Am80 reduces intercellular adhesion molecule-1 (ICAM-1) expression in dermal microvascular endothelial cells.

Since cell adhesion molecules, especially ICAM-1, are involved in the fibrotic process of BLM-treated mice (Matsushita *et al.*, 2007; Taniguchi *et al.*, 2015; Yoshizaki *et al.*, 2010), we looked at the effect of Am80 on the expression of ICAM-1 in BLM-treated mice. As expected, *Icam1* mRNA expression in the skin lesions of BLM-treated mice was significantly reduced by Am80 treatment (Figure 4a). As shown in immunohistochemistry, an inhibitory effect of Am 80 was remarkably seen on dermal microvascular endothelial cells of BLM-treated mice (Figure 4b). Am80 also modestly and inconsistently attenuated BLM-induced ICAM-1 expression in dermal fibroblasts and infiltrating lymphocytes. Consistent with these *in vivo* data, Am80 moderately, but significantly, suppressed TNF-α-induced *ICAM1* mRNA expression in HDMECs (Figure 4c). Furthermore, relevant to the ICAM-1 suppression in dermal microvascular endothelial cells, BLM-induced infiltration of inflammatory cells, such as mast cells, macrophages, $CD4^+$ T cells, and $CD8^+$ T cells, was significantly attenuated by Am80 (Figure 4d). Collectively, these results indicate that Am80 prevents the infiltration of various types of inflammatory cells in the lesional skin of BLM-treated mice, at least partially by suppressing ICAM-1 expression in endothelial cells.

Am80 inhibits endothelial-to-mesenchymal transition in the lesional skin of BLM-treated mice.

Since endothelial-to-mesenchymal transition (EndoMT) provides activated fibroblasts contributing to tissue fibrosis in BLM-treated mice (Taniguchi *et al.*, 2015), we evaluated

whether Am80 influences this pathological process by immunostaining for VE-cadherin and fibroblast-specific protein 1 (FSP1), markers of endothelial cells and fibroblasts, respectively. In the absence of Am80 administration, the number of VE-cadherin/FSP1 double-positive cells around small vessels in the lower dermis was greater in BLM-treated mice than in PBS-treated mice. In contrast, the number of such cells was much smaller in BLM-treated mice exposed to Am80 than in BLM-treated mice unexposed to Am80 (Figure 5a and 5b). These results indicate that Am80 prevents the induction of EndoMT in the context of BLM-treated skin.

Relevant to EndoMT, we also evaluated pericyte coverage over dermal small blood vessels by immunostaining for α-SMA. The coverage of pericytes was gradually lessened along with the duration of BLM injection in the absence of Am80 treatment, whereas its coverage still remained to a greater extent after 4 weeks' BLM injection in the presence of Am80 treatment (Figure 5c). Importantly, the greater the number of interstitial myofibroblasts, the lesser the dermal microvessels were covered with pericytes (Figure 5c - 5e), suggesting that vascular destabilization is related to the induction of EndoMT in BLM-treated mice. Given that myofibroblasts possibly originate from resident fibroblasts, fibrocytes, and epithelial-to-mesenchymal transition as well as EndoMT, these results suggest that the prevention of EndoMT partially contributes to the impact of Am80 on the fibrotic and vascular aspects of BLM-treated mice.

Am80 directly ameliorates the TGF-β-induced pro-fibrotic phenotype of human dermal fibroblasts.

Finally, we explored if Am80 directly affects the pro-fibrotic phenotype of dermal fibroblasts *in vitro* by focusing on the expression of type I collagen and MMP-1. Am80 significantly reversed

the stimulatory effect of TGF-β1 on *COL1A2* mRNA levels and its inhibitory effect on *MMP1* mRNA levels dose-dependently in normal human dermal fibroblasts (Figure 6a). Consistently, Am80 normalized the altered expression of type I collagen and MMP-1 proteins in TGF-β1-treated normal human dermal fibroblasts (Figure 6b). Additionally, under the same condition Am80 also partially, but significantly, reduced TGF-β1-induced expression of CTGF at mRNA and protein levels (Figure 6c). Luciferase assay and actinomycin D chase analysis revealed that Am80 decreased the *COL1A2* promoter activity and the stability of *COL1A2* mRNA (Figure 6d and 6e). Thus, Am80 directly ameliorates the pro-fibrotic phenotype of activated dermal fibroblasts partly by suppressing type I collagen expression at transcriptional and post-transcriptional levels.

DISCUSSION

As hypothesized, Am80 prevented the development of dermal and hypodermal fibrosis in BLM-treated mice and TSK1 mice, respectively. To elucidate the underlying molecular mechanisms, we focused on three major pathological events related to tissue fibrosis, such as activation of immune cells, endothelial cells, and fibroblasts, in BLM-treated mice. BLM injection characteristically induces Th2/Th17-skewed immune polarization in fibrotic tissues, leading to fibroblast activation (Takahashi *et al.*, 2015; Taniguchi *et al.*, 2015), but Am80 treatment diversely reduced mRNA levels of cytokines produced by Th1, Th2, and Th17 cells in the lesional skin of BLM-treated mice. Under the same condition, the numbers of Th1, Th2, Th17, and regulatory T cells and the proportion of effector T cells were decreased, while the proportion of $CD4^+$ naïve T cells was increased in draining lymph nodes, suggesting that Am80 attenuates the inflammatory state in response to BLM-dependent tissue injury by inhibiting the

differentiation of naïve $CD4^+$ T cells into effector T cells such as Th1, Th2, and Th17 cells. In addition to the altered $CD4^+$ T cell subsets, the number of inflammatory cells, including $CD4^+$ T cells, $CD8^+$ T cells, mast cells, and macrophages, was attenuated in response to Am80 in the lesional skin of BLM-treated mice, which is likely to be at least partially attributable to the Am80-dependent suppression of ICAM-1 and MCP-1. Am80 also skewed macrophages towards an M1-like phenotype from a pro-fibrotic M2-like phenotype in the lesional skin of BLM-treated mice partly by directly acting on macrophages as proved by *in vitro* experiments. Moreover, Am80 ameliorated BLM-induced EndoMT, a potential source of myofibroblasts in this murine model. Finally, we confirmed the direct anti-fibrotic effect of Am80 on dermal fibroblasts stimulated with TGF-β1. In aggregate, these *in vivo* and *in vitro* data demonstrate that Am80 exerts a potent anti-fibrotic effect on dermal fibrosis in a BLM-induced murine SSc model through the inactivation of immune cells, endothelial cells, and fibroblasts, the three major components underlying the pathogenesis of SSc.

Am80 inhibits the differentiation of Th17 and Treg cells and enhances the differentiation of Th1 cells in collagen-induced arthritis, thereby ameliorating the clinical arthritis score and bone destruction in joints (Sato *et al.*, 2010). In experimental autoimmune myositis, Am80 promotes the differentiation of Th1 and Th2 cells, but not that of Th17 cells, thereby suppressing myositis (Ohyanagi *et al.*, 2009). On the other hand, in this study, Am80 alleviated BLM-induced dermal fibrosis and pro-fibrotic immune responses by suppressing the differentiation of $CD4^+$ T cells into effector T cells. These findings suggest that Am80 normalizes various pathological conditions through its pleiotropic effects on the differentiation and the effector functions of $CD4^+$ T cells in the context-dependent manner. However, Am80 may exert an undesirable effect under certain situations, especially infections. For instance, Am80

administration to the mice with *Trichuris muris* infection increases $CD4^+$ T cells infiltration but fails to induce the sufficient resistance to the parasites in large intestine of the mice (Hurst *et al.*, 2013). This report suggests that Am80 administration may hinder the activation of $CD4^+$ T cells in some infections as is the case with BLM-treated mice, which should be noticed in the clinical setting.

Vascular phenotype is also critical for the development of pathological tissue fibrosis. For instance, ICAM-1 deficiency significantly suppresses the development of skin sclerosis in TSK1 mice (Matsushita *et al.*, 2007) and BLM-treated mice (Yoshizaki *et al.*, 2010) by decreasing the infiltration of inflammatory cells. Based on these findings, it is likely that the decreased expression of ICAM-1 in dermal microvascular endothelial cells by Am80 treatment partially accounts for its anti-fibrotic effect on dermal fibrosis through its inhibitory effect on the infiltration of inflammatory cells, including T cells, macrophages, and mast cells, in BLM-treated mice. Importantly, our results indicate that Am80 decreases ICAM-1 expression in dermal microvascular endothelial cells directly *in vitro*. Therefore, Am80 coordinately suppresses ICAM-1 expression directly as well as indirectly owing possibly to the cumulative effects of various inflammatory cytokines.

In addition to the altered expression of cell adhesion molecules, EndoMT is another important phenomenon linking endothelial cell activation to tissue fibrosis. Together with the activation of resident fibroblasts, the recruitment of fibrocytes, and epithelial-to-mesenchymal transition, EndoMT provides activated fibroblasts in the affected organs of various pathological conditions, such as cardiac fibrosis, kidney fibrosis, and pulmonary hypertension (Arciniegas *et al.*, 2007; Chaudhuri *et al.*, 2007; LeBleu *et al.*, 2013). Skin fibrotic conditions are no exception. For instance, EndoMT is promoted in two distinct SSc animal models, BLM-treated mice and

TSK1 mice (Xu *et al.*, 2011). Therefore, the present findings indicate that Am80 ameliorates a pro-fibrotic vascular phenotype in the lesional skin of BLM-treated mice through at least two distinct mechanisms, the suppression of cell adhesion molecules and the inhibition of EndoMT.

Activated macrophages are divided into two major subsets, M1 and M2 macrophages, depending on the expression of various molecules and their functions (Mosser and Edwards, 2008). M2 macrophages secrete components of the extracellular matrix, leading to wound healing or tissue fibrosis (Kreider *et al.*, 2007), and play an important role in the skin and pulmonary involvement of SSc. Here, Am80 induced the differentiation into M1 instead of M2 macrophages *in vivo* and *in vitro*, potentially contributing to the anti-fibrotic effect of Am80 on dermal fibrosis in BLM-treated mice. However, M1-skewed differentiation of macrophages did not result in the increased expression of M1 cytokines such as IFN-γ and TNF-α. It is partially because the infiltration of macrophages into the skin was suppressed by Am80. Additionally or alternatively, the cytokine production of T cells was decreased by Am80. Given the impact of Am80 on macrophage phenotypes in BLM-treated mice, Am80 may exert an anti-fibrotic effect on the pathological tissue fibrosis, such as SSc by shifting macrophages from the M2 to the M1 phenotype.

Finally, we investigated the direct effect of Am80 on the pro-fibrotic phenotype of activated dermal fibroblasts. As demonstrated by reporter assay and actinomycin D chase analysis, Am80 suppressed *COL1A2* gene expression at both transcriptional and post-transcriptional levels. Given that retinoic acid hinders TGF-β1 signaling by decreasing Smad2/3 phosphorylation and restraining their translocation into the nucleus (Yang *et al.*, 2008), similar processes might account for the inhibitory effect of Am80 on the transcription of the *COL1A2* gene in TGF-β1-stimutated fibroblasts. As for mRNA stability, retinoic acids

down-regulate the uptake of transported amino acids, resulting in the reduction of steady state *COL1A1* mRNA levels in human dermal fibroblasts (Varga *et al.*, 1987), which may be the case with *COL1A2* mRNA under the presence of Am80. Further studies are required to elucidate the molecular mechanisms underlying the direct anti-fibrotic effect of Am80 on activated dermal fibroblasts.

In summary, this study is the first to demonstrate that Am80 has an anti-fibrotic effect on BLM-induced SSc model mice by acting on various types of cells, including fibroblasts, endothelial cells, and immune cells. Since Am80 is already used in clinical practice, the current results suggest that Am80 warrants evaluation as a novel anti-fibrotic agent for SSc.

MATERIALS AND METHODS

Mice

Wild type (WT) (C57BL/6) mice were purchased from Japan SLC Inc. (Tokyo, Japan). Female mice, 6-8 weeks old at the beginning of the PBS or BLM treatment, were used. All animal protocols were approved by the Animal Care and Use Committee of University of Tokyo.

A BLM-induced murine model of SSc

BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in PBS at the concentration of 1 mg/ml and sterilized by filtration. BLM (200 μg) or PBS was injected subcutaneously into a single location on the shaved backs of C57BL/6 mice with a 27-gauge needle. The injection was carried out consecutively for 1 week or 4 weeks dependent on the purpose of experiments.

TSK1 mice

TSK1 mice were purchased from The Jackson Laboratory. Genotyping of TSK1 mice was performed by PCR with the following primers (forward 5'-GTTGGCAACTATACCTGCAT-3' and reverse 5'-CCTTTCCTGGTAACATAGGA-3'). Am80 treatment to TSK1 mice was began at the age of 3 weeks and finished at the age of 7 weeks.

Am80 treatment

Am80 was provided by the Research Foundation ITSUU Laboratory (Tokyo, Japan). Feeding pellets were made by mixing Am80 with a commercial rodent diet, MF (Oriental Yeast; Tokyo, Japan). Approximately 1 mg/kg/day Am80 were administered to mice, according to the procedure of Miwako and Shudo (Miwako and Shudo, 2009). Administration was started 1 week before BLM or PBS subcutaneous injection.

Histological assessment and immunohistochemistry

All skin sections were taken from the paramidline, lower back region. Skin sections from TSK1 mice were taken together with hypodermal tissues. Sections were stained with hematoxylin and eosin (H&E), toluidine blue and Masson's trichrome stain. We examined dermal thickness, which was defined as the thickness of skin from the top of the granular layer to the junction between the dermis and subcutaneous fat. Immunohistochemistry was performed using antibodies directed against CD4 (BD Pharmingen, San Diego, CA), CD8 (BD Pharmingen), B220 (BD Pharmingen), F4/80 (Serotec, Eching, Germany), ICAM-1 (BD Pharmingen), and α-SMA (Sigma–Aldrich, St. Louis, MO). All sections were examined independently by two investigators in a blinded manner.

Determination of hydroxyproline content in skin tissue

Following instructions of the QuickZyme Total Collagen Assay kit (QuickZyme Biosciences, Leiden, Netherlands), 6-mm punch biopsy skin samples were hydrolyzed with 6 N HCl and collagen content was quantified.

RNA preparation and quantitative real-time reverse transcription-polymerase chain reaction

Gene expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), as described previously (Akamata *et al.*, 2014). mRNA levels were normalized to those of the *GAPDH* gene. Primer sequences are listed in Table 1.

Flow cytometric analysis

WT mice were treated with BLM for 7 days in the presence or absence of Am80, as described above. The next day, lymphocytes from lymph nodes draining the lesional skin were obtained. For intracellular cytokine staining experiments, cells were stimulated with 10 ng/ml of phorbol myristate acetate and 1 μg/ml of ionomycin (Sigma–Aldrich), in the presence of 1 μg/ml of brefeldin A (GolgiStop; BD Pharmingen) for 4 h. Staining was performed according to the protocol of the anti-mouse/rat Foxp3 staining set (eBioscience, San Diego, CA), using anti-CD4 (RM4-5; BioLegend, San Diego, CA), anti-CD25 (PC01; BioLegend), anti-IL-4 (11B11; BioLegend), anti-IL-17A (TC11.18H10; BioLegend), anti-IFN-γ (XMG1.2; BioLegend), anti-T-bet (4B10; BioLegend), anti-GATA-3 (TWAJ; eBioscience, San Diego, CA), anti-ROR-γt (B2D; eBioscience), and anti-Foxp3 (FJK-16s; eBioscience) antibodies. In cell surface marker

staining experiments, cells were stained with anti-CD4, anti-CD62L (MEL-14; BioLegend), and anti-CD44 (IM7; BioLegend) antibodies. Cells were analyzed on a FACSVerse flow cytometer (BD Biosciences, San Jose, CA).

Macrophage polarization

Thioglycollate-elicited peritoneal macrophages were prepared from WT mice as described previously (Matsumoto *et al.*, 2007). Peritoneal macrophages, cultured overnight, were polarized to M2 macrophages by stimulating with 20 ng/ml of IL-4 in the presence or absence of Am80 for 24 h. Total RNA was isolated from cultured cells and analyzed by qRT-PCR. Additionally, macrophages were stained with anti-CD204 (NSU; R & D systems, Minneapolis, MN) and anti-CD206 (C068C2; BioLegend) antibodies and quantified using flow cytometry.

Cell culture of THP-1 cells

THP-1 cells were purchased from the American Type Culture Collection (Rockville, MA). THP-1 cells in log phase growth were primed with 100 ng/ml of IFN-γ (R&D Systems) for 6 h and stimulated with 100 ng/ml of lipopolysaccharides (InvivoGen, San Diego, CA) in the presence or absence of Am80 for 24 h. Total RNA was isolated from cell lysates, as described above.

Cell culture of human dermal microvascular endothelial cells

HDMECs were purchased from Lonza Ltd. (Basel, Switzerland). HDMECs were treated with Am80 at the indicated concentrations or an equal amount of DMSO, 1 h before the stimulation with 0.5 ng/ml of TNF-α. Cells were cultured for an additional 24 h. Total RNA was isolated

from cell lysates as described above.

Immunofluorescence staining of skin sections

Staining was performed on skin sections of BLM-treated mice used in the above-mentioned experiments, as described previously (Chrobak *et al.*, 2013), using goat anti-mouse VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-mouse FSP1 (Abcam, Cambridge, MA) primary antibodies and examined using Biozero BZ-8100 (KEYENCE, Osaka, Japan).

Cell culture of human fibroblasts

Human dermal fibroblasts were obtained by skin biopsy from the affected area (dorsal forearm) of healthy donors and cultured as described previously (Akamata *et al.*, 2014). Experiments using human samples were performed according to the Declaration of Helsinki and approved by the ethical committee of the University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all patients and healthy controls.

Immunoblotting

Human dermal fibroblasts were stimulated with TGF-β1 in the presence or absence of Am80 for 24 h. Cell lysates were prepared and subjected to immunoblotting, as described previously (Noda *et al.*, 2014) .

Promoter assay

The -772 *COL1A2*/Lux constructs, consisting of a *COL1A2* fragment (+58 to -772 bp relative to the transcription start site) linked to the synthetic firefly luciferase, were prepared from the -772

COL1A2/chloramphenicol acetyltransferase construct which is described previously (Ihn *et al.*, 1996). Normal dermal fibroblasts were transfected with this construct and the promoter activity was determined as described previously (Noda *et al.*, 2014).

Analysis of mRNA stability

To investigate the stability of *COL1A2* mRNA, gene transcription was arrested by the addition of 1.0 μg/ml of actinomycin D (Sigma-Aldrich) to the cell culture medium. Total RNA was extracted from fibroblasts, and *COL1A2* mRNA levels were analyzed by qRT-PCR.

Statistical analysis

Distributions of two unmatched groups were statistically compared using the Mann-Whitney U-test. Significance was defined as a P value of < 0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Am80 ameliorates skin fibrosis in BLM-induced SSc model mice and TSK1 mice.
(a) Skin sections of BLM- or PBS-treated mice in the presence or absence of Am80 administration were stained with hematoxylin & eosin stain (left panels) and Masson's trichrome stain (right panels). The vertical bars show dermal thickness. Representative images are shown (original magnification, × 100) and data of dermal thickness are summarized as a graph. (b) Collagen contents were measured by hydroxyproline assay in each group of mice. (c) mRNA levels of molecules related to extracellular matrix metabolism, such as *Col1a1, Col1a2, Col3a1, Col5a1,* and *Mmp13*, in the lesional skin were assessed by qRT-PCR. (d) mRNA levels of growth factors, such as *Ctgf* and *Tgfb1*, were assessed by qRT-PCR. (e) CTGF expression was evaluated by immunohistochemistry. Arrows indicate CTGF-positive cells. (f) Skin sections of tight skin 1 (TSK1) or wild type (WT) mice treated or untreated with Am80 were stained with hematoxylin & eosin stain (left panels) and Masson's trichrome stain (right panels). The vertical bars show dermal thickness. Representative images are shown (original magnification, × 100) and data of dermal thickness are summarized as a graph. (g) Collagen contents were measured by hydroxyproline assay in each group of mice. Each graph indicates mean ± SD of the indicated parameters (n = 6–12 per group; $*P < 0.05$).

Figure 2. Am80 administration reduces T cell activation.
(a) *Il4, Il6, Il10, Il13, Il17a, Ifng, Tnfa*, and *Mcp1* mRNA levels in the lesional skin of mice were measured by qRT-PCR. (b) Total cell number of the bilateral axillary and inguinal lymph nodes was measured by the flow cytometer. (c) The numbers of IFN-γ-, IL-4-, and IL-17A-positive $CD4^+$ T cells and the numbers of $CD4^+$ T cells expressing the transcription factor T-bet, GATA-3, and ROR-γt were evaluated by the flow cytometry. (d) The number of $Foxp3^+CD4^+CD25^+$ regulatory T cells was determined by the flow cytometer. (e) Proportions of naïve T cells and

effector memory T cells among $CD4^+$ T cells were assessed by the flow cytometry. Each graph represents mean ± SD of the indicated parameters (n = 5–10 per each group, $*P < 0.05$). Representative fluorescence-activated cell sorting plots are shown.

Figure 3. Am80 shifts macrophages from M2 to M1 polarization.

(a, b) *Ym1* and *Fizz1* mRNA levels in lesional skin samples of mice (a) and in IL-4-stimulated peritoneal macrophages with or without Am80 pretreatment (b) were assessed by qRT-PCR. (c, d) Cell surface expression levels of CD204 and CD206 were analyzed by the flow cytometry in peritoneal macrophages stimulated with IL-10 and TGF-β1 (c). The average expression levels of CD204 and CD206 are represented as relative mean fluorescence intensity (MFI) (d). (e, f) mRNA expression levels of *Inos* and *Il12a* in the lesional skin of mice (e) and those of *INOS* and *IL12A* mRNA levels in THP-1 cells stimulated with IFN-γ and lipopolysaccharides (f) were assessed by qRT-PCR. Each graph represents mean ± SD of the indicated parameters (n = 4–8 per group; $*P < 0.05$).

Figure 4. Am80 reduces the expression of ICAM-1 on dermal microvascular endothelial cells.

(a, c) *Icam1* mRNA expression in the lesional skin of mice (a) and *ICAM1* mRNA expression in TNF-α-stimulated human dermal microvascular endothelial cells in the presence or absence of Am80 (c) were assessed by qRT-PCR. (b) Lesional skin sections of mice were stained with anti-ICAM-1 antibody. Representative images are shown. (d) Skin sections were stained with antibodies against F4/80, CD4, and CD8 and with toluidine blue, and the numbers of positive cells per high power field were counted. Each graph represents mean ± SD of the indicated parameters (n = 4–8 per group; $*P < 0.05$).

Figure 5. Am80 inhibits EndoMT in the lesional skin of BLM-treated mice.

(a, b) Immunofluorescence staining for FSP1 (green), VE-cadherin (red), and DAPI (blue) was performed with skin sections (a). The number of FSP1/VE-cadherin double-positive cells was counted under × 200 magnification (b). (c, d) Skin sections were stained for α-smooth muscle actin (α-SMA). α-SMA-positive cells in vascular walls (c) and α-SMA positive spindle cells in dermis (d) were recognized as pericytes and myofibroblasts, respectively. (e) The number of α-SMA positive myofibroblasts was counted under × 200 magnification. Each graph represents mean ± SD of the number of positive cells (n = 4–8 per group; $*P < 0.05$).

Figure 6. Am80 has an anti-fibrotic effect on normal human fibroblasts.

(a) Normal human fibroblasts were stimulated with TGF-β1 for 24 h in the presence or absence of Am80 treatment. *COL1A2* and *MMP1* mRNA levels were determined by qRT-PCR. (b) The protein levels of type I collagen and MMP1 were assessed by immunoblotting. Representative images are shown. (c) *CTGF* mRNA levels and CTGF protein levels in TGF-β1-stimulated fibroblasts with or without Am80 treatment were assessed by qRT-PCR and immunoblotting, respectively. (d) The transcriptional activity of the *COL1A2* promoter was determined by luciferase reporter assay. (e) The stability of *COL1A2* mRNA in TGF-β1-stimulated normal human dermal fibroblasts with or without Am80 treatment was assessed by actinomycin D chase analysis. Each graph represents mean ± SD of the indicated parameters (n = 4–6 cell lines per group; $*P < 0.05$, $**P < 0.01$). The values below each blot represent the relative levels of target molecules normalized by loading controls with densitometry.