Molecular Biology of the Cell
Vol. 15, 4467–4475, October 2004

Insufficient Folding of Type IV Collagen and Formation of Abnormal Basement Membrane-like Structure in Embryoid Bodies Derived from Hsp47-Null Embryonic Stem Cells D

**Yasuhiro Matsuoka,[*] Hiroshi Kubota,[*†] Eijiro Adachi,[‡] Naoko Nagai,[*§]
Toshihiro Marutani,[*] Nobuko Hosokawa,[*†] and Kazuhiro Nagata[*†||]**

[*]Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8397, Japan; [†]Core Research for Evolution Science and Technology, Japan Science and Technology Agency, Japan; and [‡]Department of Molecular Morphology, Kitasato University Graduate School of Medicine, Sagamihara, Kanagawa 228-8555, Japan

Submitted January 20, 2004; Revised May 25, 2004; Accepted July 6, 2004
Monitoring Editor: Peter Walter

Hsp47 is a molecular chaperone that specifically recognizes procollagen in the endoplasmic reticulum. *Hsp47*-null mouse embryos produce immature type I collagen and form discontinuous basement membranes. We established *Hsp47*$^{-/-}$ embryonic stem cell lines and examined formation of basement membrane and production of type IV collagen in embryoid bodies, a model for postimplantation egg-cylinder stage embryos. The visceral endodermal cell layers surrounding *Hsp47*$^{-/-}$ embryoid bodies were often disorganized, a result that suggested abnormal function of the basement membrane under the visceral endoderm. Rate of type IV collagen secretion by *Hsp47*$^{-/-}$ cells was fourfold lower than that of *Hsp47*$^{+/+}$ cells. Furthermore, type IV collagen secreted from *Hsp47*$^{-/-}$ cells was much more sensitive to protease digestion than was type IV collagen secreted from *Hsp47*$^{+/+}$ cells, which suggested insufficient or incorrect triple helix formation in type IV collagen in the absence of Hsp47. These results indicate for the first time that Hsp47 is required for the molecular maturation of type IV collagen and suggest that misfolded type IV collagen causes abnormal morphology of embryoid bodies.

INTRODUCTION

Collagen is one of the most abundant proteins of the extracellular matrix (Kuhn *et al.*, 1987); 26 subclasses of collagen molecules, types I through XXVI, have been identified (Kuhn *et al.*, 1987; Prockop and Kivirikko, 1995; Sato *et al.*, 2002). Although the fibril-forming types I and III collagen are abundant in most extracellular matrices, type IV collagen, a nonfibrillar network-forming collagen (Timpl *et al.*, 1981; Kuhn *et al.*, 1987), is the major component of basement membrane (BM). Collagen molecules contain helical domains, known as collagenous domains, that are composed of X-Y-Gly triplet repeats; often, the Y residue is hydroxyproline (Kuhn *et al.*, 1987; van der Rest M, 1991). The type IV procollagen molecule consists of three domains; 7S, a short, N-terminal triple-helical domain; NC1, a noncollagenous, C-terminal C-propeptide domain; and a long triple-helical collagenous domain centrally (Kuhn *et al.*, 1987).

During collagen biosynthesis, procollagen chains interact with several endoplasmic reticulum (ER)-resident molecular chaperones and protein folding catalysts (Lamande and Bateman, 1999), including the 47-kDa heat shock protein (Hsp47) (Nagata, 1996), the 78-kDa glucose-regulated protein (GRP78, also called BiP) (Chessler and Byers, 1993), protein disulfide isomerase (PDI) (Wilson *et al.*, 1998), and prolyl 4-hydroxylase (P4H) (Chessler and Byers, 1992; Walmsley *et al.*, 1999). Formation of triple helixes within collagenous domains proceeds from the C terminus to the N terminus (Bachinger *et al.*, 1980; Engel and Prockop, 1991; Bulleid *et al.*, 1997), and hydroxylation of proline residues at the Y positions stabilizes the triple helixes of procollagen (Uitto and Prockop, 1974). After triple helix formation, procollagen is exported through the general secretion pathway and then mature collagen forms higher order complexes in the extracellular matrix (Timpl *et al.*, 1981; Kuhn *et al.*, 1987).

Hsp47 is a collagen-binding protein (Nagata and Yamada, 1986) that assists in the molecular maturation of procollagen (Nagai *et al.*, 2000; Tasab *et al.*, 2000). Hsp47 can bind to procollagen in vivo (Nakai *et al.*, 1990) and in vitro (Natsume *et al.*, 1994; Koide *et al.*, 2002) and preferentially binds the triple-helical region of procollagen (Koide *et al.*, 2000; Tasab *et al.*, 2000). In vitro analyses have indicated that Hsp47 recognizes ProPro-Gly triplet repeats (Koide *et al.*, 2000) and preferentially binds to ProArg-Gly triplet repeats (Koide *et al.*, 2002; Tasab *et al.*, 2002). Hsp47 dissociates from procollagen in the ER-Golgi intermediate compartment or in the *cis*-Golgi during transport to the Golgi apparatus (Nakai *et*

D The online version of this article contains supplemental material accessible through http://www.molbiolcell.org.

[§] Present address: Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan.

[||] Corresponding author. E-mail address: nagata@frontier.kyoto-u.ac.jp.

Abbreviations used: BM, basement membrane; EB, embryoid body; ER, endoplasmic reticulum; ES, embryonic stem; LIF, leukemia inhibitory factor; PDI, protein disulfide isomerase; TCA, trichloroacetic acid; VE, visceral endoderm.

al., 1992; Satoh *et al.*, 1996). We have found that *Hsp47* knockout mouse embryos cannot survive beyond 11.5 d postcoitus and that they are severely deficient in fibrillar collagen within the extracellular matrix of mesenchymal tissues (Nagai *et al.*, 2000). Fibroblasts established from *Hsp47*$^{-/-}$ mice produce immature type I collagen without a well-defined triple-helical structure (Nagai *et al.*, 2000).

Although Hsp47 binds types I through V collagen in vitro (Natsume *et al.*, 1994), little is known about its role as a molecular chaperone in type IV collagen maturation in vivo. We established embryonic stem (ES) cell lines which lack the *Hsp47* gene to examine the role of Hsp47 in the molecular maturation of type IV collagen in developing embryoid bodies (EBs), an in vitro model for postimplantation egg-cylinder–stage embryos. The morphology of the visceral endodermal (VE) cell layer is disorganized in EBs of *Hsp47*$^{-/-}$ ES cells, a finding that suggests impaired function of the BM. *Hsp47*$^{-/-}$ cells secreted type IV collagen at a much slower rate and produced misfolded type IV collagen that was susceptible to protease digestion. We discuss the essential role of Hsp47 in the productive folding of type IV collagen in vivo.

MATERIALS AND METHODS

Cell Culture

ES cells were cultured on mitomycin C-treated STO feeder cells in DMEM with high glucose and supplemented with 15% fetal bovine serum (Bioserum; CSL Limited, Victoria, Australia), 0.1 mM 2-mercaptoethanol, and 1000 U/ml leukemia inhibitory factor (LIF) (Chemicon International, Temecula, CA), as described previously (Robertson, 1987). ES cells were treated with 1 mM retinoic acid and 0.5 mM dibutyryladenosine 3′,5′-cyclic phosphate for 3 d to induce differentiation of monolayer endodermal cells.

For EB formation, ES cells were first cultured on tissue culture plates for 3 d with ES culture medium to remove residual feeder cells and then seeded on bacteriological petri dishes with ES culture medium without LIF. Cell aggregates were disrupted by hanging and dropping and were transferred to new petri dishes to maintain suspension cultures. The day on which primary cell aggregates were resuspended in new petri dishes was designated as day 0.

Isolation of Homozygous Hsp47 Knockout ES Cells

Hsp47$^{+/-}$ ES cells (Nagai *et al.*, 2000) were seeded onto feeder cells at a concentration of 2.0×10^6 cells per 10-cm dish. On the following day, these *Hsp47*$^{+/-}$ ES cells were cultured in medium that contained 12.5–17.5 mg/ml G-418 (Calbiochem-Novabiochem, San Diego, CA); cells were maintained in this medium for 8 d. Drug-resistant colonies were picked, expanded, and screened using Southern blotting to obtain clones that had undergone a second recombination event that yielded *Hsp47*$^{-/-}$ cells.

Antibodies

For Western blotting or immunoprecipitation, we used rabbit polyclonal antibodies raised against mouse Hsp47 and rat type I collagen (LSL, Tokyo, Japan), laminin (provided by Dr. Hayashi, University of Tokyo, Japan), and mouse plasma fibronectin (H6660/4731; provided by Dr. Hancock, National Institutes of Health, Bethesda, MD); rat monoclonal antibody (mAb) to the α2 chain of human type IV collagen (H22) (Sado *et al.*, 1995); and mouse mAb to chicken gizzard actin (C4) (Chemicon International). Goat anti-rabbit IgG (Biomedical Technologies, Stoughton, MA), and anti-mouse IgG and anti-rat IgG (Organon Teknika, Durham, United Kingdom) were used as secondary antibodies.

Western Blot Analysis

Proteins were extracted from ES cells, EBs, and BALB/3T3 (control) cells in cell extraction buffer that contained 0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, 5.0 mM EDTA, 1% NP-40, and protease inhibitors [2.0 mM *N*-ethylmaleimide, 2.0 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 μg/ml leupeptin and pepstatin] at 4°C. After centrifugation, soluble protein in the extract was quantified according to the method of Bradford (Bradford, 1976). Proteins were separated using 8% SDS-PAGE (Laemmli, 1970) and were blotted onto nitrocellulose filters. Filters were blocked in Dulbecco's phosphate-buffered saline containing 5% skim milk and 3% bovine serum albumin (BSA). Specific antibody binding was detected using the enhanced chemiluminescence system (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, England).

Immunostaining

EBs were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde at 4°C. Paraffin sections (4 μm) were treated with 0.3% hydrogen peroxide/methanol at room temperature for 30 min. After blocking nonspecific protein binding using 5% horse serum for 30 min, sections were incubated with rabbit antibodies against Hsp47, type IV collagen, or laminin (1:200), followed by incubation with biotinylated anti-rabbit IgG (Elite ABC kit PK-6100; Vector Laboratories, Burlingame, CA). Specific antibody binding was visualized using Elite ABC reagent (Vector Laboratories) and the Envision kit/HRP (DakoCytomation California, Carpinteria, CA). The percentage of EBs that contained a normal VE cell layer was estimated using the following four criteria for normal: 1) EB diameter was >100 μm; 2) extracellular thin layer (between the outer VE cell layer and the epiblast/inner cell mass) stained strongly with antitype IV collagen antibody; 3) VE cell layer showed normal simple epithelial morphology; and 4) >20% of the outer surface of the EB was surrounded by VE cells.

Metabolic Labeling

Ascorbic acid phosphate (136 μg/ml) was added to monolayer endodermal cells 16 h before metabolic labeling. Cells were incubated with 3.9 MBq/ml ^{35}S-labeled Met and Cys (Express ^{35}S protein labeling mixture; PerkinElmer Life and Analytical Sciences, Boston, MA) in medium containing ascorbic acid phosphate without fetal calf serum, Met, Cys, or LIF for 30 min. For pulse-labeling and -chase experiments, labeled cells were chased for appropriate periods of time in medium containing excess unlabeled Met and Cys. Soluble proteins were extracted in cell extraction buffer (described previously). For immunoprecipitation, anti-mouse type IV collagen antibody was added to cell extracts and culture media, and anti-mouse plasma fibronectin antibody was added to culture media; both were incubated at 4°C overnight. Protein A-Sepharose 4 Fast Flow resin (Pharmacia Biotechnology, Wikstroms, Sweden) was added to the mixture and resin was recovered by centrifugation. Immunocomplexes bound to the resin were washed in cell extraction buffer that had been modified by increasing the NaCl concentration to 0.4 M. Proteins were separated using 5% SDS-PAGE; gels were fixed in saturated trichloroacetic acid (TCA), soaked in 1 M sodium salicylic acid, and exposed to x-ray film.

Protease Digestion of Secreted Type IV Collagen

Monolayer endodermal cells were cultured in medium that contained ascorbic acid phosphate (136 μg/ml) and dialyzed 10% fetal calf serum in the presence of 3.7 MBq/ml L-[2,3-[^{3}H]Pro (Amersham Biosciences UK) for 10 h. Aliquots of medium containing equal amounts of TCA-insoluble radioactivity were treated with a protease mixture consisting of 100 μg/ml trypsin and 250 μg/ml chymotrypsin in 0.05 M Tris-HCl (pH 7.4), 0.2 M NaCl, and 0.25 M glucose at 37°C or 4°C. Digests of type IV collagen were analyzed using 5% SDS-PAGE; proteins were fixed using 30% methanol/10% acetic acid, and gels were soaked in EN^{3}HANCE (PerkinElmer Life and Analytical Sciences) and exposed to x-ray film.

Binding of Type IV Collagen to Fibronectin

Secreted type IV collagen was labeled with L-[2,3-[^{3}H]Pro (described previously) by using a shortened labeling time of 6 h. Bovine plasma fibronectin (Itoham Foods, Hyogo, Japan) was coupled with cyanogen bromide-activated-Sepharose 4B (Pharmacia Biotechnology). Fibronectin-coupled beads (20 μl) were mixed with cell culture medium (200 μl), incubated for 2 h at 4°C with gentle mixing, and washed in 0.05 M HEPES (pH 7.5) containing 0.15 M NaCl and 2.5 mM EDTA. Bound proteins were extracted by boiling for 5 min in Laemmli's sample buffer supplemented with 0.1 M dithiothreitol. Extracted proteins were separated using 5% SDS-PAGE; proteins were fixed using 30% methanol/10% acetic acid, and gels were treated with EN^{3}HANCE and exposed to x-ray films. Type IV collagen radioactivity was quantified using the software program NIH Image, version 1.62.

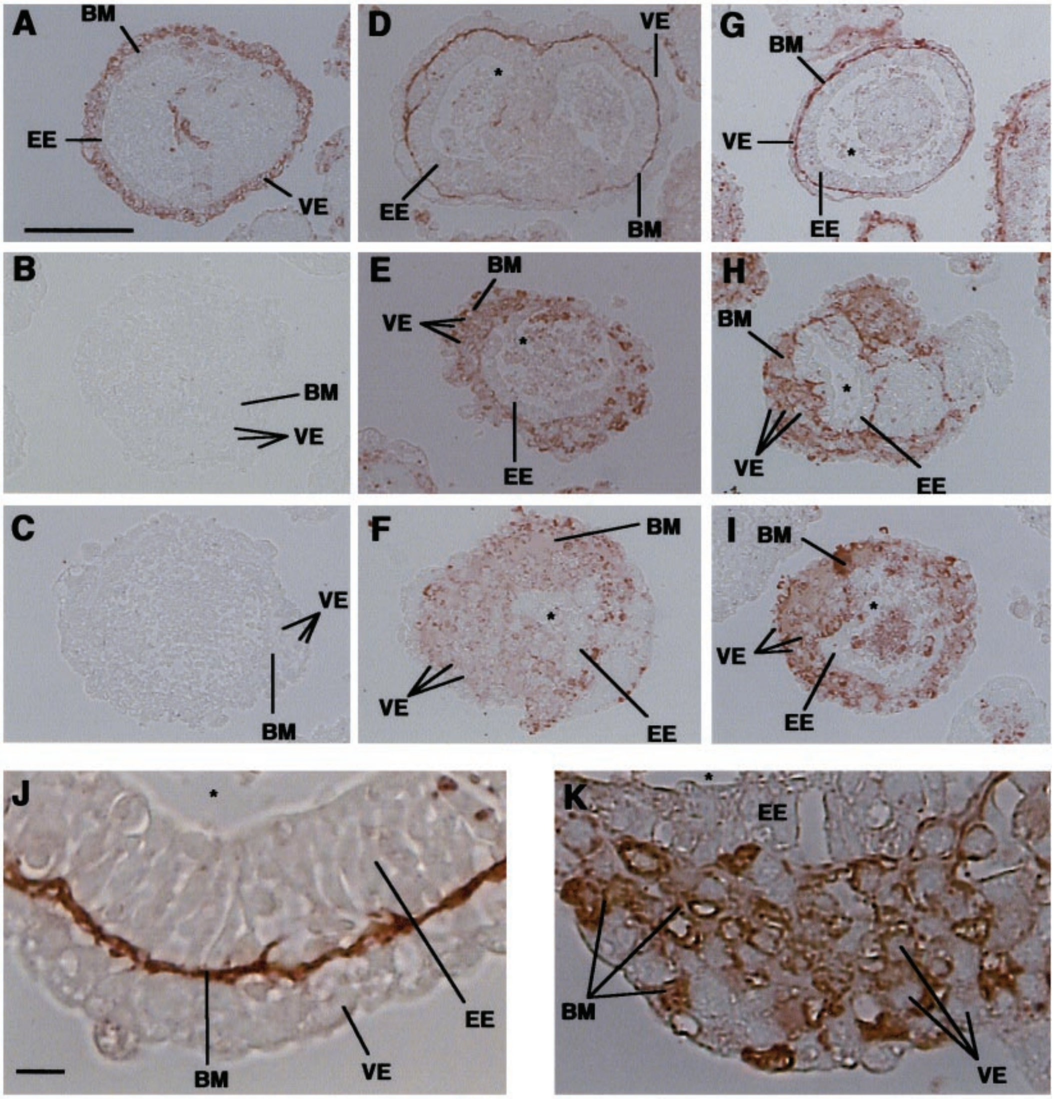
Northern Blot Analysis

Total RNA was extracted from cultured cells by the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987) by using TRIzol (Invitrogen, Carlsbad, CA) and separated using formaldehyde/agarose gel electrophoresis. RNA was blotted onto nylon filters (GeneScreen Plus; PerkinElmer Life and Analytical Sciences). Filters were hybridized with a ^{32}P-labeled human proα1(IV) cDNA fragment (Pihlajaniemi *et al.*, 1985) or a β-actin cDNA fragment (Gunning *et al.*, 1983), washed in two times in SSC at 65°C, and exposed to x-ray films.

RESULTS

Abnormal Morphology of VE Cell Layer in EBs of Hsp47$^{-/-}$ *ES Cells*

BM has been reported to be discontinuously disrupted in *Hsp47*-null mouse embryos (Nagai *et al.*, 2000). We first



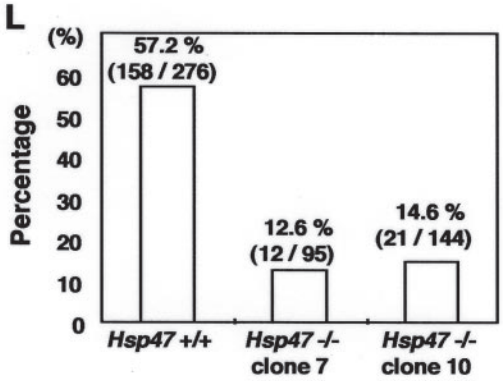


Figure 1. VE cell layer shows disorganized morphology in EBs of *Hsp47*$^{-/-}$ ES cells. The distribution of Hsp47 (A–C), type IV collagen (D–F, J, and K), and laminin (G–I) in EBs differentiated from *Hsp47*$^{-/-}$ and *Hsp47*$^{+/+}$ ES cells at day 8 were analyzed by immunostaining. (A, D, G, and J) *Hsp47*$^{+/+}$. (B, E, H, and K) *Hsp47*$^{-/-}$ clone 7. (C, F, and I) *Hsp47*$^{-/-}$ clone 10. (L) Percentage of EBs that contained normal morphology of VE cell layers adjacent to BM-like structures (see *Materials and Methods* for details) was determined. Bars, 100 μm (A–I); and 10 μm (J and K). EE, epiblast epithelium; asterisk, cavity.

examined the morphology of *Hsp47*$^{-/-}$ EBs by immunostaining of sections (Figure 1) and found that the morphology of VE cell layers composing outer surface of EBs are abnormal in *Hsp47*$^{-/-}$ EBs. At day 8, anti-Hsp47 antibody strongly stained VE cell layers in *Hsp47*$^{+/+}$ EBs (Figure 1A), whereas no staining was observed in *Hsp47*$^{-/-}$ EBs, confirming the absence of Hsp47 protein (Figure 1, B and C). *Hsp47*$^{+/+}$ and *Hsp47*$^{-/-}$ EBs both contained BM-like structures that separate the outer VE cell layer from the inner epiblast cells (Figure 1, D–K). In both genotypes, most EBs formed cavities (Figure 1, D–K), which suggested that differentiation of EBs were not affected by absence of Hsp47 at this stage of development.

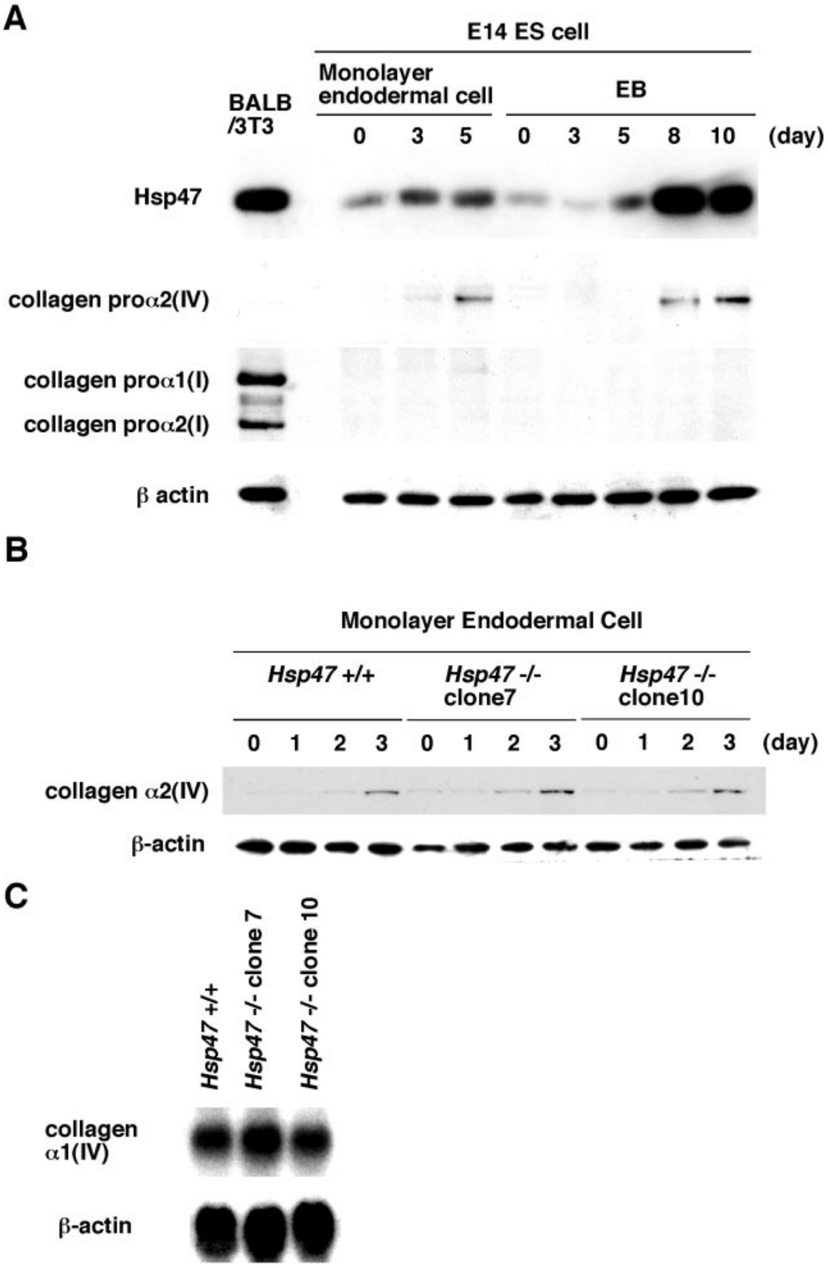


Figure 2. Expression of Hsp47 and type IV collagen in differentiating ES cells and EBs. (A) Expression of Hsp47 correlates with that of type IV collagen in monolayer endodermal cells and in EBs differentiated from *Hsp47*$^{+/+}$ ES cells. Total soluble proteins in monolayers of differentiating *Hsp47*$^{+/+}$ ES cells, *Hsp47*$^{+/+}$ EBs, and BALB/3T3 cells was analyzed by Western blotting by using specific antibodies. (B) Type IV collagen in soluble proteins extracted from monolayers of differentiating *Hsp47*$^{+/+}$ and *Hsp47*$^{-/-}$ ES cells were analyzed. (C) Northern blot analysis of type IV collagen mRNA in monolayers of differentiating *Hsp47*$^{+/+}$ and *Hsp47*$^{-/-}$ ES cells.

However, we found significant difference in the morphology of VE cell layer situated along the BM-like structures. In most *Hsp47*$^{-/-}$ EBs, the VE cell layers exhibited disorganized morphology, and weak dispersed staining with anti-type IV collagen and anti-laminin antibodies was observed at the extracellular regions (Figure 1, E, F, H, I, and K). In contrast, the VE cell layers exhibited smooth simple epithelium-like morphology in *Hsp47*$^{+/+}$ EBs (Figure 1, D, G, and J). Most *Hsp47*$^{+/+}$ EBs contained thin BM-like structures strongly and continuously stained with antitype IV collagen and anti-laminin antibodies. In two *Hsp47*$^{-/-}$ ES clones, normal VE cell layer epithelial morphology was seen only in 13% (12 of 95) and 15% (21 of 144) of EBs, whereas normal morphology was seen in 57% (158 of 276) of *Hsp47*$^{+/+}$ EBs (Figure 1L). Because the BM is important for maintaining epithelial cell layers, the abnormal morphology of the VE cell layer adjacent to the BM in *Hsp47*$^{-/-}$ EBs may be due to a defect in BM function.

Expression of Type IV Collagen Is Not Affected by the Absence of Hsp47

Because the morphology of VE cell layers along the BM-like structures was disordered in *Hsp47*$^{-/-}$ EBs (Figure 1) and Hsp47 is known to recognize type IV collagen in vitro (Natsume *et al*., 1994), we next examined expression levels of type IV collagen, a component of BM, in *Hsp47*$^{-/-}$ cells during differentiation (Figure 2). Using Western blotting, we found Hsp47 in *Hsp47*$^{+/+}$ monolayer endodermal cells at day 3 and in *Hsp47*$^{+/+}$ EBs at day 8. The level of Hsp47 and type IV collagen both increased over the course of differentiation (Figure 2A), a result that is consistent with previous observations that indicated Hsp47 and type IV collagen are

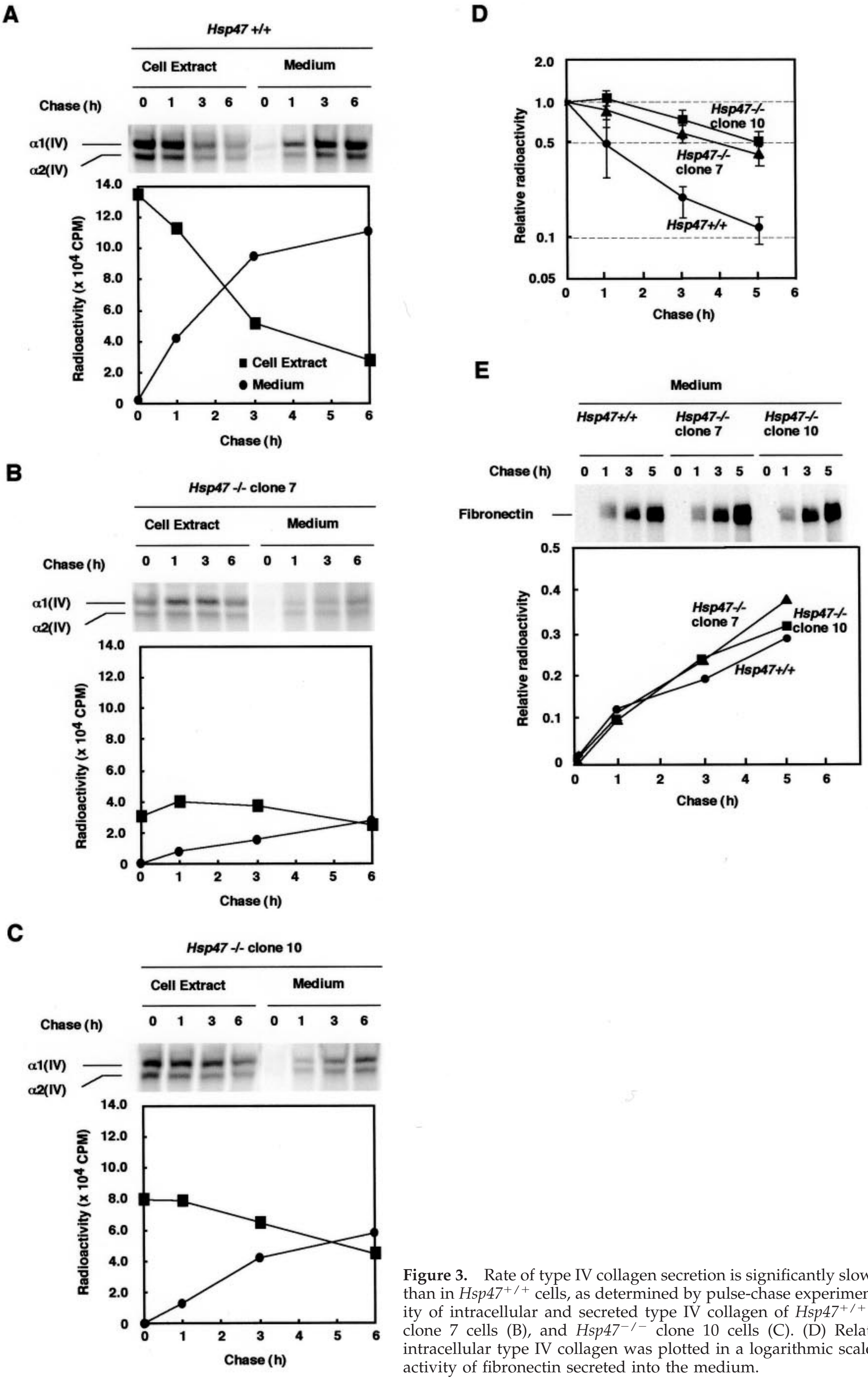


Figure 3. Rate of type IV collagen secretion is significantly slower in *Hsp47*$^{-/-}$ cells than in *Hsp47*$^{+/+}$ cells, as determined by pulse-chase experiment. (A–C) Radioactivity of intracellular and secreted type IV collagen of *Hsp47*$^{+/+}$ cells (A), *Hsp47*$^{-/-}$ clone 7 cells (B), and *Hsp47*$^{-/-}$ clone 10 cells (C). (D) Relative radioactivity of intracellular type IV collagen was plotted in a logarithmic scale (n = 3). (E) Radioactivity of fibronectin secreted into the medium.

both expressed in early-stage mouse embryos and in differentiating F9 cells (Leivo *et al.*, 1980; Takechi *et al.*, 1992; Nagai *et al.*, 2000). In contrast, type I collagen was not detected at any stage. These results suggest that the upregulation of Hsp47 during ES cell differentiation may play a role in the production of type IV collagen.

The level of type IV collagen and of type IV collagen mRNA was similar between *Hsp47*$^{-/-}$ and *Hsp47*$^{+/+}$ cells (Figure 2, B and C). In both cell types, most type IV collagen was present in the NP-40–soluble fraction (unpublished data), which suggests that type IV collagen does not aggregate in these cells. These results indicate that disruption of the *Hsp47* gene does not affect the level of expression of type IV collagen.

Type IV Collagen Secretion Rate Is Significantly Slower in Hsp47$^{-/-}$ *Cells*

To examine whether the secretion of type IV collagen is affected by the absence of Hsp47, we determined the secretion rate of type IV collagen by pulse-label and -chase experiments by using ^{35}S-Met and ^{35}S-Cys. Intracellular and secreted type IV collagens were detected by immunoprecipitation by using the specific antibody (Figure 3). The decrease in the intracellular amounts of type IV collagen directly corresponded to the increase in the amounts of type IV collagen secreted into the medium both in *Hsp47*$^{+/+}$ and *Hsp47*$^{-/-}$ cells (Figure 3, A–C). The level of labeled intracellular type IV collagen decreased during chase periods, and the rate of decrease was fourfold slower in *Hsp47*$^{-/-}$ cells than in *Hsp47*$^{+/+}$ cells (Figure 3D), which was consistent with the rate of increase in the type IV collagen secreted into the medium, that is, the rate of secretion was significantly higher in *Hsp47*$^{+/+}$ cells compared with that in *Hsp47*$^{-/-}$ cells (Figure 3, A–C). In contrast, the secretion of fibronectin analyzed as a control was not affected by the absence of Hsp47 (Figure 3E), which indicated that general secretion pathways were not impaired by the disruption of *Hsp47* gene. These results indicate that the absence of Hsp47 specifically caused a marked decrease in the rate of secretion of type IV collagen, a finding that is consistent with the previous observation that the secretion of type IV collagen was enhanced in Hsp47 overexpress cells (Tomita *et al.*, 1999; Rocnik *et al.*, 2002).

Type IV Collagen Secreted by Hsp47$^{-/-}$ *Cells Is Susceptible to Protease Digestion*

Unfolded collagen molecules are more sensitive to protease digestion than are those with correct triple-helical structures (Dolz *et al.*, 1988; Nagai *et al.*, 2000). We compared the protease sensitivity of type IV collagen derived from differentiating ES cells by digesting the collagen by using a mixture of trypsin and chymotrypsin to address the molecular features of secreted collagen (Figure 4). Type IV collagen molecules of 210 and 200 kDa secreted by *Hsp47*$^{+/+}$ cells yielded a 190-kDa fragment probably consisting of triple-helical domain after protease treatment at 37°C for 5 min. In contrast, type IV collagen molecules secreted by *Hsp47*$^{-/-}$ cells that were treated under the same conditions were undetectable by SDS-PAGE (Figure 4). Complete digestion of type IV collagen secreted by *Hsp47*$^{-/-}$ cells also was observed after treatment at 4°C for 5 min (unpublished data). In contrast, protease sensitivity for intracellular type IV collagen of *Hsp47*$^{-/-}$ cells was similar to that of *Hsp47*$^{+/+}$ cells (Supplemental Data 1), suggesting that properly folded type IV collagen is rapidly secreted from the ER and only minor portion was remained in the *Hsp47*$^{+/+}$ cells. These results indicate that type IV collagen secreted by *Hsp47*$^{-/-}$ cells is not in the form with a correctly folded triple-helix, even at temperatures well below the melting point of normal collagen. These results clearly show that Hsp47 is essential for triple-helix formation of type IV collagen.

Type IV Collagen Secreted from Hsp47$^{-/-}$ *Cells Binds with High Affinity to Fibronectin*

Fibronectin is an extracellular matrix protein that interacts with various types of collagen (Engvall *et al.*, 1978; Jilek and Hormann, 1978). Interestingly, fibronectin preferentially binds via ionic interaction (Vuento *et al.*, 1982) to denatured, unfolded collagen (gelatin) rather than to native collagen (Engvall and Ruoslahti, 1977; Engvall *et al.*, 1982). We therefore used fibronectin as a high-affinity indicator of denatured or misfolded collagen. Binding of secreted type IV collagen to fibronectin was examined using fibronectin-coupled Sepharose beads (Figure 5). Fibronectin-Sepharose bound a much higher (10-fold) quantity of type IV collagen, which had been secreted by *Hsp47*$^{-/-}$ cells compared with that secreted by *Hsp47*$^{+/+}$ cells (Figure 5, A and C). Similarly, heat-denatured type IV collagen exhibited marked increase (14-fold) in binding to fibronectin-Sepharose (Figure 5, B and D), a result that confirmed that fibronectin binds to denatured type IV collagen with high affinity. These results support our earlier observation; type IV collagen secreted by *Hsp47*$^{-/-}$ cells is not correctly folded.

DISCUSSION

In this study, we found that type IV collagen secreted by *Hsp47*$^{-/-}$ cells is much more susceptible to protease diges-

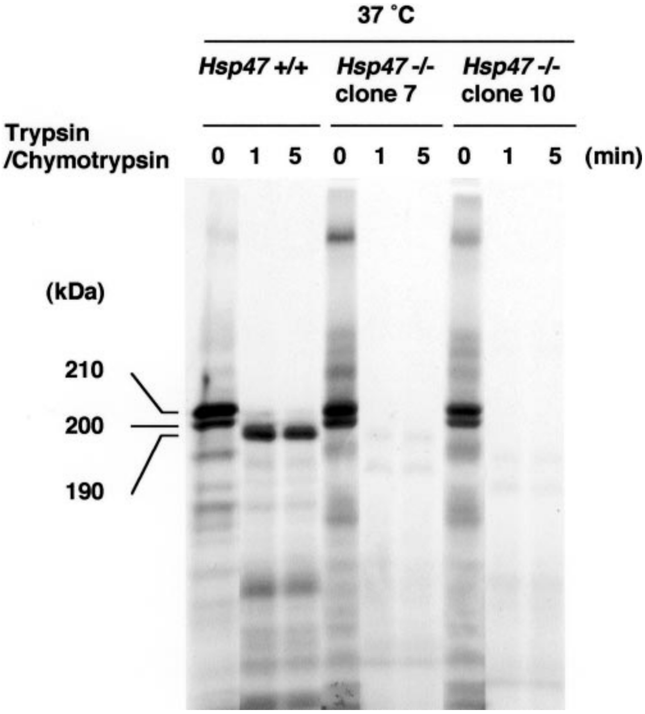


Figure 4. Type IV collagen secreted from *Hsp47*$^{-/-}$ cells is susceptible to protease digestion. Differentiating ES cells were cultured in the presence of L-[2,3-[^{3}H]Pro, and aliquots of medium containing equivalent amounts of TCA-insoluble radioactivity were treated with a mixture of trypsin and chymotrypsin at 37°C for indicated periods. The medium before and after protease digestion was analyzed by SDS-PAGE.

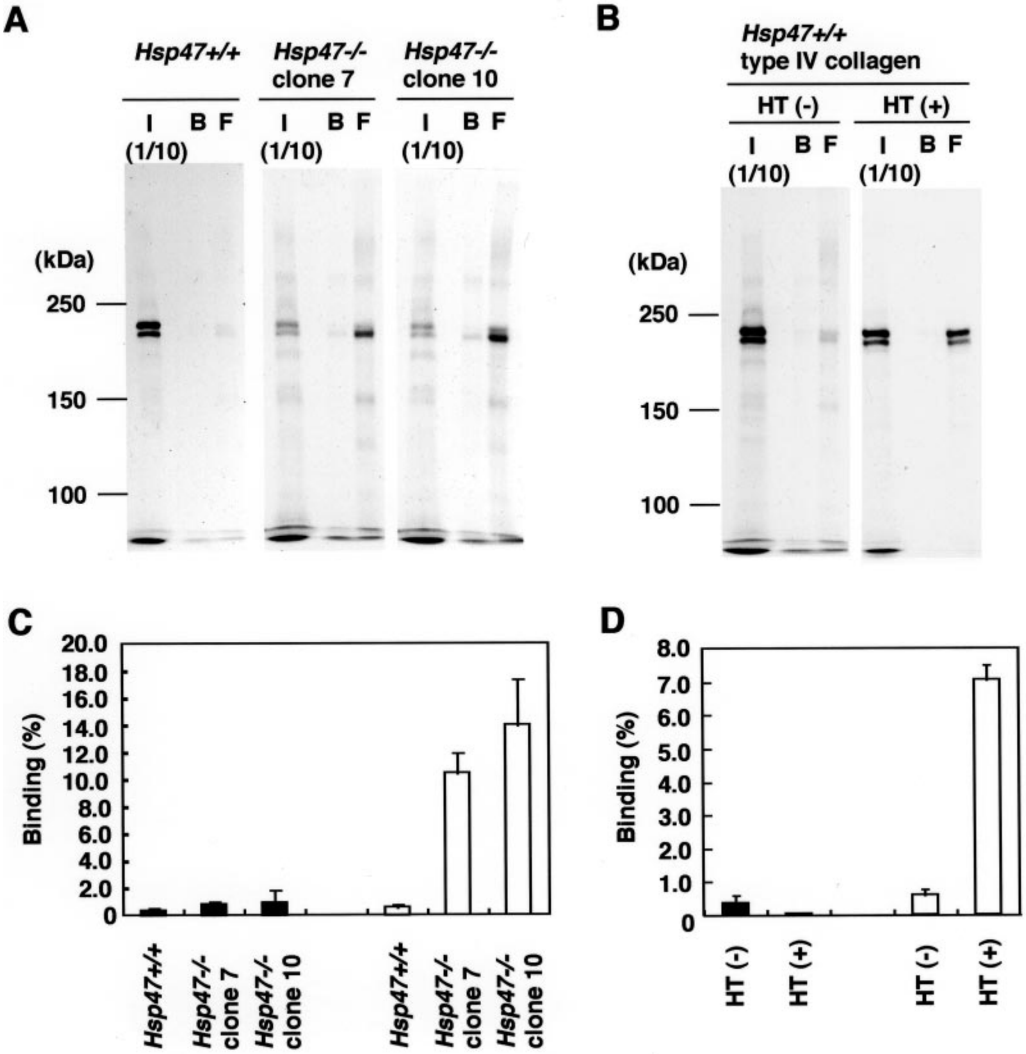


Figure 5. Type IV collagen secreted from *Hsp47*$^{-/-}$ cells exhibits higher affinity to fibronectin than that secreted from *Hsp47*$^{+/+}$ cells. Culture medium containing proteins labeled with L-[2,3-[^{3}H]Prowas mixed with fibronectin-Sepharose beads. After washing, proteins bound to the beads were analyzed by SDS-PAGE. (A) Binding of type IV collagen secreted from *Hsp47*$^{+/+}$ or *Hsp47*$^{-/-}$ cells to fibronectin. (B) Binding of type IV collagen secreted from *Hsp47*$^{+/+}$ cells after heat treatment. The input applied to SDS-PAGE was 10% of total sample. (C and D) Quantification of type IV collagen binding to fibronectin beads (including means and standard deviations of four experiments). Type IV collagen binding to BSA-Sepharose is shown by solid bars, and type IV collagen binding to fibronectin-Sepharose is shown by open bars. HT, heat-treatment; I, input; B, fraction bound to BSA-Sepharose; F, fraction bound to fibronectin-Sepharose.

tion than that secreted by *Hsp47*$^{+/+}$ cells (Figure 4), a finding that is consistent with our previous results for type I collagen (Nagai *et al*., 2000). Type IV collagen molecules secreted by *Hsp47*$^{-/-}$ cells were completely digested by trypsin/chymotrypsin at 37°C and at 4°C, whereas type IV collagen secreted by *Hsp47*$^{+/+}$ cells were not digested completely, suggesting that collagen secreted by *Hsp47*$^{-/-}$ cells is unfolded, even at temperatures that are well below the melting point of normal collagen. These results clearly show that Hsp47 plays an essential role in the productive folding of type IV collagen.

Type IV collagen secreted by *Hsp47*$^{-/-}$ cells readily binds fibronectin at an affinity similar to that of fibronectin binding to heat-denatured type IV collagen (Figure 5). Because fibronectin preferentially binds denatured forms of collagen such as gelatin (Engvall and Ruoslahti, 1977; Engvall *et al*., 1982), these observations support the idea that type IV collagen secreted by *Hsp47*$^{-/-}$ cells is unfolded configuration. It is noteworthy that the α2 chain secreted from *Hsp47*$^{-/-}$ cells preferentially bound to fibronectin, whereas the α1 and α2 chains of heat-denatured collagen exhibited a similar binding activity to fibronectin (Figure 5, A and B). Folding and/or denaturing states may be different in the type IV collagen secreted from *Hsp47*$^{-/-}$ cells from those of heat-denatured one.

We also revealed here that secretion of type IV collagen was significantly delayed in the absence of Hsp47 (Figure 3), although the absence of Hsp47 does not affect the expression of type IV collagen (Figure 1). Delayed secretion is consistent with previous observations that indicated that secretion of type I collagen is enhanced with overexpression of Hsp47 (Tomita *et al*., 1999; Rocnik *et al*., 2002). Quality control in the ER is accomplished by cooperation among several ER-resident proteins (Hammond and Helenius, 1995; Ellgaard *et al*., 1999) that interact with unfolded proteins and retain them in the ER until they adopt correct conformation. During collagen synthesis, PDI associates with the C-propeptides of monomeric procollagen chains before initial chain assembly (Wilson *et al.*, 1998), which may maintain them in the unfolded state until the trimer formation is initiated in the C-propeptide region (Bottomley *et al.*, 2001). Thus, these ER chaperones, including PDI, may cause delayed secretion of type IV collagen in the absence of Hsp47.

Triple helix formation occurs from the C terminus to the N terminus (Bachinger *et al.*, 1980; Engel and Prockop, 1991; Bulleid *et al.*, 1997), and hydroxylation of proline residues at the Y position of X-Y-Gly repeats by P4H helps to stabilize the triple-helical structure (Berg, 1973). Hsp47 preferentially binds the triple-helical form of procollagen rather than the monomeric α-chain (Koide *et al.*, 2000; Tasab *et al.*, 2000, 2002). Hsp47 dissociates from procollagen during transport from the ER to the Golgi apparatus, presumably at the ER-Golgi intermediate compartment or at the *cis*-Golgi (Nakai *et al.*, 1992; Satoh *et al.*, 1996). Although Hsp47 is reported to prevent formation of collagen fibrils and aggregation of collagen molecules in vitro (Thomson and Ananthanarayanan, 2000; Tasab *et al.*, 2002), we suggest that Hsp47 has another role in facilitating productive folding of collagen in the ER.

We previously reported that *Hsp47* knockout mouse embryos cannot survive beyond 11.5 d postcoitus (Nagai *et al.*, 2000). These embryos are severely deficient in collagen fibril formation, and *Hsp47*$^{-/-}$ fibroblasts established from *Hsp47* knockout mouse embryos produce immature type I collagen that does not adopt an appropriate triple-helical conformation. In addition to these abnormalities, BMs of *Hsp47*$^{-/-}$ embryonic tissues are discontinuous. Hsp47 is therefore an important molecular chaperone during murine development. However, the molecular mechanisms that produce disrupted BMs were not clarified. From the data presented in this study, we suggest that the collagen fibril deficiency is caused by misfolding of type IV collagen. The BM that contains misfolded type IV collagen may be more fragile than one that contains properly folded type IV collagen.

Expression of Hsp47 correlates with expression of various types of collagen (Nagata and Yamada, 1986; Nakai *et al.*, 1990; Takechi *et al.*, 1992; Masuda *et al.*, 1994); here, we showed that during differentiation of ES cells, expression of Hsp47 correlated with that of type IV collagen but not with that of type I collagen during the differentiation of ES cells (Figure 2), consistent with previous observations that both Hsp47 and type IV collagen are expressed in early-stage mouse embryos and in differentiating F9 cells (Leivo *et al.*, 1980; Takechi *et al.*, 1992; Nagai *et al.*, 2000). The up-regulation of Hsp47 during ES cell differentiation may facilitate the folding and assembly of type IV collagen.

Although histochemical analysis indicated that *Hsp47*$^{-/-}$ EBs contained type IV collagen in the BM-like structure beneath the VE cell layer, the VE cell layer adjacent to this structure exhibited disorganized morphology (Figure 1). The percentage of *Hsp47*$^{-/-}$ EBs with normal VE cell morphology was only one-fourth that of *Hsp47*$^{+/+}$ EBs. These results suggest that the BM-like structures in *Hsp47*$^{-/-}$ EBs are functionally abnormal, which is consistent with the observation that BMs are discontinuous in *Hsp47*$^{-/-}$ mouse embryos (Nagai *et al.*, 2000), because EBs are an in vitro model for postimplantation egg-cylinder stage embryos. The type IV collagen that is secreted by *Hsp47*$^{-/-}$ cells into the extracellular matrix may not be competent to form a meshwork in BMs.

Type IV collagen is important for BM function. For example, Alport syndrome is a genetic disease caused by any of >50 different mutations in the gene encoding the type IV collagen α5 chain, mutations that include single-base mutations and large deletions (Hudson *et al.*, 1993). These mutations cause abnormal structure and function of the type IV collagen, resulting in derangement of BMs and defects in kidney function. Moreover, mouse embryos that lack the laminin γ1 subunit lack BMs and die by day 5.5 postcoitus (Smyth *et al.*, 1999).

The results of this report combined with those of our previous report (Nagai *et al.*, 2000) clearly show that Hsp47 in vivo is indispensable for productive folding of collagen types I and IV. Although Hsp47 can bind to collagen types I through V in vitro (Natsume *et al.*, 1994), the in vivo chaperone function of Hsp47 for other types of collagen has not yet been established, because early-stage embryos die. To address this issue, we are now analyzing the role of Hsp47 in vivo by making mice with conditionally disrupted Hsp47 gene by adopting Cre-LoxP system.

ACKNOWLEDGMENTS

We thank Dr. M. Hosokawa for helpful discussion, and Drs. Sado, Hancock, and Hayashi for antibodies to type IV collagen, fibronectin, and laminin, respectively.

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