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*Original Research*

# Enhancement of fibrinolytic activity of bovine aortic endothelial cells by ginsenoside $Rb_2$[1]

LIU Jian-Wen[2], WEI Dong-Zhi, DU Chang-Bin, ZHONG Jian-Jiang

*State Key Laboratory of Bioreactor Engineering, Institute of Biochemistry, East China University of Science and Technology, Shanghai 200237, China*

**KEY WORDS** vascular endothelium; *Panax ginseng*; saponins; plasminogen activators; fibrinolysis; tretinoin

## ABSTRACT

**AIM:** The effect of ginsenoside $Rb_2$ purified from *Panax ginseng* on fibrinolytic activity of bovine aortic endothelial cells (BAEC) was investigated. **METHODS:** Cellular plasminogen activator (PA) level of the lysates was measured by the chromogenic substrate S-2403. Fibrin underlay technique was carried out to observe fibrinolysis by growing endothelial cells in the culture medium. Cell viability was then determined by measurement of the activity of mitochondrial dehydrogenase. The ability of $Rb_2$ of potentiating cellular PA activity was investigated by measuring the amounts of PA and PA inhibitor-1 (PAI-1) in the culture medium using zymography and reverse zymography. Changes in the expression of urokinase-type PA (uPA), uPA receptor, and PAI-1 mRNA in BAEC after treatment with $Rb_2$ were analyzed by Northern blot. **RESULTS:** $Rb_2$ enhanced cellular PA activity in a concentration-and time-dependent manner. Treatment of BAEC with $Rb_2$ 10 mg/L for 9 h resulted in a 3.5-fold increase of PA activity without a marked cytotoxic effect, as shown by LDH levels in culture. Increased PA levels caused the increase in surface plasmin levels as observed by fibrin underlay technique. $Rb_2$ greatly or moderately increased the amount of urokinase-type PA (uPA) or its inhibitor (PAI-1), present in the culture medium, whereas saponin did not influence mRNA levels of uPA, its surface receptor, and PAI-1, suggesting that $Rb_2$ may stimulate the secretion of uPA without enhancing its gene expression. The enhancement of PA levels by retinoic acid alone, a stimulator of PA synthesis, was potentiated by the simultaneous addition of ginsenoside $Rb_2$ 1 mg/L. Therefore, $Rb_2$ might exert a strong synergism in the synthesis of cellular PA in BAEC. **CONCLUSION:** Ginsenoside $Rb_2$ enhanced the PA activity levels in BAEC as well as the surface plasmin activity of BAEC. $Rb_2$ may stimulate the secretion of uPA without enhancing the gene expression of uPA, uPA receptor (uPAR), and PAI-1.

## INTRODUCTION

Vascular endothelial cells play key roles in the maintenance of fibrinolysis. They produce and secrete two immunologically distinct plasminogen activators (PA), tissue-type PA (tPA) and urokinase-type PA (uPA), in different ratios depending upon the origin of the cells. Endothelial cells also produce and secrete an inhibitor of PA, PA inhibitor-1 (PAI-1) that rapidly inhibits the activity of both PA[1]. Hence, the balance between the production of PA and PAI-1 from endothelial cells is important to maintain a normal fibrinolytic status. It has been demonstrated that fibrinolysis reactions proceed on the surface of the cells and /or extracellular matrix and that these surface reactions are physiologi-

[1] Supported by Shanghai Key Discipline.
[2] Correspondence to Prof LIU Jian-Wen. Phn 86-21-6425-3715. Fax 86-21-6425-0068. E-mail liujian@ecust.edu.cn
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cally significant[2,3]. Although the primary physiological role of plasmin is to dissolve fibrin clots, another important role of plasmin, especially cell-associated plasmin is to generate the active form of other enzymes[4] and cytokines[5]. Furthermore, the physiological relevance of cellular PA/plasmin in cell migration or in new capillary blood vessels (angiogenesis) has been pointed out[6].

Ginsenoside-$Rb_2$ is one of the dammarane-type saponins, which do not induce hemolysis, extracted from the root of *Panax ginseng* C A Meyer. Ginsenoside sapanins have been shown to possess various biological activities such as anti-hyperlipidemia[7], immunomodulatory, anti-tumor[8-10], antimetastatic, and anti-angiogenic activities[11,12]. It has been reported that saponins derived from different origins have adjuvanticity and anti-inflammatory effects[13,14]. However, to the best of our knowledge, there has been no report describing the effect of saponins on the fibrinolytic activity of the endothelial cells.

In the present study, we examined the effect of ginsenosides $Rb_2$, derived from *Panax ginseng*, on PA/plasmin levels of bovine aortic endothelial cells (BAEC) in cultures.

## MATERIALS AND METHODS

**Materials** Ginsenosides $Rb_2$ was kindly provided by Korea Tobacco and Ginseng Corporation as well as by Japan Korea Red Ginseng Co, Ltd (Tokyo, Japan). The purity of ginsenosides $Rb_2$ was 97.8 %. The chemical structure of $Rb_2$ is shown in Fig 1. Stock solution of $Rb_2$ was prepared in the 1:1 mixture of dimethyl sulfoxide ($Me_2SO$) and ethanol, and serially diluted into culture medium to yield final concentrations of 0.499 and 0.001 % in ethanol and $Me_2SO$, respestively. These concentrations of ethanol and $Me_2SO$ did not affect production or activity of PA (data not shown).

**Treatment of BAEC with saponins** BAEC were isolated and grown in minimal essential medium (MEM) containing 10 % new calf serum. After cells were grown to confluence, the cultures were rinsed with phosphate-buffered saline (PBS), pH 7.4 and incubated in serum-free MEM containing 0.1 % BSA (MEM-BSA) plus either 0.499 % ethanol-0.001 % $Me_2SO$ or various concentrations of $Rb_2$. At the indicated time points, the medium was aspirated, and the cultures were washed with PBS and lysed with 0.5 % Triton X-100 in Tris-HCl 0.1 mol/L , pH 8.1, then stored at -20 °C until assay of cellular PA levels, or lysed with guanidinium isothiocyanate solution and stored at -80 °C until isolation of RNA.

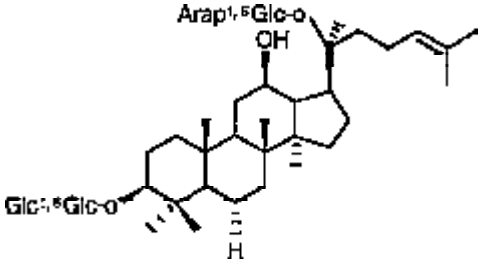


**Fig 1. Chemical structure of ginsenoside $Rb_2$.**

**Assay of cellular PA activity** Cellular PA level of the lysates was measured using the chromogenic substrate S-2403, as described previously[15] and expressed as urokinase (UK) units ($kU \cdot g^{-1}$ protein) in the sample. Protein concentration was measured by BCA (Pierce) assay using BSA as the standard.

**Assay of lactate dehydrogenase (LDH) activity** After BAEC were grown to confluence in 96-well culture plates, the cultures were rinsed with PBS and incubated in serum-free MEM-BSA containing various concentrations of $Rb_2$. At the indicated time points, the medium was collected and the activity of LDH present in each culture medium was measured using a kit obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The amount of resulting diformazan was determined by reading the absorbance at 570 nm and the LDH activity was expressed by Wroblewski units[16].

**Zymography and reverse zymography** Zymography and reverse zymography were carried out as described previously[15]. After proteins in the culture medium were separated by SDS-polyacrylamide gel electrophoresis, the gels were washed with 2.5 % Triton X-100 and NaCl 9 g/L, applied onto fibrin-agar gels containing plasminogen without (zymography) or with (reverse zymography) urokinase, and incubated at 37 °C. The uPA and PAI-1 were visualized, respectively, as either a lysis band in the zymography or a lysis-resistant band in the reverse zymography.

**Isolation of RNA and Northern blot analysis** Total RNA was extracted from cells treated or untreated with $Rb_2$ using acid guanidinium isothiocyanated through 1 g/L agarose-formaldehyde-gel electrophoresis and transferred to Hybond$^N$nylon membranes (Amersham) according to the published protocols[17]. Membranes were hybridized with cDNA for bovine uPA, bovine uPA receptor (gifts from Dr Wolf-Dieter

SCHLEUNING, Research Laboratories of Achering AG, Berlin Germany), human PAI-1 (gift from Dr David J LOSKUTOFF, The Scripps Research Institute, La Jolla, CA), and rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase. The cDNA probes were labeled with [$\alpha$-$^{32}$P] dCTP (DuPont) via random priming using the kit supplied from Boehringer Mannheim. Conditions for hybridization and washing were described previously[18]. Autoradiographies were performed using Fujix BSA 2000 Bio-imaging analyzer (Fuji Photo-Film, Tokyo, Japan).

**Fibrin underlay technique** Fibrin underlay technique was carried out to observe fibrinolysis by endothelial cells grown in the culture medium by the method described previously[19]. The dissolution of a fibrin layer plated under the cells was observed from the surroundings of each cell, reflecting surface plasmin activity.

**Statistical analysis** Each point in every figure represented mean±SD from three similar determinations. Student's *t*-test was used to evaluate the significance between groups and the criterion of statistical significance was taken as $P<0.05$.

## RESULTS

**Effect of $Rb_2$ on PA level in BAEC** In order to know whether saponins affect fibrinolytic property of endothelial cells, the effect of addition of purified $Rb_2$ on cellular PA level in BAEC culture was determined. After confluent BAEC cultures were incubated with serum-free medium containing the indicated amounts of $Rb_2$ for 9 h, cell lysates were prepared, and the levels of PA activity in the lysate were measured (Fig 2, panel A). Saponin $Rb_2$ increased PA activity levels in a concentration-dependent manner. $Rb_2$ 10 mg/L increased PA levels about 3.5-fold (Fig 2, panel A). But at a higher concentration, 100 mg/L, PA level was decreased. The decline seemed to be ascribed to the cytotoxic effect of $Rb_2$, as total protein content of BAEC cultures was reduced after the treatment with $Rb_2$ 100 mg/L (Fig 2, panel B). In addition, the levels of LDH that is known as a cytotoxic protein marker, in the culture medium which was increased 6-fold at $Rb_2$ 100 mg/L (Fig 3). However, ginsenosides $Rb_2$ 1 mg/L, which enhanced PA level, neither declined protein contents, nor increased LDH leakage. Fig 4 depicted the time course of augmentation of PA activity with $Rb_2$ 1 mg/L. Between 4-12 h, ginsenoside $Rb_2$ increased PA levels almost proportionally to the period of treatment. Thereafter, $Rb_2$ continued to enhance PA level up to 72 h. These data

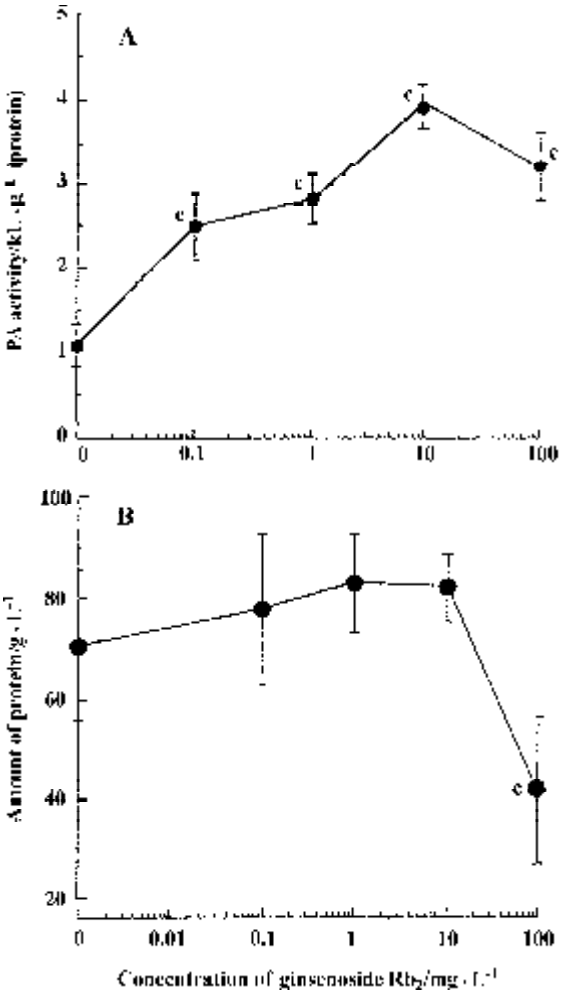


**Fig 2. Concentration-dependent enhancing effect of ginsenoside $Rb_2$ on PA activity levels in BAEC (A) and amount of protein (B). $n$=3. Mean±SD. $^cP<0.01$ *vs* control (0 mg·L$^{-1}$).**

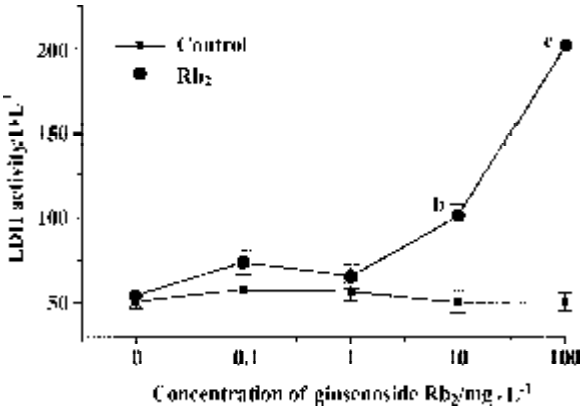


**Fig 3. Effect of ginsenoside $Rb_2$ on the leakage of LDH from BAEC. $n$=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.**

indicated that ginsenosides $Rb_2$ 1 mg/L could enhance PA activity levels in BAEC. Especially, ginsenoside $Rb_2$

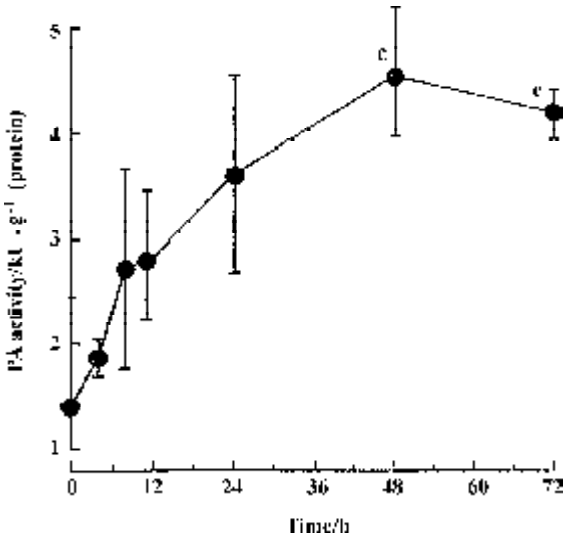


**Fig 4. Time course of enhancement of PA levels induced by ginsenoside $Rb_2$ 1 mg/L in BAEC. $n$=3. Mean±SD. $^cP$<0.01 *vs* control.**

1 mg/L exerted this activity without a marked cytotoxic effect.

**Enhancement of cell surface plasmin level by $Rb_2$** In the presence of a constant amount of plasminogen, changes in PA activity were accompanied by the similar changes in cell surface plasmin levels. Therefore, it was likely that $Rb_2$-induced PA activity provoked the elevation of cell surface plasmin levels in BAEC. To confirm this, the fibrin underlay technique was employed (Fig 5). This assay system could detect the dissolution of a fibrin layer, over which endothelial cells were grown in a culture medium. Seven days after starting the incubation, dissolution area was observed in the control dish (A), reflecting the expression of surface PA/plasmin in BAEC culture. The dissolution area was seen in the dishes containing ginsenoside $Rb_2$ 5 mg/L. Because cells were localized around the center or below to the right, dissolution area expanded ununiformly. The result indicated that $Rb_2$ enhanced surface plasmin activity of BAEC (Fig 5).

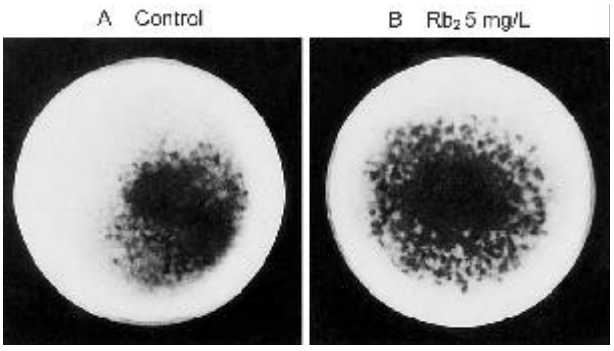


**Fig 5. Dissolution of fibrin layer by BAEC in the absence (A) and the presence of ginsenosides $Rb_2$ 5 mg/L (B). The medium was changed every 2 days.**

**Changes in mRNA levels of PA and PAI-1 after the treatment with $Rb_2$** To analyze the ability of $Rb_2$ to potentiate cellular PA activity, we measured the amounts of PA and PAI-1 in the culture medium using zymography and reverse zymography. The result was shown in Fig 6. Following treatment of BAEC with ginsenoside $Rb_2$ 5 mg/L, PAI-1 band ($M_r$=55 000) was significantly enlarged (upper bands), whereas the PAI-1 band ($M_r$=50 000) was moderately enlarged (lower bands). However, $Rb_2$ did not affect mRNA level of uPA, uPA receptor (uPAR), and PAI-1 as assessed by Northern blot analysis (Fig 7). Radioactivity of each band was detected on an image analyzer (data not shown), which did not show significant differences between them. These results indicated that $Rb_2$ up-regulated both uPA and PAI-1 levels without enhancing their mRNA levels.

**Synergism between $Rb_2$ and retinoic acid on BAEC PA levels** Because $Rb_2$ up-regulated fibrinolytic levels of BAEC without stimulating biosynthesis of PA, it was likely that $Rb_2$ might exert a synergism between factors that stimulate the biosynthesis of PA. We examined this hypothesis using a combination of $Rb_2$ and retinoic acid (RA), which was shown to be a potent

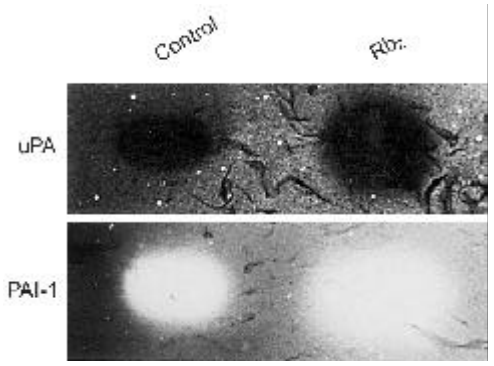


**Fig 6. Effect of ginsenoside $Rb_2$ on secretion of uPA and PAI-1 from BAEC. Confluent BAEC cultures grown in 10-cm dishes were treated with ginsenosides $Rb_2$ 5 mg/L in 6 mL of serum-free MEM for 9 h. Culture medium was collected and concentrated 50-fold on Centrcon and Microcon concentrators (Amicon, Danvers, MA). The amounts of PA and PAI-1 in the concentrate were measured by zymography (upper black bands) or by reverse zymography (lower white bands).**

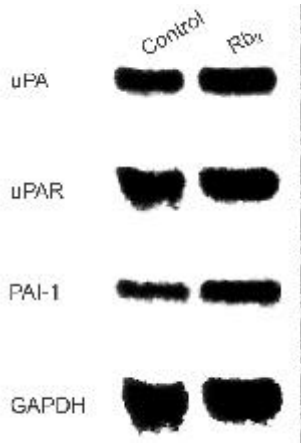


**Fig 7. Northen blot analysis of uPA, uPA receptor, and PAI-1 mRNA level in BAEC after treatment with $Rb_2$.**

stimulator of PA synthesis[20, 21]. RA 0.01 μmol/L enhanced the effect of ginsenoside $Rb_2$ 0.1-10 mg/L on PA activity (Fig 8A) and $Rb_2$ 0.1 mg/L enhanced the effect of RA 0.001-1 μmol/L on PA activity (Fig 8B). Synergistic effect was observed between ginsenoside $Rb_2$ and RA in enhancing the PA levels.

## DISCUSSION

The present paper demonstrated that ginsenosides $Rb_2$ purified from *Panax ginseng,* enhanced fibrinolytic activity of BAEC *in vitro*. $Rb_2$ increased PA level at subcytotoxic concentrations (ie 1-5 mg/L, 9-12 h), as shown by the leakage of LDH from the cells. $Rb_2$ enhanced both PA and PAI-1 levels without alteration of their mRNA levels, suggesting that the effect of ginsenoside $Rb_2$ may be related to the change of the permeability of the cell membrane. Because the induction of PA was more significant than that of PAI-1, the total PA activity would be increased. Therefore, it appears that there are preferences among molecules to permeate membrane by saponin and that the secretion of PA is preferentially increased than that of PAI-1. It is notable that ginsenoside $Rb_2$ and RA showed a strong synergism in the enhancement of cellular PA levels. This was supposed to be due to that RA enhanced the biosynthesis of PA[20, 21] and $Rb_2$ stimulated its secretion as shown in the present report. The synergism suggests the advantage that we can lower the concentrations of $Rb_2$ and RA to less cytotoxic concentrations of each other to enhance cellular fibrinolytic levels.

We have reported that the enhancement of cellular PA/plasmin levels by RA causes the activation of latent

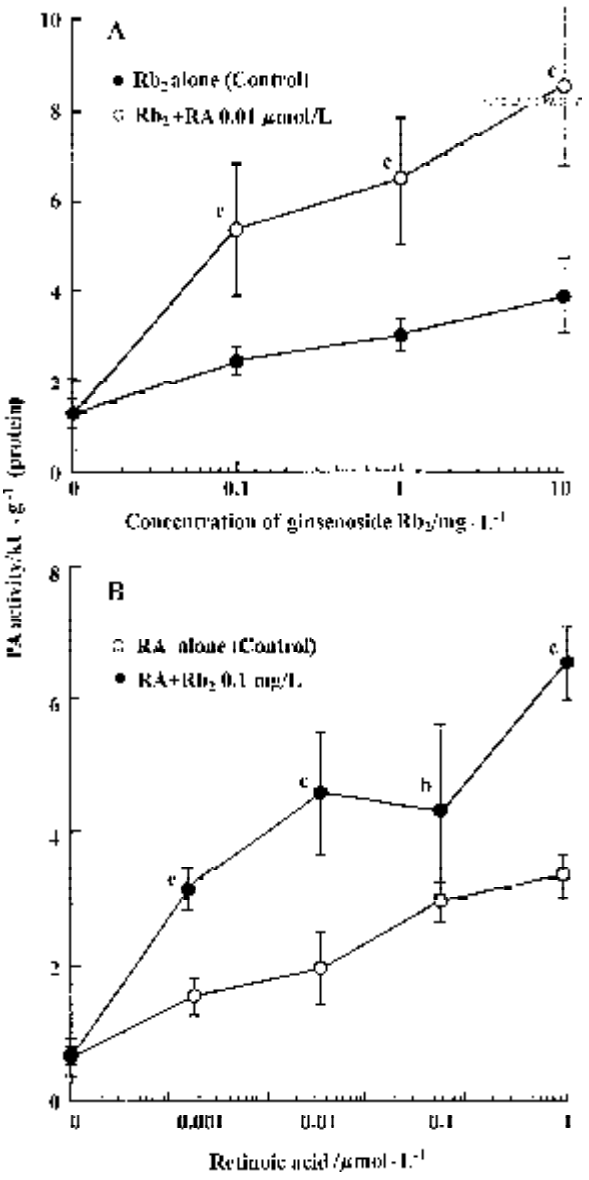


**Fig 8. Effect of simultaneous addition of ginsenoside $Rb_2$ and retinoic acid (RA) on BAEC PA levels. Confluent BAEC were treated for 9 h either with various concentrations of $Rb_2$ in the presence and absence of RA 0.01 m mol/L (A) or with various concentrations of RA in the presence and the absence of $Rb_2$ 0.1 mg/L (B). Cells lysates were prepared and the PA activities were determined as before. $n$=3. Mean±SD. $^bP$<0.05, $^cP$<0.01 *vs* control.**

TGF-β and that the resultant active TGF-β functions to inhibit *in vitro* migration and growth of BAEC[5,18]. Recently, we found that RA induced suppression of the angiogenesis, which was observed on chorioallantoic membranes, seemed to be mediated by the formation of TGF-β at focal sites. Therefore, it might be possible that, anti-angiogenic activity of ginsenoside saponins reported previously[11,12] might also be mediated by the

formation of TGF-β. We are now examining this hypothesis using both *in vitro* cell cultures as well as *in vivo* chorioallantoic membranes.

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