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SUPPLEMENTARY MATERIALS

Materials and Methods

Immunocytochemistry

Mouse 3T3-L1 preadipocytes were incubated in DMEM containing 10% FBS and 5 μg/ml insulin in the presence of 10 μM rosiglitazone or 5 μM CDDO for 7 days. Fibroblasts were pretreated with 2.5 μM CDDO for 15 min, followed by incubation in media with 10 ng/ml TGF-β2 for 24 h. At the end of the incubation, cells were fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), incubated with primary antibodies against perilipin (1: 400 dilution, Abcam) or Type I collagen (1:200, Southern Biotechnology) for 120 min, followed by Alexa® 594-conjugated secondary antibodies (Invitrogen) for 60 min. Nuclei were identified by 4'-6-diamidino-2-phenylindole (DAPI) staining. Non-immune IgG was used as a negative control in each experiment. Following stringent washing, slides were examined under a Zeiss UV Meta 510 confocal microscope (Carl Zeiss, Jena, Germany). Each experiment was repeated at least three times with consistent results.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from cultured cells or mouse skin biopsies using Quick RNA™ Miniprep (Zymo Research, Irvine, CA), and reverse transcriped for real-time quantitative PCR (qPCR) using SuperScript First-strand synthesis system (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Real-time qPCR reactions were performed on an ABI-Prism 7300 sequence detection PCR machine (Applied Biosystem, Forster City, CA) [1] using PCR primers

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shown in Table S1. mRNA expression levels normalized with *Gapdh* or *36B4* levels in each sample were determined by calculating $\Delta\Delta C_{t.}$

Western analysis

At the end of the incubation periods, cultures were harvested, and equal amounts of whole cell lysates (5–15 µg) were subjected to electrophoresis in tris–glycine 4–15% gradient gels [1]. Membranes were incubated with the following primary antibodies: Type I collagen (1: 400, Southern Biotechnology, Birmingham, AL), phospho-Smad2, E-cadherin, phospho-Akt^{S473}, total Akt (both 1:1000, Cell Signaling Technology, Danvers, MA), Smad1/2/3, histone H4, vimentin (both 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (1:3000, Invitrogen), followed by appropriate secondary antibodies. Antigen–antibody complexes were visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL). To quantify bands intensity normalized to loading controls, ImageJ software based analysis was used (http://rsb.info.nih.gov/ij/).

Plasmids and transient transfection assays

The PPRE-luc contains 3 tandem copies of the PPAR response element (PPRE) sequence of the acyl-coenzyme A oxidase gene linked to thymidine kinase and luciferase genes [2]. $[SBE]_4$-TK-Luc contains 4 copies of the consensus Smad-binding element linked to thymidine kinase and luciferase genes [3]. 376Col1A2-luc contains 376 bp of the human Col1A2 promoter and 58 bp of the transcribed sequence linked to the luciferase gene [4]. Fibroblasts at early confluence were transiently transfected with reporter constructs or appropriate empty vectors using Lipofectamine™ LTX (Invitrogen, Carlsberg, CA). Following incubation with CDDO and TGF-βfor 24 h, cultures were harvested and whole cell lysates were assayed for

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their luciferase activities using the dual-luc reporter assay system (Promega, Madison, WI) [5]. pRL-TK Renilla luciferase (pRL-TK-Luc) was used in each experiment as an internal control and experiments were performed in triplicate.

Cell migration assay

Confluent monolayers of A549 cells were incubated in serum-free medium for 12 h, scratch wounds were induced using standard p1000 pipette tips, and cell migration was monitored for up to 48 h by phase contrast microscopy. Gap lengths were determined at indicated intervals at three different sites/sample.

Histochemical and immunohistochemical analysis

Consecutive 4-µm serial sections of paraffin-embedded skin tissue from mice were stained with hematoxylin and eosin (H&E) or Trichrome [6]. Dermal thickness, defined as the distance between the epidermal-dermal and dermal-subcutaneous adipose junctions, was determined at five random locations/slide for each mouse [7]. The total collagen content in skin samples was determined by hydroxyproline assays [8]. For quantification of myofibroblasts, skin sections were immunostained with anti-αSMA antibodies (Sigma-Aldrich) followed by biotinylated secondary antibodies (all Vector labs, Peterborough, UK). Spindle-shaped cells positive for αSMA in the dermis were counted in six randomly chosen high-power fields by two observers in a blinded manner [9].

Results

CDDO attenuates TGF-β-induced epithelial-mesenchymal transition

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Epithelial-mesenchymal transition (EMT) has been suggested to play an important role in fibrosis, we therefore examined how CDDO modulated EMT using human A549 epithelial cells. In the presence of TGF-β, these alveolar epithelial cells readily differentiated into a mesenchymal phenotype with characteristic loss of the epithelial cell marker E-cadherin, and concurrent expression of Type I collagen, vimentin and connective tissue growth factor (Fig. S1 A and B). Pretreatment of the epithelial cells with CDDO markedly reduced the stimulation of *COL1A1, CTGF* and vimentin, and partially abrogated the loss of E-cadherin induced by TGF-β. Moreover, enhanced migration of epithelial cells induced by TGF-β was also significantly attenuated by CDDO (Fig. S1C).

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Figure legends

FigureS1. ***CDDO attenuates epithelial-mesenchymal transition and migration induced by TGF-β.***

A, B. A549 epithelial cells were preincubated with CDDO (2.5 μM) followed by with TGF-β (5 ng/ml) for 72 h. A. RNA was examined by real-time qPCR. The results, normalized with GAPDH, represent the means ± SD of triplicate determinations from a representative experiment. Open bars, DMSO; closed bars, CDDO. * $p<0.05$. B. Western analysis of whole cell lysates. Bands were quantitated by densitometry. Relative levels normalized with GAPDH are shown below the images. Representative images. C. In vitro wound healing assays. Right panel, scratch widths were determined at three different sites/sample. The results are means ± SD of triplicates from an experiment representative of three. * $p < 0.05$.

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Table S1 **Primers used for real-time quantitative PCR**

Human	GAPDH	forward	CATGAGAAGTATGACAACAGCCT
		reverse	AGTCCTTCCACGATACCAAAGT
	Col1A1	forward	GCTGGTGTGATGGGATTC
		reverse	GGGAACACCTCGCTCT
	Col1A2	forward	CGGACGACCTGGTGAGAGA
		reverse	CATTGTGTCCCCTAATGCCTT
	α-SMA	forward	CAGGGCTGTTTTCCCATCCAT
		reverse	GCCATGTTCTATCGGGTACTTC
	CTGF	forward	AGCTGACCTGGAAGAGAACATTAAG
		reverse	GATAGGCTTGGAGATTTTGGGAGTA
Mouse	GAPDH	forward	ATCTTCTTGTGCAGTGCCAGC
		reverse	GTTGATGGCAACAATCTCCAC
	PPARγ2	forward	AACTCTGGGAGATTCTCCTGTTGA
		reverse	TGGTAATTTCTTGTGAAGTGCTCATA
	FABP4	forward	CACCGCAGACGACAGGAAG
		reverse	GCACCTGCACCAGGGC

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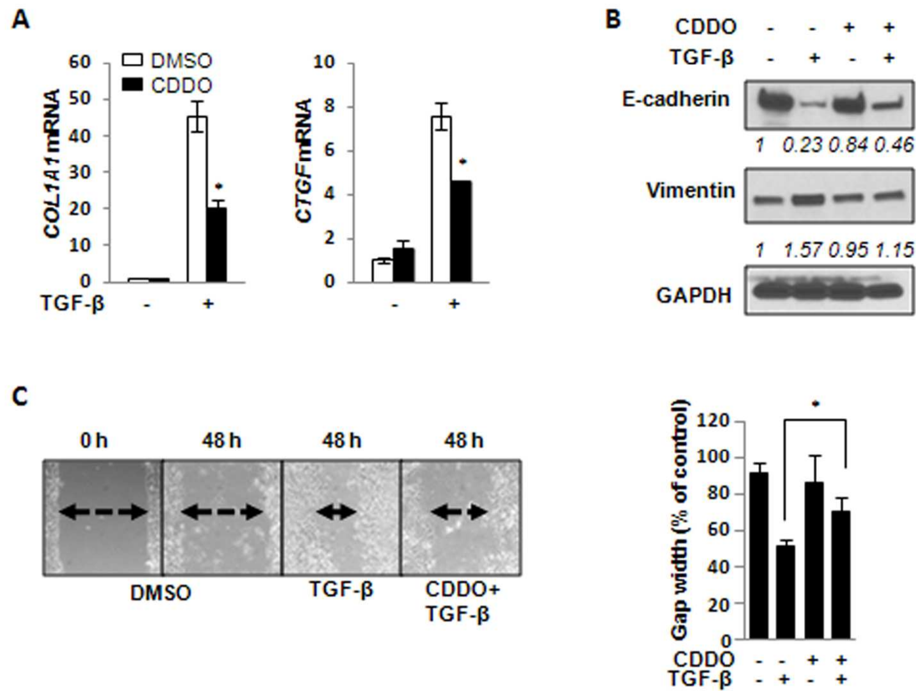

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