Materials and methods

Proteins and Antibodies

Antibodies against human bFGF, TGF-β1 were purchased from Serotec Ltd. Antibodies against VEGF, EGF were purchased from CHEMICON International, Inc. Antibody against PlGF and human VEGF ELISA were purchased from R&D systems, Inc. Recombinant human bFGF, recombinant human EGF were purchased from CytoLab Ltd.

Extraction of Placenta

Porcine placenta was collected from Animal Technology institute Taiwan. Fresh porcine placenta was washed with cold phosphate-buffered saline (PBS, pH 7.4), slashed in a blender for 30 sec. Mashed placenta was immersed in extraction buffers (25% w/v) and stirred for 24 h at 4°C. Extraction buffers used for theses studies included distilled water, 0.1 M NaCl, 1 M $MgCl_2$, and 2 M urea, each prepared in 50 mM Tris-HCl. The extraction mixture was then centrifuged at 5000 rpm (revolutions per minute) for 30 min at 4°C and the supernatant collected (Beckman). The supernatant was dialyzed (3 kDa) and concentrated using Amicon Ultrafiltration Cell (cut-off size, 1 kDa, Millipore Corp.), and then stored at -80°C prior to use. Samples were maintained at 4°C throughout any processing. The protein concentration of porcine placental extract was determined by the method of Bradford with bovine serum albumin as the protein standard. Human placenta collected from Taiwan Adventist Hospital was extracted by the same token.

Protein Electrophoresis and Western Blot

Extracts were mixed in 4 X sample buffer, boiled for 5 min, and then subjected to 15% SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) in Tris-Glycine-SDS running buffer

(Hoefer scientific instruments). After proteins were transferred onto PVDF (polyvinylidene difluoride) membrane in transfer buffer (Bio-Rad semi-dry transfer units), the membrane was blocked with 5% non-fat milk for 2 h at room temperature. Washed three times with 50 ml of PBST and incubated with the following primary antibodies: 1:5000 dilution of anti-mouse anti-TGF-β1; 1:500 dilution of anti-mouse anti-bFGF; 1:5000 dilution of anti-mouse anti-EGF antibody. Following washing with PBST for three times, blot was probe with peroxidase-conjugated horseradish antibody against mouse IgG at 1:2000 dilution. After incubation for 1 h at room temperature, the protein bends were identified by developing the blot with DAB substrate (Sigma-Aldrich, Inc.) or ECL (enhacing chemiluminescence) kit (Amersham Corp.).

Enzyme-link immunosorbent assay (ELISA)

96-well plates were coated with 100μl of PBS containing placental extracts and standard (bFGF, EGF, PlGF) and were incubated overnight at 4°C. After wash with PBS, the remaining protein binding sites were blocked by incubating with 1% BSA for 1 h at room temperature. Primary antibodies were then added to the sample complexes for 1 h incubation at 37°C followed by reaction with second antibodies for 1 h at 37°C. The substrate solution tetramethylbenzidine (TMB, Sigma-Aldrich, Inc.) was added, incubated for 30 min at room temperature, and the staining reaction was stopped by adding 1M H_2SO_4. The absorbance was read at 450 nm (Bio-Rad model 550 microplate reader). VEGF ELISA (R&D systems, Inc.) used as directed by manufacturer.

Cell Culture

The mouse fibroblasts cell line, NIH3T3 (BCRC No 60008, Hsinchu, Taiwan) were maintained in DMEM supplemented with 10% calf serum (CS, Gibco Life Technologies, lot: 726289). The human skin keratinocyt cell line, HaCaT cells were cultured in DMEM

supplemented with 10% fetal calf serum (FCS, Gibco Life Technologies, lot: 715480). HaCaT cells were a generous gift from Dr. Chen SL, Department of Microbiology and Immunology, National Defense Medical Center. Under these culture conditions, the fibroblasts and keratinocytes remain proliferative and undifferentiated. Calf pulmonary artery endothelial cells were grown in MEM supplemented with 20% FCS. CPAE cells were a generous gift from Dr. Lin MT, Department of Biochemistry, National Cheng-Kung University. All the cells were grown at 37°C in the presence of 5% CO_2 and were split by trypsinization.

Proliferation Assay

NIH3T3 fibroblasts (4×10^4), HaCaT keratinocytes (4×10^4), or CPAE cells (2×10^4) were seeded in 24-well plates and allowed to attach for 3 h at 37 °C, respectively. Then, we subjected cells in serum free medium starvation for 24 h, which should have brought most of cells into G0 phase of cell cycle. The absence of serum in cell culture medium mimics the conditions of the healing adult wounds in vivo. In addition, the serum-free conditions were also chosen, to ensure that no factors present in serum could influence the results. After starvation, the medium was replaced with DMEM containing 0.5% serum with various concentrations of porcine placental extracts. Cells cultured in medium containing only 0.5% serum to maintain the minimal physiological condition served as negative control. Cells cultured in medium containing 15% serum served as positive control. After incubation at 37°C for 24 h, 1 mg/ml MTT [3-(4,5-dimethythiazol-2-yl)- 2,5-diphenyltetrazolium bromide] solution was added. After incubation at 37°C for 4 h, MTT solution was carefully decanted to remove untransformed MTT. DMSO was used to dissolve formazan crystals. The absorbance was read at 540nm (Bio-Rad model 550 microplate reader).

Flow cytometic analysis

2×10^5 NIH3T3 cells were seeded in 6-well plate and allowed to attach for 3 h at 37 °C. Then, we subjected cells in serum free medium starvation for 24 h, which should have brought most of cells into G0 phase of cell cycle. After incubation with different placental extracts for 20 hr, cells were harvested in FACS tube. 70 % ethanol was added to cells and incubated at -20°C overnight. Ethanol was then removed by centrifugation at 1000 rpm for 10 min and DNA of the cells was stained with 1 ml of PI staining solution and incubated at room temperature for 30 min protecting from light. Analyzed cells in S phase immediately by CyFlow® SL flow cytometer and analyzed data with FloMax® software (Partec GmbH).

Scratch Assay

24-well plates were coated overnight with 10 μg/ml fibronectin and blocked with 1% BSA. Cells (10^5 cells/well) were plated in DMEM only for 3 h. Monolayer cultures were then wounded with a pipette tip and medium was replaced with DMEM/0.5% serum and placental extracts. After incubation at 37°C for 24 h, cells were photographed at 100× adjacent to a reference line etched on the bottom of the plate (Nikon COOLPIX 4500).

Cell Attachment Assay

96-well plates were coated with 100μl of PBS containing placental extracts and 5 μg/ml fibronectin and then were incubated overnight at 4°C. After rinsing the wells three times with PBS, the remaining protein binding sites were blocked by incubating with 1% BSA for 1h at room temperature. Cells (10^5 cells/well) in 100 μl of Hepes-Tyrode buffer were added to the wells, which were washed three times with PBS previously. After incubating at 37°C for 1 h, the wells were gently rinsed three times with PBS to remove unbound cells. Adherent cells were quantified with a colorimetric reaction using endogenous cellular phosphatase activity by adding 100 μl of substrate/lysis solution to each well. After incubating at 37°C for 1 h, the

reaction stopped by the addition of 50 μl 1N NaOH and read with a spectrophotometer at 405 nm (Bio-Rad model 550 microplate reader). Since fibronectin was important cell adhesion protein that mediated various aspects of cellular associations with ECM through a variety of integrin-ligand interaction (Ayad S et al., 1994), we toke fibronectin as a standard. Standard curves were linear with cell number from 10^1 to 10^5 cells.

Gelatin Zymography

The gelatinase activity was analyzed by gelatin zymography as described by Liota. For studies on MMPs, HaCaT cells seeded at a density of10^6cells in 10-cm Petri-dish were washed three times with PBS to remove residual growth factors, soluble gelatinases, and inhibitors provided by serum or directly secrted by the cells. Then, cells were starved in serum-free medium for 24 h. To avoid the effects of serum growth factors, cell were cultured in serum-free medium supplemented with various placental extracts for another 24 h. Serum-free conditioned medium samples were collected. Medium samples were mixed with 2X SDS sample buffer and then incubated at 37°C for 10 min, and subjected to 10% SDS-PAGE containing 0.1% bovine skin gelatin. All sample volumes were adjusted for total culture medium volumes. After electrophoresis, the gel was rinsed in 2.5% Triton-X 100 for 1 h, and incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ for 18 h at 37˚C. The gel was stained with 0.5% Coomassie blue R 250 in 40% methanol, 10% acetic acid and destained in the same solution, but without Coomassie blue. Gelatinolytic activity was visualized as a clear band against a dark background of stained gelatin. This is the most sensitive method for identification of MMP2 and MMP9. MMP2 is detected by the clear band appearing at 72 kDa and MMP9 at 92 kDa. The amount of lysis of substrate gel (sum intensity) was quantitated using the Kodak Digital Science 1D Image Analysis Software.

Extraction of Total RNA

Total RNA was isolated from about 3×10^6 keratinocytes, using a modification of the single-step method described by Chomczynski and Sacchi, involving 5-min incubation of the total cell pellets with 1 ml of REzol^{TM} C&T reagent (PROtech Technologies, Inc.). The cell pellets were disrupted by repetitive pushpull. Cell lysates were transferred to RNase-free sterile Eppendorf tubes, and RNA was extracted over 0.2 ml of chloroform by centrifugation (12,000 *g*, 15 min, 4°C). RNA was precipitated from the supernatant phase with 0.5 ml of isopropyl alcohol and washed in 70% (v/v) ethanol. The optical density of RNA resuspended in sterile water was recorded ($A_{260\ nm/280\ nm}$ ratio: 1.8–2.0) (OPRON-300 UV/VIS spectrophotometer).

Reverse Transcription-Polymerase Chain Reaction

Aliquots of 5 μg of total RNA were reverse-transcribed in RT buffer, using oligo(dT) primers and a cDNA synthesis kit used according to the manufacturer's instructions. cDNA was immediately amplified by PCR with MMP-9, and MMP-1 primers (Table 2). Commercial actin primers were run in parallel PCR tests as a control for PCR and RNA extraction efficiency. Aliquots of 25 μl of PCR products in bromphenol blue solution were run together with a scale of DNA ladders. Flood the 1% agarose gel with an ethidium bromide solution (1 μg/ml), and allow it to stain for 1 minute, then destain the gel in ddH_2O for 30 minutes or longer. The bands were visualized under UV light and photographed.

Statistical analysis

Results were presented as the mean ± standard deviation. Experimental results were statistically analyzed using Student's t-test with Microsoft Excel software. *P* values <0.05 were regarded as indicating significant differences.