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Overexpression of TNF- α converting enzyme in fibroblasts augments dermal fibrosis after inflammation

Short running title: Overexpression of TACE and dermal fibrosis

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Abstract

TNF- α converting enzyme (TACE) can cleave transmembrane proteins, such as TNF- α , TNF receptors, and epidermal growth factor receptor (EGFR) ligands, to release the extracellular domains from the cell surface. Recent studies have suggested that overexpression of TACE may be associated with the pathogenesis of inflammation and fibrosis. To determine the roles of TACE in inflammation and fibrosis, TACE transgenic (TACE-Tg) mice, which overexpressed TACE systemically, were generated. Since the transgene-derived TACE was expressed as an inactive form, no spontaneous phenotype developed in TACE-Tg mice. However, the transgene-derived TACE could be converted to an active form by furin *in vitro* and by phorbol myristate acetate (PMA) *in vivo*. Subcutaneous injection of PMA into mice induced inflammatory cell infiltration 1 day later and subsequent dermal fibrosis 7 days later. Interestingly, the degree of dermal fibrosis at day 7 was significantly higher in TACE-Tg mice than in wild-type mice. Correspondingly, PMA increased the expression of type I collagen in the primary culture of dermal fibroblasts derived from TACE-Tg mice. Furthermore, phosphorylated EGFR was increased in the fibroblasts by the PMA treatment. The collective findings suggest that TACE overexpression and activation in fibroblasts could shed off putative EGFR ligands. Subsequently, the soluble EGFR ligands could bind and activate EGFR on fibroblasts, and then increase the type I collagen expression resulting in induction of dermal fibrosis. These results also suggest that TACE and EGFR on fibroblasts may be novel therapeutic targets of dermal fibrosis, which is induced after diverse inflammatory disorders of the skin.

Introduction

TNF- α converting enzyme (TACE), which belongs to a disintegrin and metalloproteinase (ADAM) family, can cleave transmembrane proteins to release the extracellular domains from the cell surface (1, 2). Initially produced as an inactive 120 kDa protein, the N-terminus prodomain is removed by furin at the trans-golgi-network, and consequently TACE is converted to an active form of 100 kDa protein (3-6). The active form of TACE is transported to the plasma membrane and binds to its substrates on the cell surface. Substrates of TACE include TNF- α , TNF receptors, and epidermal growth factor receptor (EGFR) ligands.

When focusing on the role of TNF- α in inflammation, it is considered that TACE contributes to promote inflammation by increasing soluble TNF- α . However, it is also considered that TACE plays a role in the suppression of inflammation by decreasing membrane-type TNF receptors and producing soluble TNF receptors, which can work as decoy receptors. These concepts seem contradictory, but TACE really functions to maintain the physiological homeostasis. The expression of TACE substrates is strictly regulated in a time-dependent manner during the inflammation process.

On the other hand, it has been demonstrated that rat collagen antibody-induced arthritis and lipopolysaccharide (LPS)-induced acute lung injury can be treated by TACE inhibitors (7, 8). Recently, Terao and colleagues have demonstrated that murine bleomycin-induced scleroderma could be treated also by TACE inhibitors (9). These findings suggest that TACE may be critically involved in the pathogenesis of these inflammatory and fibrous connective tissue diseases. However, the precise mechanism of the implication of TACE in inflammation and fibrosis has not been revealed. This study aimed to clarify the role of TACE in the

pathogenesis of inflammation and fibrosis using TACE transgenic (TACE-Tg) mice, which could overexpress TACE in the systemic organs.

Materials and Methods

Generation of TACE-Tg mice

The transgene for generation of TACE-Tg mice contained the full length mouse TACE cDNA, which connected the Flag tag to the 3' region. The connection of the Flag tag rendered distinction of the transgene-derived TACE from the endogenous TACE. The construct was inserted into pCAGGS vector containing the β -actin promoter, which could bring systemic expression of the transgene. Then, the pCAGGS vector carrying the transgene was microinjected into fertilized eggs of BDF1 mice at Genome Information Research Center, Research Institute of Microbial Disease, Osaka University (Osaka, Japan). Four founder mice obtained were mated with C57BL/6 mice (Japan Clea, Tokyo, Japan), and then the offspring mice were backcrossed into C57BL/6 mice more than 6 times. Among them, one stable line of TACE-Tg mice with heterozygous transgene insertion was served for this study. Age-matched wild-type (WT) C57BL/6 mice were used for the control. Experiments using mice were done in accordance with the Guidelines for the care and use of laboratory animals in Hokkaido University.

Real-time RT-PCR

For RNA extraction from mouse tissues, RNeasy Mini kit (Qiagen, Hilden, Germany) was used. After digestion of contaminated genomic DNA by DNase I, RNA was reverse transcribed to cDNA using Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The expression of TACE mRNA was quantified by real-time RT-PCR using QuantiTect SYBR Green PCR kit (Qiagen). The primer sequences for TACE were as follows: 5'-ATCTGAAGAGTTTGTTCGTCGAG-3' (sense) and 5'-TCCACGGCCCATGTATTTAT-3' (antisense). PCR was run on ABI

Prism 7000 (Applied Biosystems, Carlsbad, CA) as follows; after denaturation at 95 °C for 10 min, 40 cycles of reaction at 95 °C for 15 sec and at 56 °C for 60 sec were carried out. For the internal control, the expression of hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) was monitored. The primer sequences for HPRT-1 were as follows: 5'-TGGAAAGAATGTCTTGATTGTTGAA-3' (sense) and 5'-AGCTTGCAACCTTAACCATTTTG-3' (antisense).

Western blotting

The mouse tissues were homogenized in lysis buffer (0.1% sodium dodecyl sulfate (SDS), 1% Nonidet-P40, 0.5% sodium deoxycholate, 100 µg/ml phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, protease inhibitor cocktail (Complete Mini, Roche, Basel, Switzerland)). The lysates adjusted ranging from 10 to 40 µg/lane were fractionated on 7.5% SDS polyacrylamide gel and then transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK). After blocking by TBS-T (0.1% Tween-20 in Tris-buffered saline) containing 2% non-fat milk, the membranes were incubated with 1:5000 dilution of the anti-TACE antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:5000 dilution of the anti-Flag antibody (Sigma-Aldrich, St. Louis, MO) overnight at 4 °C. After 3 times wash by TBS-T, the membranes were next incubated with 1:25000 dilution of the peroxidase-labelled secondary antibodies (GE Healthcare) overnight at 4 °C. Protein bands were detected using ECL Advance Western Blotting Detection kit (GE Healthcare). The anti-TACE antibody used in this study could react with the C-terminus of mouse TACE; thus, it could detect both the inactive and active forms.

Activation of TACE by furin *in vitro*

The skin tissues obtained from TACE-Tg and WT mice were lysed in the furin

assay buffer (100 mM HEPES (pH 7.5), 0.5% Triton X-100, 1 mM CaCl₂, 1 mM 2-mercaptoethanol). The lysates (140 µg/100 µl) were incubated with recombinant human furin (Sigma-Aldrich) at respective concentrations of 0, 0.1, and 1 unit/µl for 1 hour at 30 °C. The samples were then fractionated on 7.5% SDS polyacrylamide gel, and western blotting was performed using the anti-TACE or anti-Flag antibodies.

Measurement of TACE activity

The skin lysates treated by furin were subjected for measurement of TACE activity. The TACE activity was measured using SensoLyte 520 TACE Activity Assay kit (AnaSpec, Fremont, CA) and Varioskan Flash Microplate Multimode Readers (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Primary culture of dermal fibroblasts

The back skin of TACE-Tg and WT mice were turned inside out and 3 mm pieces of the dermis were excised, put on flat dishes, and then incubated in RPMI-1640 (Sigma-Aldrich) containing 20% fetal calf serum, 50 µg/ml streptomycin, and 50 U/ml penicillin. Several days later, spindle-shaped cells migrated and proliferated around the skin pieces. After removal of the skin pieces, the cells were used as primary culture of dermal fibroblasts. Experiments were conducted using the cells at 3-5 passages.

Stimulation of dermal fibroblasts by PMA

The primary culture of dermal fibroblasts was stimulated by phorbol myristate acetate (PMA, LC Laboratories, Woburn, MA) at respective concentrations of 0, 6.4, 64, and 640 nM. After 30 min incubation at 37 °C, the cells were lysed, and then the lysates were served for the anti-TACE or anti-Flag immunoblotting.

Subcutaneous injection of PMA

TACE-Tg and WT mice (10 weeks old, female) with shaved back skin were subcutaneously injected with 0.08 µg PMA in 0.1 ml PBS (1300 nM) using 29G syringe needle. As control, the same volume of PBS without PMA was injected subcutaneously.

Histological evaluation

At 1 and 7 days after the inoculation, the skin sites with PMA-injection and PBS-injection were excised as 6 mm round-shape pieces, fixed in formalin, and then subjected for Hematoxylin and Eosin (HE) staining. The samples at 7 days were also subjected for Elastica Masson (EM) staining. Dermal thickening ratio was calculated as follows: 1) thickness of dermis was measured at 3 random points of the sites with PBS-injection, 2) mean thickness at the sites with PBS-injection was calculated, 3) thickness of dermis was measured at 3 random points of the sites with PMA-injection, and 4) dermal thickness ratios were calculated by dividing the thickness of dermis at the PMA-injection sites by the mean dermal thickness at the sites with PBS-injection.

Expression of type I collagen

To evaluate fibrosis in the molecular level, the expression of type I collagen (collagen 1A1) was examined by real-time RT-PCR. First, at 7 days, the skin sites with PMA-injection and PBS-injection were excised as 6 mm round-shape pieces, RNA was extracted from the tissues, and then real-time RT-PCR was performed as described above. The primers for collagen 1A1 were as follows: 5'-GAGCCCTCGCTTCCGTACTC-3' (sense) and 5'-TGTTCCCTACTCAGCCGTCTGT-3' (antisense). Next, the primary culture of dermal fibroblasts was treated by PMA at respective concentrations of 0, 20, 160, and

1300 nM. After 4 hours incubation at 37 °C, RNA was extracted from the cells, and then the expression of collagen 1A1 was examined similarly by real-time RT-PCR.

TACE inhibition assay

The fibroblasts derived from TACE-Tg mice were treated by 1300 nM of PMA with or without 25 µg/ml of TAPI-0 (Enzo Life Sciences, Farmingdale, NY) at 37 °C. TAPI-0 can inhibit TACE and other matrixmetalloproteases, which major target is TACE (10). The concentration of TAPI-0 was adopted according to the literature (11). After 4 hours incubation, RNA was extracted from the cells, and then the expression of collagen 1A1 was examined by real-time RT-PCR as described above.

Detection of phosphorylated EGFR

To determine if EGFR was activated by PMA, phosphorylation of EGFR was examined. The primary culture of dermal fibroblasts was treated by PMA at respective concentrations of 0, 20, 160, and 1300 nM. After 1 hour of incubation at 37 °C, RNA and cell lysates were extracted. The RNA was then served for RT-PCR using the EGFR primers (sense: 5'-GAACTGGGCTTAGGGAACTGC-3', antisense: 5'-CATTGGGACAGCTTGGATCAC-3'), and the lysates were served for western blotting using the anti-phosphorylated EGFR antibody (Phospho-EGF Receptor (Tyr1068), Cell Signaling Technology, Tokyo, Japan). RT-PCR was carried out as described above. As internal controls, the expression of HPRT1 and the amount of actin detected by the anti-actin antibody (Chemicon International, Temecula, CA) were monitored.

Statistics

Data were presented as mean $\pm$ standard deviation. Student's *t*-test was

applied for statistical analysis. The p -value of less than 0.05 was considered to be significant.

Results

Overexpression of TACE in TACE-Tg mice

The TACE mRNA and protein expressions in the systemic organs of 6-week-old TACE-Tg and WT mice were evaluated by real-time RT-PCR and western blotting, respectively. The TACE mRNA expression in all organs examined was higher in TACE-Tg mice than in WT mice, though the expression level was variable among organs (Figure 1a). The top 5 organs with the highest level of expression of TACE mRNA included the muscle, pancreas, heart, skin, and intestine. In WT mice, the TACE mRNA expression was relatively high in the pancreas and skin. The TACE protein expression, which was detected by the anti-TACE immunoblotting, well reflected the mRNA expression (Figure 1b). These findings indicated the overexpression of TACE in TACE-Tg mice compared to WT mice. The anti-Flag immunoblotting suggested that the difference in the amount of TACE between TACE-Tg and WT mice was attributable to the expression of the transgene-derived TACE. Remarkably, most of the transgene-derived TACE protein was detected as the 120 kDa inactive form.

No spontaneous phenotype in TACE-Tg mice

Comparison of histology of systemic organs between 6-week-old TACE-Tg mice and WT mice revealed no remarkable difference (Supplemental Figure 1). The TACE-Tg mice kept for up to 2 years showed no spontaneous development of a specific phenotype. This might be consistent with the presence of the most of all transgene-derived TACE protein as the inactive form in TACE-Tg mice.

Activation of TACE by furin

ADAM family molecules, including TACE, undergo proteolysis to the active form by protein convertases (3, 4). The inactive TACE of 120 kDa protein is removed the N-terminus prodomain and then converted to the active form of 100 kDa protein by furin *in vivo* (5, 6). To determine the catalytic capacity of transgene-derived TACE, the tissue lysates of skin from TACE-Tg and WT mice were incubated with furin *in vitro*. The western blotting using the anti-TACE and anti-Flag antibodies revealed that the transgene-derived TACE protein could be converted to the 100 kDa active form by furin in a dose-dependent manner (Figure 2a). Compatible with these findings, the TACE activity in the samples from TACE-Tg mice was increased by furin dose-dependently, and the increased TACE activities in the samples from TACE-Tg mice exhibited significantly higher levels than those in WT samples (Figure 2b).

Activation of TACE by PMA *in vitro*

Administration of furin into mice is difficult because furin exclusively functions in the cytoplasm *in vivo*. In this study, alternative stimulation, which can convert TACE to the active form, was sought; hence, PMA was employed. When the primary culture of dermal fibroblasts was stimulated by PMA, the transgene-derived TACE protein was effectively converted to the active form (Figure 3a). Although the TACE activity in WT fibroblasts was significantly increased by PMA dose-dependently as well as that in the TACE-Tg fibroblasts, the increased amount of active TACE in WT samples seemed to remain at an undetectable level of the anti-TACE immunoblotting (Figure 3b).

Activation of TACE by PMA *in vivo*

To determine that PMA could convert TACE to the active form *in vivo*, PMA was subcutaneously injected into TACE-Tg and WT mice, and then skin samples were

obtained 1 and 7 days after the inoculation. Western blotting using the anti-TACE and anti-Flag antibodies revealed that the active TACE of 100 kDa protein increased in the sites of PMA-injection in TACE-Tg mice at day 1. The amount of the active TACE in the sites of PMA-injection in WT mice did not reach detection level (Figure 4a). The TACE activation recovered to the unstimulated level at 7 days after the PMA-injection even in TACE-Tg mice. The TACE activity in the skin tissues of WT mice was significantly increased by PMA at day 1 as well as that in the TACE-Tg samples, though the increased amount of active TACE in the WT samples still remained at an undetectable level of the anti-TACE immunoblotting (Figure 4b).

Augmented dermal fibrosis after PMA-induced inflammation in TACE-Tg mice

One day after the PMA-injection, a severe infiltration of polymorphonuclear cells was observed in the subcutaneous tissue. Variety and degree of inflammatory cell infiltration were equivalent between TACE-Tg and WT mice (Figure 5). Thickening of the dermis and scar formation in the subcutaneous tissue were observed at the inflammation sites 7 days after the PMA-injection (Figure 6a-6h). The dermal thickening ratio (PMA-injection site/PBS-injection site) was significantly higher in TACE-Tg mice than in WT mice (Figure 6i). Correspondingly, the mRNA expression of type I collagen in the skin 7 days after the PMA-injection was relatively higher in TACE-Tg mice than in WT mice, though there was no statistical significant difference (Figure 6j). Notably, the type I collagen expression at the sites of PMA-injection reached 2-fold level of the PBS-injected sites in TACE-Tg mice; whereas, the expression at PMA-injection sites was equal to that of the PBS-injection sites in WT mice.

Induction of type I collagen expression in dermal fibroblasts by PMA

To elucidate the hypothesis that the quantitative difference of TACE in dermal fibroblasts between TACE-Tg and WT mice was attributable to the degree of dermal fibrosis after the PMA-induced inflammation, the primary culture of dermal fibroblasts was stimulated by PMA *in vitro*, and then the mRNA expression of type I collagen was examined by real-time RT-PCR. As a result, the levels of type I collagen expression were up-regulated by PMA dose-dependently in dermal fibroblasts of TACE-Tg mice, though the expression was not altered by the PMA-treatment in dermal fibroblasts of WT mice (Figure 7a). In addition, the induction of type I collagen by PMA was significantly inhibited by the TACE inhibitor, TAPI-0 (Figure 7b). These findings suggested that the overexpression and activation of TACE in dermal fibroblasts could promote the type I collagen expression.

Increase of phosphorylated EGFR in dermal fibroblasts by PMA

It has been shown that the expression of type I collagen was driven by the EGFR signal in fibroblasts (12). To determine the activation of EGFR, the primary culture of dermal fibroblasts was treated by PMA, and then the expressions of EGFR and phosphorylated EGFR were examined. As a result, phosphorylated EGFR was increased by the PMA-treatment dose-dependently, and the degree was higher in TACE-Tg mice than in WT mice (Figure 7c).

Discussion

Association of the TACE expression with the pathogenesis of inflammation and fibrosis has been documented in animal models (7-9). In humans, TACE has been also demonstrated to be involved in the pathogenesis of inflammatory and fibrous connective tissue diseases, such as rheumatoid arthritis (13, 14) and systemic sclerosis (SSc) (15). Bohgaki *et al.* reported that TACE was overexpressed in peripheral blood monocytes of patients with early stage of SSc, and that the TACE expression was decreased by treatment. These findings suggest that the overexpression of TACE in monocytes might be critically implicated in the development of SSc. On the other hand, it remains elusive how TACE in organ cells can be implicated in the pathogenesis of inflammation and fibrosis.

In the present study, TACE-Tg mice were generated in order to answer the question (Figure 1). Since majority of the transgene-derived TACE were expressed as an inactive form, no spontaneous phenotype occurred in TACE-Tg mice (Supplemental Figure 1). However, furin could convert the transgene-derived inactive TACE to active form; thus, TACE-Tg mice were regarded as inducible models of TACE overexpression and activation (Figure 2). Interestingly, furin failed to activate endogenous TACE unlike the transgene-derived TACE. Although the reason should be revealed by further studies, it is possible that undetermined factors in tissue lysates interfered with the measurement of TACE activity when inactive TACE were converted to active form in the tissue lysates. The amount of the putative inhibitory factors seemed to be enough to mask the TACE activity in WT samples mostly, but insufficient to mask that in TACE-Tg samples with overexpression of TACE.

Since furin functions exclusively in the cytoplasm *in vivo*, we employed PMA as stimulant to induce TACE activation in TACE-Tg mice (Figure 3). As a result, the

overexpression and activation of TACE in fibroblasts were demonstrated to augment dermal fibrosis after inflammation (Figures 4-6). The subcutaneous injection of PMA into TACE-Tg mice activated TACE in the tissue 1 day later and induced subsequent dermal fibrosis 7 days later. Since PMA-induced activation of TACE already returned to the baseline level at day 7, it remained unclear whether the TACE overexpression and activation at day 1 were critically associated with the increased fibrosis at day 7. Although further studies are needed to clarify the association, it is possible that the TACE-dependent type I collagen induction at an early state in inflammation could make an orientation toward subsequent fibrosis. Interestingly, the degree of dermal fibrosis 7 days after the PMA-injection was significantly higher in TACE-Tg mice than in WT mice, though the degree of inflammatory cell infiltration at day 1 was comparable between the two. These findings suggest that the overexpression of TACE is related to fibrosis after inflammation rather than inflammation itself.

There is a controversy over the contribution of TACE to tissue fibrosis. Terao *et al.* demonstrated that TACE contributed to dermal fibrosis using murine bleomycin-induced scleroderma model (9). This finding corresponds to our results. On the contrary, Leco *et al.* reported that lung emphysema, an opposite phenotype of fibrosis, developed in tissue inhibitor of metalloproteinase 3 (TIMP-3)-deficient mice (16). Since TIMP-3 functions as a TACE inhibitor *in vivo*, TIMP-3 deficient mice have been documented as a TACE activation model (17, 18). Therefore, this finding suggests that TACE plays an opposite role in induction of tissue fibrosis and is contradictory to our results. However, TIMP-3-deficient mice are not necessarily an ideal model of TACE activation *in vivo* because TIMP-3 inhibits not only TACE but also other metalloproteinases.

To confirm the contribution of TACE overexpression in fibroblasts to dermal fibrosis, the expression of type I collagen using primary culture of dermal fibroblasts

was examined in this study. Results indicated that PMA effectively activated TACE and subsequently increased expression of the type I collagen in the fibroblasts derived from TACE-Tg mice (Figure 7a). Furthermore, the induction of type I collagen by PMA was significantly inhibited by the TACE inhibitor (Figure 7b). These findings support our conclusion that TACE overexpression and activation in fibroblasts could contribute to dermal fibrosis. However, this does not necessarily mean that TACE exclusively regulates the PMA-induced type I collagen expression in dermal fibroblasts because TAPI-0 is not a specific inhibitor of TACE.

The substrates of TACE involved in the process of dermal fibrosis after the PMA-induced inflammation have not been identified. However, the amount of phosphorylated EGFR was increased by the PMA-treatment of dermal fibroblasts (Figure 7c). Since the expression of type I collagen could be driven by the EGFR signal (12), TACE activated by PMA could shed off putative EGFR ligands on the surface of fibroblasts. Subsequently, the soluble EGFR ligands could bind and activate EGFR on fibroblasts through the autocrine and paracrine pathways and increase the type I collagen expression resulting in induction of dermal fibrosis. With regards to the EGF signaling pathway, EGFR ligands, including transforming growth factor α (TGF- α), heparin-binding EGF (HB-EGF), amphiregulin, and epiregulin, may be the candidates shed by TACE. Among them, amphiregulin is known to be expressed in fibroblasts (19); thus, this molecule is the next target in our continuing study.

The EGFR expression has been reported to be up-regulated in dermal fibroblasts of SSc patients (20). In the present study, the EGFR expression in TACE-Tg-derived dermal fibroblasts did not appear to be increased by PMA *in vitro*. This could be interpreted by the short duration of PMA stimulation (1 hour stimulation) in the experiments. In addition, murine bleomycin-induced lung fibrosis could be suppressed by the EGFR tyrosine kinase inhibitor (21). These findings

suggest that the EGF signaling pathway comes to a great interest as the mechanism bridging the TACE expression and the pathogenesis of fibrosis.

In summary, the collective findings suggest the possibility that overexpression of TACE in fibroblasts could contribute to the pathogenesis of dermal fibrosis after inflammation. Further studies are needed to reveal the process leading to fibrosis; however, our data suggest that TACE and EGFR on fibroblasts may be novel therapeutic targets of dermal fibrosis, which is induced after diverse inflammatory disorders of the skin.

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Figure legends**Figure 1. TACE mRNA and protein expressions in TACE-Tg and WT mice**

The mRNA expressions of TACE in systemic organs of 6-week-old TACE-Tg and WT mice were quantified using real-time RT-PCR (a). The expression level in each organ was standardized by the level in the skin of WT mice. The expression of TACE protein was examined by western blotting (b). Lysates of systemic organs from 6-week-old TACE-Tg and WT mice were served for the anti-TACE and anti-Flag immunoblotting. Experiments were repeated 3 times, and similar results were reproduced. Representative data are shown.

Figure 2. TACE activation by furin *in vitro*

Lysates of the skin from 6-week-old TACE-Tg and WT mice were incubated with furin at respective concentrations of 0, 0.1, and 1 unit/ μ l for 1 hour at 30 °C. The samples were then subjected for the anti-TACE and anti-Flag immunoblotting (a). Experiments were repeated 3 times, and similar results were reproduced. Representative data are shown. TACE activities in the samples (TACE-Tg: n=3, WT: n=3) were measured using SensoLyte 520 TACE Activity Assay kit. N.C. represents the spontaneous cleavage of TACE substrates in the kit. * $p < 0.05$, ** $p < 0.01$.

Figure 3. TACE activation by PMA *in vitro*

The primary culture of dermal fibroblasts derived from TACE-Tg and WT mice was stimulated by PMA at respective concentrations of 0, 6.4, 64, and 640 nM. After 30 min incubation at 37 °C, the cells were lysed, and then the lysates were served for the anti-TACE and anti-Flag immunoblotting (a). Experiments were repeated 3 times, and similar results were reproduced. Representative data are shown. PC:

positive control (furin-treated TACE-Tg skin lysates used in Figure 2). TACE activity in each sample (n=3, in each group) was measured using SensoLyte 520 TACE Activity Assay kit (b). *p<0.05, **p<0.01.

Figure 4. TACE activation by PMA *in vivo*

PMA (0.08 µg/0.1 ml PBS, 1300 nM) was injected subcutaneously into TACE-Tg and WT mice using 29G syringe needle. As a control, the same volume of PBS without PMA was injected subcutaneously. Lysates were obtained from the sites with PMA-injection and PBS-injection, respectively, at day 1 and day 7. Western blotting was performed using the anti-TACE and anti-Flag antibodies (a). Experiments were repeated 3 times, and similar results were reproduced. Representative data are shown. TACE activity in each sample (n=3, in each group) was measured using SensoLyte 520 TACE Activity Assay kit (b). **p<0.01.

Figure 5. Subcutaneous inflammatory cell infiltration at sites with PMA-injection

One day after the PMA-injection, the sites with PMA-injection were excised from TACE-Tg (n=5) and WT (n=8) mice, and then subjected for HE staining (a, b, and c: TACE-Tg; d, e, and f: WT; bar: 100 µm). Representative photos are shown.

Figure 6. Dermal fibrosis at sites with PMA-injection

Seven days after the PMA-injection, the sites with PMA-injection (b, d, f, and h) and the sites with PBS-injection (a, c, e, and g) were excised from TACE-Tg (n=5) and WT (n=8) mice, and then subjected for HE (a-d) and EM (e-h) staining (a, b, e, and f: TACE-Tg, n=5; c, d, g, and h: WT, n=8). Representative photos are shown. Dermal thickening ratios were calculated as follows: dermal thickness measured at 3 random points of the sites with PMA-injection (arrows in panels b and d)/mean value of dermal

thickness measured at 3 random points of the sites with PBS-injection (arrows in panels a and c), and then were compared between TACE-Tg and WT mice (i). The skin tissues at sites with PMA-injection and PBS-injection were obtained at day 7, the expression of type I collagen (collagen 1A1) was examined by real-time RT-PCR, and then fold increase by the PMA-injection was compared between TACE-Tg (n=5) and WT (n=8) mice (j). *p<0.05.

Figure 7. Increased type I collagen and phosphorylated EGFR in fibroblasts by PMA

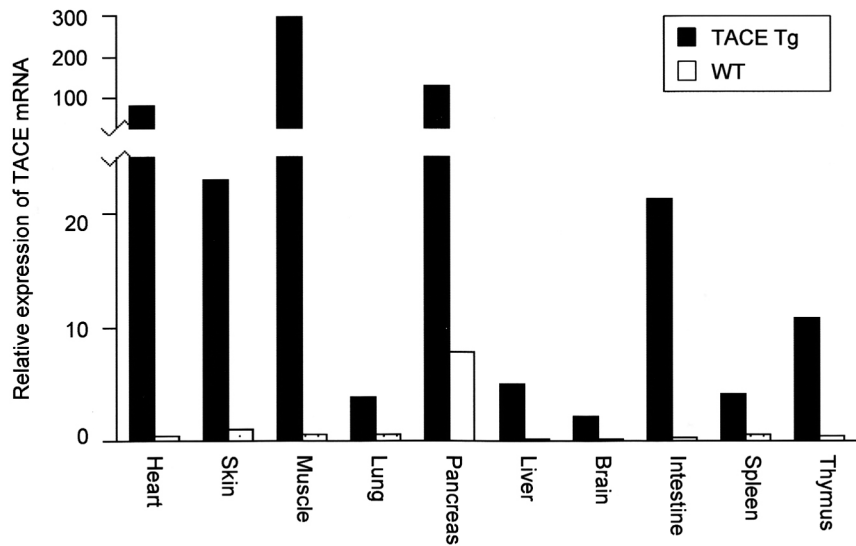
The primary culture of dermal fibroblasts (TACE-Tg: n=3, WT: n=3) was treated by PMA at respective concentrations of 0, 20, 160, and 1300 nM. After 4 hours incubation at 37 °C, RNA was extracted from the cells, and then the expression of type I collagen (collagen 1A1) was examined by real-time RT-PCR (a). The fibroblasts derived from TACE-Tg mice were treated by 1300 nM of PMA with or without 25 µg/ml of TAPI-0 at 37 °C. After 4 hours incubation, RNA was extracted from the cells, and then the expression of type I collagen (collagen 1A1) was examined by real-time RT-PCR (b). The PMA-induced increase of type I collagen (collagen 1A1) was set as 100%. *p<0.05. To determine the activation of EGFR, the expressions of EGFR and phosphorylated EGFR were examined (c). The primary culture of dermal fibroblasts was treated by PMA at respective concentrations of 0, 20, 160, and 1300 nM. After 1 hour incubation at 37 °C, RNA and cell lysates were extracted. The RNA was served for RT-PCR using the HPRT1 and EGFR primers. The lysates adjusted ranging from 10 to 40 µg/lane were fractionated on 7.5% SDS polyacrylamide gel, and then transferred onto PVDF membranes. After blocking by TBS-T containing 1% non-fat milk, the membranes were incubated with 1:2500 dilution of the anti-phosphorylated EGFR (p-EGFR) antibody overnight at 4 °C. After 3 times wash by TBS-T, the

membranes were next incubated with 1:25000 dilution of the peroxidase-labelled secondary antibodies overnight at 4 °C. Protein bands were detected using ECL Advance Western Blotting Detection kit. As an internal control, the amount of actin was monitored by the anti-actin antibody. Experiments were repeated 3 times, and similar results were reproduced. Representative data are shown.

Supplemental Figure 1. Histological findings in systemic organs in TACE-Tg mice

No abnormal finding in the heart (a), lung (b), liver (c), kidney (d), pancreas (e), intestine (f), skin (g), muscle (h), joint (i), thymus (j), spleen (k), and brain (l) of 6-week-old TACE-Tg mice. Representative photos are shown.

a



b

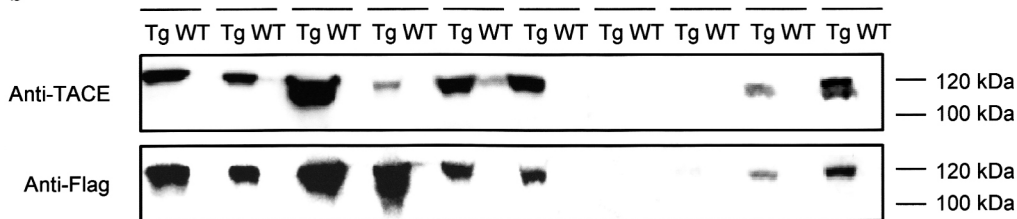


Figure 1

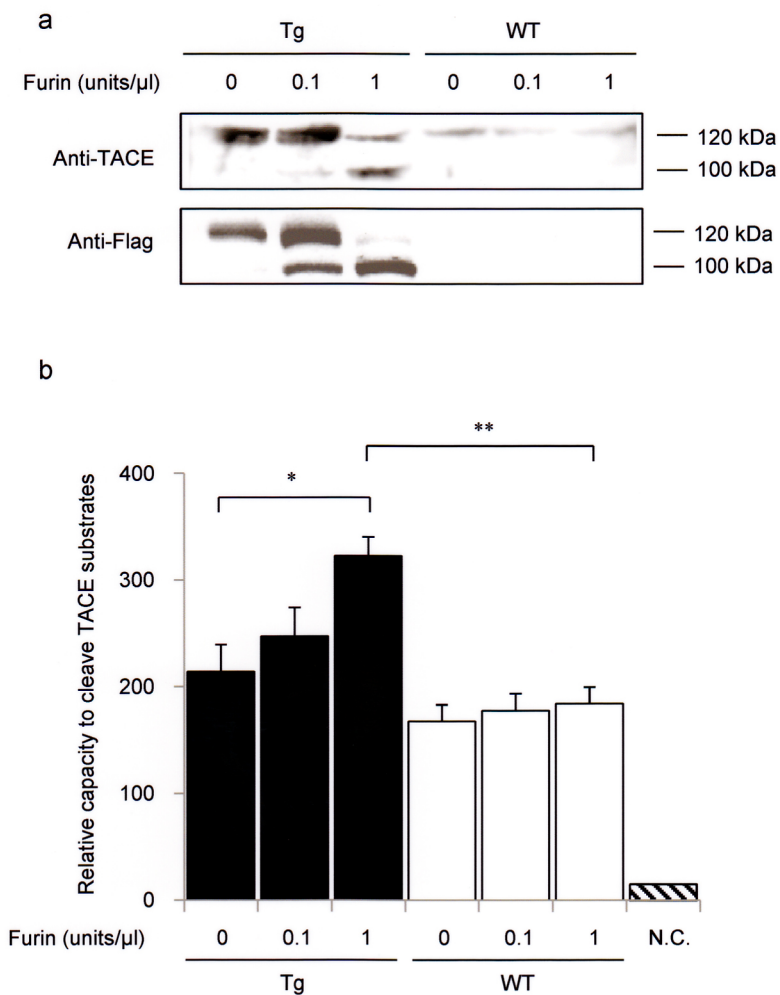


Figure 2

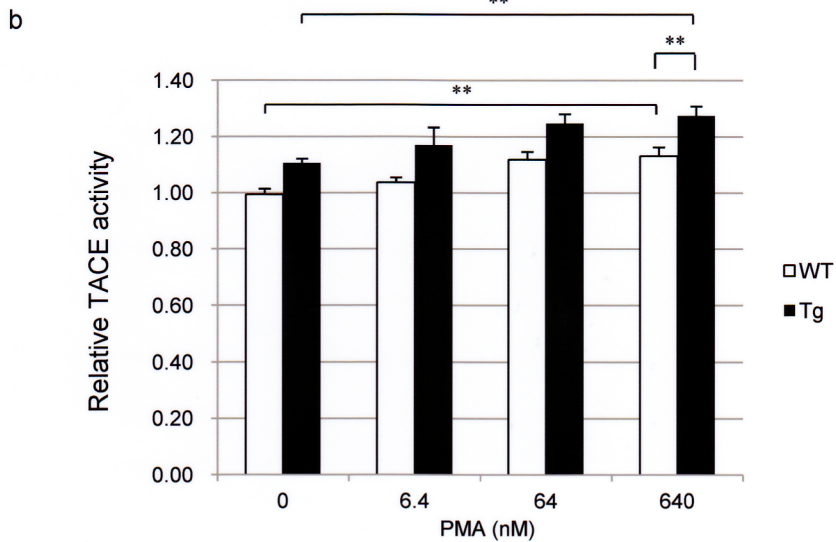
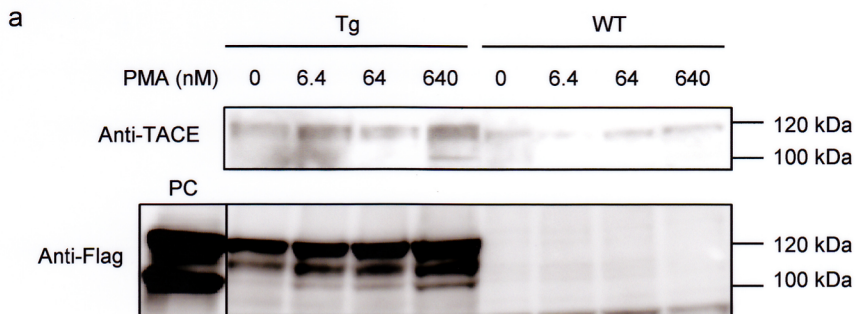


Figure 3

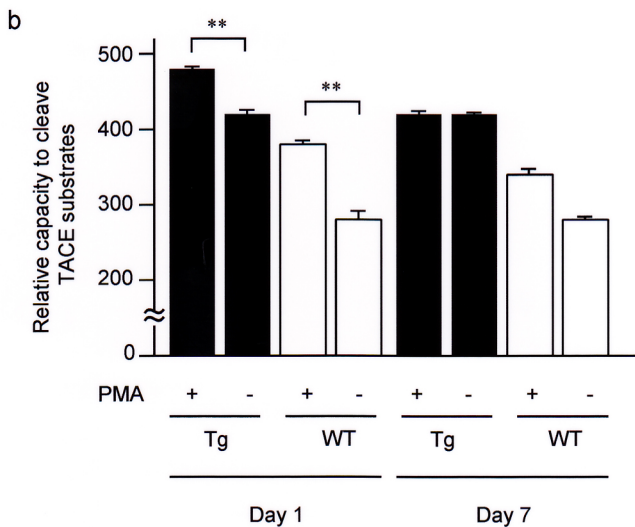
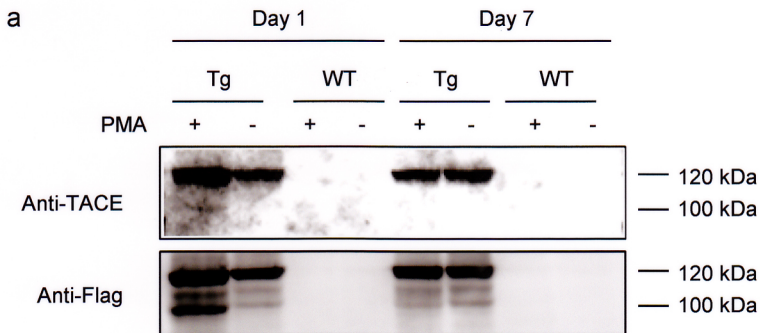


Figure 4

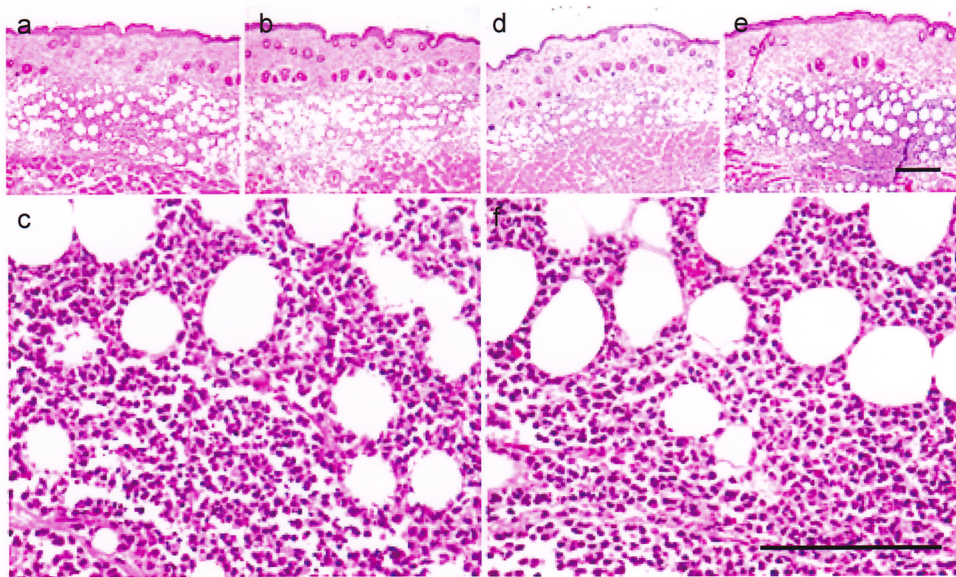
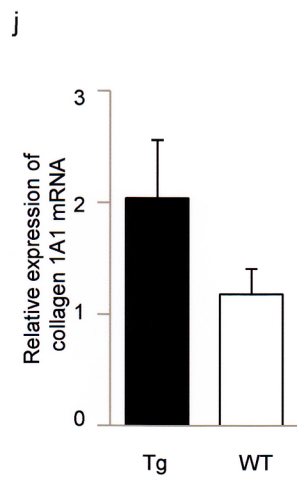
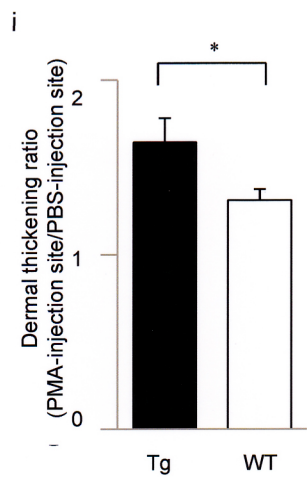
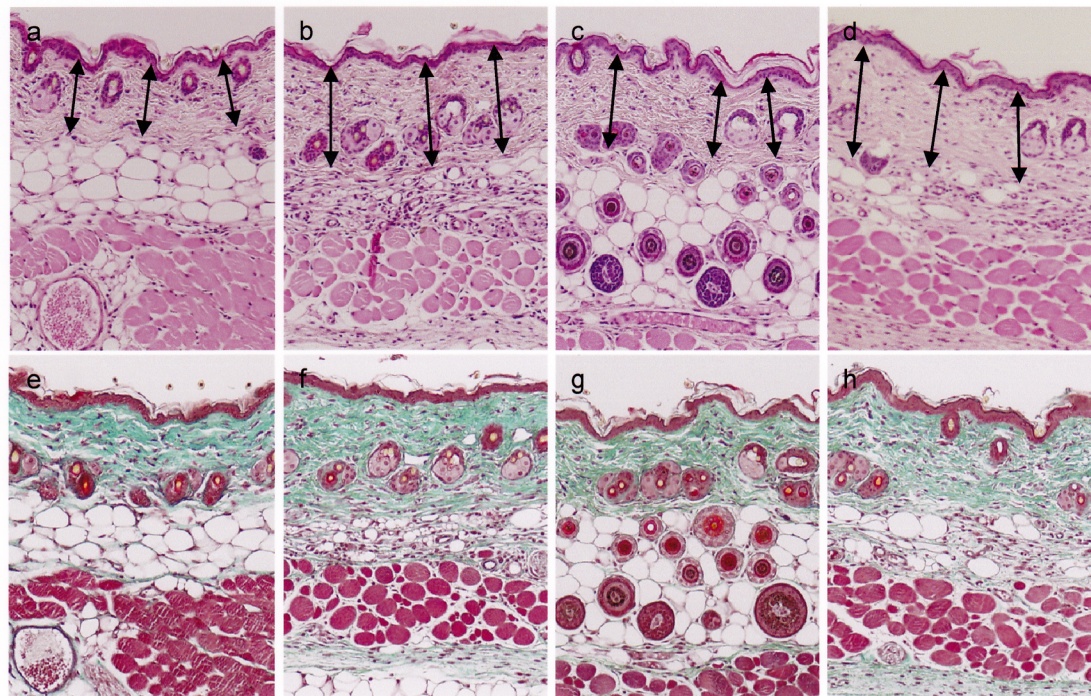
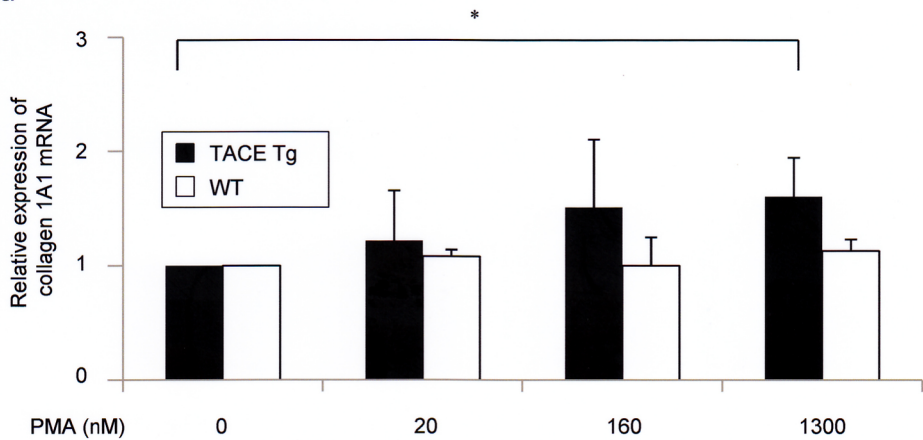


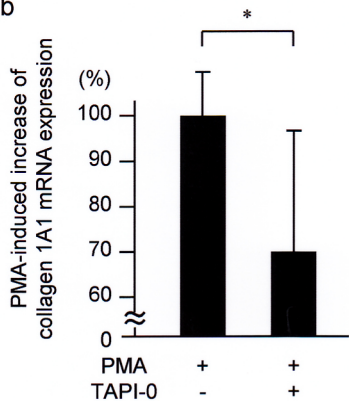
Figure 5



a



b



C

