Deregulation of AP-1 Proteins in Collagen Gel-induced Epithelial Cell Apoptosis Mediated by Low Substratum Rigidity

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In this study, we established that collagen gel, but not collagen gel coating, induced apoptosis exclusively in epithelial cell lines, which indicated that low substratum rigidity might trigger cell apoptosis. To confirm this, we used collagen gels with different rigidities due to cross-linking or physical disruption of collagen fibrils caused by sonication. We found that collagen gel-induced apoptosis was inversely correlated with substratum rigidity. Low substratum rigidity collagen gel-induced apoptosis was neither prevented by Bcl-2 overexpression nor preceded by mitochondrial release of cytochrome c. This suggested that the mitochondrial pathway was not involved in low substratum rigidity-induced apoptosis. Low substratum rigidity activated c-Jun N-terminal kinase (JNK) within 4 h, but it also rapidly down-regulated c-Jun within 1 h and triggered persistent aberrant expression of c-Fos for at least 24 h. Either reduced c-Jun expression or c-Fos overexpression induced apoptosis in several epithelial cells. Inhibiting low substratum rigidity-induced JNK activation prevented aberrant c-Fos expression but only partially blocked low substratum rigidity-induced apoptosis. Taking these results together, we conclude that low substratum rigidity collagen gel induced apoptosis in epithelial cells and that deregulated AP-1 proteins mediated that apoptosis, at least in part.

The physical properties of extracellular matrix influenced the locomotion (1, 2), survival (3), gene expression, and proliferation (4) of cells. Several methods have been developed to study the effects of the mechanical properties of extracellular matrix on cell behaviors. Type I collagen-coated polyacrylamide gel with different rigidities was used to study the functions of substratum flexibility on cell responsiveness, such as propagative forces, migration, and apoptosis (2, 5). Elastic micro-patterned substrates were used to study force generation and focal adhesion assembling in cells (6). In addition, a micro-fabrication strategy to generate micrometer-scale rods of an elastomeric polymer, polydimethylsiloxane, was used to study the traction force generated by cells (7).

Fibril collagen is the most abundant extracellular matrix in normal interstitium. Due to its fibril nature, collagen gel is the most commonly used three-dimensional scaffolding material for biological studies. In the past, we used hydrated fibril collagen as a three-dimensional culture model to study cystogenesis and branching tubulogenesis (8–11). We frequently found that epithelial cells developed apoptosis in collagen gel (8). In addition, we found that collagen gel overlay induced apoptosis only in epithelial cells (12). Subsequent findings indicated that collagen gel overlay triggered FAK proteolysis, which was associated with MDCK cell apoptosis (13). Here we examined whether the physical properties of collagen gel triggers cell apoptosis. Collagen gel extracted from the tail tendons of older rats showed more cross-linking of collagen fibrils than those from younger rats (10, 14). To prepare collagen gel with different physical properties, we extracted collagen from the tail tendons of rats of different ages, and then we modified the collagen gel rigidity using sonication (15, 16). Collagen gel is a viscoelastic material with non-linear elasticity whose shear mechanical properties are assessable using a rheometer, a well established tool (17, 18). We used modified methods of parallel plating to determine the viscoelastic modulus of collagen gel.

To elucidate the signal mechanism underlying low substratum rigidity-induced epithelial cell death, we investigated the traditional cell-death pathway. The mitochondrial pathway did not seem to be involved, however. On the other hand, we found that low substratum rigidity activated the JNK group of MAP kinases, also known as stress-activated protein kinases. Previous studies (19–21) reported that the JNK protein kinases

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2 To whom correspondence should be addressed: Tel: 886-6-236-3535 (ext. 5425); Fax: 886-6-236-2780; E-mail: mtj@ncku.edu.tw.
3 The abbreviations used are: MDCK, Madin-Darby canine kidney cells; shRNA, short hairpin RNA; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; SEM, scanning electron microscope; MEM, minimal essential medium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; BAEc, bovine aortic endothelial cell; PBS, phosphate-buffered saline; Pa, Pascal; z, benzylxoycarbonyl; fmk, fluoromethyl ketone; MEKK, MAP kinase/extracellular signal-regulated kinase kinase.
phosphorylated transcriptional activation domains of the AP-1 family proteins.

The AP-1 family consists of several groups of bZIP domain (bZIP is basic region leucine zipper) proteins: the Jun, Fos, and ATF-2 subfamilies (22). C-Jun overexpression induced apoptosis in 3T3 fibroblasts (23). In addition, JNK/c-Jun signaling was necessary for the apoptotic response in certain neuronal cell types (24, 25). On the other hand, c-Jun knock-out mouse embryonic fibroblast cells exhibited defects in proliferation and increases in UV-induced apoptosis (26, 27). Consistent with cell culture studies, the lack of c-Jun resulted in massive apoptosis of hepatoblasts and erythroblasts in the developing mouse liver in vivo (28). The c-fos proto-oncogene, the other major AP-1 protein member, encoded a nuclear protein that dimerized with Jun family proteins (29–31) to form a transcription factor complex (32). The Fos protein has been implicated as a key molecule in cell proliferation (22, 32), differentiation (33, 34), and transformation (35). In addition to a primary role in normal development and cellular growth, c-Fos protein has been associated with apoptotic cell death induced by anti-proliferative conditions (36, 37). In this study, we explored the novel role of AP-1 deregulation in low rigidity collagen gel-induced epithelial cell apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cultures**—MDCK, LLC-PK1, BS-C-1, NMuMG, NRK-52E, HK-2, HEK 293, and Chang liver cells were purchased from ATCC and regularly maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). BS-C-1 cells were cultured in modified Eagle’s medium (MEM) supplemented with 10% FCS. HK-2 cells were cultured in MEM plus methanol-soluble factor with 5 ng/ml EGF, 40 ng/ml bovine pituitary extract and supplemented with 10% FCS. Chang liver cells were cultured in MEM supplemented with 10% FCS. Bovine aortic endothelial cells (BAECs) were a gift from Dr. Wen-Chan Chang, NIH/3T3 cells were a gift from Dr. Hsiao-Shen Liu, U-373MG were a gift from Dr. Ji-Jing Chaung, and four oral cancer cell lines (OC-2, DOK, SSC-25, and HSC-3) encoded a nuclear protein that dimerized with Jun family proteins (32). The Fos protein has been implicated as a key molecule in cell proliferation (22, 32), differentiation (33, 34), and transformation (35). In addition to a primary role in normal development and cellular growth, c-Fos protein has been associated with apoptotic cell death induced by anti-proliferative conditions (36, 37). In this study, we explored the novel role of AP-1 deregulation in low rigidity collagen gel-induced epithelial cell apoptosis.

**Preparation of Hydrated Collagen Gel**—Type I collagen was prepared from rat tail tendons according to the established procedure (10, 38). The final concentration of type I collagen stock was 1% (wet weight) dissolved in 0.025 N acetic acid. To prepare collagen gel, 3 vol of collagen stock was mixed with 5.7 × DMEM (1 vol), 2.5% NaHCO3 (0.5 vol), 0.1 M HEPES (1 vol), 0.17 M CaCl2 (0.1 vol), 1 N NaOH (0.1 vol), and 4.3 vol of 1× culture medium (DMEM plus 10% FCS) under chilled conditions. The mixture was dispensed on culture dishes and kept at room temperature to allow gelation. After gelation, each culture was overlaid with 2 ml of culture medium that was replaced every other day. To prepare collagen gel-coated dishes, collagen gel mixtures were dispensed in culture dishes and then aspirated to allow only a thin sheet of collagen to cover the dish. The collagen gel-coated dishes were semi-air-dried in a culture hood. Every culture plate was freshly prepared before it was used in an experiment.

**DNA Extraction and Electrophoresis**—The method of extracting low molecular genomic DNA has been previously described (39). Briefly, cultured cells were extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris (pH 7.4) and phenol/chloroform three times. The DNA was precipitated in propanol and electrophoresed in 1.5% agarose gel. Finally the DNA was visualized using ethidium bromide staining under UV light. 

**Hoechst 33258 Staining and Assessing Nuclear Areas**—LLC-PK1 cells cultured on culture dishes or collagen gel for 24 h were washed twice with PBS and then fixed with 2% paraformaldehyde in the gel. After they had been washed, the cells were permeabilized using buffer containing 0.1% Triton X-100 and then stained with Hoechst 33258 (5 mg/ml) for 1 h in the dark. Finally, the stained nuclei were visualized under a fluorescence microscope (BX-51, Olympus, Tokyo, Japan). To assess nuclear areas, cells were cultured under different conditions for 24 h and subjected to Hoechst 33258 staining, and then the nuclear area was evaluated under a fluorescence microscope with imaging software (Image-Pro Plus 6.0, Media Cybernetics, Inc., Silver Spring, MD).

**Analysis of Apoptosis Ratio**—The apoptosis ratio was assessed using flow cytometry with propidium iodide as previously described (8, 12). After cells were seeded on collagen gel for different time periods, the gel was removed and treated with 0.2% collagenase at 37 °C for 10 min. The cell mixtures collected were washed with PBS and fixd in 70% alcohol. After fixation, the cells were treated with RNase (100 mg/ml PBS) and stained with propidium iodide (40 mg/ml PBS). The mixed cells were incubated in the dark at room temperature for 30 min and analyzed using propidium iodide excitation set at 488 nm. Data were analyzed using CellQuest software.

**Western Blotting**—We used the following antibodies to assess protein levels: caspase 2 (Santa Cruz Biotechnology), procaspase 3 (Upstate Biotechnology), Bcl-2 (DAKO), cytchrome c (BD Pharmingen), cleaved caspase 3 and caspase 9 (Cell Signaling Technology), caspase 8 (Oncogene), JNK and phosphorylated JNK (Cell Signaling), c-Jun (BD Transduction), c-Fos (Upstate Biotechnology), and horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Santa Cruz Biotechnology). Protein concentration was measured using the Lowry method (40). For Western blotting, 30 or 50 μg of cell homogenate from specific samples was resolved using 10% SDS-PAGE and electrophoretically blotted onto nitrocellulose paper. The nitrocellulose paper was incubated with the specific antibody listed above, and then immunocomplexes were detected using horseradish peroxidase-conjugated IgG, and, finally, the immunocomplexes were made visible using fluorography with an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences).
AP-1 Deregulation in Low Rigidity-induced Apoptosis

Treatment with Caspase or MAP Kinase Inhibitors—Cells were pretreated for 1 h and then seeded onto a culture dish, a collagen gel-coated dish, or on collagen gel with the caspase inhibitors z-VAD and DEVD-fmk (Calbiochem-Novabiochem) and JNK inhibitor SP600125 (Tocris). At various time points, cells were harvested and the apoptosis ratio was assessed using FACScan analysis.

Scanning Electron Microscopy—LLC-PK1 cells cultured for 48 h on a dish with normal culture, a collagen gel-coated dish, or collagen gel were rinsed twice with PBS solution. The cells were fixed with 2% buffered paraformaldehyde for 1 h. After fixation, the cells were rinsed twice with PBS to remove paraformaldehyde. Samples were dehydrated using incubation with gradient alcohol from 50% to 95% for 10 min under each concentration. In the final step, absolute alcohol was used to complete dehydration three times for 5 min. Samples were critical point-dried in liquid CO2 solution and then coated with a thin layer of gold-particle film. Finally the samples were visualized under a scanning electron microscope (SEM, Hitachi S5200).

Quantification of Collagen Gel Rigidity—The detailed protocol of the quantification of collagen gel rigidity was previously described (41). In brief, the viscoelastic properties of collagen gel were analyzed using a rheometer (AR1000, TA Instruments Ltd., West Sussex, UK) using cone-plate geometry with a 2° angle and a 60-mm diameter cone. To avoid the fracture of collagen gel and to increase the adhesion force between the collagen fiber network and the cone-plate geometry, neutralized collagen solution was first poured onto a pre-cooled plate disk at 5 °C, and then the cone was moved to approach the solution. After connecting the cone and solution, the temperature was shifted to 37 °C for 25 min to allow the collagen solution to gel. While we measured the viscoelasticity of the gel, the temperature was held at 25 °C. The dynamic shear storage modulus (G’), also called dynamic rigidity (42), and loss modulus (G”) of gels were acquired with angular frequency ranging from 0.1 to 10 rads/s. The maximum strain was set at 5% according to the requirements, and as suggested (43), of within a 10% linear viscoelastic range for shear measurements.

Plasmid Constructs and Transfections—The cDNA encoding c-Jun or c-Fos was generated using PCR from a 1-month-old mouse cerebrum cDNA library. Plasmid expressing full-length c-Jun or c-Fos was constructed using standard molecular cloning techniques. The expressing sequence was constructed by inserting pEGFP-N1 (BD Biosciences Clontech) into pSUPER vector (44) (kindly provided by Dr. R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands). To generate the shRNA expression vector pSUPER-Mmc-Jun/EGFP, the pSUPER/EGFP was digested with BglII and HindIII, and an annealed oligonucleotide duplex against the sequence of 5’-CGCAGCAGTTGCAAAAGT-3’ for murine c-Jun (45) was ligated into the vector.

Confocal Microscopy and Real-time Fluorescence Images—LLC-PK1, NMuMG, or HeLa cells were transiently transfected with EGFP-containing plasmid for the indicated time points. For a vital stain assay, cells were pre-stained with propidium iodide (200 μg/ml in culture medium) for 30 min before fixation. For fluorescence images, cells were rinsed twice with PBS solution, fixed with 4% buffered paraformaldehyde for 15 min, and then blocked with super blocking buffer (Pierce) for 1 h. To detect c-Jun expression, cells were stained with mouse anti-c-Jun antibody (BD transduction) for 1 h. Cells were rinsed five times with PBS. Finally, the cells were stained with anti-mouse IgG conjugated with Alexa 594 (Molecular Probes) for 1 h. The fluorophore was excited using a laser at 488 or 594 nm and detected at emission spectrum (520 or 640 nm) using a photomultiplier with a ×40 water-immersion objective lens (HCX APO L ×40/0.90 W-U. V-1) for display as a high resolution image. Finally the stained results were visualized under a confocal microscope (SP-2, Leica Mikrosysteme Vertrieb GmbH, Bensheim, Germany). Images were taken using Leica SP-2 software. For time-lapse fluorescence recording, NMuMG cells were transiently transfected with shRNA-containing EGFP expression plasmid and recovered 4 h after transfection. The cells were moved from an incubator to a culture system on confocal microscopy (FV1000, Olympus), in which the temperature was kept at 37 ± 0.5 °C, and 5% CO2 was injected into PBS to maintain the CO2 concentration and moisture in the chamber. The EGFP was excited using an argon laser at 488 nm and detected at emission spectrum (520 nm) using a photomultiplier with a ×40-inverted objective lens (Uplan Apo ×40/1.0 oil, Olympus) to display as a high resolution image for 10-min intervals (scan speed: 3.928 s/frame). Images were taken or composed (video) using imaging software (FV10-SW, Olympus).

Statistics—All data are expressed as means ± S.E. of at least three independent experiments. One-way analysis of variance was used to test for statistical differences. Statistical significance was set at p < 0.05.

RESULTS

Characterization of LLC-PK1 Cell Death Induced by Fibril Collagen Gel—LLC-PK1 and HEK 293 cells were cultured for 24 h on normal culture dishes or 0.3% type I collagen gel prepared from the tail tendons of 1-month-old rats. Collagen gel did not alter the morphology of HEK 293 cells. However, LLC-PK1 cells cultured on collagen gel showed distinct morphological changes. These cells formed contracted cell islands with many disintegrated vesicles (Fig. 1A). An analysis of the DNA extracted from LLC-PK1 cells cultured on collagen gel showed a DNA ladder pattern, indicating that collagen gel-induced cell death was apoptosis (Fig. 1B). In addition, an assessment of the
integrity of cell nuclei using Hoechst 33258 staining revealed nuclear condensation as well as fragmentation in LLC-PK1 cells cultured on collagen gel (Fig. 1C). The results of the FACSscan analysis of the nuclear size also showed, after 48 h, a significantly ($p < 0.001$) increased (56.4 ± 9.8%) sub-G$_0$ population in LLC-PK1 cells cultured on collagen gel, but only a <7% increase in cells cultured on a normal dish (Fig. 1D). To examine whether other types of extracellular matrix had similar effects, LLC-PK1 cells were cultured on dishes pre-treated with fibronectin, laminin, vitronectin, or Matrigel for 48 h, and the apoptosis ratio was assessed. None of these extracellular matrices triggered apoptosis in LLC-PK1 cells (data not shown). However, when LLC-PK1 cells were cultured on 0.3% type III collagen gel for 48 h, they displayed approximately similar levels of apoptosis (Fig. 1E). Because both type I and type III collagen belong to the fibril collagen family, these data suggested that collagen gel-induced apoptosis may be caused by the physical nature of the three-dimensional gel. To determine whether collagen gel-induced apoptosis was cell-type specific, we cultured epithelial (NMuMG, BS-C-1, MDCK, and NRK-52E), endothelial (BAECs), mesenchymal (HEK 293 and NIH-3T3), and tumor cells (HK-2, Chang liver, U-373 MG, OC-2, DOK, SSC-25, and HSC-3) on type I collagen gel for 48 h and assessed the apoptosis ratio using FACSscan analysis. We found that only polarized cells, including epithelial and endothelial cells, had elevated levels of apoptosis. Only polarized cells, including epithelial and endothelial cells, had elevated levels of apoptosis on collagen gel (Fig. 1F). In contrast, mesenchymal as well as tumor cells showed little apoptosis when they were cultured on collagen gel.

**Collagen Gel Coating Alleviated Collagen Gel-induced Apoptosis**

To determine whether collagen gel-induced apoptosis in epithelial cells was caused by its physical or chemical nature, we used collagen gel-coated dishes that exerted a chemical impact similar to that of collagen.
Collagen gel coating alleviated collagen gel-induced apoptosis—We attributed the cross-linking of collagen fibril to the substratum rigidity of collagen gel. Collagen gels prepared from rat tail tendons of different ages have different mechanical properties due to the cross-links of collagen fibrils (9, 14). To determine whether the age of the collagen fibril affects collagen gel-induced apoptosis, we cultured LLC-PK1 cells on 0.3% collagen gels prepared from the tail tendons of 1-, 4-, or 16-month-old rats. Cells cultured on 1-month-old collagen gel showed active pulling of collagen fibrils, indicating that cross-links of collagen fibrils might be disrupted by cell retraction. The cell pulling of collagen fibrils was clearly reduced by 4-month-old collagen gel (Fig. 3A). The morphology of cells cultured on collagen gel of different ages was further examined using SEM, which showed that LLC-PK1 cells cultured on 1-month-old collagen gel formed a cell island with little cell extension (Fig. 3B). We also observed cells actively pulling collagen fibrils. However, cells cultured on 4-month-old collagen gel showed cell extension manifested by the extensive lamellipodia and membrane ruffle on the outer rim of the cell island. In addition, a FACScan analysis demonstrated that the age of the tail tendon used to make the collagen gel was inversely related to the level of collagen gel-induced apoptosis (Fig. 3C). These findings indicated that augmentation of the rigidity of collagen gel produced by an increase in the cross-links of collagen fibers significantly inhibited collagen gel-induced apoptosis.

Reduction of the Rigidity of Collagen Gel by Sonication Increased Collagen Gel-induced Apoptosis—Sonication may affect the rigidity of collagen gel by disrupting the structure of collagen fibrils (16). To test this possibility, we treated collagen stock solutions with sonication of different durations (1, 2, and 4 min) and then allowed them to gel. We found that the gel time rose in proportion to the length of sonication. We examined the ultrastructure of sonicated collagen gel using SEM and found that cross-linking levels of collagen fibrils were inversely proportional to the sonication time (Fig. 4A). This finding indicated that sonication may reduce the rigidity of collagen gel. To test whether lowering the rigidity of collagen gel alters the level of apoptosis in LLC-PK1 cells, we used collagen gel pretreated with 1, 2, and 4 min of sonication. The FACScan analysis showed that sonication dose-dependently increased collagen gel-induced apoptosis (Fig. 4B), which meant that low-
The substratum rigidity of collagen gel increased the level of apoptosis. **Quantification of Collagen Gel Rigidity**—To detect the rigidity of collagen gel, we used both a rheometer and a dynamic mechanical analyzer. The dynamic rigidity, in Pascals (Pa), of collagen gel extracted from 8-month-old rat tails was 123.43 ± 7.99 Pa, from 4-month-old rat tails was 79.16 ± 6.73 Pa, and from 1-month-old rat tails was 14.25 ± 0.92 Pa. After sonication for 1, 2, or 4 min, the dynamic rigidity of 0.3% collagen gel from 1-month-old rat tails dose-dependently decreased to 7.84 ± 1.01 Pa, 6.25 ± 0.98 Pa, and 5.21 ± 1.12 Pa, respectively. In addition, the results of the dynamic rigidity of collagen gel, assessed using a dynamic mechanical analyzer, were similar, which confirmed that the age of the tail tendon used to make the collagen gel affected the rigidity of the collagen gel (41). In summary, our findings indicated that lowering the substratum rigidity of collagen gel triggered a higher rate of apoptosis in epithelial cells.

**The Rate of Epithelial Cell Apoptosis Was Inversely Proportional to the Nuclear Area of Cells and the Rigidity of Collagen Fibrils**—Cell tension regulated by cytoskeletal organization may control cellular as well as nuclear morphology. The area of the nucleus may reflect the degree of cell extension. To understand whether cell extension is associated with epithelial cell survival/apoptosis, we cultured LLC-PK1, NMuMG, NRK-52E, and BS-C-1 cells on collagen gel prepared from 1- or 8-month-old rat tails and assessed their nuclear size and apoptosis ratio. The nuclear area was smaller in all cells cultured on collagen gel (Fig. 5A). A reduction in cross-links of collagen fibrils caused a decrease in the nuclear area. On the other hand, the apoptosis ratio remained very low when cells were cultured on a dish. A reduction in the cross-links of collagen fibrils triggered apoptosis in all epithelial cells examined (Fig. 5B). The apoptosis ratio was inversely associated with the size of the nuclear area in all cells examined (Fig. 5C). Interestingly, there seemed to be a lower limit of nuclear area for each cell line to maintain survival. When the nuclear area of the cell was below this limit, the apoptosis ratio was significantly higher (p < 0.02 for all cell lines). These findings taken together strongly suggest that low substratum rigidity collagen-fibril-induced epithelial cell apoptosis might result from inhibiting cell extension, which indicated that extension is required for the survival of epithelial cells.
AP-1 Deregulation in Low Rigidity-induced Apoptosis

Neither Mitochondria nor Caspase 3 Was Involved in Collagen Gel-induced Apoptosis—Bcl-2 is important for preventing apoptosis induced by the mitochondrial pathway (47). To see whether Bcl-2 blocks collagen gel-induced apoptosis, we used LLC-PK1 and MDCK cells overexpressing Bcl-2. We found that Bcl-2 overexpression did not inhibit collagen gel-induced apoptosis in these cells (Fig. 6A). To determine whether the release of cytochrome c from mitochondria was involved in collagen gel-induced apoptosis, LLC-PK1 cells were cultured on culture dishes or collagen gel for 24 h. We analyzed the mitochondrial and cytosolic fractions of cell lysates for cytochrome c levels. We found that collagen gel did not alter the distribution of cytochrome c levels in either mitochondrial or cytosolic fractions (Fig. 6B), which indicated that mitochondrial pathways were not involved in collagen gel-induced apoptosis.

To see which caspases were involved in collagen gel-induced apoptosis, LLC-PK1 cells were cultured on culture dishes, collagen gel-coated dishes, and collagen gel. Western blotting showed that collagen gel had not activated caspase-2, -8, or -9, the initiators and mediators of apoptosis (Fig. 6C). We did find, however, that caspase 3 was activated within 8 h in cells seeded on collagen gel. Various caspase inhibitors were used to evaluate which caspase cascade was involved in collagen gel-induced apoptosis. Pan-caspase inhibitor z-VAD partially inhibited collagen gel-induced apoptosis, but caspase-3-specific inhibitor DEVD-fmk did not (Fig. 6D).

Low Substratum Rigidity Induced Down-regulation of c-Jun and Aberrant Expression of c-Fos—The activation of JNK and its downstream signals reflects the environmental stress on cells. To determine whether low substratum rigidity activates JNK and its downstream signaling, we used Western blotting to detect JNK, phosphorylated JNK, and AP-1 proteins. When LLC-PK1 cells were cultured on collagen gel, JNK was activated within 4 h and continued for 18 h (Fig. 7A). However, the downstream signal molecule, c-Jun, was down-regulated within 1 h and gradually disappeared because of the low substratum rigidity. In contrast, c-Fos was consistently expressed over 24 h because of the low substratum rigidity (Fig. 7B). Similar results were found in NMuMG cells (Fig. 7C). We examined other members of the AP-1 family protein and found that JunD and SP-1 were down-regulated because of the low substratum rigidity but that ATF-2 levels were not significantly different in cells cultured in culture dishes, collagen gel-coated dishes, or collagen gel (supplemental Fig. S1). In contrast, HeLa cells that survived low substratum rigidity showed no c-Jun degradation (data not shown). Increasing substratum rigidity, by increasing the age of the rat tail tendons used to make the collagen gel, partially reversed the c-Jun degradation in NMuMG cells (Fig. 7D). These findings indicated that the down-regulation of c-Jun and aberrant expression of c-Fos might be involved in low substratum rigidity-induced apoptosis.

We assessed the time course changes of p-JNK, JNK, c-Jun, and c-Fos expression in BAECs cultured on dish, collagen gel-coated dish, or collagen gel (data not shown). Low rigidity of collagen gel induced JNK activation in both LLC-PK1 cells and BAECs. The activation of JNK was induced in LLC-PK1 cells within 4 h and in BAECs within 1 h. Low rigidity of collagen gel induced a decrease in c-Jun level within 1 h in LLC-PK1 cells or BAECs (data not shown). However, low rigidity-induced decrease in c-Jun was not further down-regulated in BAECs from 8 to 18 h and on the other hand the decrease was reversed (data not shown). This might be the reason why low rigidity-induced apoptosis in BAECs was markedly lower than that in epithelial cells. In addition, low rigidity-induced aberrant expression of c-Fos was found in LLC-PK1 cells but not in BAECs, which could be another reason why low rigidity-induced relatively lower apoptosis in BAECs.

**shRNA Inhibited c-Jun Expression-induced Cell Death in NMuMG Cells—**Because low substratum rigidity degraded c-Jun, we wanted to test whether c-Jun degradation induced cell death in epithelial cells. We used shRNA to down-regulate c-Jun expression in NMuMG cells and then visualized cell viability using vital stain. Cells with green fluorescence (Fig. 8A, *panel a*, *left panel*) indicated positive transfection of control or shRNA for c-Jun; the expression of c-Jun in the same cells is shown in the *right panel*. Higher expression of shRNA completely inhibited the expression of c-Jun. Vital stain indicated that shRNA-transfected cells underwent cell death (Fig. 8A, *panel b*). We also used a time-lapse recording method to observe the process of cell death in transfected cells under a confocal microscope (Fig. 8B and supplemental video). Cell death was seen (Fig. 8B) within 10 h of shRNA transfection, but there was no detectable cell death in cells transfected with EGFP control or in cells with low shRNA expression. The transfection rate of control EGFP in NMuMG cells was quite constant, with a maximum ratio of ~10%. However, the percentage of cells that expressed shRNA 8 h after transfection remained very low (Fig. 8C, *panel a*), possibly because cells transfected with shRNA underwent cell death because of reduced c-Jun levels. To test this possibility, we quantified the cell-death ratio of cells transfected with shRNA by counting the propidium iodide positively stained cells (Fig. 8C, *panel b*). We found that...
the cell-death ratio in cells expressing shRNA increased over time. These findings indicated that shRNA down-regulation of c-Jun triggered cell death in NMuMG cells.

Overexpression of Either c-Fos or c-Jun Induced Cell Death in Various Cells—Because low substratum rigidity also induced aberrant expression of c-Fos, we wanted to see whether c-Fos overexpression resulted in cell death. NMuMG cells were transfected with c-Jun and c-Fos. We found that overexpression of both c-Jun and c-Fos induced cell death not only in NMuMG cells (Fig. 9A) but also in LLC-PK1 and HeLa cells (Fig. 9B). Overexpression of c-Jun increased the cell-death ratio from 17% to 48% in HeLa cells, from 23% to 44% in LLC-PK1 cells, and from 20% to 50% in NMuMG cells at 12 and 24 h, respectively. Overexpression of c-Fos increased the cell-death ratio from 8% to 32% in HeLa cells, 17% to 34% in LLC-PK1 cells, and 18% to 26% in NMuMG cells at 12 and 24 h, respectively. Interestingly, we found that JNK inhibitor suppressed the aberrant expression of c-Fos (Fig. 9C, panel a). However, inhibiting JNK expression and the aberrant expression of c-Fos reduced the apoptosis ratio only ~20% (Fig. 9C, panel b). JNK activation might be responsible for the aberrant expression of c-Fos. According to these results, levels of c-Jun and c-Fos are tightly regulated in epithelial cells.

DISCUSSION
In this study, we demonstrated that low substratum rigidity, the physical property of fibril collagen, induced apoptosis in epithelial cells but not in fibroblasts or transformed cells. Normal epithelial cells could not extend on collagen gel due to inadequate cross-linking of collagen fibrils; their configuration was reminiscent of the morphology of cells grown on a very limited area of fibronectin (48). In both conditions, maintenance of cell extension is required for cell survival. We showed that collagen gel constructed from the tendons of older rat tails resulted in greater cell extension and significantly attenuated collagen gel-induced apoptosis. Cell extension in cells grown on collagen gel prepared from older rat tail tendons was not as great at that in cells grown on collagen gel-coated dishes. Therefore, cells grown on collagen gel extracted from 16-month-old rats had a higher rate of apoptosis than cells grown on collagen gel-coated dishes (24 ± 1% versus 14 ± 0.5%). Because these results are consistent with observations that cell geometry governs cellular life and death, the model presented here is useful for studying the signal transduction mechanism through which low rigidity induces apoptosis in epithelial cells.

Cells are able to sense the rigidity of their environments and change their motility through integrins and FAK (5, 49, 50). The tensional forces are generated by cytoskeletons that resist forces from the extracellular matrix (51, 52). The force applied
or generated through cell matrix interactions altered cell morphology, migration, and differentiation (53–55). Previous data also showed that extracellular matrix rigidity influenced the strengthening of integrin-cytoskeleton linkages (56). Cultured on collagen gel, epithelial cells not only displayed restricted cell extension but also disorganized actin filaments due to low substratum rigidity (data not shown), which is consistent with recent studies (5, 7) showing that altering the balance of physical forces transmitted from the cell surface changed the cytoskeletal structure of the cells. Tensegrity (57) is a model that explains how a cell stabilizes itself mechanically by balancing contractive forces. In our model, we hypothesize that, because collagen fibers did not provide enough strength for cell extension, epithelial cells developed apoptosis because they lost tensegrity.

Sonication breaks triple helices into short segments but does not change their structures (15, 16). In the present study, sonication prolonged gelation time and may have increased the lateral aggregation of collagen fibrils. Sonication also significantly decreased cross-linking of collagen fibrils, which contributed to the reduction in rigidity of collagen gel. Under the assumption that the nuclear area was proportionally correlated to cell extension, we assessed the nuclear area of cells. We found that substratum rigidity and the nuclear area were positively correlated and that the degree of cell extension was inversely correlated. Our data are compatible with the results of the recent study by Storm et al. (18). However, results on the rigidity of collagen gel, different by about one order, were found when an electromechanical computer-controlled indenter was used in another study (58). The difference might have been caused by different detection methods. Nevertheless, our data, obtained using a rheometer, showed that collagen gel rigidity increased directly with the age of the rat tail tendons used to make the gel and decreased after sonication and that the apoptosis ratio was inversely proportional to the substratum rigidity of the gel. In general, lowering substratum rigidity below 100 Pa significantly up-regulated epithelial cell apoptosis. We have not, however, determined the threshold of substratum rigidity for triggering epithelial-cell apoptosis.

The morphology of cells cultured on low substratum rigidity collagen gel is reminiscent of that of cells deprived of cell-matrix interactions, so called “homeless cell death” or “anoikis” (59). Both low rigidity-induced cell death and homeless cell death are exclusive characteristics of epithelial cells. The anoikis signal is mediated through the activation of caspase-8, caspase-3, MEK-1, and phospho-JNK (60, 61) or Bim (62), and is blocked by Bcl-2 overexpression. This contrasts with our finding that Bcl-2 overexpression did not prevent low substratum rigidity-induced apoptosis. We also found that low rigidity down-regulated FAK (data not shown), which was not observed in the anoikis model. In addition, we found that the mitochondrial pathway was not involved in low rigidity-induced apoptosis and that pan-caspase inhibitor only partially blocked this apoptosis. These findings taken together indicate that low substratum rigidity collagen gel-induced apoptosis is not mediated by the mechanism that mediates anoikis. A novel apoptosis-inducing pathway seems to be involved in low substratum rigidity collagen gel-induced apoptosis.

AP-1 proteins were activated by MAP kinases, and the phosphorylated AP-1 proteins resulted in dimerization, which in turn triggered the expression of genes associated with cell growth or survival (63). We found that low substratum rigidity activated JNK, but not p38 or ERK, within 4 h (data not shown). Under stress, caused by, for example, UV or drug treatment, the activation of JNK and downstream amplification of c-Jun have been considered cues for apoptosis. In addition, JNK activation may trigger the mitochondrial apoptosis pathway that increases Bcl-2 phosphorylation. The present study shows that low substratum rigidity activated JNK without triggering cytochrome c release from mitochondria. In addition, we demonstrated that low rigidity rapidly down-regulated c-Jun before JNK was activated. The study also showed that low substratum rigidity did not alter c-Jun mRNA levels and that low substratum rigidity-induced down-regulation of c-Jun was mediated by post-transcriptional regulation. Ample evidence has shown that the polyubiquitination of c-Jun and subsequent degradation by the 26 S proteasome may contribute to the degradation of c-Jun (64, 65). Whether low substratum rigidity-induced down-regulation of c-Jun is mediated through this pathway remains to be investigated. In the present study, c-Jun down-regulation caused apoptosis in NMuMG cells. In vivo study showed that a c-Jun deficiency resulted in embryonic lethality. Although embryonic fibroblasts were cultured from c-Jun-deficient mice, they lasted for only two passages. These cells easily developed apoptosis, because they accumulated spontaneous DNA damage (66). Lowering the rigidity of collagen gel decreased the expression level of c-Jun and increased apoptosis in epithelial cells. On the other hand, transformed cells cultured on collagen...
gel showed persistent c-Jun expression and evaded low substratum rigidity-induced apoptosis. Taking these findings together, we speculate that the degradation of c-Jun is important in low substratum rigidity-induced apoptosis in epithelial cells.

Although experiments attempting to maintain c-Jun expression in cells cultured on collagen gel have been tried, they have been very difficult, because cells transfected with c-Jun have all developed apoptosis regardless of cell type.

Low substratum rigidity also induced persistent expression of c-Fos, which is mediated by activated JNK; inhibiting JNK activation reduced c-Fos expression. We examined whether persistent expression of c-Fos is involved in low substratum rigidity-induced epithelial cell apoptosis. In the present study, c-Fos overexpression induced cell death in many epithelial cell types, which is consistent with the findings in photoreceptor cells (67) and ganglion cells (68). Normally, cells express c-Fos only transiently. In experiments using white light-induced apoptosis, inducing persistent c-Fos expression is required for chromosome condensation and DNA fragmentation. Depletion of c-Fos can prevent light damage-induced apoptosis morphology in the retina (69). These findings suggest the importance of persistent c-Fos expression in apoptosis.

In this study, we found that only epithelial cells are more susceptible to collagen gel-induced apoptosis than mesenchymal and tumor cells. Among all extracellular matrices examined, only type I and type III collagen gel induced apoptosis in epithelial cells. Because type I and type III collagen are fibril in nature, our data indicate that collagen gel-induced apoptosis may be related to their fibril characteristics. Under physiological conditions, epithelial cells do not contact with collagen fibril directly. In fact, there is a sheet of basement membrane that separates epithelium from direct contact with the interstitial tissues. Therefore, the function of basement membrane could be considered as a natural shield, in addition to playing roles in growth and differentiation for epithelium. On the other hand, fibroblast or transformed cells do not develop apoptosis when they contact directly with fibril collagen. Fibroblast belongs to the mesenchymal cells. Under the physiological con-

**FIGURE 8.** Down-regulation of c-Jun expression induced cell death in NMuMG cells. A, cultured NMuMG cells were transiently transfected with 5 μg/ml EGFP plasmid or c-Jun shRNA containing an EGFP sequence for 4 h. Eight hours after transfection, cells were either fixed and immunostained with c-Jun antibody and secondary antibody conjugated with tetramethyl rhodamine isothiocyanate fluorescent dye (a), or vitally treated with propidium iodide (PI, 200 μg/ml) for 1 h (b), and then observed under a microscope. Green fluorescence indicates cells with positive transfection. Red fluorescence in a indicates c-Jun expression and in b indicates the propidium iodide-positive stained cells. Higher expression of c-Jun shRNA completely inhibited the expression of c-Jun and resulted in cell death. B, cultured NMuMG cells were transiently transfected with 5 μg/ml EGFP plasmid (a) or c-Jun shRNA containing an EGFP sequence (b) for 4 h. Cells were then traced under a fluorescence time-lapse microscope, and fluorescence images were taken at the indicated time points. Cells with c-Jun shRNA developed fragmentation of the cell corpse. C, cultured NMuMG cells were transiently transfected with 5 μg/ml EGFP plasmid or c-Jun shRNA containing an EGFP sequence for 4 h. After transfection, the cells were treated with propidium iodide (200 μg/ml) for 1 h, fixed at the indicated time points, and observed under fluorescence microscopy. Ten randomly selected fields of transfected cells and non-transfected cells were counted under a microscope at ×100 magnification at each time point. The transfection rate (transfected cells/total cells) (a) and transfected cell-death ratio ((propidium-iodide-positive cells in transfected cells/total transfected cells)) (b) were quantified (*, p < 0.05; **, p < 0.02).
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FIGURE 9. Overexpression of c-Fos or c-Jun induced cell death in various epithelial cells. A, cultured NMuMG cells were transiently transfected with 5 μg/ml EGFP plasmid, c-Jun, or c-Fos plasmid containing an EGFP sequence for 4 h. Eight hours after transfection, cells were vitally treated with propidium iodide (PI) 200 μg/ml for 1 h. After they had been fixed, the cells were observed under fluorescence microscopy. Green fluorescence indicates cells with positive transfection. Red fluorescence indicates propidium iodide-positive cells. Overexpression of c-Jun or c-Fos resulted in cell death. B, LLC-PK1, NMuMG, and HeLa cells were transfected with EGFP, c-Fos, and c-Jun and vitally stained with propidium iodide as described in A. The percentage of propidium iodide-positive cells in the transfected cell population was assessed 12 and 24 h after transfection. C, NMuMG cells were cultured on normal culture dishes, collagen gel-coated dishes, and collagen gel for 8 h with or without U0126 (50 μM) or SP600125 (20 μM) treatment. The expression level of c-Fos was assessed using Western blotting (a). NMuMG cells were cultured on collagen gel for 8 h with or without SP600125, and the apoptosis ratio was assessed using FACS analysis. Inhibiting aberrant c-Fos partially blocked low substratum rigidity-induced apoptosis (*, p < 0.05).

conditions, they grow in type I collagen-enriched environments. We showed that fibroblasts grown on collagen gel exhibited elongated shape. They may change the architectural state of the cytoskeleton to evade the low rigidity collagen gel-induced apoptosis. Fibroblast or transformed cells are also more resistant to homeless cell death than epithelial cells. Recent reports show that expression of certain oncogene in epithelial cells conferred resistance to anoikis (70, 71). Ras, for example, is a common oncogene that overexpresses or mutates in many malignant cancer cells (72). It could auto-transduce the growth factor downstream signals for cell survival. Ras also governs the cytoskeleton-altering proteins in the cytoskeletal control in the cells. It is plausible that transformed cells may actively change their cytoskeletal construction upon contacting to matrix of low rigidity. Our findings are important for tumor biology, because invasive tumor must have acquired this phenotype to survive their paths in interstitium. We postulate that one of the characteristics that invasive cancer cells acquire during transformation is to escape collagen gel-induced apoptosis.

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REFERENCES

1. Bernstein, L. R., and Liotta, L. A. (1994) Curr. Opin. Oncol. 6, 106–113
2. Dembo, M., and Wang, Y. L. (1999) J. Biophys. 76, 2307–2316
3. Wang, H. B., Dembo, M., and Wang, Y. L. (2000) Am. J. Physiol. 279, C1345–C1350
4. Juliano, R. L., and Haskell, S. J. (1993) J. Cell Biol. 120, 577–585
5. Pelham, R. J., Jr., and Wang, Y. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13661–13665
6. Balaban, N. Q., Schwarz, U. S., Rivelone, D., Goichberg, P., Tzur, G., Sabanay, I., Mahalu, D., Safran, S., Bershadsky, A., Addadi, L., and Geiger, B. (2001) Nat. Cell Biol. 3, 466–472
7. Tan, J. L., Tien, J., Pirone, D. M., Gray, D. S., Bhatdiraju, K., and Chen, C. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14844–14849
8. Lin, H. H., Yang, T. P., Jiang, S. T., Yang, H. Y., and Tang, M. J. (1999) Kidney Int. 55, 168–170
9. Jiang, S. T., Chiang, H. C., Yang, T. P., Chuang, W. J., and Tang, M. J. (1999) Kidney Int. 56, 92–103
10. Jiang, S. T., Yang, T. P., Huang, J. J., Hsu, C. C., and Tang, M. J. (2000) Kidney Int. 57, 1539–1548
11. Jiang, S. T., Chuang, W. J., and Tang, M. J. (2000) Kidney Int. 57, 1860–1867
12. Tang, M. J., Hu, J. J., Lin, H. H., Chiu, W. T., and Jiang, S. T. (1998) Am. J. Physiol. 275, C921–C931
13. Wang, Y. K., Lin, H. H., and Tang, M. J. (2001) Am. J. Physiol. 280, C1440–C1448
14. Pokharna, H. K., Monnier, V., Boja, B., and Moskowitz, W. (1995) J. Orthop. Res. 13, 13–21
15. Betsch, D. F., and Baer, E. (1980) Biorheology 17, 83–94
16. Giraud-Guille, M. M. (1989) Biol. Cell 67, 97–101
17. Hsu, S., Jamieson, A. M., and Blackwell, J. (1994) Biorheology 31, 21–36
18. Storm, C., Pastore, J. J., MacKintosh, F. C., Lubensky, T. C., and Janmey, P. A. (2005) Nature 435, 191–194
19. Minden, A., Lin, A., Smeal, T., Derijard, B., Cobb, M., Davis, R., and Karin, M. (1994) Mol. Cell. Biol. 14, 6683–6688
20. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
21. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
22. Angel, P., and Karin, M. (1991) Biochim. Biophys. Acta 1072, 129–157
23. Bosy-Wetzel, E., Bakiri, L., and Yaniv, M. (1997) EMBO J. 16, 1695–1709
24. Watson, A., Eilers, A., Lallemend, D., Kyriakis, J., Rubin, L. I., and Ham, J. (1998) J. Neurosci. 18, 751–762
25. Le-Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F. X., Green, D. R., and Karin, M. (1999) Mol. Cell. Biol. 19, 751–763
26. Behrens, A., Sibilia, M., and Wagner, E. F. (1999) Nat. Genet. 21, 326–329
27. Wisdom, R., Johnson, R. S., and Moore, C. (1999) EMBO J. 18, 188–197
28. Eferl, R., Sibilia, M., Hilberg, F., Fuchsbieler, A., Kufferath, I., Guertler, B., Zenz, R., Wangner, E. F., and Zatloukal, K. (1999) J. Cell Biol. 145,
AP-1 Deregulation in Low Rigidity-induced Apoptosis

1049–1061
29. Ryder, K., and Nathans, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8464–8467
30. Ryder, K., Lanahan, A., Perez-Albuerne, E., and Nathans, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1500–1503
31. Hirai, S., Ryseck, R., Mecha, F., Bravo, R., and Yaniv, M. (1989) EMBO J. 8, 1433–1439
32. Curran, T., and Franza, B. R., Jr. (1988) Cell 55, 395–397
33. Distel, R. J., Ro, H. S., Rosen, B. S., Groves, D. L., and Spiegelman, B. M. (1987) Cell 49, 835–844
34. Ruther, U., Wagner, E. F., and Muller, R. (1985) EMBO J. 4, 1775–1781
35. Lee, M. S., Yang, J. H., Salehi, Z., Armstrong, P., Chen, L. S., Jay, G., and Rhim, J. S. (1993) Oncogene 8, 387–393
36. Colotta, F., Polentarutti, N., Sironi, M., and Mantovani, A. (1992) J. Biol. Chem. 267, 18278–18283
37. Smeyne, R. J., Vendrell, M., Hayward, M., Baker, S. J., Miao, G. G., Schilling, K., Robertson, L. M., Curran, T., and Morgan, J. I. (1993) Nature 363, 166–169
38. McAteer, J. A., and Cavanagh, T. J. (1982) J. Tissue Cult. Methods 7, 117–122
39. Eastman, A. (1995) Methods Cell Biol. 46, 41–55
40. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 251, 165–175
41. Wu, C. C., Ding, S. J., Wang, Y. H., Tang, M. J., and Chang, Y. H. (2003) J. Mol. Cell Biol. 159, 695–705
42. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 537–546
43. Rich, K. A., Zhan, Y., and Blanks, J. C. (1997) J. Neurobiol. 39, 232–239
44. Nateri, A. S., Riera-Sens, L., Costa, C. D., and Behrens, A. (2004) Exp. Cell Res. 295, 253–262
45. Mooney, D. J., Langer, R., and Ingber, D. E. (1998) Trends Cell Biol. 8, 51–54
46. Choquet, D., Felsenfeld, D. P., and Sheetz, M. P. (1997) Cell 88, 39–48
47. Inger, D. E. (1993) J. Cell Sci. 104, 613–627
48. Paszek, M. J., Zahir, N., Johnson, K. R., Lakins, J. N., Rozenberg, G. I., Gefen, A., Reinhart-King, C. A., Margulies, S. S., Dembo, M., Boettiger, D., Hammer, D. A., and Weaver, V. M. (2005) Cancer Cell 8, 241–254
49. Frisch, S. M., and Francis, H. (1994) J. Cell Biol. 124, 619–626
50. Ruoslahti, E., and Reed, J. C. (1994) Cell 77, 477–478
51. Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. (1994) J. Cell Biol. 127, 537–546
52. Reginato, M. J., Mills, K. R., Paulus, J. K., Lynch, D. K., Sgroi, D. C., Debnath, J., Muthuswamy, S. K., and Brugge, J. S. (2003) Nat. Cell Biol. 5, 733–740
53. Whitmarsh, A. J., and Davis, R. J. (1996) J. Mol. Med. 74, 589–607
54. Nateri, A. S., Riera-Sens, L., Costa, C. D., and Behrens, A. (2004) Science 303, 1374–1378
55. Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y. C., and Karin, M. (2004) Science 306, 271–275
56. MacLaren, A., Black, E. J., Clark, W., and Gillespie, D. A. (2004) Mol. Cell. Biol. 24, 9006–9018
57. Rich, K. A., Zhan, Y., and Blanks, J. C. (1997) J. Neurobiol. 32, 593–612
58. Oshitari, T., Dezawa, M., Okada, S., Takano, M., Negishi, H., Horie, H., Savada, H., Takahisa, T., and Adachi-Usami, E. (2002) Invest. Ophthal. Vis. Sci. 43, 2442–2449
59. Wenzel, A., Grimm, C., Marti, A., Kueng-Hitz, N., Hafezi, F., Niemeyer, G., and Reme, C. E. (2000) J. Neurosci. 20, 81–88
60. Yasuda, A., Adachi, M., Okuda, H., Nishiroya, Y., Takamura, T., Hareyama, M., Takayama, S., Reed, J. C., and Imai, K. (1999) Oncogene 18, 2681–2686
61. Spannghathasan, M., and Jothy, S. (2000) Pathol. Int. 50, 273–279
62. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999) Nature 400, 464–468