

## The effects of placental extract on fibroblast proliferation

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### Synopsis

Human placental extract is used in the treatment of skin wrinkles and wounds. To date, no studies have evaluated the effects of placental extract on dermal fibroblast proliferation. To investigate the effects of placental extract versus ascorbic acid on fibroblast proliferation and transforming growth factor (TGF)- $\beta$ 1 expression, cultured human fibroblasts were treated with placental extract (0, 0.08, 0.16, 0.32, and 0.64%) or L-ascorbic acid-2-phosphate magnesium (0, 0.01, 0.1, 1.0, and 10 mM). Fibroblast proliferation was determined by MTT assay, and TGF- $\beta$ 1 protein expression was analyzed by ELISA. The proliferation of fibroblasts increased significantly after treatment with placental extract at concentrations of 0.32 and 0.64% and with L-ascorbic acid-2-phosphate magnesium at concentrations of 1.0 and 10 mM. Placental extract demonstrated no significant effects on TGF- $\beta$ 1 expression; however, TGF- $\beta$ 1 expression significantly increased after treatment with ascorbic acid at concentrations of 1.0 and 10 mM. Placental extract and ascorbic acid had similar effects on fibroblast proliferation; however, placental extract did not significantly increase TGF- $\beta$ 1 protein expression.

### INTRODUCTION

Human placenta is a rich reservoir of bioactive molecules, including hormones, proteins, lipids, nucleic acids, glucosaminoglycans, amino acids, vitamins, and minerals. It possesses anti-inflammatory (1), anti-anaphylactic, antioxidative, anti-melanogenic (2), melanizing (3,4), moisturizing, and collagen-synthesizing properties, and is generally well tolerated in antiaging, rejuvenation, and aesthetic treatments. Human placenta has been used to treat many skin conditions, including melasma, freckles, wrinkles, atopic dermatitis, xerosis cutis, striae distensae, and wounds.

Ascorbic acid (vitamin C) is an important regulator of collagen expression and appears to increase collagen biosynthesis in an age-dependent manner, serving as a potent stimulator for types I and III collagen expression in dermal fibroblasts. Transforming growth factor (TGF)- $\beta$  plays a central role in the fibrogenic response of mesenchymal

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cells to injury; it may also be involved in the deposition of connective tissue matrix proteins in diverse states such as angiogenesis and organogenesis, as well as in pathological fibrotic states (5,6). TGF- $\beta$ 1 induces collagen synthesis and has been shown to be upregulated in scars. In addition, TGF- $\beta$ 1 has been shown to promote the growth of human fibroblasts into stratified layers, mimicking *in vivo* fibroplasia (7,8). Previous reports have described the anti-melanogenic effects of placental extract; however, in a Medline search of the literature published between 1957 and 2006, we found no studies evaluating the effects of placental extract on fibroblast proliferation associated with collagen synthesis.

We investigated the effects of placental extract on fibroblast proliferation and TGF- $\beta$ 1 expression. To validate our findings, we performed experiments on both placental extract and ascorbic acid, which has been shown to affect fibroblast proliferation. Placental extract increased fibroblast proliferation but did not significantly increase TGF- $\beta$ 1 expression compared to the controls.

## MATERIALS AND METHODS

### HUMAN SKIN FIBROBLAST CULTURE

Primary cultures of normal human skin fibroblasts were established from newborn prepuce in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine 2 mM, penicillin 100 U/ml, and streptomycin 100  $\mu$ g/ml at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. The fibroblasts were cultured to 90% confluence and then subcultured. For all assays, fibroblasts at fourth passages were used.

### TREATMENT OF FIBROBLASTS WITH PLACENTAL EXTRACTS AND ASCORBIC ACID

Cultured human fibroblasts were treated with placental extracts (Melsmon Pharmaceutical Co. Ltd., Tokyo, Japan) and ascorbic acid (L-ascorbic acid-2-phosphate magnesium; Sigma, St. Louis, MO). We stored the placental extract under temperatures between 2° and 8°C, and carried out the experiments as soon as the extract was received from the manufacturer, although the valid period is two years according to the instructions. The fibroblasts were exposed to various concentrations of placental extract (0, 0.08, 0.16, 0.32, and 0.64%) and ascorbic acid (0, 0.01, 0.10, 1, and 10 mM). We regarded cultured human fibroblasts without any treatment as the control. All experiments were performed independently three times.

### CELL PROLIFERATION ASSAY

Cell proliferation was determined by MTT assay. A CellTiter 96 aqueous proliferation assay kit (Promega, Madison, WI) was used for the cell viability test in accordance with the manufacturer's instructions. Fibroblasts were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well and were incubated for 48 h. The fibroblasts were then treated with placental extract (0.08, 0.16, 0.32, and 0.64%) and ascorbic acid (0.01, 0.1, 1, and 10 mM) and incubated for 24 h. A total of 20  $\mu$ l of CellTiter reagent containing tetrazolium

and an electron-coupling reagent was added to each well and catalyzed by mitochondrial dehydrogenase enzymes in metabolically active cells. The plates were incubated for 4 h and the colorimetric absorbance was recorded at 570 nm using a microplate reader. The results were expressed as a percentage of the controls.

#### MEASURING TGF- $\beta$ 1 PROTEIN EXPRESSION BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Fibroblasts were seeded at a density of  $1 \times 10^6$  cells/ml per 100-mm dish and cultured for 48 h. The culture medium was replaced by DMEM without FBS three days before the experiment. The fibroblasts were then treated with different concentrations of placental extract and ascorbic acid for 24 h. Culture supernatants were collected, centrifuged at  $1000 \times g$  for 5 min, and stored at  $-80^\circ\text{C}$  until an ELISA was performed for TGF- $\beta$ 1. The volumes of culture supernatants were adjusted to  $1 \times 10^6$  cells/ml. TGF- $\beta$ 1 protein was quantified using a total human TGF- $\beta$ 1 ELISA kit (R&D Systems, Minneapolis, MN). Briefly, the ELISA plates were coated with 100  $\mu\text{l}$  of antihuman TGF- $\beta$ 1 antibodies that had been diluted in  $1 \times$  Voller's buffer (pH 9.6) and stored at  $4^\circ\text{C}$  overnight. Test samples were activated with 1 N HCl for 10 min and neutralized with 1.2 N NaOH/0.5 M HEPES at room temperature. The plates were washed, and the samples were added in duplicate to individual wells and incubated at room temperature for 2 h. After three washes, 100  $\mu\text{l}$  of biotinylated antibodies diluted in PBS (pH 7.4) containing 0.05% Tween 20 was added for 1 h. After washing, 100  $\mu\text{l}$  of streptavidin-horseradish peroxidase (HRP) conjugate that had been diluted to 1:20,000 in a dilution buffer was added for 1 h. After a final wash, 200  $\mu\text{l}$  of the HRP substrate tetramethylbenzidine dihydrochloride and hydrogen peroxide in 0.05 M phosphate-citrate buffer (pH 5.0) were added for 30 to 60 min. The reaction was stopped by adding 50  $\mu\text{l}$  of 1 M sulfuric acid, and the absorbance at 450 nm was determined with an EMax microplate reader (Molecular Devices, Sunnyvale, CA). Protein levels were determined by comparing the absorbences produced by the test samples versus those produced by the standards. Existing levels of TGF- $\beta$ 1 in placental extract were excluded in the measures of the placental extract-treated groups.

#### STATISTICAL ANALYSIS

Statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL). Student's two-tailed *t*-test was used to evaluate the differences between the study groups. *P* values less than 0.05 were considered statistically significant.

## RESULTS

#### EFFECTS OF PLACENTAL EXTRACT AND ASCORBIC ACID ON FIBROBLAST PROLIFERATION

To clarify the effects of placental extract on fibroblast proliferation, fibroblasts were treated with placental extract at concentrations of 0, 0.08, 0.16, 0.32, and 0.64% and ascorbic acid at concentrations of 0, 0.01, 0.1, 1.0, and 10 mM. Placental extract concentrations of 0.08 and 0.16% (% of controls,  $103.4 \pm 5.9\%$  and  $104.9 \pm 3.4\%$ , respectively) did not show a significant effect on fibroblast proliferation compared to the

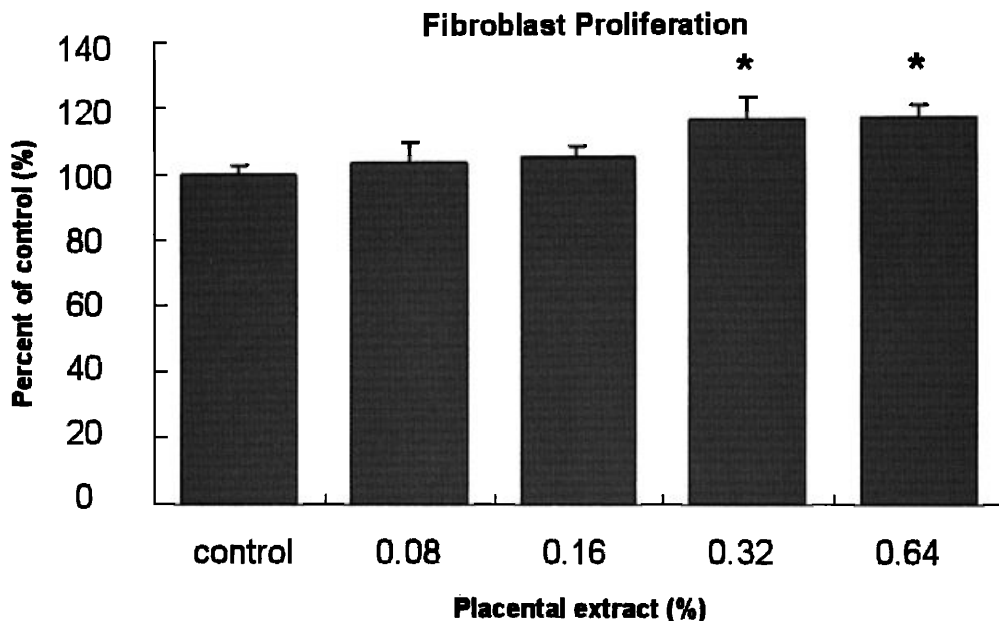
controls ( $100 \pm 2.7\%$ ); however, concentrations of 0.32 and 0.64% ( $116.3 \pm 6.4\%$  and  $116.7 \pm 3.3\%$ , respectively) showed a significant effect on fibroblast proliferation compared to the controls ( $p < 0.05$ ) (Figure 1). Ascorbic acid concentrations of 0.01 and 0.1 mM ( $110.2 \pm 9.4\%$  and  $110.6 \pm 27.5\%$ , respectively) did not show a significant effect on fibroblast proliferation compared to the controls ( $100 \pm 3.1\%$ ); however, concentrations of 1.0 and 10 mM ( $120.9 \pm 24.1\%$  and  $135.3 \pm 33.6\%$ , respectively) showed a significant effect on fibroblast proliferation compared to the controls ( $p < 0.05$ ) (Figure 2).

#### EFFECTS OF PLACENTAL EXTRACT AND ASCORBIC ACID ON TGF- $\beta$ 1 EXPRESSION

The expression of TGF- $\beta$ 1 was determined by ELISA. No significant differences in TGF- $\beta$ 1 expression were observed after treatment with placental extract at concentrations of 0.08, 0.16, 0.32, and 0.64% ( $99.9 \pm 3.7\%$ ,  $99.4 \pm 7.2\%$ ,  $100.2 \pm 6.4\%$ , and  $106.9 \pm 0.9\%$ , respectively) compared to the controls ( $100 \pm 4.2\%$ ) ( $p < 0.05$ ). Moreover, TGF- $\beta$ 1 expression did not increase significantly when fibroblasts were treated with a concentration of placental extract greater than 0.64%. Conversely, a significant increase in TGF- $\beta$ 1 expression occurred after treatment with ascorbic acid at concentrations of 1.0 and 10 mM ( $107.3 \pm 9.7\%$  and  $120.33 \pm 17.8\%$ , respectively) compared to the controls ( $100 \pm 5.4\%$ ) ( $p < 0.05$ ). The results are shown graphically in Figures 3A and 3B.

#### DISCUSSION

The activity of human placental extract has become a matter of increasing interest, and



**Figure 1.** The effects of placental extract on fibroblast proliferation. Fibroblasts were treated with placental extract at concentrations of 0.08, 0.16, 0.32, and 0.64%. Concentrations of 0.32 and 0.64% resulted in significant differences in fibroblast proliferation compared to controls. \*Significantly different from controls ( $p < .05$ ).

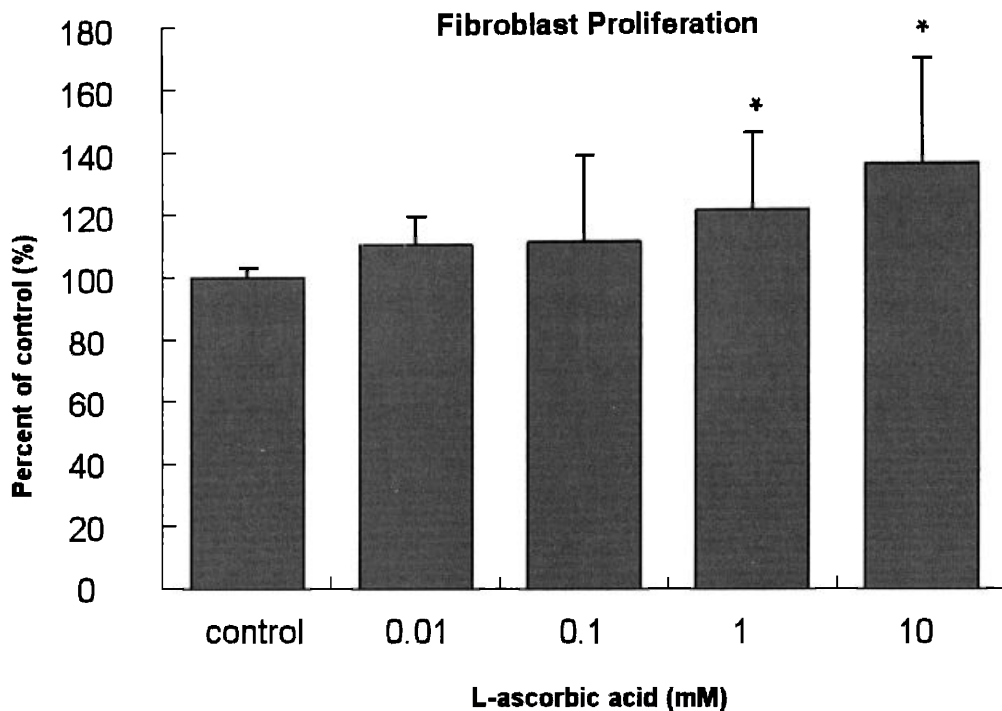
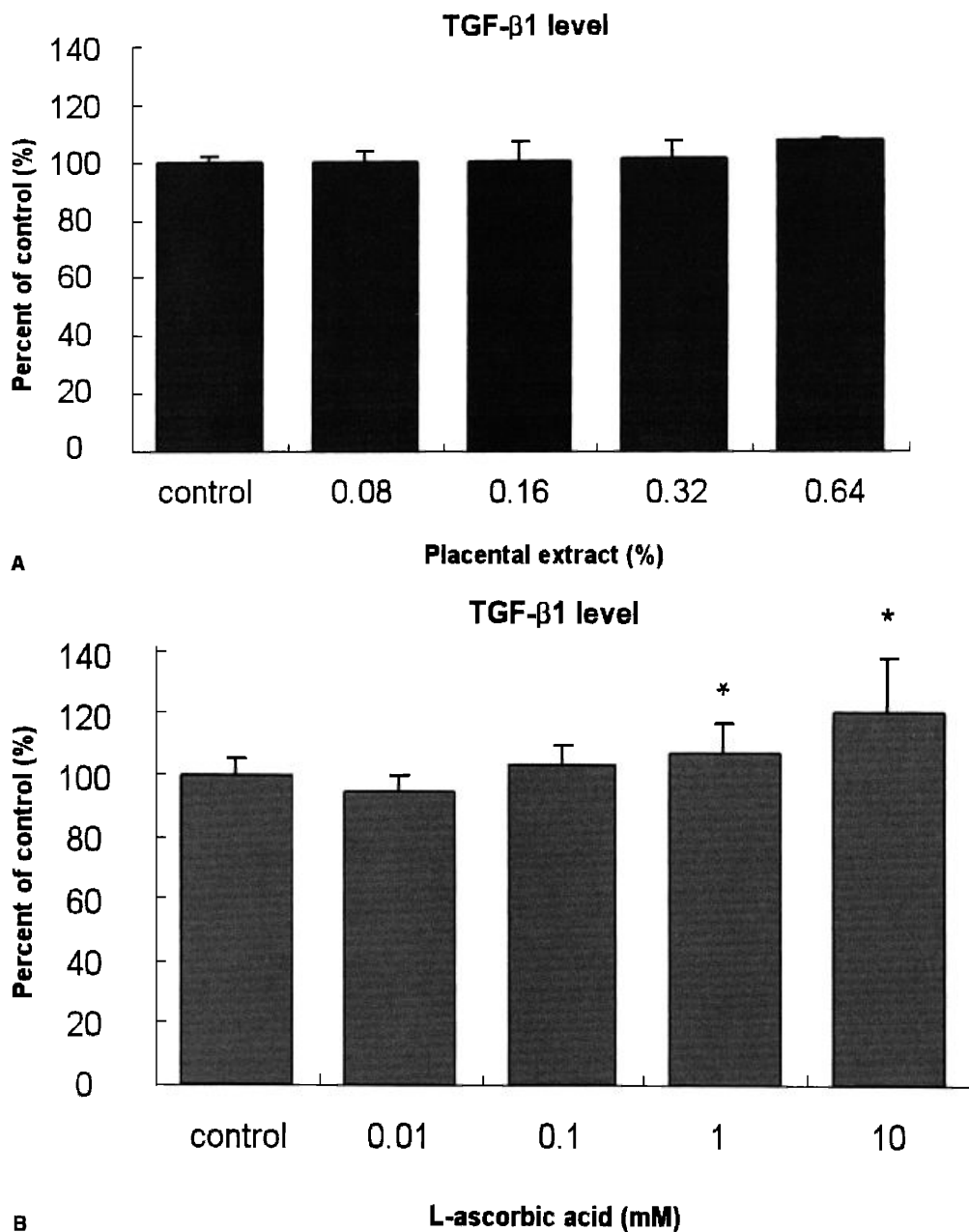


Figure 2. The effects of ascorbic acid on fibroblast proliferation. Fibroblasts were treated with placental extract at concentrations of 0.01, 0.1, 1.0, and 10 mM. Concentrations of 1.0 mM and 10 mM resulted in significant differences in fibroblast proliferation compared to controls. \*Significantly different from controls ( $p < .05$ ).

it has been shown to increase peripheral circulation, stimulate cell respiration and tissue metabolism, reduce inflammation, prevent allergies and pigmentation, promote granulation and removal of old keratin, moisturize, and eliminate activated oxygen. Thus, many dermatologists are interested in its therapeutic properties. Human placenta is an extremely rich reservoir of bioactive molecules. The presence of bioactive peptides in human placenta, such as endothelin (ET)-1 (9,10), adrenocorticotrophic hormone (ACTH) (11,12), and sphingolipids (13,14) is well documented (15). ET-1 is a versatile peptide that demonstrates significant mitogenic (16), dendricity-inducing (17,18) and melanogenic (19) activity in melanocytes. ACTH has been reported to play an important role in melanogenesis (20). Sphingolipids and their metabolites act as crucial second messenger molecules that control the rheostatic switch that balances cell growth promotion and inhibition signals (21–23).

Human placenta has been used in cosmetics and skin-care soaps due to its anti-inflammatory, anti-anaphylactic, antioxidative, anti-melanogenic, melanizing, moisturizing, and collagen-synthesizing properties. In a previous study, we investigated the effects of placental extract on the proliferation and melanogenesis of pigment cells and showed that placental extract may be an effective agent in the treatment of pigment disorders aggravated by ultraviolet light (24). In a separate *in vitro* study, Sarkar *et al.* showed that placental protein/peptide fraction-mediated increases in tyrosinase expression occurred via transcriptional upregulation, which stimulated melanogenesis in



**Figure 3.** TGF- $\beta$ 1 protein expression determined by ELISA. (A) No significant difference in TGF- $\beta$ 1 expression was observed after treatment with placental extract at all concentrations. (B) A significant increase in TGF- $\beta$ 1 expression was seen after treatment with ascorbic acid at 1.0 and 10 mM compared to controls. \*Significantly different from controls ( $p < .05$ ).

B16F10 cells and primary melanocytes (29). Although the literature describes the anti-melanogenic effects of placental extract, we were not able to find any studies on the effects of placental extract on fibroblast proliferation-associated collagen synthesis. Thus, we compared the effects of placental extract on fibroblast proliferation with those of

ascorbic acid (L-ascorbic acid-2-phosphate magnesium), a known stimulator of collagen synthesis. The growth factors contained in placental extract include hepatocyte growth factor, nerve growth factor, epidermal growth factor, fibroblast growth factor, colony-stimulating factor, insulin-like growth factor, TGF, and interleukin 1–4 (25). TGF- $\beta$ 1 is a potent stimulator of collagen synthesis and has been shown to increase collagen synthesis via pre- and posttranslational mechanisms (26,27). Activated TGF- $\beta$ 1 down-regulates the expression of collagen-degrading matrix metalloproteinases (MMP-1), and can play an important role in collagen synthesis via fibroblast proliferation. Ascorbic acid has been reported to be involved in a spectrum of disease states (28). As a co-factor for collagen synthesis, ascorbic acid is one of the micronutrients that is important for wound and burn healing, and appears to increase collagen biosynthesis in an age-independent manner.

In the present study, we examined the effects of both placental extract and ascorbic acid on fibroblast proliferation, and our findings show that fibroblasts proliferated at specific concentrations of each agent. In addition, we evaluated the influence on TGF- $\beta$ 1, which has been associated with fibroblast proliferation. Fibroblast proliferation is stimulated by several growth factors such as fibroblast growth factor, epidermal growth factor, and transforming growth factor. We only measured transforming growth factor beta-1, known to increase the synthesis of collagen. Thus, we did not investigate factors other than TGF- $\beta$ 1. Further studies are required to clarify whether other factors may contribute to placental extract-induced fibroblast proliferation. We observed significant increases in TGF- $\beta$ 1 expression in the cultured human fibroblasts treated with ascorbic acid, but not in cultured human fibroblasts treated with placental extract. Collagen synthesis requires ascorbic acid as a co-factor and is influenced by various cytokines produced by many cells. In our study, the placental extract-induced fibroblast proliferation appeared to be mildly influenced by TGF- $\beta$ 1 or stimulated by factors other than TGF- $\beta$ 1. The benefits of placental extracts have been overstated and excessively publicized in comparison to the actual established clinical results. The efficacy of placental extract remains controversial, and placental extract must be used appropriately, in accordance with the findings of clinical investigations. Although the extract may have use in the treatment of skin wrinkles and wounds, further investigation is required to elucidate its safety and efficacy in comparison with other agents.

## REFERENCES

- (1) T. K. Sur, T. K. Biswas, L. Ali, and B. Mukherjee, Anti-inflammatory and anti-platelet aggregation activity of human placental extract, *Acta Pharmacol. Sin.*, **24**, 187–192 (2003).
- (2) H. Itho, Functions and whitening effect of placenta, *Fragrance J.*, **6**, 67–71 (1990).
- (3) S. Mallick, S. K. Mandal, and R. Bhadra, Human placental lipid induces mitogenesis and melanogenesis in B16F10 melanoma cells, *J. Biosci.*, **27**, 243–249 (2002).
- (4) P. Pal, S. Mallick, S. K. Mandal, *et al.*, A human placental extract: *In vivo* and *in vitro* assessments of its melanocyte growth and pigment-inducing activities, *Int. J. Dermatol.*, **41**, 760–771 (2002).
- (5) R. A. Ignatz and J. Massague, Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix, *J. Biol. Chem.*, **261**, 4337–4345 (1986).
- (6) D. R. Edwards, G. Murphy, J. J. Reynolds, *et al.*, Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor, *EMBO J.*, **6**, 1899–1904 (1987).
- (7) R. A. Clark, G. A. McCoy, J. M. Folkvord, and J. M. McPherson, TGF-beta 1 stimulates cultured

- human fibroblasts to proliferate and produce tissue-like fibroplasia: A fibronectin matrix-dependent event, *J. Cell. Physiol.*, 170, 69–80 (1997).
- (8) A. B. Campaner, L. M. Ferreira, A. Gragnani, *et al.*, Upregulation of TGF-beta 1 expression may be necessary but is not sufficient for excessive scarring, *J. Invest. Dermatol.*, 126, 1168–1176 (2006).
- (9) B. M. Wilkes, M. Susin, P. F. Mento, Localization of endothelin-1-like immunoreactivity in human placenta, *J. Histochem. Cytochem.*, 41, 535–541 (1993).
- (10) A. Benigni, F. Gaspari, S. Orisio, *et al.*, Human placenta expresses endothelin gene and corresponding protein is excreted in urine in increasing amounts during normal pregnancy, *Am. J. Obstet. Gynecol.*, 164, 844–848 (1991).
- (11) A. Liotta, R. Osathanondh, K. J. Ryan, and D. T. Krieger, Presence of corticotrophin in human placenta: Demonstration of *in vitro* synthesis, *Endocrinology*, 101, 1552–1558 (1977).
- (12) T. Shibasaki, E. Odagiri, K. Shizume, and N. Ling, Corticotrophin releasing factor-like activity in human placental extracts. *J. Clin. Endocrinol. Metab.*, 55, 384–386 (1982).
- (13) P. Strasberg, A. Grey, I. Warren, and M. Skomorowski, Simultaneous fractionation of four placental neutral glycosphingolipids with a continuous gradient, *J. Lipid Res.*, 30, 121–127 (1989).
- (14) S. B. Levery, E. D. Nudelman, M. E. K. Salyan, and S. Hakomori, Novel tri- and tetrasialosylpoly-N-acetyllactosaminyl gangliosides of human placenta: Structure determination of pentadeca- and eicosaglycosylceramides by methylation analysis, fast atom bombardment mass spectrometry and 1H-NMR spectroscopy, *Biochemistry*, 28, 7772–7781 (1989).
- (15) P. Strasberg, A. Grey, I. Warren, and M. Skomorowski, Simultaneous fractionation of four placental neutral glycosphingolipids with a continuous gradient, *J. Lipid Res.*, 30, 121–127 (1989).
- (16) G. Imokawa, Y. Yada, and M. Miyagishi, Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes, *J. Biol. Chem.*, 267, 24675–24680 (1992).
- (17) T. Horikawa, D. A. Norris, J. J. Yohn, *et al.*, Melanocyte mitogens induce both melanocyte chemokinesis and chemotaxis, *J. Invest. Dermatol.*, 104, 256–259 (1995).
- (18) M. Hara, M. Yaar, and B. A. Gilchrist, Endothelin-1 of keratinocyte origin is a mediator of melanocyte dendricity, *J. Invest. Dermatol.*, 105, 744–748 (1995).
- (19) G. Imokawa, M. Miyagishi, and Y. Yada, Endothelin-1 as a new melanogen: Coordinated expression of its gene and tyrosinase gene in UVB-exposed human epidermis, *J. Invest. Dermatol.*, 105, 32–37 (1995).
- (20) G. Hunt, C. Todd, S. Kyne, and A. J. Thody, ACTH stimulates melanogenesis in cultured human melanocytes, *J. Endocrinol.*, 140, R1 (1994).
- (21) Y. A. Hannun, The sphingomyelin cycle and the second messenger function of ceramide, *J. Biol. Chem.*, 269, 3125–3128 (1994).
- (22) S. Spiegel and S. Milstein, Sphingolipid metabolites: Members of a new class of lipid second messengers, *J. Membr. Biol.*, 146, 225–237 (1995).
- (23) S. M. Mandala, R. Thornton, Z. Tu, *et al.* Sphingolipid base-1-phosphate phosphatase: A key regulator of sphingolipid metabolism and stress response, *Proc. Natl. Acad. Sci. USA*, 95, 150–155 (1998).
- (24) H. J. Kim, J. W. Lee, Y. I. Kim, and M. H. Lee, The effect of placental extract on the expression of tyrosinase, TRP-1 and TRP-2 in SK30 melanoma cells, *Korean J. Dermatol.*, 41(12), 1612–1618 (2003).
- (25) M. Presta, P. Mignarti, D. E. Mullins, and D. A. Moscatelli, Human placental tissue stimulates bovine capillary endothelial cell growth migration and protease production, *Biosci. Rep.*, 5, 783–790 (1985).
- (26) C. L. Phillips, S. Tajima, and S. R. Pinnell, Ascorbic acid and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) increase collagen biosynthesis via different mechanisms: Coordinate regulation of pro alpha 1(I) and pro alpha 1(III) collagens, *Arch. Biochem. Biophys.*, 295, 397–403 (1992).
- (27) R. P. Penttinen S. Kobayashi and P. Bornstein, Transforming growth factor  $\beta$  increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability, *Proc. Natl. Acad. Sci. USA*, 85, 1105–1108 (1988).
- (28) H. E. Sauberlich, Pharmacology of vitamin C, *Annu. Rev. Nutr.*, 14, 371–391 (1994).
- (29) C. Sarkar, S. K. Singh, S. K. Mandal, *et al.* Human placental protein/peptides stimulate melanin synthesis by enhancing tyrosinase gene expression, *Mol. Cell. Biochem.*, 285, 133–142 (2006).