., Aoki, T., Fujimoto, T. et al. (1997). Afadin: a novel actin filament-binding protein with one PDZ domain localized at cadherin-based cell-to-cell adherens junction. *J. Cell Biol.* **139**, 517-528.

Masuda, H., Fukumoto, M., Hirayoshi, K. and Nagata, K. (1994). Coexpression of the collagen-binding stress protein HSP47 gene and the alpha 1(I) and alpha 1(III) collagen genes in carbon tetrachloride-induced rat liver fibrosis. *J. Clin. Invest.* **94**, 2481-2488.

Masuda, H., Hosokawa, N. and Nagata, K. (1998). Expression and localization of collagen-binding stress protein Hsp47 in mouse embryo development: comparison with types I and II collagen. *Cell Stress Chaperones* **3**, 256-264.

Matsuoka, Y., Kubota, H., Adachi, E., Nagai, N., Marutani, T., Hosokawa, N. and Nagata, K. (2004). Insufficient folding of type IV collagen and formation of abnormal basement membrane-like structure in embryoid bodies derived from Hsp47-null ES cells. *Mol. Biol. Cell* **15**, 4467-4475.

Mayer, U., Kohfeldt, E. and Timpl, R. (1998). Structural and genetic analysis of laminin-nidogen interaction. *Ann. N. Y. Acad. Sci.* **857**, 130-142.

McLaughlin, S. H. and Bulleid, N. J. (1998). Molecular recognition in procollagen chain assembly. *Matrix Biol.* **16**, 369-377.

Mori, K. (2000). Tripartite management of unfolded proteins in the ER. *Cell* **101**, 451-454.

Moriyama, T., Kawada, N., Ando, A., Yamauchi, A., Horio, M., Nagata, K., Imai, E. and Hori, M. (1998). Up-regulation of HSP47 in the mouse kidneys with unilateral ureteral obstruction. *Kidney Int.* **54**, 110-119.

Nagai, N., Hosokawa, M., Itohara, S., Adachi, E., Matsushita, T., Hosokawa, N. and Nagata, K. (2000). Embryonic lethality of molecular chaperone Hsp47 knockout mice is associated with defects in collagen biosynthesis. *J. Cell Biol.* **150**, 1499-1506.

Nagata, K. (2003). HSP47 as a collagen-specific molecular chaperone: function and expression in normal mouse development. *Seminars Cell Dev. Biol.* **14**, 275-282.

Nagata, K. and Hosokawa, N. (1996). Regulation and function of collagen-specific molecular chaperone, HSP47. *Cell Struct. Funct.* **21**, 425-430.

Nagata, K. and Yamada, K. M. (1986). Phosphorylation and transformation sensitivity of a major collagen-binding protein of fibroblasts. *J. Biol. Chem.* **261**, 7531-7536.

Naitoh, M., Hosokawa, N., Kubota, H., Tanaka, T., Shirane, H., Sawada, M., Nishimura, Y. and Nagata, K. (2001). Upregulation of HSP47 and collagen type III in the dermal fibrotic disease, Keloid. *Biochem. Biophys. Res. Commun.* **280**, 1316-1322.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A. and Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**, 98-103.

Natsume, T., Koide, T., Yokota, S., Hirayoshi, K. and Nagata, K. (1994). Interactions between collagen-binding stress protein HSP47 and collagen. *J. Biol. Chem.* **269**, 31224-31228.

Nozaki, J., Kubota, H., Yoshida, H., Naitoh, M., Goji, J., Yoshinaga, T., Mori, K., Koizumi, A. and Nagata, K. (2004). The endoplasmic reticulum stress response is stimulated through the continuous activation of transcription factors ATF6 and XBP1 in Ins2+/Akita pancreatic beta cells. *Genes Cells* **9**, 261-270.

Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E. and Mori, M. (2001). Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc. Natl. Acad. Sci. USA* **98**, 10845-10850.

Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E. and Mori, M. (2002). Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J. Clin. Invest.* **109**, 525-532.

Poschl, E., Schlotzer-Schrehardt, U., Brachvogel, B., Saito, K., Ninomiya, Y. and Mayer, U. (2004). Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development* **131**, 1619-1628.

Prockop, D. J., Sieron, A. L. and Li, S. W. (1998). Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. *Matrix Biol.* **16**, 399-408.

Saga, S., Nagata, K., Chen, W. T. and Yamada, K. M. (1987). pH-dependent function, purification, and intracellular location of a major collagen-binding glycoprotein. *J. Cell Biol.* **105**, 517-527.

Sato, K., Yomogida, K., Wada, T., Yorihuzi, T., Nishimune, Y., Hosokawa, N. and Nagata, K. (2002). Type XXVI collagen, a new member of the collagen family, is specifically expressed in the testis and ovary. *J. Biol. Chem.* **277**, 37678-37684.

Satoh, M., Hirayoshi, K., Yokota, S., Hosokawa, N. and Nagata, K. (1996). Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen. *J. Cell Biol.* **133**, 469-483.

Sauk, J. J., Norris, K., Hebert, C., Ordonez, J. and Reynolds, M. (1998). Hsp47 binds to the KDEL receptor and cell surface expression is modulated by cytoplasmic and endosomal pH. *Connect. Tissue Res.* **37**, 105-119.

Schnieke, A., Harbers, K. and Jaenisch, R. (1983). Embryonic lethal mutation in mice induced by retrovirus insertion into the alpha 1(I) collagen gene. *Nature* **304**, 315-320.

Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M. and Edgar, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol.* **144**, 151-160.

Sunamoto, M., Kuze, K., Tsuji, H., Ohishi, N., Yagi, K., Nagata, K., Kita,

T. and Doi, T. (1998). Antisense oligonucleotides against collagen-binding stress protein HSP47 suppress collagen accumulation in experimental glomerulonephritis. *Lab. Invest*. **78**, 967-972.

Tasab, M., Batten, M. R. and Bulleid, N. J. (2000). Hsp47: a molecular chaperone that interacts with and stabilizes correctly folded procollagen. *EMBO J*. **19**, 2204-2211.

Timpl, R., Dziadek, M., Fujiwara, S., Nowack, H. and Wick, G. (1983). Nidogen: a new, self-aggregating basement membrane protein. *Eur. J. Biochem*. **137**, 455-465.

Umebayashi, K., Fukuda, R., Hirata, A., Horiuchi, H., Nakano, A., Ohta,

A. and Takagi, M. (2001). Activation of the Ras-cAMP signal transduction pathway inhibits the proteasome-independent degradation of misfolded protein aggregates in the endoplasmic reticulum lumen. *J. Biol Chem*. **276**, 41444-41454.

Urano, F., Bertolotti, A. and Ron, D. (2000). IRE1 and efferent signaling from the endoplasmic reticulum. *J. Cell Sci*. **113**, 3697-3702.

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L. and Ron, D. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev*. **12**, 982-995.