·Basic Research·

# Inhibition of β1-integrin on apoptosis of rabbit corneal epithelial cells

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## Abstract

• AIM: To investigate the effect of β1-integrin overexpression on the apoptosis of rabbit corneal epithelial cells and the related mechanism.

• METHODS: The plasmid expressing β1-integrin-GFP fusion protein was constructed by polymerase chain reaction (PCR), and this plasmid (β1 group) or the empty vector (mock group) was transfected into rabbit corneal epithelial cells, respectively. The expression of β1-integrin-GFP fusion gene was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. The adhesion of transfected cells to extracellular matrix (ECM) proteins was determined by adhesion assay. The apoptosis of rabbit corneal epithelial cells was assayed by Hoechst 33342 staining and DNA ladder. The phosphorylation of mitogen-activated protein (MAP) kinase was examined by Western blot.

• RESULTS: Rabbit corneal epithelial cells overexpressing β1-integrin-GFP fusion gene were successfully established. Compared with mock group, β1-integrin transfection significantly promoted the adhesion of rabbit corneal epithelial cells to ECM proteins such as laminin, fibronectin, collagen I and collagen IV. β1-integrin overexpression inhibited apoptosis and induced MAP kinase phosphorylation in rabbit corneal epithelial cells ($P<0.05$).

• CONCLUSION: These data suggest that overexpression of β1-integrin confers resistance to apoptosis in rabbit corneal epithelial cells, and MAP kinase pathway may play an important role in this process.

• KEYWORDS: corneal epithelial cells; β1-integrin; apoptosis; MAP kinase

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## INTRODUCTION

Corneal transplantation utilizing cultured corneal epithelial cells *in vitro* overcomes the unavailability of donor corneas and postoperative transplantation rejection of penetrating keratoplasty, which shows its broad prospects. However, how to overcome cellular apoptosis *in vitro*, and obtain corneal epithelial cell with long-term survival and proliferation has been the bottleneck of corneal cell transplantation and the topic of great interest to researchers. β1-integrin family is one of the factors that most correlated to the survival of epithelial cells [1]. In the present article we investigated the possibility and mechanism of inhibition of corneal epithelial cell apoptosis by the overexpression of β1-integrin, and provided theoretical basis for the prevention of corneal epithelial cell apoptosis and clinical application of corneal cell transplantation.

## MATERIALS AND METHODS

**Reagents and Materials** HamsF12 and DMEM (Gibco, Langley, OK, USA) mixed at 1:1,with 100mL/L fetal bovine serum (FBS) (Gibco), 100U/mL penicillin, 100mg/L streptomycin and 0.02g/L glutamine then added and pH value adjusted to be 7.2-7.4, served as cell culture solution. Phosphorylated specific anti-ERK, p38 MAPK, JNK antibody, and total anti-ERK, p38 MAPK, JNK antibody (Cell Signaling, Danvers, MA, USA).

### Methods

**Rabbit corneal epithelial cell culture** Eyeballs of adult Holland rabbits weighing 2.5-3.0kg were extracted aseptically and washed with normal saline after they were executed with air embolism. Corneal tissue which contains only epithelium and stroma was separated on the superclean blenches, and then was digested in 3mL 2.5g/L trypsin at

4℃ overnight. On the next day corneal epithelium was scraped gently with scraper and washed in Hanks solutions for three times. 2mL 100mL/L FBS contained culture solution were then added and when cells dispersed evenly, they was transferred to 24- and 6-well culture plates and incubated in 50mL/L $CO_2$ incubator at 37℃. Cell concentration is adjusted to $4\times10^5$/mL with culture solution.

**Construction of the plasmid expressing β1-integrin-GFP fusion protein** Plasmid pBJ-1, provided by Dr. Y. Takada(Scripps Research Institute, USA), contains full-length encoding sequence of β1-integrin cDNA. Full-length cDNA sequence of β1-integrin lacking termination codon was obtained by polymerase chain reaction (PCR), then inserted with restriction BamH1 and XhoI restriction enzyme cutting sites at ends, and then was assembled into plasmid pT7Blue vector (Novagen, Madison, WI, USA) to form pT7GFβ1. Sequence of cDNA of β1-integrin was determined by DNA sequencer. pT7GFβ1 was digested by BamH1 and XhoI restriction enzyme, and 2.4kb fragment was recycled, and connected to the same restriction enzyme cutting site in pEGFP-N1 vector (Clonetech, Palo Alto, CA, USA) to form β1-integrin-green fluorescein protein (GFP) fusion plasmid pEGFP-N1/GFβ1, and was confirmed by restriction enzyme.

**Gene transfection** Cell concentration of the passage 2-4 cells was adjusted to $4\times10^5$/mL and these cell was transferred to the culture plates until 80% of cells fused. pEGFP-N1/GFβ expressing plasmid (β1 group) and pEGFP-N1 empty vector (mock groupwere transfected into cells using Lipofectamine gene transfection technology (GIBCO-BRL, Grand Island, NY, USA) (refering to company specifications). Transfection solution was aspirated after 12 hours of incubation in 50mL/L $CO_2$ incubator at 37℃ and was added into serum contained culture solution for 2 days incubation.

**Reverse transcription-polymerase chain reaction detection of mRNA expression of transfected gene in transfected cells** Total RNA is extracted by total RNA extraction kit (Nippon Gene, Toyama, Japan) following its instructions. RNA quality is detected by electrophoresis and optic density is detected at 260nm wavelength. All the primers are known as the gene specific by consulting Genbank. GFP primer sequence: 5'-GCAAGCTGACCCTG AAGTTCATC-3,reverse sequence:5'-GGATCTTGAAA TTC ACCTTGATGC-3', length of PCR product is 384bp. Primer sequence of β1-integrin: 5'-AGAATCCAGAGTGTCCCAC TGG-3', reverse sequence: 5'-TTCCCTCATACTTCGGA TTGA-3', length of PCR product is 238bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sequence: 5'-A CGCATTTGGTCGTATTGGG-3', reverse sequence: 5'-TG ATTTTGGAGGGATCTCGC-3', length of PCR product is 231bp. β1-integrin-GFP fusion gene expression is detected using sense β1-integrin and antisense GFP as primers. Product length is 785bp. RT-PCR is conducted by the instructions of first-strand synthesis kit (Takara, Shiga, Japan). The amplifying conditions: degeneration at 95℃ for 1 minute, annealing at 55℃ for 1 minute, extension at 72℃ for 1 minute, 30 cycles. PCR products are detected by 20g/L agarose gel electrophoresis, and observed at long-wavelength light after Ethidium bromide staining.

**Cell adhesion test** Laminin, fibronectin, type I and IV collagen coated 24-well culture plates (BD Company, Franklin Lakes, NJ, US). In the control group, culture plates was coated with 10g/L BSA. At 1 hour after 1g/L BSA blocking all the wells at 37℃. Cells transfected by β1-integrin-GFP fusion gene expression plasmid or empty vector were added to 24-well plate coated with different extracellular matrix proteins with concentration of $5\times10^4$ cells in each well. These cells were incubated in 50mL/L $CO_2$ incubator at 37℃ for 1 hour and rinsed in PBS three times and suspension cells were removed. Number of adhesion cells was evaluated by the absorbance measured by WST-1 proliferation kit.

**Apoptosis measured by Hoechst 33342 staining** $1\times10^5$ of β1-integrin transfected cells or mock transfected cells were cultured in 35mm culture dish. After 10 days, newly prepared Hoechst33342 (Wako, Osaka, Japan) was added to incubate for another 10 minutes at 37℃. Cell morphology was observed under fluorescence microscope. Cells with pyknosis or karyorrhexis were identified as apoptotic. Number of apoptotic cells in every 1 000 cell, known as apoptosis index was counted in randomly chosen 10 high power fields.

**Apoptosis detected by DNA ladder** Transfected corneal epithelial cells were collected and rinsed in PBS. After centrifuging, the supernatant was discarded. Cells were resuspended in proper volume of lysis buffer [10mmol/L ethylene diamine-N, N-tetraacetic acid (EDTA), 10mmol/L Tris (pH8.0), 5g/L Triton] for 10 minutes at 4℃. Supernatant was collected after centrifuge at 15 000r/min for 20 minutes. RNAase (100mg/L) was placed in 37℃ water bath for 1 hour. Then proteinase K (100mg/L) was added at 50℃ for 30 minutes. Solution was sedimented in isopropanol at -20℃ overnight. Centrifuge rotated at 15 000r/min for 10 minutes to get sediment which was then washed in 700mL/L ethanol twice. Solution was centrifuged followed by air drying of the sediment which was resolved in pH 8.0 TE

buffer. 20g/L agarose gel (containing 0.5mg/L EB) electrophoresis analysis was conducted for 3 hours with 1×TBE as electrophoresis buffer under persistent voltage of 50V. Results were observed under ultraviolet light and photographed.

**The phosphorylation of mitogen-activated protein kinase examined by Western blot** Corneal epithelial cells 48 hours after transfection were collected and rinsed in PBS. Supernatant was discarded after centrifuge. Cells were resuspended in proper volume of lysis buffer [50mmol/L Tris-HCl (pH 7.5), 150mmol/L NaCl, 5mmol/L EDTA (pH 8.0), 1mmol/L phenylmethylsulfonal fluoride (PMSF), 1g/L SDS, 1mg/L aproptinin, 10mg/L pepstatin, 10mg/L leupeptin, 10mg/L peptin, 10mg/L soybean trypsin inhibito], and sonicated for 45s. Supernatant was preserved at -70℃ after centrifuge at 15 000 r/min for 20 minutes at 4℃. Protein concentration was detected by BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Equal protein sample were applied to the lanes, and electrophoresed in 100g/L sodium dodecyl sulfate-polyacrylamide gel and electrophoretic transferred to polyvinylidene fluoride (PVDF) membrane. Blocking buffer was added. 1∶500 phosphor- ylated specific anti-ERK, p38 MAPK, JNK antibody, and total anti-ERK, p38 MAPK, JNK antibody were added and incubated at 4℃ overnight, and then in 1∶2 000 enzyme- labeled secondary antibody at room temperature for 1 hour. Solution was rinsed in TBS, and developed color with ECL. Film was pressed in dark room.

**Statistical Analysis** Data were analyzed by SPSS 12.0 statistical package (SPSS, Chicago, IL, USA). ANOVA and Dunnett *t* was used for intergroup comparison. $P<0.05$ was considered to be significant.

## RESULTS

**β1-integrin-GFP Fusion Gene Expression in Epithelial Cells** Figure 1A shows the RT-PCR of mock transfected cells and representative β1-integrin transfected cells. mRNA expression of GFP in the two groups was detected, and 785bp strip was found in β1 group, which indicated that β1 GFP gene was successfully transferred into corneal epithelial cells and expressed in the mRNA level. Moreover, mRNA expression level of β1-integrin and GFP-fused detected in β1 group was obviously up-regulated compared to mock group. Increased β1-integrin mRNA was also found in β1-integrin transfected cells compared to mock group. Figure 1B showed in Western blot, 140 000Da band (which represents β1-integrin and GFP-fused protein) and 113 000Da band (which represents β1-integrin protein) were both found

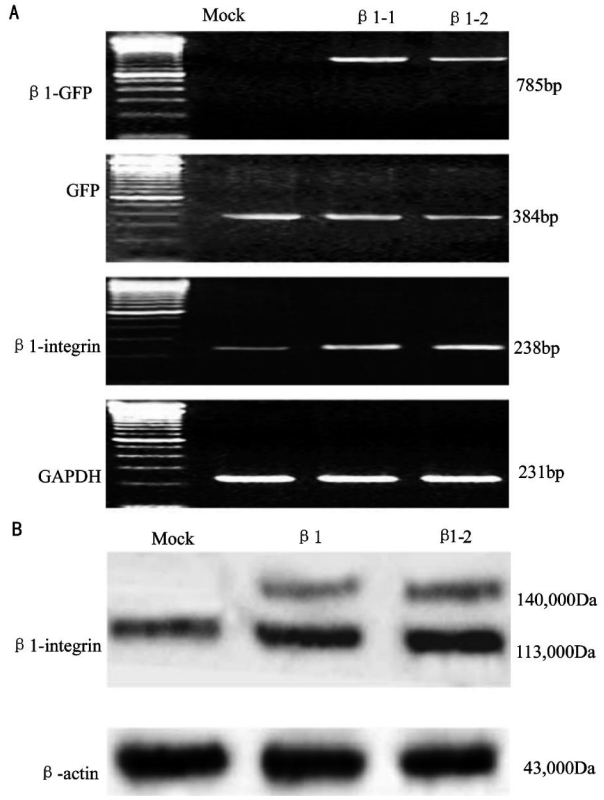


**Figure 1 The expression of transgene in transfected cells detected by RT-PCR and Western blot** A:RT-PCR. β1-integrin and GFP-fused mRNA expression was detected in β1-integrin transfected cells. Increased β1-integrin mRNA was also found in β1-integrin transfected cells compared to mock transfected cells;B: Western blot. β1-integrin and GFP-fused protein expression was detected in β1-integrin transfected cells

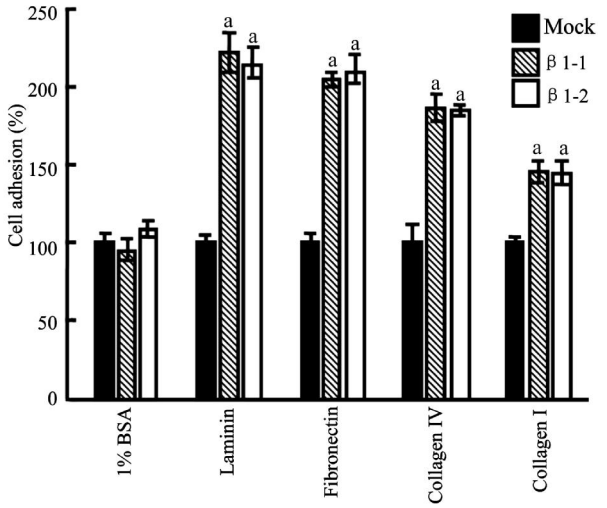


**Figure 2 The adhesive ability of transfected cells to extra-cellular matrix (ECM) proteins ($F=29.198$, $P<0.001$).** Compared with mock transfected cells, the adhesive ability to ECM proteins but not 10g/L BSA was increased in β1-integrin transfected cells. $^{a}P<0.05$, *vs* mock cells

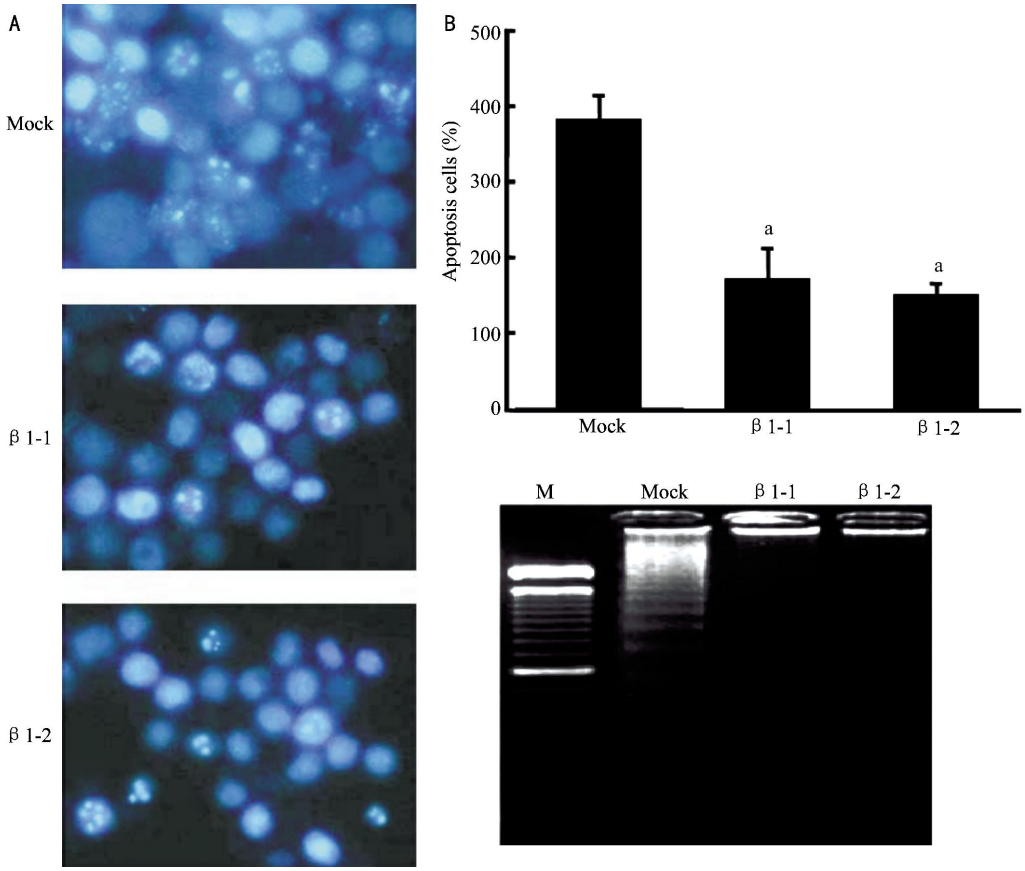


**Figure 3 The result of apoptosis in transfected cells** A:Hoechst 33342 staining;B:The apoptosis cells percentage of mock transfected cells and β1-integrin transfected cells;C:DNA ladder. Compared with mock transfected cells, β1-integrin transfected cells showed resistance to apoptosis after 6-10 days transfection

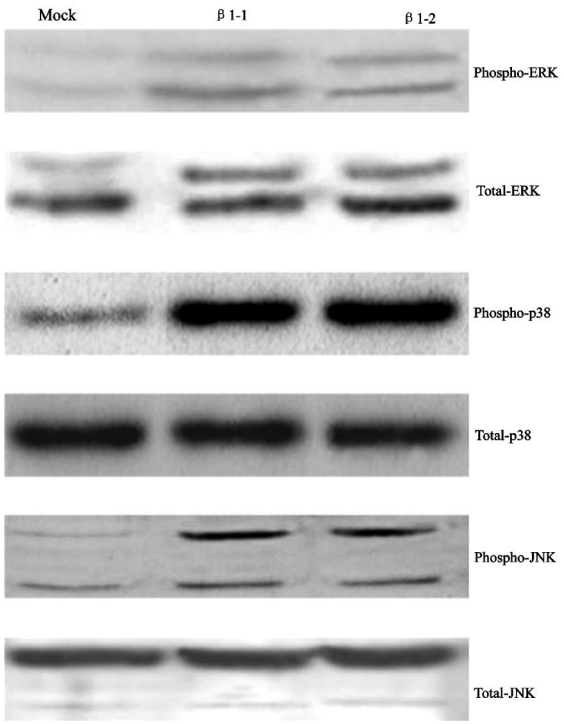


**Figure 4 The result of mitogen -activated protein kinase (MA -P) kinase phosphorylation in transfected cells** β1-integrin and GFP-fused gene transfection induced the phosphorylation of MAP kinase in RCE cells

in β1 group, while only 113000Da band was found in mock group, which indicates fused expression of β1-integrin and GFP-fused protein in corneal epithelial cells.

**Impact of β1-integrin-GFP Gene Transfection to the Adhesive Force of Corneal Epithelial Cell *In Vitro*** Figure 2 shows increased adhesive ability of transfected cells to ECM proteins but not 10g/L BSA in β1 group than in mock group ($P<0.05$).

**Impact of β1 -integrin -GFP Gene Transfection to Corneal Epithelial Cell Apoptosis** Within 5 days, apoptosis percentage was the same in mock and β1 group. At 6-10 days after transfection, apoptosis was inhibited in β1 group (Figure 3).

**Impact of β1 -integrin -GFP Gene Transfection to MAP kinase in Corneal Epithelial Cell** Figure 4 shows that β1-integrin and GFP-fused gene transfection induced the phosphorylation of three MAP kinases, indicating MAP kinase plays an important role in resistence of β1-integrin to apoptosis.

## DISCUSSION

The precondition of corneal cell transplantation is obtaining corneal epithelial cell capable of long-term survival and

proliferation *in vitro*. However, apoptosis has always been the pressing issue during the process of cell culture *in vitro*. β1-integrin family is the major cellular surface receptor mediating extracellular matrix signal, and is related to the cell proliferation, differentiation, apoptosis and migration [2]. Overexpression of β1-integrin by the way of transfecting β1-integrin-GFP gene into corneal epithelial cell significantly increases the adhesive force of corneal epithelial cell to ECM, which indicates certain biological function of overexpressed β1-integrin in corneal epithelial cell. And GFP is currently an ideal molecular probe analyzing protein function and dynamics at cellular level. RT-PCR and Western blot confirm that insertion and expression of exogenous GFP gene. It provides an ideal cell model for observing and localizing the distribution and transportation of β1-integrin real time in viable cells. It has advantages including simplicity, specificity and lower cost over the traditional way of studying integrin by ECM protein coated culture plate.

Although there has been reports that β1-integrin is closely related to resistance to apoptosis in certain cell-type cells [3-5]. However, the study on relationship of β1-integrin and corneal epithelial cell apoptosis has been in the initial stage. Esco *et al* [6] found that when anti-laminin antibody acting on corneal epithelial cell, and blocking intragenous and exogenous laminin, mass apoptosis occurred in primary cell. Adding laminin could obviously resist apoptosis, which indicates β1-integrin as laminin receptor is possible related to the apoptosis resistence. Our results proved the possibility of overexpression of β1-integrin inhibits corneal epithelial cell apoptosis. It is reported that the mechanism of β1-integrin resisting apoptosis is related to the up-regulation of Bcl-2, activation of MAP and PI-3 kinase [7-9]. Our data for the first time proves that β1-integrin overexpression induced MAP kinase phosphorylation in corneal epithelial cells, and β1-integrin mediated MAP kinase phosphorylation is possibly the vital mechanism of overexpression of β1-integrin inhibiting corneal epithelial cell apoptosis. MAP kinase presents with wide catalytic acitivity. It regulates gene transcription, cell growth and apoptosis through phosphar-ylating residue of transacting actor in the nucleus. It is key enzyme in the apoptosis pathway [10]. We will further focusing on the role of three members of MAP kinase, i.e. ERK, p38 and JNK, played in β1-integrin anti-apoptosis effect to investigate the molecular mechanism of β1-integrin inhibiting corneal epithelial cell apoptosis.

Our study found β1-integrin overexpression in corneal epithelial cell by transfecting β1-integrin-GFP gene can inhibit corneal epithelial cell apoptosis *in vitro* during which phosphorylation of MAP kinase may play an important role. β1-integrin overexpression is an indispensable factor of improving long-term survival of corneal epithelial cells and provides important theoretical basis for the prevention of corneal epithelial cell apoptosis and clinical application of corneal cell transplantation.

**REFERENCES**

1 Pajoohesh-Ganji A, Pal-Ghosh S, Simmens SJ, Stepp MA. Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. *Stem Cells* 2006;24:1075-1086

2 Hasenson S, Maatta M, Rousselle P, Kekkawa Y, Miner JH, Tervo T, Virtamen I. The immortalized human corneal epithelial cells adhere to laminin-10 by using Lutheran glycoproteins and integrin alpha3beta1. *Exp Eye Res*2005;81:415-421

3 Park CC, Zhang H, Pallavicini M, Gray JW, Baehner G, Park CJ, Bissell MJ. Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and *in viva Cancer Res*2006;66:1526-1535

4 Wang R, Li J, Lyte K, Yashpal NK, Fellows F, Goodyer CG. Role for beta1 integrin and its associated alpha3, alpha5, and alpha6 subunits in development of the human fetal pancreas. *Diabetes*2005;54:2080-2089

5 Pinkse GG, Voorhoeve MP, Noteborn M, Terpstra OT, Bruijn JA, De Heer E. Hepatocyte survival depends on beta1-integrin-mediated attachment of hepatocytes to hepatic extracellular matrix. *Liver Int*2004;24:218-226

6 Esco MA, Wang Z, McDermott ML, Kurpakus-Wheater M. Potential role for laminin 5 in hypoxia-mediated apoptosis of human corneal epithelial cells. *J Cell Sci*2001;114:4033-4040

7 Tiberio R, Marconi A, Fila C, Fumelli C, Pignatti M, Krajewski S, Giannetti A, Reed JC, Pincelli C. Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner. *FEBS Lett* 2002;524:139-144

8 Aoudjit F, Vuori K. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene*2001;20:4995-5004

9 Jin A, Kurosu T, Tsuji K, Mizuchi D, Arai A, Fujita H, Hattori M, Minato N, Miura O. BCR/ABL and IL-3 activate Rap1 to stimulate the B-Raf/MEK/Erk and Akt signaling pathways and to regulate proliferation, apoptosis, and adhesion. *Oncogene*2006;25:4332-4340

10 Lu L, Wang L, Shell B. UV-induced signaling pathways associated with corneal epithelial cell apoptosis. *Invest Ophthalmol Vis Sci*2003;44:5102-5109