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Oral administration of pineapple glucosylceramide improves defective epidermal barrier function by restoring diminished level of TGF-β in the skin

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21

Abstract

It was reported that oral administration of glucosylceramide (GlcCer) from plants ameliorates skin barrier function. GlcCer is a type of glycosphingolipid that exists widely as a component of the cell membrane. However, the mechanism of improvement by dietary GlcCer is not completely understood. The aim of the present study was to investigate the mechanism of GlcCer in the improvement of skin barrier function. Five-week-old male Hos:HR-1 mice were divided into three groups fed on standard diet, unsaturated fatty acids-deficient (HR-AD) diet, and HR-AD diet supplemented with 0.1% P-GlcCer diet. Skin barrier function was evaluated by transepidermal water loss (TEWL) value and wrinkle formation. The effect of transforming growth factor-β (TGF-β) on skin barrier function and collagen content in skin was examined using exogenous TGF-β and its neutralizing antibody. TEWL value and wrinkle formation were decreased by oral administration of pineapple (P)-GlcCer. Not only the TGF-β contents in serum and skin but also hydroxyproline content reduced by the HR-AD diet were restored by P-GlcCer administration. Exogenous TGF-β ameliorated both skin barrier function and collagen contents. However, the improvement of skin barrier function by P-GlcCer was cancelled and collagen content in skin was also decreased by TGF-β antibody injection. Recovery of TGF-β content by P-GlcCer contributed to the

production of collagen in skin, resulting in improved skin barrier function.

Key Words

atopic dermatitis; collagen; glucosylceramide; pineapple; transforming growth factor-β

Abbreviations

AP-1, activator protein-1; AD, atopic dermatitis; ECM, extracellular matrix; FMOC, 9-fluorenylmethylchloroformate; GlcCer, glucosylceramide; HPLC, high-performance liquid chromatography; HR-AD, unsaturated fatty acids-deficient; MMP, matrix metalloproteinase; OPA, *o*-phthalaldehyde; P-GlcCer, pineapple glucosylceramide; PMSF, phenylmethylsulfonyl fluoride; SE, standard error; TEWL, transepidermal water loss; TGF-β, transforming growth factor-β; TLR, toll-like receptor

Introduction

The skin is continuously exposed to environmental influences, and it provides an effective barrier between an organism and the environment by preventing physical, chemical, and microbial damage [1]. Skin is made up of three cutaneous layers consisting of the epidermis (top layer), dermis (middle layer), and subcutaneous tissue (deepest layer) [2]. Dermis contains extracellular matrix (ECM) proteins such as collagen, elastic fibers, fibronectin, glycosaminoglycans, and proteoglycans produced by fibroblasts, which are the main cell type of the dermis [3]. Collagen is the most abundant protein in ECM [4].

Generally, wrinkle formation results from degradation of collagen in skin [5]. Therefore, it is considered that promotion of collagen synthesis may be able to prevent wrinkle formation and enhance skin barrier function. However, there are few reports that food components improve the skin barrier function by increasing collagen content in skin.

Transforming growth factor (TGF)-β is a cytokine that regulates cell differentiation, migration, and proliferation, and TGF-β plays a crucial role in maintaining skin homeostasis [6]. It is well known that TGF-β promotes collagen production in skin [2,7], and moreover, TGF-β is known to promote keratinocyte

71 migration, which is essential for the reconstruction of the cutaneous barrier after skin
72 injury [8,9]. However, it is not well understood whether TGF-β improves skin barrier
73 function.

74 Atopic dermatitis (AD) is a common chronic skin disease characterized by severe
75 itching [10]. Recently, it has been recognized that skin barrier defects are also important
76 in AD [11]. In fact, the skin of AD patients has impaired skin barrier function even in
77 non-lesional skin [12]. It was shown that basal transepidermal water loss (TEWL)
78 values, which are an index of the function of the skin barrier, increased by 2–5-fold in
79 AD patients skin [13]. Therefore, it is thought that improvement of skin barrier function
80 is important in the treatment of AD.

81 It was reported that oral administration of glucosylceramide (GlcCer) from plants
82 improves skin barrier function [14]. GlcCer is a type of glycosphingolipid that exists
83 widely as a component of the cell membrane [15]. Hos:HR-1 hairless mice are known
84 as an animal model for observing dry skin and wrinkle formation such as AD [16].
85 AD-like symptoms can be induced in these mice by long-term feeding of an unsaturated
86 fatty acids-deficient (HR-AD) diet [17]. Recently, Kuwata *et al*. has reported that not
87 pineapple GlcCer (P-GlcCer) itself but the metabolite ameliorated dry skin symptoms in
88 Hos:HR-1 mice fed HR-AD diet, and P-GlcCer exhibited the improvement of the skin

89 barrier function accompanied with the enhancement of *Il23a* expression in the small
90 intestine [18]. However, the mechanism of epidermal barrier function improvement by
91 P-GlcCer is not completely understood. In this study, we investigated the improvement
92 mechanism of skin barrier function by oral administration of pineapple GlcCer
93 (P-GlcCer) in Hos:HR-1 mice fed with an HR-AD diet.
94
95 **Materials and Methods**
96 **Reagents**
97 P-GlcCer (purified by HPLC according to Kuwata *et al*. [18]) was kindly donated
98 by Maruzen Pharmaceuticals Co., Ltd (Hiroshima, Japan). L-Hydroxyproline was
99 -purchased from Wako Pure Chemical Industries (Osaka, Japan).
100 9-Fluorenylmethylchloroformate (FMOC) and aprotinin were obtained from Sigma (St.
101 Louis, MO, USA). Acetone, β-mercaptoethanol, ethyl ether, boric acid, sodium
102 hydroxide, *o*-phthalaldehyde (OPA), iodoacetamide, acetonitrile, glacial acetic acid,
103 leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Nacalai
104 Tesque (Kyoto, Japan). These chemicals were special or HPLC grade. Other chemicals
105 and reagents were ordinary commercial and guaranteed products.
106

Mice

Male Hos:HR-1 hairless (4-week-old) mice were purchased from Japan SLC (Shizuoka, Japan). All mice were housed in plastic cages in a temperature- and humidity-controlled room (23 ± 2℃ and 50 ± 10% relative humidity), maintained on a 12 h light-dark cycle, and acclimated for 7 days before the experiments. All animal experiments were approved and treated according to the established rules and the guidelines approved for animal use and care at Kobe University (The Guidelines for the Care and Use of Experimental Animals of Rokkodai Campus, Kobe University) (approval number: 25-06-04).

***In vivo* model in mice for dry skin syndrome**

Hos:HR-1 mice were divided into three groups (n=5), and fed a Labo MR stock (Nosan, Kanagawa, Japan) as standard diet, HR-AD diet (Nosan), or HR-AD diet supplemented with 0.1% (w/w) P-GlcCer for 4 weeks. The mice fed standard diet and the HR-AD diet were injected with recombinant TGF-β (17.5 ng/mouse) (R&D, Minneapolis, MN, USA) by intraperitoneal injections every day for 4 weeks. In addition, a neutralizing monoclonal antibody against TGF-β (10 μg /mouse) (eBioscience, San Diego, CA, USA) was injected intravenously three times at 1 week intervals. As an

125 isotype control for the TGF-β antibody, an isotype-matched mouse IgG antibody (10 μg
126 /mouse) (Life Technologies, Tokyo, Japan) was injected. Mice were allowed free access
127 to food and water for 4 weeks. After 4 weeks of feeding, the mice were euthanized
128 under anesthesia. Whole blood was obtained by using cardiocentesis. Skin tissues were
129 collected by cutting the back skin of mice. Whole blood and skin tissues were
130 immediately used for the quantification of TGF-β and hydroxyproline contents.
131
132 **Measurement of skin barrier function**
133 To evaluate the skin barrier function, TEWL values, which increase when skin
134 barrier function is disturbed, were determined, and wrinkle formation in the dorsal
135 epidermis was observed. All mice had TEWL values measured using a Tewameter
136 TM300 (Courage & Khazaka, Cologne, Germany) in standard conditions (23 ± 2°C and
137 50 ± 10% relative humidity) every week. Before sacrifice, photographs were taken of
138 the dorsal areas of the mice. Wrinkle formation was investigated by analysis of the
139 photographs.
140
141 **Contents of TGF-β in serum and skin**
142 Whole blood was left undisturbed for 30 min at room temperature, and

143 subsequently centrifuged at 10,000 rpm at 4°C for 10 min. Supernatants were collected
144 as blood serum. Serum samples were stored at -80°C until analysis. Serum TGF-β levels
145 were measured using a ELISA (Promega, Madison, WI, USA) in accordance with the
146 manufacturer's recommended protocol. After the weight of skin tissue was measured, it
147 was homogenized in ice-cold tissue lysis buffer (20 mM Tris buffered saline containing
148 1% NP-40, 10% glycerol, and protease inhibitor (1 mM PMSF, 10 μg/ml aprotinin, and
149 1 μg/ml leupeptin]) (10 mg tissue/ml buffer). After centrifugation at 1,500 g at room
150 temperature for 15 min, the concentration of TGF-β in the supernatant was measured in
151 the same manner as for serum.
152
153 **RNA isolation and quantitative Real-Time PCR**
154 Total RNA was extracted from all organs using Sepasol RNA I super (Nacalai
155 Tesque). cDNA synthesis was performed using a High Capacity cDNA Reverse
156 Transcription kit (Applied Biosystems, Foster City, CA, USA) in accordance with the
157 manufacturer's protocol. Quantitative PCR assays were analyzed using Applied
158 Biosystem 7500 Fast Real-Time PCR system with TaqMan gene expression assay.
159 TaqMan gene expression assay were purchased from Applied Biosystems; β-actin
160 (Mm00607939_s1) and mouse TGF-β (Mm01178820_m1).

161

162 **Skin tissue preparation for collagen contents analysis**

163 Measurement of hydroxyproline, which is considered as a collagen-specific
164 amino acid, was carried out using the method reported by Hutson *et al*[19]. Skin tissues
165 were crushed in liquid nitrogen using a Multi-beads shocker (Yasui Kikai, Osaka,
166 Japan) and kept at -80°C overnight. After weighing, the crushed skin tissue was
167 incubated sequentially in 70% ethanol, 100% ethanol, and 100% acetonitrile twice at
168 room temperature for 15 min in each solvent to release the lipids. After removal of the
169 acetonitrile layer, the pellets were dried and reweighed. The pellets were suspended in
170 6 M HCl (4.8 ml) and then placed on a 110°C heat block for 18 h to hydrolyze *in vacuo*.
171 The hydrolysates were cooled at room temperature and concentrated using a lyophilizer
172 (Taitec, Saitama, Japan). The residues were dissolved with 1 ml of water and neutralized
173 with 6 M NaOH to pH 9.5±1.0. This solution was used for high-performance liquid
174 chromatography (HPLC) analysis.

175

176 **Quantification of collagen contents in skin by high-performance liquid**
177 **chromatography**

178 Hydroxyproline were converted to a sensitive fluorescent derivative with

179 FMOC. Nine hundred microliters of sample or hydroxyproline standard solutions (25,
180 50, 100, 250, or 500 μM in water) were transferred to glass test tubes and 200 μl borate
181 buffer (0.7 M boric acid, pH 9.5 adjusted with NaOH) was added. Then 100 μl OPA
182 solution (50 mg OPA dissolved in 1 ml acetonitrile with 26 μl β-mercaptoethanol) was
183 added. After incubation for 1 min, 100 μl iodoacetamide reagent (140 mg
184 iodoacetamide in 1 ml acetonitrile) was added. To allow the reaction to proceed for 1
185 min, 300 μl 5 M FMOC in acetone was added. The react vials were vortexed at after
186 each addition. After incubation for 2 min, 2 ml ethyl ether was added to all the test tubes
187 and mixed well for 30 sec. The organic layer was removed and the wash was repeated
188 twice. Then, 50 μl of the filtered aliquots were analyzed by HPLC. The fluorescent
189 sample solutions were subjected to HPLC (LaChrom Elite HPLC system, Hitachi,
190 Tokyo, Japan). The column, a 5 μm Mightysil RP-18 GP Aqua 250 mm × 4.6 mm
191 column (Kanto Chemical, Tokyo, Japan), was maintained at 35°C. The mobile phase
192 was composed of 65:35 3% glacial acetic acid buffered with sodium acetate (pH 4.3):
193 acetonitrile delivered at a flow late of 1 ml/min, and its peak was detected at a
194 wavelength of 265 nm.
195
196 **Statistical analysis**

197 Data are expressed as the mean ± standard error (SE). Statistical analysis was
198 performed using a Tukey-Kramer multiple comparisons test to determine differences
199 between groups. A P value <0.05 was considered significant.
200
201 **Results**
202 **Effect of dietary pineapple glucosylceramide on transepidermal water loss value**
203 **and wrinkle formation**
204 TEWL value and wrinkle formation were examined as indices of dry skin. Figure
205 1A shows changes in TEWL value after oral administration of P-GlcCer. The TEWL
206 value was significantly increased by the HR-AD diet compared with the standard diet.
207 However, TEWL values of the mice fed P-GlcCer were significantly decreased to
208 almost the same level as the standard diet. In addition, wrinkle formation induced by the
209 HR-AD diet was decreased by the oral administration of P-GlcCer (Fig. 1B).
210 Histological observation showed that the increase in the epidermal thickness induced by
211 HR-AD diet was suppressed by oral administration of P-GlcCer (Fig. 1C). These results
212 suggested that P-GlcCer improved skin barrier function.
213
214 **Changes in TGF-β contents in serum and skin by oral administration of pineapple**

glucosylceramide

It was reported that exogenous TGF-β suppressed AD-like skin lesion in mice [20]. To investigate whether TGF-β is involved in the improvement of skin barrier function by P-GlcCer, TGF-β contents in serum and skin were measured at 28 days after administration. TGF-β contents in serum and skin (Fig. 2A and B, respectively) were decreased by the HR-AD diet compared with the standard diet. However, the decreased TGF-β content with the HR-AD diet recovered after oral administration of P-GlcCer to the same levels as with the standard diet. Hydroxyproline contents which result from the hydrolysis of collagen in skin were measured. As shown in Fig. 3, hydroxyproline contents were decreased by the HR-AD diet compared with the standard diet. However, the content was restored by P-GlcCer to the same levels as standard diet as well as TGF-β contents in serum and skin (Fig. 2A and B).

Involvement of TGF-β produced by pineapple glucosylceramide in the improvement of unsaturated fatty acids-deficient diet inducible dry skin

The results in Fig. 2 led to the hypothesis that the TGF-β content in serum and/or skin affected skin collagen production. To prove this hypothesis, mice fed with the HR-AD diet were injected intraperitoneally with TGF-β to increase TGF-β content

233 in blood every day for 28 days. Injection of TGF-β showed changes in neither in the
234 growth of the mice nor in hepatic fibrosis (data not shown). It was confirmed that
235 exogenous TGF-β injection significantly increased TGF-β contents in both serum (Fig.
236 3A) and skin (Fig. 3B). In addition, it was revealed that exogenous TGF-β prevented
237 an increase in TEWL values (Fig. 3C) and decrease in hydroxyproline contents in skin
238 (Fig. 3D) despite feeding of the HR-AD diet. Moreover, wrinkle formation was
239 ameliorated (Fig. 3E). These results suggested that increased TGF-β in blood promotes
240 collagen production in skin and improves skin barrier function. To reaffirm the
241 importance of serum TGF-β levels, a TGF-β neutralizing antibody was injected
242 intravenously. As shown in Fig. 4A, an isotype-matched mouse IgG antibody did not
243 show any influence because the TGF-β content in serum was the same between the
244 control and IgG in each diet. TGF-β content in the serum was apparently reduced to the
245 same level as in mice fed on the P-GlcCer + HR-AD diet as a standard diet, by the
246 injection of TGF-β neutralizing antibody (Fig. 4A hatched bar). As shown in Fig. 54B,
247 reduced TEWL values of mice fed P-GlcCer + HR-AD diet were significantly
248 increased to almost the same levels as the HR-AD diet by the intravenous injection of
249 the TGF-β neutralizing antibody. The hydroxyproline content of mice fed the P-GlcCer
250 + HR-AD diet was significantly decreased by the TGF-β neutralizing antibody to

almost the same level as mice fed the HR-AD diet (Fig. 4C). As shown in Fig. 4D, wrinkle formation was increased by TGF-β neutralizing antibody administration in mice fed P-GlcCer + HR-AD. These results suggested that TGF-β recovery by P-GlcCer treatment was related to improved skin barrier function.

Discussion

The number of patients with AD has increased in recent years [21]. The most common symptom of AD is dry skin [22]. Hos:HR-1 mice fed a HR-AD diet are known to exhibit AD-like skin symptoms [17]. Although some studies reported that dietary GlcCer from plants improves skin barrier function [14,23], the mechanism is not well understood. Moreover, no studies have reported the effect of P-GlcCer in relation to skin barrier function. In this study, we investigated the effect of P-GlcCer on skin barrier function in Hos:HR-1 mice fed on a HR-AD diet.

It has been reported that collagen can be classified into more than 20 different types [24]. Of these many types of collagen, type I collagen is the most abundant in skin, comprising 80% of all collagen present [25,26]. The major amino acid components of type I collagen are glycine (33%), proline and hydroxyproline (21%), and alanine (11%) [27]. Taken together, the reduction in collagen in mice fed on a HR-AD diet was

probably an effect on type I collagen in skin.

In this study, it was found that P-GlcCer improved TEWL values and wrinkle formation, which are ascribed to the skin barrier function in Hos:HR-1 mice fed on a HR-AD diet, and the recovery of the reduced collagen content in skin from the HR-AD diet. Although it has not been reported that TGF-β production is induced by GlcCer, the reduced TGF-β levels by the HR-AD diet were restored by oral administration of P-GlcCer in serum and skin. TGF-β is known to be secreted by various cells such as platelets, fibroblasts, keratinocytes, and macrophages [28-30]. In particular, it is well known that macrophages produce TGF-β through toll-like receptor (TLR) 4 [31], and because ceramide is known as a TLR4 agonist, macrophages stimulated by P-GlcCer through TLR4 may produce TGF-β [32].

The content of skin collagen exhibited similarly with TGF-β content in skin. These results were in agreement with the previous report that TGF-β promoted collagen production in skin [2,7]. It has been unclear whether or not TGF-β affects the improvement of skin barrier function. However, our study suggested that TGF-β recovery by P-GlcCer increased collagen content in skin and was related to the improvement of skin barrier function.

In summary, this study demonstrated that TGF- β recovery by P-GlcCer

contributed to the production of collagen in skin, thereby resulting in improved skin barrier function. This indicated that P-GlcCer is a promising alternative therapeutic strategy of skin improvement. In addition, further studies are needed to elucidate the mechanism how P-GlcCer promoted TGF- β secretion through intestinal tract including linkage of collagen synthesis.

Conflict of interest

The authors have declared no conflicts of interest.

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390 **Figure legends**

391

392 Fig. 1 Effect of pineapple glucosylceramide on skin barrier function in Hos:HR-1 mice
393 fed the unsaturated fatty acids-deficient diet.
394 Hos:HR-1 mice were divided into three groups and fed a standard diet, an unsaturated
395 fatty acids-deficient (HR-AD) diet, or HR-AD supplemented with 0.1% (w/w)
396 pineapple glucosylceramide (P-GlcCer) for 4 weeks. (A) TEWL values on day 28. (B)
397 Wrinkle formation on day 28. The panels are representatives of mouse dorsal skin. (C)
398 Hematoxylin-eosin staining of mouse dorsal skin sections on day 28. Images are shown
399 at 100× magnification. Scale bars, 500 μm. Values represent means ± SE (n=5). *:
400 $p<0.05$ vs HR-AD (n=5)

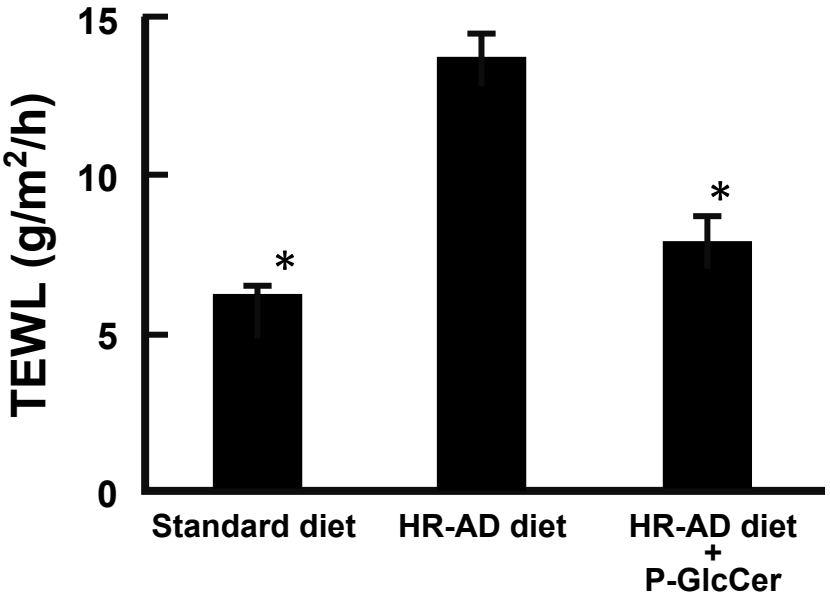
401

402 Fig. 2 Effect of pineapple glucosylceramide on transforming growth factor-β and
403 hydroxyl proline level in Hos:HR-1 mice fed the unsaturated fatty acids-deficient diet.
404 Hos:HR-1 mice were divided into three groups and fed a standard diet, an unsaturated
405 fatty acids-deficient (HR-AD) diet, or HR-AD diet supplemented with 0.1% (w/w)
406 pineapple glucosylceramide (P-GlcCer) for 4 weeks. (A) Transforming growth factor-β
407 (TGF-β) levels in serum on day 28. (B) TGF-β levels in skin on day 28. (C) Hydroxy

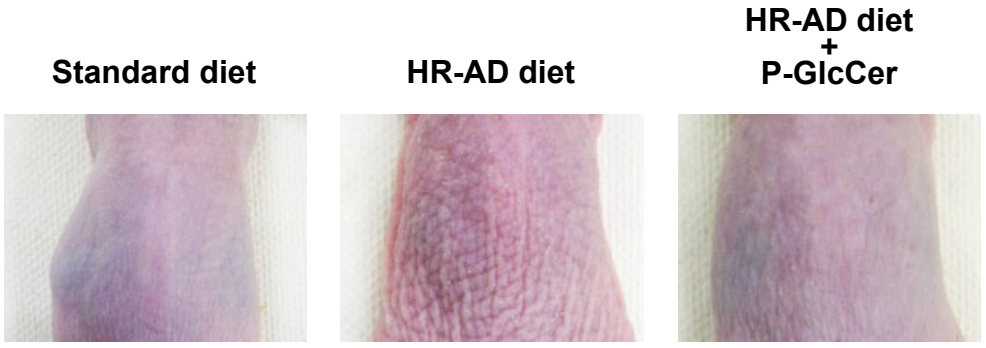
408 proline contents in skin on day 28. Values represent means ± SE (n=5). Groups that do
409 not share a letter are significantly different. $p<0.05$
410
411 Fig. 3 Effect of exogenous transforming growth factor-β on skin barrier function and
412 collagen contents in skin.
413 Hos:HR-1 mice were divided into five groups (n=5): standard diet group, the
414 unsaturated fatty acids-deficient (HR-AD) diet group, or HR-AD diet supplemented
415 with 0.1% (w/w) pineapple glucosylceramide (P-GlcCer) group. The mice fed the
416 standard diet and the HR-AD diet were injected intraperitoneally with recombinant
417 transforming growth factor-β (TGF-β) (17.5 ng/mouse/day) every day for 4 weeks. (A)
418 TGF-β level in serum. (b) TGF-β level in skin. (C) transepidermal water loss (TEWL)
419 value on day 28. (D) Hydroxyproline content in skin. (E) Wrinkle formation on day 28.
420 The panels are representatives of the mouse back epidermis. (F) Animal experimental
421 design. Values represent means ± SE (n=5). Groups that do not share a letter are
422 significantly different. $p<0.05$
423
424 Fig. 4 Effect of transforming growth factor-β neutralizing antibody on skin barrier
425 function and collagen contents in skin.

426 Hos:HR-1 mice were divided into twelve groups (n=5): standard diet group, unsaturated
427 fatty acids-deficient (HR-AD) diet group, and HR-AD diet supplemented with 0.1%
428 (w/w) pineapple glucosylceramide (P-GlcCer) group each with or without transforming
429 growth factor-β (TGF-β) neutralizing antibody (10 μg) for 4 weeks. As an isotype
430 control, an isotype-matched mouse IgG antibody (10 μg) was injected intravenously.
431 (A) TGF-β levels in serum. (B) transepidermal water loss (TEWL) values on day 28.
432 (C) Collagen content in skin. (D) Wrinkle formation on day 28. The panels are
433 representatives of the mouse back epidermis. (F) Animal experimental design. Values
434 represent means ± SE (n=5). Groups that do not share a letter are significantly different.
435 $p<0.05$.

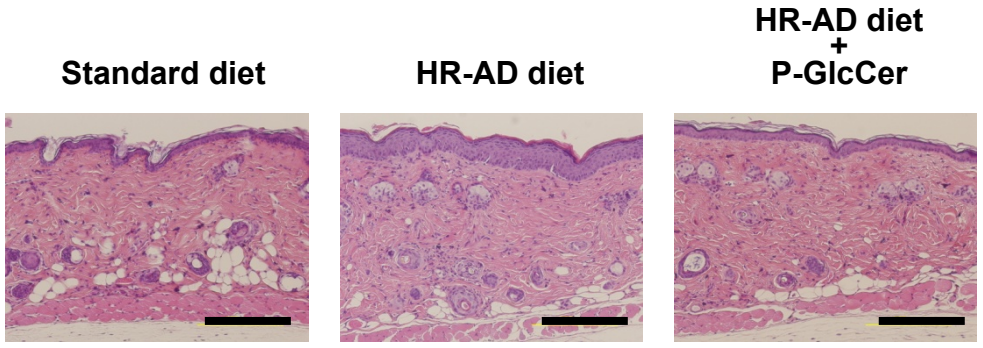
A



B

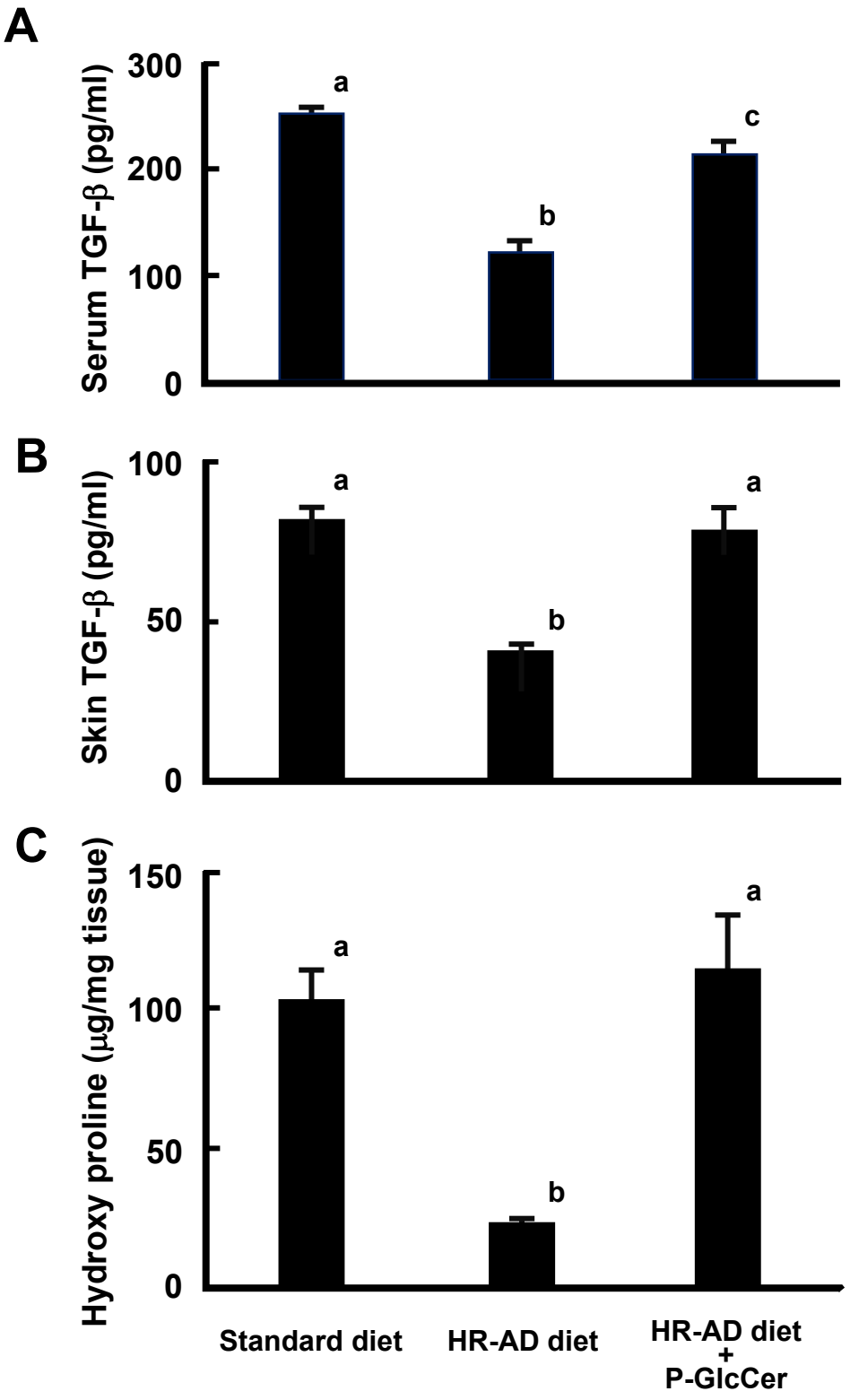


C



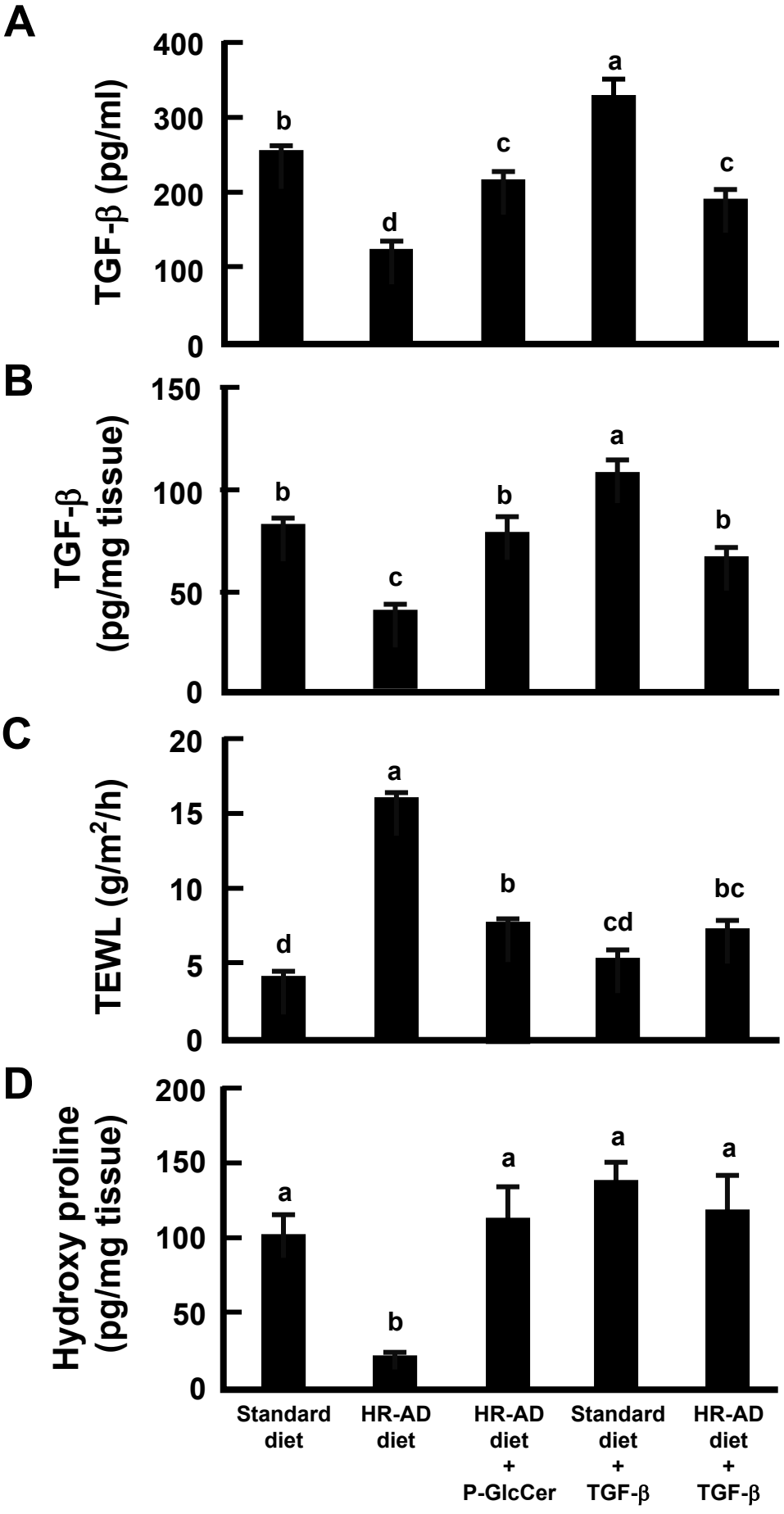
up

Oka et al., Fig. 1

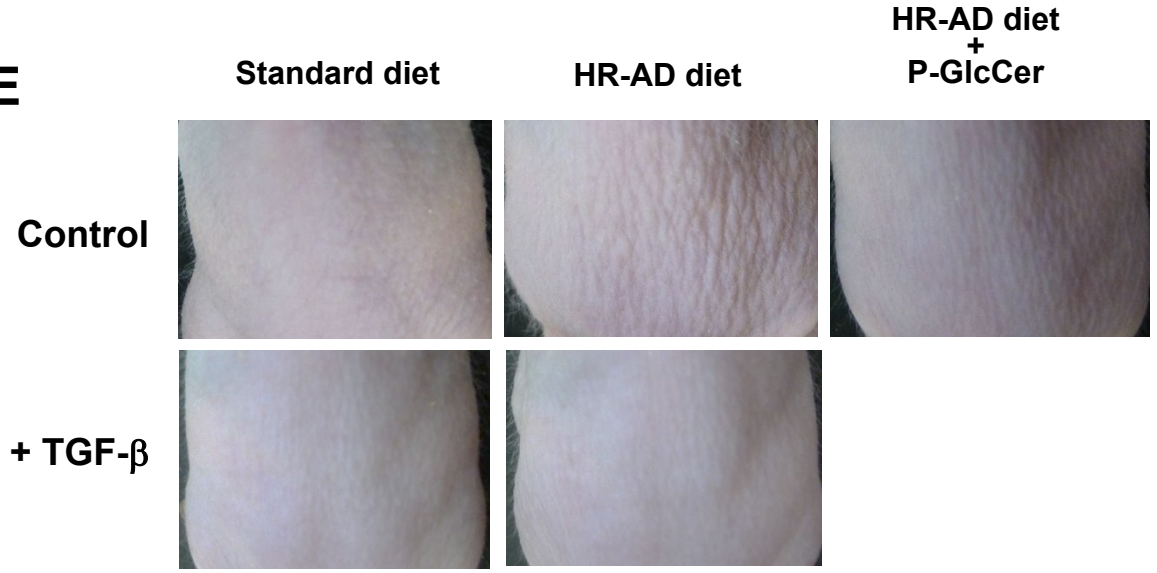


up

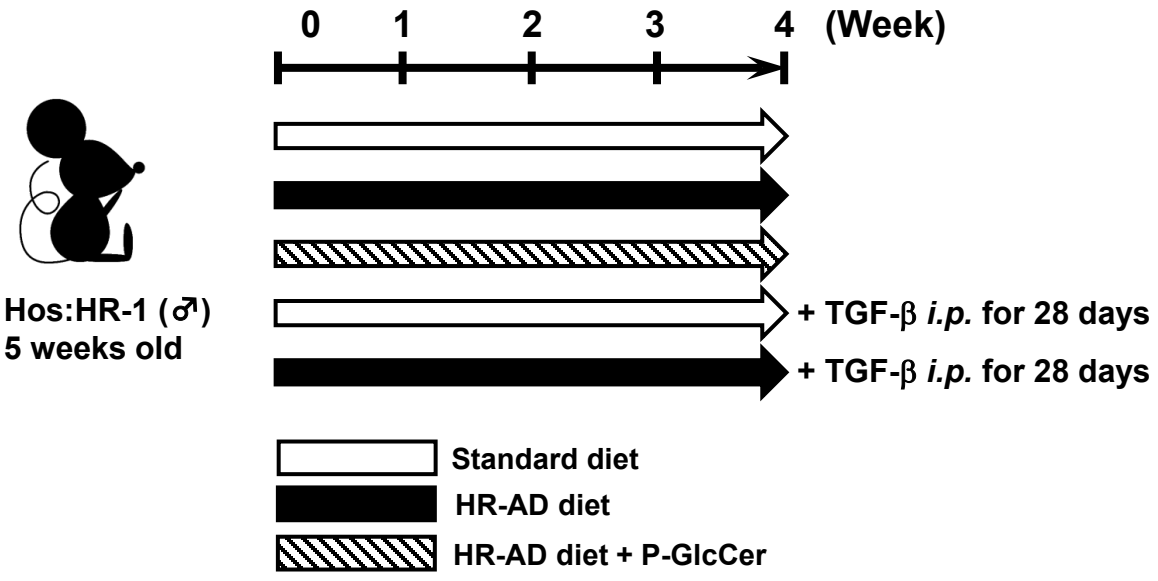
Oka et al., Fig. 2


A
TGF-β (pg/ml)
400
300
200
100
0
b
d
c
a
c
B
TGF-β
(pg/mg tissue)
150
100
50
0
b
c
b
a
b
C
TEWL (g/m²/h)
20
15
10
5
0
d
a
b
cd
bc
D
Hydroxy proline
(pg/mg tissue)
200
150
100
50
0
a
b
a
a
a
Standard diet
HR-AD diet
HR-AD diet + P-GlcCer
Standard diet + TGF-β
HR-AD diet + TGF-β

E

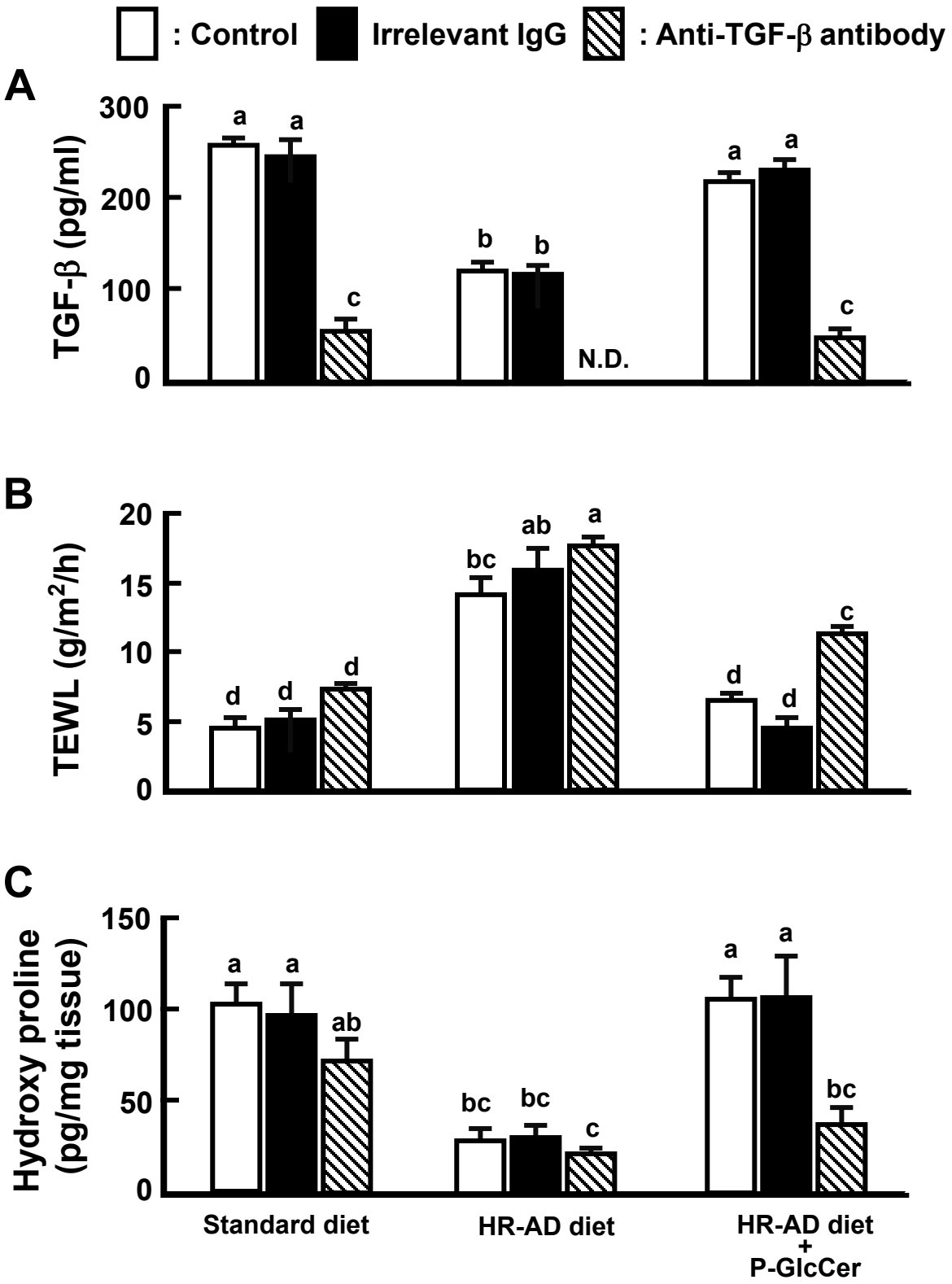


F

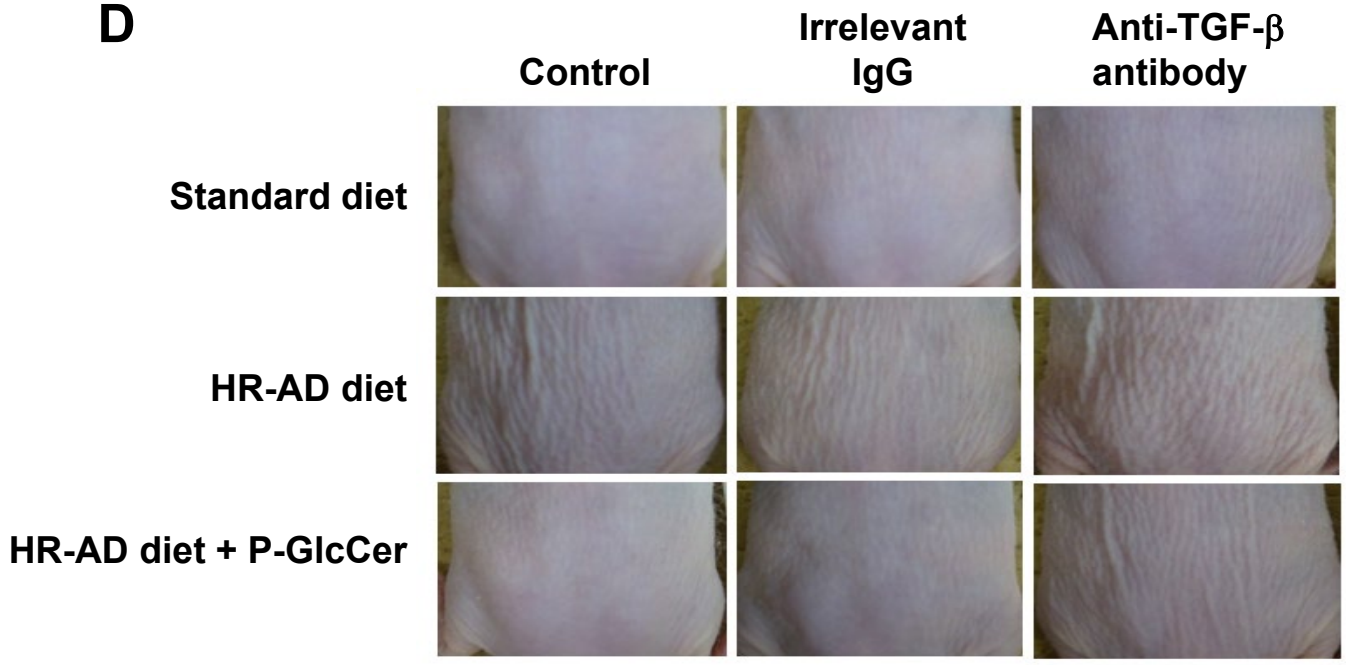


up

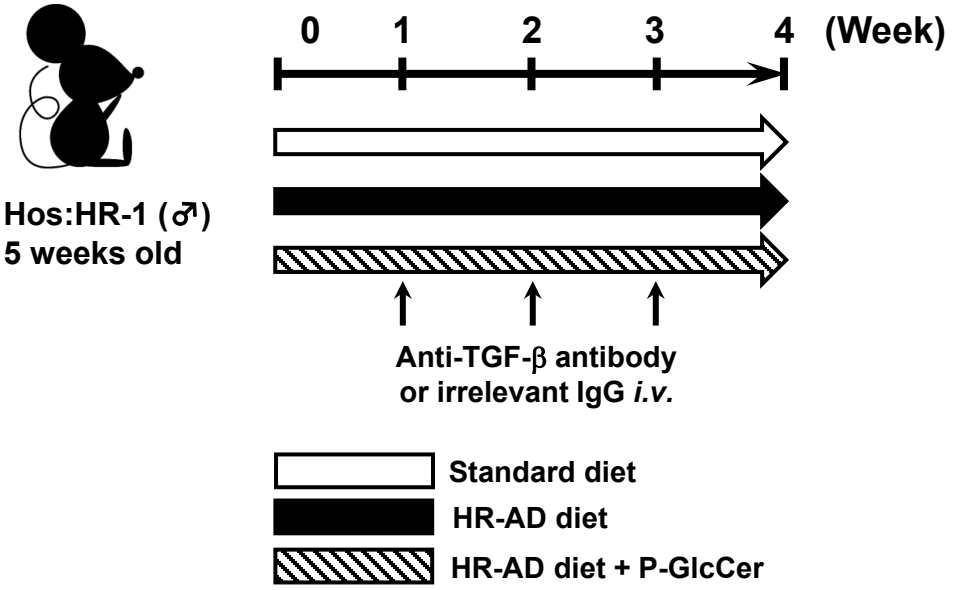
Oka et al., Fig. 3


: Control
Irrelevant IgG
: Anti-TGF-β antibody
A
TGF-β (pg/ml)
300
200
100
0
a
a
c
b
b
N.D.
a
a
c
B
TEWL (g/m²/h)
20
15
10
5
0
d
d
d
bc
ab
a
d
d
c
C
Hydroxy proline (pg/mg tissue)
150
100
50
0
a
a
ab
bc
bc
c
a
a
bc
Standard diet
HR-AD diet
HR-AD diet + P-GlcCer

D



E



up

Oka et al., Fig. 4