

Embryonic Lethality of Molecular Chaperone Hsp47 Knockout Mice Is Associated with Defects in Collagen Biosynthesis

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Abstract. Triple helix formation of procollagen after the assembly of three α -chains at the C-propeptide is a prerequisite for refined structures such as fibers and meshworks. Hsp47 is an ER-resident stress inducible glycoprotein that specifically and transiently binds to newly synthesized procollagens. However, the real function of Hsp47 in collagen biosynthesis has not been elucidated in vitro or in vivo. Here, we describe the establishment of *Hsp47* knockout mice that are severely deficient in the mature, propeptide-processed form of $\alpha 1(I)$ collagen and fibril structures in mesenchymal tissues. The molecular form of type IV collagen was also affected, and basement membranes were discontinuously disrupted in the homozygotes. The homozygous

mice did not survive beyond 11.5 days postcoitus (dpc), and displayed abnormally orientated epithelial tissues and ruptured blood vessels. When triple helix formation of type I collagen secreted from cultured cells was monitored by protease digestion, the collagens of *Hsp47*^{+/+} and *Hsp47*^{+/-} cells were resistant, but those of *Hsp47*^{-/-} cells were sensitive. These results indicate for the first time that type I collagen is unable to form a rigid triple-helical structure without the assistance of molecular chaperone Hsp47, and that mice require Hsp47 for normal development.

Key words: gene targeting • extracellular matrix • collagen fibril • basement membrane • type I collagen

Introduction

As many as 19 types of collagen have been discovered, and each of them has been shown to have a different function. Interstitial collagens like type I and type III are major constituents of the extracellular matrix (ECM)¹ that supports

body structure. Nonfibrillar collagen type IV is exclusively found in basement membranes where it provides a scaffold for other ECM components. Others, known as FACIT (fibril-associated collagens with interrupted triple helices) collagens, such as types IX, XII, and XIV, associate with the surfaces of fibril collagens and modify their interactive properties. All collagens have characteristic Gly-Xaa-Yaa (glycine-any amino acid-any amino acid) triplet repeats in their amino acid sequences and form unique triple-helical structures. Mutations that impair the formation of this structure have been identified as the cause of various collagen-related diseases, including osteogenesis imperfecta and Ehlers-Danlos syndrome (Davidson and Berg, 1981; Prockop and Kivirikko, 1995). The formation

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¹Abbreviations used in this paper: ECM, extracellular matrix; ES, embryonic stem; dpc, days postcoitus; P-4H, prolyl 4-hydroxylase; pC, collagen chain with C-propeptide; RT, reverse transcription.

of rigid triple helix structures should, thus, be important for collagen function.

During protein synthesis, many proteins known as molecular chaperones interact and assist folding of newly synthesized polypeptides. ER-resident chaperones, including BiP, calnexin, and calreticulin, are involved in folding and translocation of a number of secretory and membrane proteins (for reviews, see Bergeron et al., 1994; Hebert et al., 1995), including collagens. In contrast, Hsp47 is unique in terms of its substrate specificity; that is, it binds exclusively to procollagens and collagens (for reviews, see Nagata 1996, 1998). The expression and accumulation of various types of collagens are strictly and spatio-temporally regulated in various tissues during mammalian development. The expression of Hsp47 in various cell lines and tissues is closely correlated with the expression levels of various types of collagens under nonstressful conditions (Leivo et al., 1980; Masuda et al., 1994, 1998).

Hsp47 was shown to transiently bind to newly synthesized procollagen, and to dissociate from procollagen during its transport from the ER to the cis-Golgi compartment (Sato et al., 1996). When cells were treated with α , α' -dipyridyl to inhibit prolyl 4-hydroxylation, the secretion of procollagen was prevented and procollagen bound to Hsp47 was retained in the ER (Nakai et al., 1992; Sato et al., 1996). From these corroborations, Hsp47 has been considered to function as a collagen-specific molecular chaperone and provide a quality control mechanism specific for procollagen maturation (Nagata, 1996, 1998). However, the precise role of Hsp47 as a molecular chaperone in collagen biosynthesis/maturation and mouse development has not yet been elucidated. To address this point, we disrupted *Hsp47* alleles by homologous recombination in mice by taking advantage of the fact that the *Hsp47* gene exists as a single copy in vertebrates, including human, mouse, rat, chicken, and zebrafish. Here, we show that the disruption of Hsp47 caused severe abnormality in the accumulation of properly processed mature molecules of type I collagen in the embryos, resulting in embryonic lethality before 11.5 days postcoitus (dpc). The truncated form of type IV collagen was also not discovered in *Hsp47*^{-/-} embryos.

Collagen fibers and basement membranes were hardly detected in knockout mice that displayed abnormally oriented epithelial tissues and ruptured blood vessels. We also report here the establishment of *Hsp47*^{-/-} fibroblastic cell lines, and show the importance of Hsp47 in the formation of rigid triple helix of type I procollagen by monitoring the protease sensitivity of secreted procollagen.

Materials and Methods

Gene Targeting

A gene targeting vector was constructed as follows: first, a 9-kb BamHI genomic DNA fragment containing exon 3–6 of the C57BL/6J mouse *Hsp47* gene was subcloned, and a neomycin-resistance gene expression cassette, MC1NeoPA (Stratagene), was then inserted between the two XhoI sites of the *Hsp47* gene fragment. The 5' flank/neo/3' flank was excised at the KpnI cleavage sites and subcloned into pMCDT-A (Yagi et al., 1990). This targeting vector was linearized and electroporated into E14 embryonic stem (ES) cells derived from mouse strain 129/Ola. ES cells carrying the disrupted allele were microinjected into blastocysts of mouse

strain C57BL/6J to produce chimeric mice. Heterozygotes were subsequently bred with both C57BL/6J and ICR to produce mouse lines of C57BL/6J \times 129/Ola and ICR \times 129/Ola hybrid background, respectively.

Reverse Transcription (RT)-PCR

Total RNA was isolated from 9.5 dpc embryos and RT-PCR was done with an RNA-PCR kit (TaKaRa) using the following primers: Hsp47 forward, 5'-CTGCAGTCCATCAACGAGTGGGC-3'; Hsp47 reverse, 5'-ATGGCGACAGCCTTCTTCTGC-3'; β -actin forward, 5'-CTAAG-GCCAACCGTGAAAAGA-3'; β -actin reverse, 5'-AGAGGCATACAGGGACAGCA-3'.

Western Blotting

Proteins extracted from whole embryo at 9.5 dpc were separated by electrophoresis through an 8% SDS-polyacrylamide gel, transferred to nitrocellulose filters (Schleicher and Schell), and probed with a mouse monoclonal anti-Hsp47 antibody (SPA470; StressGen Biotechnologies), rabbit polyclonal antitype I collagen C-telopeptide (LF-67; Fisher et al., 1995), antilaminin, antifibronectin serum, rat monoclonal anti- α 2(IV) collagen antibody (A22; Sado et al., 1995), or mouse monoclonal anti α 1(III) collagen antibody. Peroxidase-conjugated secondary antibodies were used and immunocomplexes were revealed by an ECL detection reagent (Amersham Pharmacia Biotech).

Histological Analysis

Embryos were excised for histological examination, fixed for 1 h in 10% neutralized formalin, and embedded in paraffin. 4- μ m thick sections were stained with Gomori's silver impregnation method for reticular fiber. For electron microscopic observation, samples from the *Hsp47* null embryos and their normal littermates were processed routinely as described (Tsunenaga et al., 1998).

Establishment of *Hsp47*-deficient Cells and Protease Digestion of Secreted Collagen

Hsp47^{+/+}, *Hsp47*^{+/-}, and *Hsp47*^{-/-} cell lines were established from 8.5 dpc embryos by the method adopted for the establishment of 3T3 cell line as described (Todaro and Green, 1963), and genotype analysis was performed by Southern and Northern blot analyses. Protease digestion of secreted collagen was performed as follows. Cells were labeled with 50 μ l/ml of L-[2, 3-³H]-proline for 8 h, and the medium containing equal amount of TCA-insoluble radioactivity was used for protease digestion with a mixture of 100 μ g/ml trypsin and 250 μ g/ml chymotrypsin for 1–5 min at 37°C. Aliquots were treated with 50 μ g/ml pepsin overnight at 4°C. Transient transfection of murine *Hsp47* cDNA was performed with lipofectamine (GIBCO BRL) as specified by the manufacturer. 50 μ g/ml of L-ascorbic acid phosphate magnesium salt *N*-hydrate was included in all the experiments.

Results

The *Hsp47* gene was disrupted in murine ES cells by the use of the targeting vector shown in Fig. 1 a. Heterozygous mice, which appeared phenotypically normal, were intercrossed to generate homozygous *Hsp47*^{-/-} mice. Homozygosity for the *Hsp47* mutation resulted in embryonic lethality in both C57BL/6 \times 129/Ola and ICR \times 129/Ola genetic backgrounds. Although homozygous new born mice and embryos after 11.5 dpc were never obtained, the null mutant embryos were recovered with the expected Mendelian ratios at 9.5 dpc and 10.5 dpc (data not shown). The homozygosity of *Hsp47*^{-/-} mice was determined by Southern blotting using 9.5 dpc embryos (Fig. 1 b). The absence of *Hsp47* expression in the null mutant embryo was confirmed at the mRNA level by RT-PCR (Fig. 1 c), and at the protein level by Western blot analysis (Fig. 1 d). The number of somites was consistently smaller in *Hsp47* homozygotes than in the wild/heterozygotic littermates from 9.5 dpc (Table I) and neural tube closure was delayed

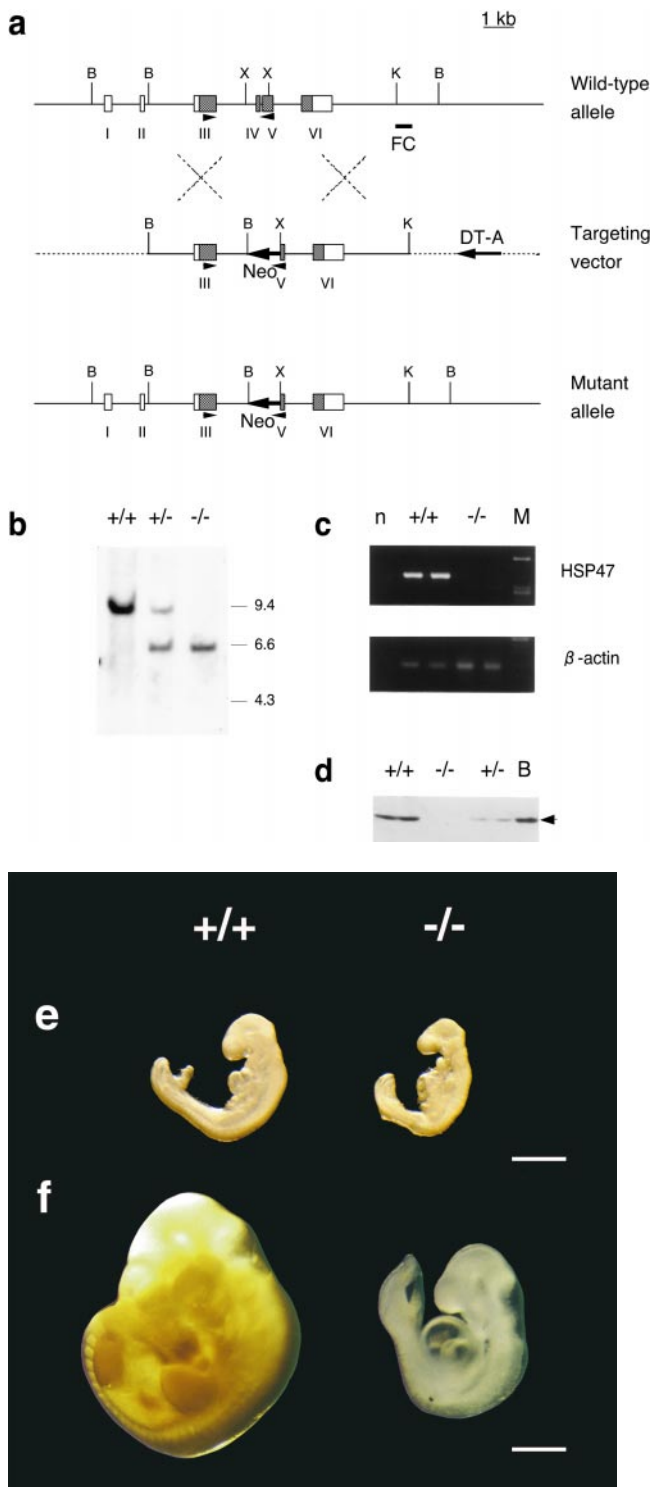


Figure 1. Targeted disruption of the *Hsp47* gene and characterization of the null phenotype. **a**, Homologous recombination with the targeting vector deletes exon IV and part of exon V, and simultaneously inserts a neomycin-resistance gene. Arrows indicate the orientation of neomycin-resistance gene and DT-A cassettes. Arrowheads indicate the location of primers used in RT-PCR assay. B, BamHI; K, KpnI; X, XhoI. **b**, Southern blot analysis demonstrating the genotypes of the offspring. A 3' flanking probe shown as FC in **a** detects a 9-kb BamHI fragment in wild-type genomic DNA and a 6-kb fragment in the targeted allele in mice. Targeted ES clones were confirmed using a 5' exter-

Table I. *Hsp47* Deficiency Was Accompanied with Developmental Delay

Gestational age	Somites		
	+/+	+/-	-/-
	<i>n</i>	<i>n</i>	<i>n</i>
E10.0	30.8 ± 0.4	30.4 ± 0.5	24.3 ± 0.6
E9.5	27.0 ± 0.8	26.6 ± 1.3	22.0 ± 0.4
E9.0	20.5 ± ND	20.6 ± 0.5	18.7 ± 2.1

The number of somites of each genotype was counted under a light microscope. More than three embryos, except E9.0 wild-type, were examined for each point and SDs are indicated. ND, not determined.

(data not shown), suggesting growth retardation in the knockout embryos. At 10.5 dpc, the mutant embryos were more translucent compared with their wild-type littermates (Fig. 1 f), probably reflecting the low cell density of their bodies, and were so fragile that they could not be manipulated with forceps. The body was shrunken, and the number of erythrocytes decreased before death (data not shown).

During development, type IV collagen first appears at the blastocyst stage (Leivo et al., 1980). The interstitial types I and III collagens were first detected in the eight-day embryos and were closely codistributed in tissues of mesodermal origin, as well as in basement membranes (Leivo et al., 1980). Northern blot analysis revealed that the amount of *Hsp47* mRNA was lower in heterozygous embryos than in wild-type embryos, and that *Hsp47* mRNA was not expressed in homozygotes (data not shown). However, wild-type, heterozygous, and homozygous embryos did not display any significant differences in the levels of $\alpha 1(I)$ and $\alpha 1(III)$ collagen mRNA (data not shown).

In contrast to Northern analysis, Western blot analysis (Fig. 2) revealed that the mature, propeptide-cleaved $\alpha 1(I)$ chain (Fig. 2, ***) was hardly detectable in *Hsp47* deficient mice. Instead, the levels of immature procollagen and its intermediately processed forms (Fig. 2, * and **), especially the collagen chain with C-propeptide (pC) form of $\alpha 1(I)$ collagen (Fig. 2, **), were higher in *Hsp47* deficient mice than in the wild-type or heterozygous littermates. In normal mouse tissues, a low molecular weight form of type IV collagen was detected as previously reported (Iwata et al., 1996), but this form of $\alpha 2(IV)$ collagen (arrow) was undetectable in the mutant embryos (Fig. 2, middle). Type II collagen could not be detected, probably because it was poorly expressed at this stage of development (data not shown). No differences in the molecular forms of type III collagen, laminin (Fig. 2, LMN), or fibronectin (Fig. 2, FN) were evident between *Hsp47*+/+,

nal probe (not shown). **c**, RT-PCR analysis was performed for the offspring using primers shown in **a**. n, Denotes the lane of negative control in which reactions are performed without RNA. **d**, Immunoblot analysis of Hsp47 was performed for wild-type, heterozygous, and homozygous null mice. B shows the positive control lane with the extract of Balbc/3T3 cells. Lateral views of 9.5 dpc (**e**) and 10.5 dpc (**f**) *Hsp47*^{-/-} homozygous embryos (right) and wild type (left). Embryos were observed before (**f**) and after (**e**) fixation with 10% formaldehyde. Bars, 1 mm.

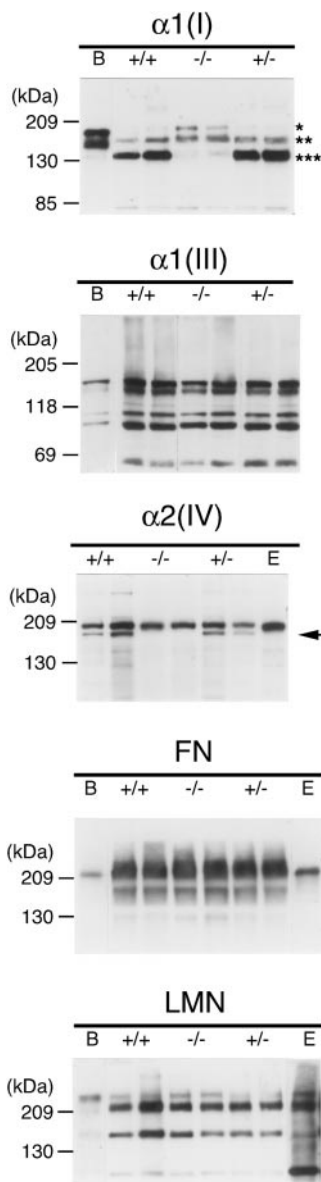


Figure 2. Western blot analyses using specific antibodies against $\alpha 1(I)$, $\alpha 1(III)$, and $\alpha 2(IV)$ chains of collagen, fibronectin (FN), and laminin (LMN) were performed for proteins prepared from 9.5 dpc whole embryos. * and *** in blots for $\alpha 1(I)$ collagen indicate pro- and mature $\alpha 1(I)$ chains, respectively. ** indicates the pC form of $\alpha 1(I)$ chain, because this band is not recognized by the anti-N-propeptide antibody (data not shown).

Hsp47^{+/-}, and *Hsp47*^{-/-} mice, at least as detected by Western blot analysis.

To investigate whether the differences observed by Western blot analysis were associated with any abnormalities in fibrillar structure in vivo, silver impregnation analysis was performed. Though fibrillar structures were obviously evident at the periphery of neural tube and in the mesenchymal tissue (Fig. 3 a) and beneath of the surface ectoderm (Fig. 3 c) of wild-type embryos, almost no staining was seen in *Hsp47*^{-/-} homozygotes at 9.5 dpc (Fig. 3, b and d) or 10.5 dpc (data not shown). The signals of periodic acid-methenamine silver (PAM) staining (data not shown) were very weak in *Hsp47* deficient embryos, as well as in yolk sacs, indicating serious damage to the basement membranes. The neuroepithelial cell distribution was disordered, and nuclei of neuroepithelial cells undergoing cell division (Fig. 3, a and b, arrowheads) were abnormally present not only near the apical surfaces, but also internally. This may have been caused by the disorder to

the basement membrane. Electron microscopic examination of *Hsp47*^{+/-} embryos at 9.5 dpc (Fig. 3 e) showed that the lamina densa of the ectoderm was a continuous sheet, and that a few collagen fibrils, ~20 nm in diameter, had formed bundles, corresponding to the reticular fibers shown in Fig. 3 c. Some of the bundles ran obliquely and approached the lamina densa, whereas others were distributed under the lamina densa. However, only a limited number of collagen fibrils were observed in the lamina fibroreticularis and stroma of *Hsp47*^{-/-} embryos, and the laminae densa of the ectoderm and neural tube were interrupted frequently (Fig. 3 f). These observations suggested impaired formation of basement membranes in homozygous embryos, which was also confirmed by silver impregnation analysis (Fig. 3 b). The disorder in ECM formation might explain other tissue malformations such as the fracture of endothelial cells surrounding blood vessels (Fig. 3 g). The infiltration of neuroepithelial cells into the neural tube beyond the basement membrane suggests impairment of the barrier function of the basement membrane in homozygous embryos (Fig. 3 h).

If *Hsp47* functions as a collagen-specific molecular chaperone, then *Hsp47* disruptants should secrete collagens with aberrant conformations. To investigate this possibility, we examined the conformation of the collagen triple helix using protease sensitivity as a probe. When the collagen in the culture media of *Hsp47*^{+/-} and *Hsp47*^{-/-} fibroblastic cells was digested with proteases, $\alpha 1(I)$ and $\alpha 2(I)$ chains were resistant to digestion, indicating protease-resistance (Fig. 4 a). However, when the medium from *Hsp47*^{+/-} cells was heated at 50°C for 15 min before protease digestion, both bands disappeared after protease treatment, indicating that the heat treatment destabilized the triple helix. The collagen secreted from *Hsp47*^{-/-} cells exhibited the same protease sensitivity as the heat-denatured one, suggesting that collagens with an abnormal triple helix are secreted from *Hsp47*^{-/-} cells. When *Hsp47* cDNA was transiently transfected into these *Hsp47*^{-/-} cells, the secreted collagen became resistant to protease treatment (Fig. 4 b). This result demonstrated that *Hsp47* functions as a molecular chaperone to ensure the rigid triple-helical conformation of type I collagen.

Discussion

The importance of individual types of collagens in the development and morphogenesis of mouse embryos has been demonstrated by several mutant mouse studies (Lohler et al., 1984; Li et al., 1995; Cosgrove, 1996; Liu et al., 1997). Mutations of various types of collagen genes have also been revealed to cause various diseases, including osteogenesis imperfecta, Alport syndrome, Ehlers-Danlos syndrome, and so on. *Hsp47* has been considered to be a collagen-specific molecular chaperone. However, the real function of *Hsp47* has never been demonstrated directly to date. To elucidate the importance of *Hsp47* in collagen biosynthesis and its involvement in the developmental processes, we developed the mice and cell lines lacking *Hsp47* gene by homologous recombination.

The phenotypes of *Hsp47*^{-/-} mice resembled those of *Mov13* mice in terms of the embryonic lethality (Schnieke et al., 1983; Lohler et al., 1984), but were more severe.

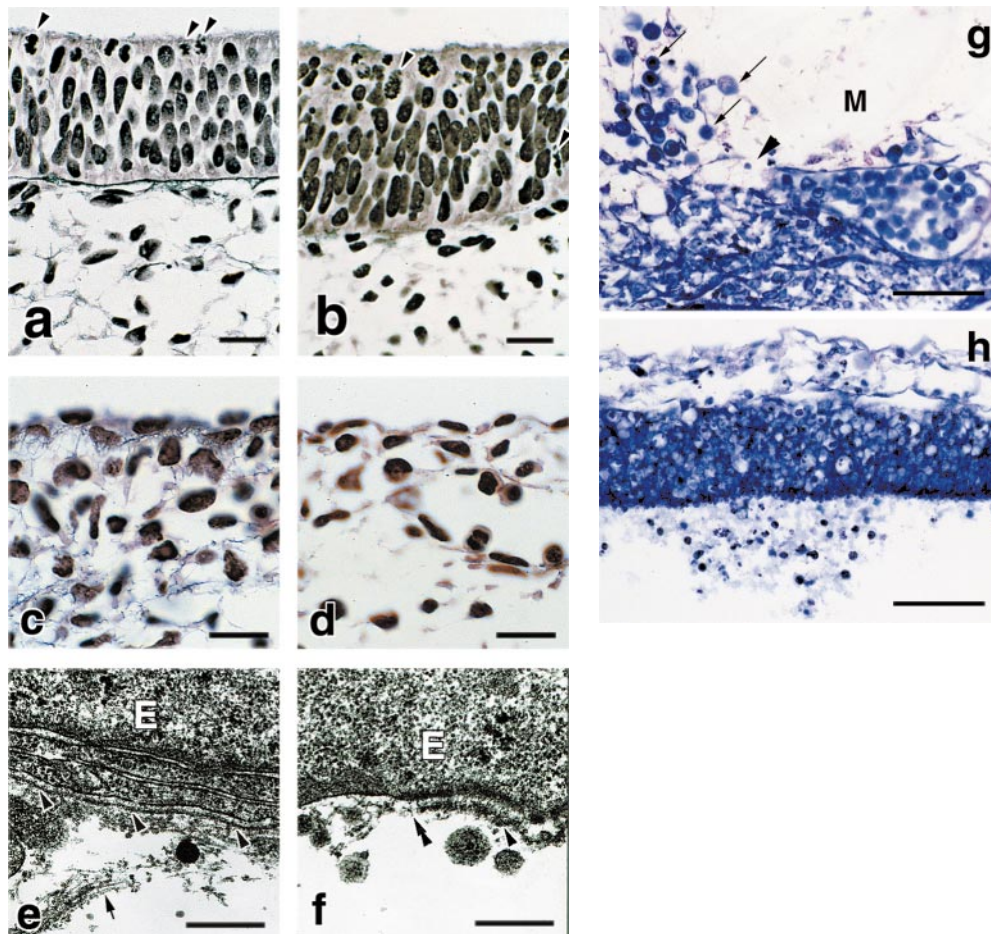


Figure 3. Silver impregnation analysis of 9.5 dpc wild-type (a and c) and homozygous (b and d) embryos. Neuroepithelium and underlying mesenchyme (a and b), and surface ectoderm and underlying mesenchyme (c and d) were analyzed. *Hsp47*^{-/-} embryos had poorly developed reticular fibers in their mesenchymal tissues (b and d) compared with wild-type embryos (a and c). The basement membrane was hardly detected between the neuroepithelium of the neural tube and the surrounding mesenchymal tissue (b) of the *Hsp47*^{-/-} embryos, whereas it was clearly detected as black staining in wild-type embryos (a). Neuroepithelium organization was disordered and cell division (arrowheads) was abnormally (closer to the mesenchymal tissue) detected in the cell layer of the neuroepithelium in *Hsp47*^{-/-} embryos (b). Bars, 20 μm. Electron microscopic observation of the ectoderm in wild-type (e) and *Hsp47*^{-/-} embryos (f). In e, a bundle of collagen fibrils (arrow) can be seen to

reach out towards the lamina densa and to be distributed in the lamina fibroreticularis. The lamina densa (arrowhead) appears as a continuous sheet with a width of ~30 nm positioned under the ectoderm (E) and the lamina lucida. The lamina densa (arrowhead) of the ectoderm (E) was interrupted (double arrowhead) and collagen fibrils were hardly discernible in *Hsp47*^{-/-} embryos (f). The lamina densa seen in *Hsp47*^{-/-} embryos was 30–50 nm in width, thicker than that in *Hsp47*^{+/+} embryos. Bar, 100 nm. Giemsa staining of *Hsp47*^{-/-} embryos of 10.5 dpc shows the discontinuity of endothelial cells surrounding blood vessels (g, arrowhead) and the leakage of erythropoietic cells (g, arrows). Neuroepithelial cells also were also shown to leak out of the neural tube (h) by Giemsa staining. Bar, 50 μm.

Mov13 mice, with the exception of osteoblastic lineages, are defective in type I collagen production and die before 13.5 dpc (or 16.5 dpc in rare cases) with the mesenchymal necrotic cell death phenotype. The growth of Mov13 and wild-type embryos could not be distinguished until 12 dpc, whereas growth retardation and deficient fibril formation was already apparent in *Hsp47*^{-/-} embryos at 9.5 dpc. The more severe phenotypes of *Hsp47* disruptants may reflect pleiotropic roles for Hsp47 during the maturation of not only type I procollagen, but other procollagens as well, which can be supported by the fact that Hsp47 can interact with types I to V collagens in vitro (Natsume et al., 1994). Indeed, PAM staining (data not shown) and electron microscopic observations (Fig. 3, e and f) indicated that the formation of the basement membrane, whose most important constituent is type IV collagen, was impaired in *Hsp47* disrupted embryos. This is consistent with the fact that type IV collagen had the altered character as shown in Western blot analysis (Fig. 2).

As shown in Fig. 4, type I procollagens secreted from *Hsp47*^{-/-} cells were sensitive to protease digestion,

whereas those secreted from *Hsp47*^{+/+} or *Hsp47*^{+/-} cells were resistant to such treatments. As the digestion by the combination of trypsin and chymotrypsin is a well established method for detecting the formation of the rigid triple helix of procollagen, the result shown in Fig. 4 demonstrated that the procollagen secreted from *Hsp47*^{-/-} cells did not form correctly aligned triple helices. Furthermore, the fact that the transfection of *Hsp47* gene into *Hsp47*^{-/-} cells resulted in the restoration of triple helix formation of the procollagen clearly suggests the important role of Hsp47 in the correct triple helix formation of procollagens in the ER. Hsp47 has been shown to interact preferentially with the triple-helical conformation of procollagens (Koide et al., 2000; Tasab et al., 2000), whereas prolyl 4-hydroxylase (P-4H) interacts with the monomeric α chain. It is assumed that Hsp47 binds to and stabilizes the triple helix forms of procollagens to avoid their wasteful unfolding in the ER and that, during heat shock, expression of Hsp47 is induced to prevent thermal denaturation (Koide et al., 2000; Tasab et al., 2000). Indeed, it is reported that procollagen within the cell is more thermo-

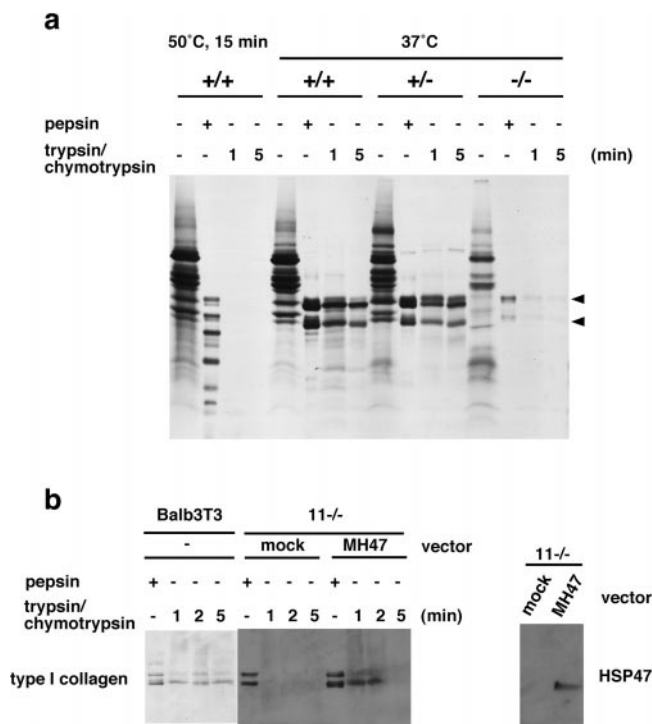


Figure 4. Altered protease-sensitivity of procollagen secreted from *Hsp47*^{-/-} cells. **a**, The culture media of *Hsp47*^{+/+}, *Hsp47*^{+/-}, and *Hsp47*^{-/-} cells labeled with L-[2, 3-³H]-proline were digested with a pepsin alone at 4°C overnight or a mixture of trypsin and chymotrypsin at 37°C for indicated periods. Arrowheads indicate the positions of collagen α 1(I) and α 2(I) chains, respectively. **b**, Transient expression of *Hsp47* cDNA in *Hsp47*^{-/-} cells restored the protease-resistance of collagen α chains secreted from the cells. Collagen chains secreted from nonlabeled cells were digested with pepsin alone or with a mixture of trypsin and chymotrypsin, and were detected by Western blotting using rabbit anti-rat α 1(I) polyclonal antiserum. The right shows the level of Hsp47 protein detected by anti-Hsp47 mAb (SPA470) after transient transfection with empty vector (mock) or murine Hsp47 expression vector (MH47).

stable than the isolated protein (Bruckner and Eikenberry, 1984), suggesting the existence of the mechanism that can prevent the collagen triple helix from heat denaturation.

Procollagen conversion to collagen is catalyzed extracellularly by procollagen N-proteinase (PNPase) and procollagen C-proteinase (PCPase), which cleave the N-propeptide and C-propeptide of procollagen, respectively (Peltonen et al., 1985). As shown in Fig. 2, pC α 1(I) and pN α 1(III) chains, as well as mature α 1(III) chains, were detected in *Hsp47*^{-/-} mice, suggesting that procollagens were secreted from the cells. The activities of these processing enzymes would not be different in *Hsp47*^{-/-} mice, since processing of type III collagen in wild-type and heterozygous mice was not different from that in homozygous mice. Rather, the conformation of procollagens in *Hsp47*^{-/-} mice may be incorrectly aligned, so that it would be incompatible with the efficient processing of the propeptides. This speculation is supported by the fact that the sensitivity to proteases are differed between procollagens

in the medium of *Hsp47*^{+/+} and *Hsp47*^{-/-} cells (Fig. 4; discussed above). The removal of the COOH-terminal propeptide of type I collagen is known to be necessary for fibril formation (Peltonen et al., 1985; Adachi et al., 1997). So the physiological effects of *Hsp47* disruption, such as the deficiency in fibril formation observed by silver impregnation and EM (Fig. 3, b, d, and f), can be explained by the accumulation of pro α 1(I) and pC α 1(I) molecules in *Hsp47*-deficient embryos. Western blotting data of Fig. 2 showed that the processing of type III procollagen occurs correctly. As discussed by Bächinger et al. (1980), one possible explanation is that type III procollagen may have an intrinsic ability to form the triple-helical structure more easily than type I collagen. However, at present, we cannot exclude the possibility that type III collagen in homozygous embryos have some conformational abnormalities that cannot be detected by Western blot analysis.

It has been widely held for a long time that collagen, after proline hydroxylation, possesses the intrinsic ability to form the triple helix structure without the intervention of any other cellular protein. However, the results in this paper showing abnormal conformation of collagen molecules secreted from *Hsp47*^{-/-} cells and accumulation of procollagen in knockout mice clearly indicate that the native collagen structure requires a specific molecular chaperone. The secretion of collagens with aberrant triple helices could result in the loss of tissue integrity and cell viability through impaired constitution of fibrils and basement membranes, which finally result in embryonic lethality. Thus, Hsp47 is an essential chaperone protein specific for collagen maturation and for normal mouse development. Although we have established in this report that targeting of Hsp47 causes aberrant biosynthesis of collagen, it should be carefully examined whether or how Hsp47 is involved in the quality control mechanism of procollagen biosynthesis.

The cyclophilin homologue, NinaA, was reported to function as a rhodopsin-specific chaperone and to be essential for rhodopsin biogenesis in *Drosophila melanogaster* using transgenic flies harboring mutant *ninaA* genes (Baker et al., 1994). Like NinaA, Hsp47 exhibits substrate specificity, but other chaperones, such as BiP, protein disulfide isomerase (PDI), calnexin, and calreticulin in the ER do not have such specificity. Thus, Hsp47 is the first substrate-specific molecular chaperone to be identified in mammals. P-4H and PDI have also been shown to act as chaperones in the biosynthetic pathway of collagens. Further study of the *Hsp47*-deficient cell line should increase our understanding of the secretory process of procollagens, that is, define how each chaperone acts in collagen biosynthesis, and allow a more refined analysis of the characteristic features of other types of secreted collagens, including their precise physical natures.

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References

- Adachi, E., I. Hopkinson, and T. Hayashi. 1997. Basement-membrane stromal relationships: interactions between collagen fibrils and the lamina densa. *Int. Rev. Cytol.* 173:73–156.
- Bächinger, H.P., P. Bruckner, R. Timpl, D.J. Prockop, and J. Engel. 1980. Folding mechanism of the triple helix in type-III collagen and type-III pN-collagen. Role of disulfide bridges and peptide bond isomerization. *Eur. J. Biochem.* 106:619–632.
- Baker, E.K., N.J. Colley, and C.S. Zuker. 1994. The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4886–4895.
- Bergeron, J.J., M.B. Brenner, D.Y. Thomas, and D.B. Williams. 1994. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* 19:124–128.
- Bruckner, P., and E.F. Eikenberry. 1984. Procollagen is more stable *in cellulo* than *in vitro*. *Eur. J. Biochem.* 140:397–399.
- Cosgrove, D. 1996. Collagen COL4A3 knockout: a mouse model for autosomal Alport syndrome. *Genes Dev.* 10:2981–2992.
- Davidson, J.M., and R.A. Berg. 1981. Posttranslational events in collagen biosynthesis. *Methods Cell Biol.* 23:119–136.
- Fisher, L.W., J.T. Stubbs, 3rd., and M.F. Young. 1995. Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins. *Acta Orthop. Scand. Suppl.* 266:61–65.
- Hebert, D.N., J.F. Simons, J.R. Peterson, and A. Helenius. 1995. Calnexin, calreticulin, and Bip/Kar2p in protein folding. *Cold Spring Harb. Symp. Quant. Biol.* 60:405–415.
- Iwata, M., Y. Sado, T. Sasaki, Y. Imamura, Y. Ninomiya, and T. Hayashi. 1996. The 160k alpha 1(IV) chain, a short form of a type IV collagen polypeptide, of bovine lens capsule retains the NC1 domain. *J. Biochem.* 120:133–137.
- Koide, T., A. Aso, T. Yorihuzi, and K. Nagata. 2000. Conformational requirements of collagenous peptides for recognition by the chaperone protein HSP47. *J. Biol. Chem.* In press.
- Leivo, I., A. Vaheri, R. Timpl, and J. Wartiovaara. 1980. Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol.* 76: 100–114.
- Li, S.W., D.J. Prockop, H. Helminen, R. Fassler, T. Lapvetelainen, K. Kiraly, A. Peltarri, J. Arokoski, H. Lui, M. Arita, 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., H. Wu, M. Byrne, S. Krane, and R. Jaenisch. 1997. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc. Natl. Acad. Sci. USA.* 94:1852–1856.
- Lohler, J., R. Timpl, and R. Jaenisch. 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.
- Masuda, H., M. Fukumoto, K. Hirayoshi, and K. Nagata. 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., N. Hosokawa, and K. Nagata. 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.
- Nagata, K. 1996. Hsp47: a collagen-specific molecular chaperone. *Trends Biol. Sci.* 21:23–26.
- Nagata, K. 1998. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. *Matrix Biol.* 16: 379–386.
- Nakai, A., M. Satoh, K. Hirayoshi, and K. Nagata. 1992. Involvement of the stress protein HSP47 in procollagen processing in the endoplasmic reticulum. *J. Cell Biol.* 117:903–914.
- Natsume, T., T. Koide, S. Yokota, K. Hirayoshi, and K. Nagata. 1994. Interactions between collagen-binding stress protein HSP47 and collagen: analysis of kinetic parameters by surface plasmon resonance biosensor. *J. Biol. Chem.* 269:31224–31228.
- Peltonen, L., R. Halila, and L. Ryhanen. 1985. Enzymes converting procollagens to collagens. *J. Cell Biochem.* 28:15–21.
- Prockop, D.J., and K.I. Kivirikko. 1995. Collagens: molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* 64:403–434.
- Sado, Y., M. Kagawa, Y. Kishiro, K. Sugihara, I. Naito, J.M. Seyer, M. Sugimoto, T. Oohashi, and Y. Ninomiya. 1995. Establishment by the rat lymph node method of epitope-defined monoclonal antibodies recognizing the six different alpha chains of human type IV collagen. *Histochem. Cell Biol.* 104: 267–275.
- Satoh, M., K. Hirayoshi, S. Yokota, N. Hosokawa, and K. Nagata. 1996. Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen. *J. Cell Biol.* 133:469–483.
- Schnieke, A., K. Harbers, and R. Jaenisch. 1983. Embryonic lethal mutation in mice induced by retrovirus insertion into the alpha 1(I) collagen gene. *Nature.* 304:315–320.
- Tasab, M., M.R. Batten, and N.J. Bulleid. 2000. Hsp47: a molecular chaperone that interacts with and stabilizes correctly-folded procollagen. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:2204–2211.
- Todaro, G.J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17:229–313.
- Tsunenaga, M., E. Adachi, S. Amano, R.E. Burgeson, and T. Nishiyama. 1998. Laminin 5 can promote assembly of the lamina densa in the skin equivalent model. *Matrix Biol.* 17:603–613.
- Yagi, T., Y. Ikawa, K. Yoshida, Y. Shigetani, N. Takeda, I. Mabuchi, T. Yamamoto, and S. Aizawa. 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. *Proc. Natl. Acad. Sci. USA.* 87:9918–9922.