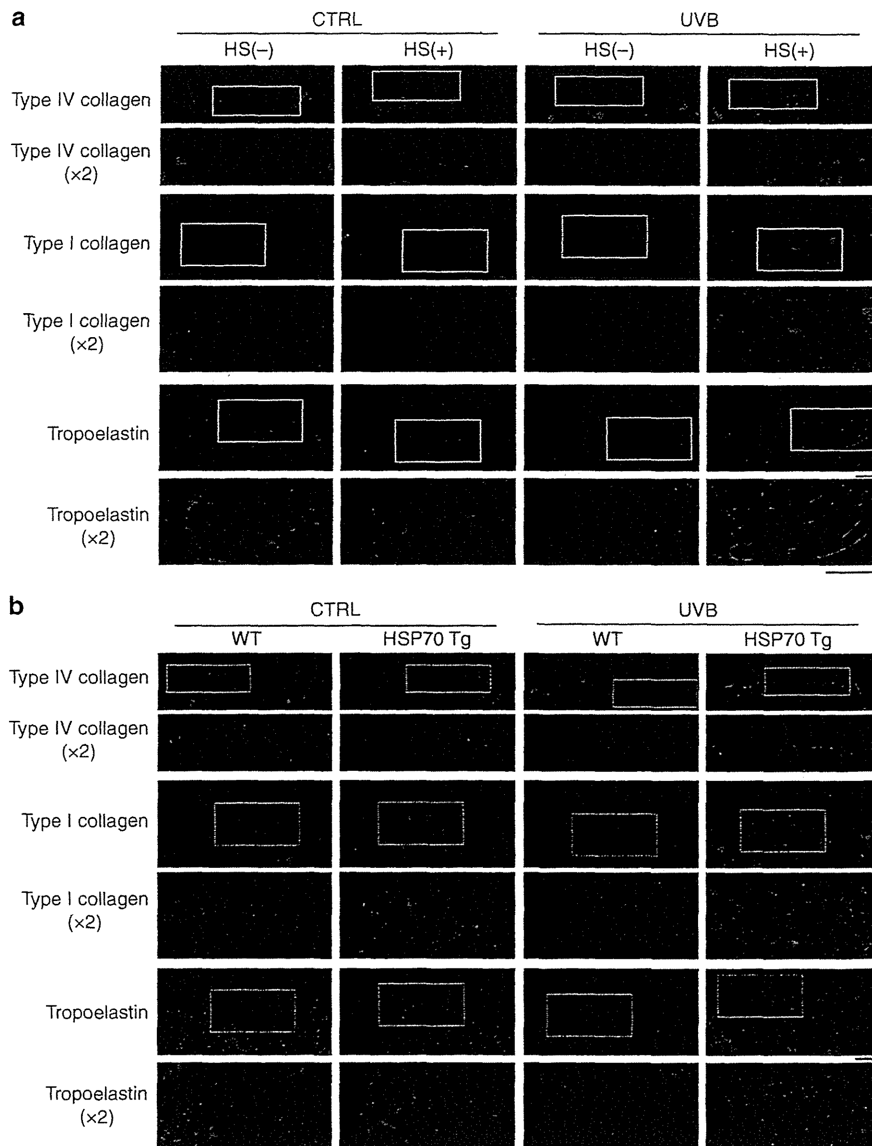

Figure 3. Effect of heat shock protein 70 (HSP70) expression on UVB-induced alteration of extracellular matrix. (**a**) Heat treatment and UVB irradiation of the dorsal skin of hairless mice were performed as described in the legends of Figure 2. (**b**) Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with UVB three times a week for 6 weeks. (**a, b**) The dorsal skin was removed 24 hours after the final UVB irradiation and subjected to immunohistochemical analysis with antibodies against type IV collagen, type I collagen, or tropoelastin. Bar = 50 μm. CTRL, control.

In relation to MMP-9, it was reported that inflammatory cells such as macrophages and neutrophils produce this protein (Kessenbrock *et al.*, 2010). We therefore used immunohistochemical analysis to examine the infiltration of these cells in the skin of UVB-treated or untreated transgenic mice expressing HSP70 or their wild-type counterparts. As shown in Figure 6j, the number of CD11b-positive cells (macrophages) or myeloperoxidase-positive cells (neutrophils) in the skin was increased by UVB irradiation in wild-type mice. This number was lower in UVB-treated transgenic mice expressing HSP70, suggesting that the expression of HSP70 inhibits the UVB-induced infiltration of macrophages and neutrophils into the skin. We also used gelatin zymography to compare the activities of MMPs in HSP70-overexpressing and wild-type macrophages, and found that the MMP-9 activity was similar between the two types of macrophages (data not shown). These results suggest that the expression of HSP70 suppresses MMP-9 activity by inhibiting the UVB-induced infiltration of macrophages and neutrophils into the skin.

DISCUSSION

Here we provide data suggesting that HSP70 is protective against UVB-induced wrinkle formation.

UVB-induced wrinkle formation requires a long period of irradiation (about 10 weeks). Thus, it is difficult to monitor UVB-induced wrinkle formation in normal mice (with hair) because a true hairless state with shaved skin can only be maintained for about 6–7 weeks. For this reason, hairless mice

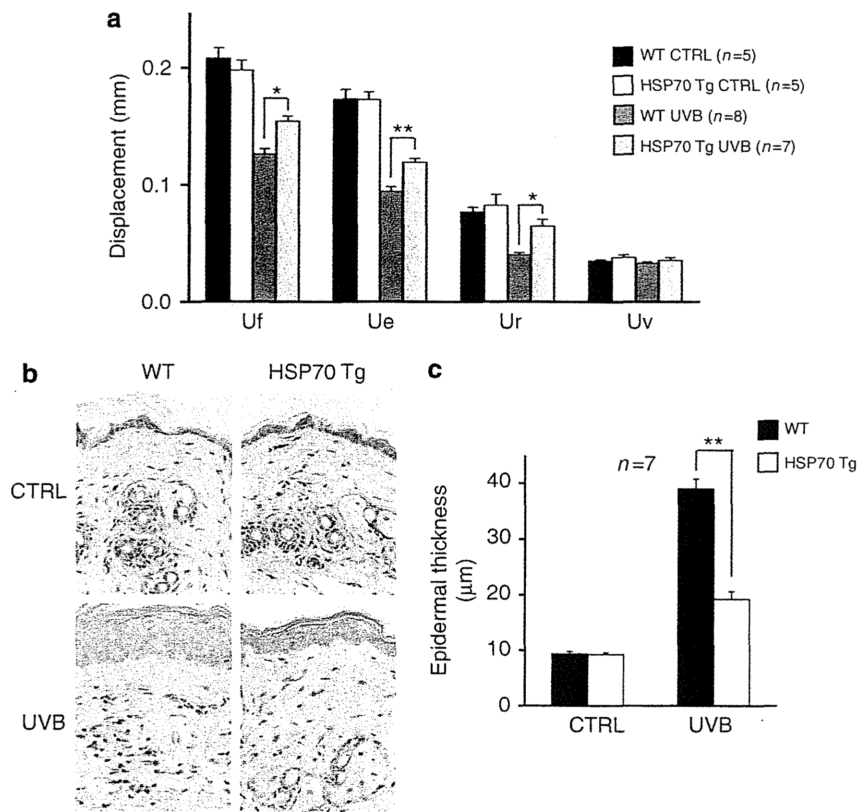

Figure 4. UVB-induced decrease in skin elasticity and epidermal hyperplasia in transgenic mice expressing heat shock protein 70 (HSP70). Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with UVB as described in the legend of Figure 3. The skin elasticity (**a**) and epidermal thickness (**b**, **c**) were measured and shown as described in the legend of Figure 2. Values are mean ± SEM. $^{**}P<0.01$; $^{*}P<0.05$. CTRL, control.

were used in the study to examine the wrinkle formation. On the other hand, transgenic mice are useful to examine the role of specific proteins in biological responses. For this reason, we studied the role of HSPs on wrinkle formation using heat treatment of hairless mice, whereas the mechanism for the protective role of HSP70 on wrinkle formation was examined in transgenic mice expressing HSP70.

It was previously reported that more severe heat treatment conditions (e.g., 43 °C for 30 or 90 minutes) than those in this study (42 °C for 5 minutes) causes skin damage such as wrinkle formation and activation of MMPs *in vivo* (Cho *et al.*, 2008, 2009; ; Kim *et al.*, 2009; Shin *et al.*, 2012). We confirmed that heat treatment of mouse skin under severe conditions (43, 44, or 45 °C for 5 minutes) but not under mild conditions (42 °C for 5 minutes) caused an increase in the amount of MMP-13 (data not shown). We also observed an increase in the amounts and/or activities of MMP-2, MMP-9, and elastase in mouse skin by heat treatment of severe conditions (data not shown). On the other hand, we found that treatment of mouse skin at 42 °C or higher for 5 minutes clearly induced the expression of HSP70 (Figure 1 and data not shown). As the purpose of this study is to examine the protective role of HSP70 induced by heat treatment on the UVB-induced wrinkle formation, we used the mild heat treatment conditions (42 °C for 5 minutes). However, it should be noted that severe heat treatment damages the skin even without concomitant exposure to UVB.

We found that mild heat treatment of the dorsal skin of hairless mice suppressed UVB-induced wrinkle formation, and decrease in skin elasticity and epidermal hyperplasia. This is an evidence showing that mild heat treatment protects against UVB-induced wrinkle formation. Furthermore, the UVB-induced decrease in skin elasticity and epidermal hyperplasia was less apparent in transgenic mice expressing HSP70 compared with wild-type mice, suggesting that the mild heat treatment suppresses UVB-induced wrinkle formation through the induction of HSP70 expression.

UVB-induced wrinkle formation is mediated by a complex mechanism involving damage to the ECM and cell death, and the resulting inflammatory responses both in the epidermis and dermis. We showed here that exposure to UVB radiation caused skin fibroblast cell death and that this was suppressed in transgenic mice expressing HSP70. We also showed that reactive oxygen species-induced cell death was suppressed in HSP70-overexpressing skin fibroblasts compared with control fibroblasts cultured *in vitro*. Similar results were observed for keratinocytes in our previous paper (Matsuda *et al.*, 2010). We also found that compared with wild-type mice, the infiltration of inflammatory cells (macrophages and neutrophils) after long-term repeated UVB irradiation of animals was suppressed in transgenic mice expressing HSP70. We previously reported that a single UVB irradiation of wild-type mice decreased the skin level of IκB-α (an inhibitor of NF-κB) and increased proinflammatory cytokines and chemokines in the

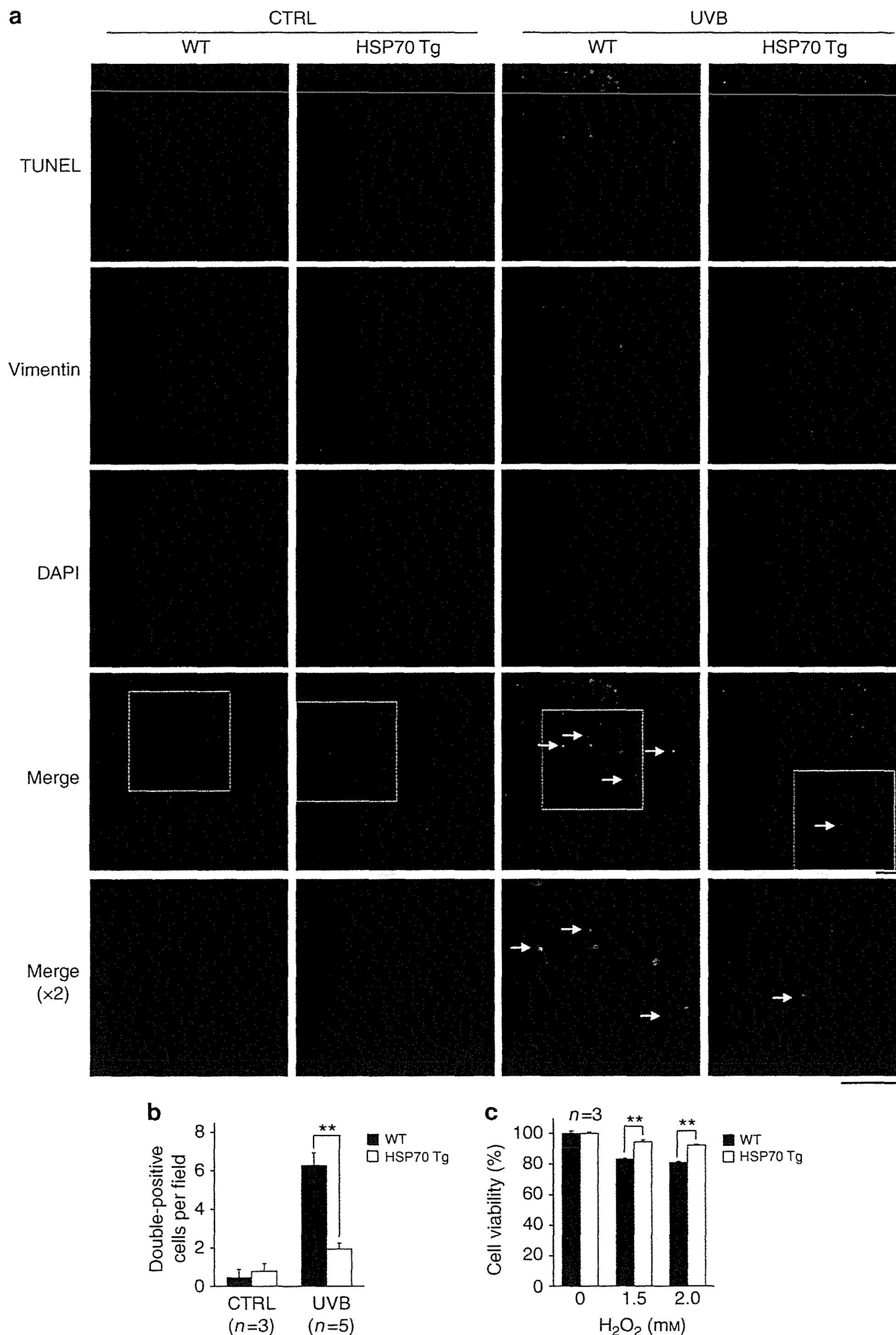

Figure 5. UVB-induced fibroblast cell death in transgenic mice expressing heat shock protein 70 (HSP70). (**a**, **b**) Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were irradiated with 180 mJ cm^{-2} UVB, and the dorsal skin was removed after 24 hours. (**a**) Sections were subjected to TUNEL assay, immunohistochemical analysis with antibody against vimentin, and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (double-positive cells are shown by arrows; bar = 50 μm). (**b**) The number of double-positive cells (TUNEL and vimentin expression) was counted. (**c**) Primary cultures of skin fibroblasts prepared from HSP70 Tg and WT were treated with the indicated concentrations of hydrogen peroxide for 1 hour and cultured for 23 hours. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method. Values are mean ± SEM. $^{**}P<0.01$. CTRL, control.

skin, and that these inflammatory responses were suppressed in transgenic mice expressing HSP70 (Matsuda *et al.*, 2010). Taken together, these results suggest that the cytoprotective and anti-inflammatory activities of HSP70 contribute to the suppression of UVB-induced phenomena related to wrinkle formation (decrease in skin elasticity and epidermal

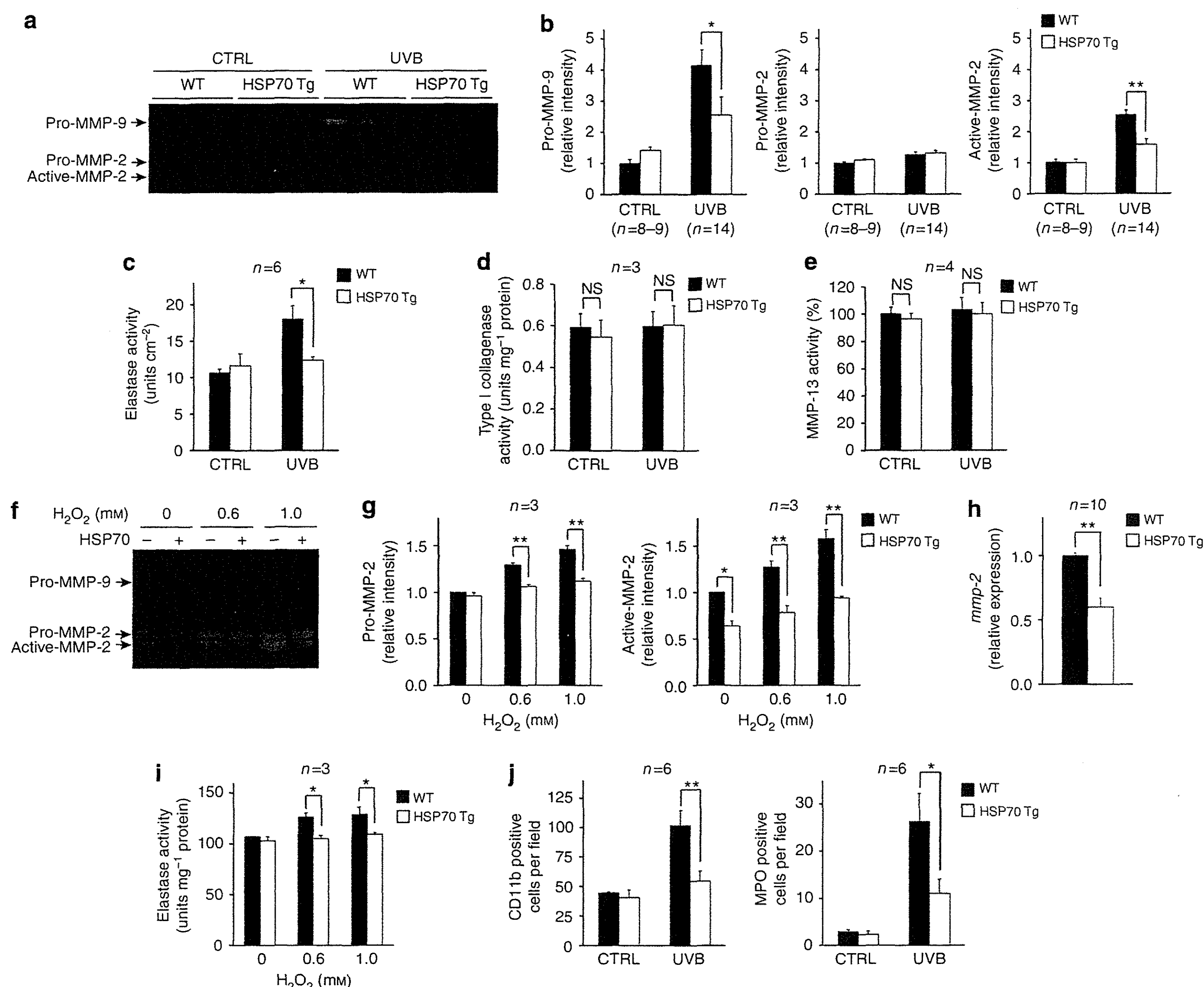

Figure 6. Effect of heat shock protein 70 (HSP70) overexpression on matrix metalloproteinases (MMPs) and elastase activities. (**a–e**, **j**) Mice were irradiated with UVB as described in the legend of Figure 3. (**f–i**) Primary skin fibroblasts were treated with hydrogen peroxide as described in the legend of Figure 5. Dorsal skin extracts (**a**) or culture media (**f**) were subjected to gelatin zymography assay, and the band intensity was determined (**b**, **g**). Elastase (**c**), type I collagenase (**d**), or MMP-13 (**e**) activity in dorsal skin extracts or elastase activity in cells (**i**) was measured. The messenger RNA expression was analyzed (**h**). The dorsal skin was subjected to immunohistochemical analysis for CD11b or myeloperoxidase, and the number of positive cells was counted (**j**). Values are mean ± SEM. ** $P<0.01$; * $P<0.05$. CTRL, control; NS, not significant; WT, wild type.

hyperplasia) in transgenic mice expressing HSP70 and wild-type mice exposed to heat treatment.

Immunohistochemical analysis suggested that the level of type I collagen was decreased by UVB irradiation in control mice and that this decrease was partially suppressed in mice concomitantly exposed to mild heat treatment, or in transgenic mice expressing HSP70. This analysis also showed that fine basal membrane of the epidermis and collagen and elastic fibers were disrupted by UVB irradiation; this damage was partially suppressed in mice concomitantly exposed to mild heat treatment or in transgenic mice expressing HSP70. We also found that the activities of MMP-2, MMP-9, and elastase were increased by UVB irradiation and that these activities were lower in UVB-treated transgenic mice expressing HSP70 than in wild-type mice. These results suggest that MMP-2, MMP-9, and elastase have important roles in the HSP70-dependent protection against UVB-induced disruption of the ECM. As the overexpression of HSP70 in primary cultures of skin fibroblasts suppressed the expression and activity of MMP-2 and the activity of elastase, HSP70 seems to directly suppress the expression and/or activity of these proteases. On the other hand, the expression of HSP70 did not affect the activity of MMP-9 in macrophage primary cultures, suggesting that the decreased MMP-9 activity in UVB-treated transgenic mice expressing HSP70 may be owing to the suppression of infiltration of inflammatory cells into the skin.

We recently found that the artificial expression of HSP70 suppresses melanin production both *in vivo* and *in vitro* (Hoshino *et al.*, 2010). We also reported that HSP70 expression suppresses both UVB-induced cellular and DNA damage

and production of reactive oxygen species (Matsuda *et al.*, 2010). As UV-induced modest melanin production has an important role in protecting the skin against UV-dependent damage (Kobayashi *et al.*, 1998), our results suggest that HSP70 inducers could serve as hypopigmenting agents (skin whitening agents) without worsening UV-induced skin damage. The results of this study suggest that such HSP70 inducers could also be beneficial for reducing UV-induced wrinkle formation.

MATERIALS AND METHODS

Animals

The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and were approved by the Animal Care Committee of Kumamoto University and Keio University.

UVB irradiation and heat treatment

Animals were exposed to UVB radiation with a double bank of UVB lamps (peak emission at 312 nm, VL-215LM lamp, Vilber Lourmat, Paris, France). This UV lamp mainly emits UVB but emits a small level of UVA. Animals were placed under deep anesthesia with chloral hydrate (250 mg kg^{-1}), and the fur (except in the case of hairless mice) was removed with electric clippers before the first UVB irradiation.

The dorsal skin of hairless mice was exposed to heated water at 42 °C or 37 °C (control) for 5 minutes for heat treatment.

Wrinkles were formed as a consequence of long-term, repeated exposure to UVB radiation (three times a week for 6 or 10 weeks) as described previously (Inomata *et al.*, 2003) with some modifications. Briefly, in the case of hairless mice, the initial dose of UVB was set at 36 mJ cm^{-2}, which was subsequently increased weekly to 54, 72, 90, 108, 126, 144, 162, and finally 180 mJ cm^{-2} (180 mJ cm^{-2}, both at week 9 and week 10; a total of 10 weeks). In the case of transgenic mice expressing HSP70 and the wild-type mice (C57/BL6), the initial dose was set at 36 mJ cm^{-2}, which was subsequently increased weekly to 54, 72, 108, 144, and 180 mJ cm^{-2} (a total of 6 weeks).

Wrinkle scoring, image analysis of skin replicas, and measurement of skin elasticity

Evaluation of wrinkle formation was performed by both visual wrinkle scoring and image analysis of skin replicas as described previously (Inomata *et al.*, 2003; Tsukahara *et al.*, 2004). Skin elasticity was measured with a Cutometer 575 meter (Courage + Khazaka) as described previously (Agache *et al.*, 1980; Elsner *et al.*, 1990; Tsukahara *et al.*, 2001).

Real-time reverse-transcriptase–PCR analysis

Total RNA was extracted from cultured cells using the RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Real-time reverse-transcriptase–PCR analysis was performed as described (Hoshino *et al.*, 2010). The primer sequence would be provided on request.

Histological and immunohistochemical analyses and TUNEL assay

Histological examination (hematoxylin and eosin staining), immunohistochemical analysis, and TUNEL assay were performed as described (Matsuda *et al.*, 2010).

Gelatin zymography and measurement of enzyme activity

The proteolytic activity of MMP-2 and MMP-9 was assessed by SDS-PAGE using zymogram gels containing 0.1% (w/v) gelatin, as described previously (Taraboletti *et al.*, 2000). Elastase activity was measured using *N*-succinyl-tri-alanyl-p-nitroanilide (Peptide Institute, Osaka, Japan) as a substrate, as described previously (Tsukahara *et al.*, 2004). Type I collagenase activity or MMP-13 activity was measured using the type I collagenase assay kit (Primary Cell, Hokkaido, Japan) or the SensoLyte MMP-13 assay kit (AnaSpec, San Jose, CA), respectively.

Statistical analysis

All values are expressed as the mean ± SEM. Two-way analysis of variance followed by the Tukey's test was used to evaluate differences between more than two groups. Differences were considered to be significant for values of $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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