Keratinocyte growth factor accelerates compensatory growth in remnant lung after trilobectomy in rats

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Abstract

Objective: In rats, pulmonary resection is followed by lung compensatory growth. However, the molecular mechanism underlying lung compensatory growth remains unclear. Keratinocyte growth factor (KGF) is expressed in lung tissue and is considered a possible mitogen for lung epithelial cells. The objectives of this study were to define the role of KGF and its receptor (KGFR) in rat lung compensatory growth after trilobectomy and the effect of exogenous KGF expression gene transfection.

Methods: Adult Lewis rats were used. Right trilobectomy was performed in operation group and sham thoracotomy in sham group. In operation group, KGF-FLAG or FLAG expression vector was transfected directly into the lung by electroporation. Expression of KGF and KGFR, and alveolar cell proliferation index using proliferating cell nuclear antigen (PCNA) were measured in the right lung at day 14 after operation.

Results: PCNA, KGF and KGFR expressions in lung epithelial cells were significantly increased at day 4 after trilobectomy. Transfection of KGF-FLAG expression vector resulted in further significant enhancement of PCNA at day 4 after trilobectomy; however, the transfection of FLAG expression vector did not alter the enhancement of PCNA. Exogenous expression of KGF in remnant lung by electroporation significantly augmented epithelial proliferation and decreased the average air space distance [mean linear intercept (Lm)].

Conclusion: Our results implicate KGF in the induction of alveolar epithelial cell proliferation for compensatory lung growth, and that overexpression of KGF in remnant lung by electroporation significantly augmented lung epithelial proliferation.

Word count; 239 words

Ultramini-abstract: KGF is a member of fibroblast growth factor family. In rats subjected to trilobectomy, PCNA, KGF and KGF-R were overexpressed in lung epithelial cells of remnant lung 4 days later. KGF gene transfection by electroporation in remnant lung significantly augmented epithelial proliferation and reduced the average air space distance.

Word count; 49 words

Introduction

The lung undergoes compensatory growth after lung injury or surgical resection. The growth process involves increasing lung volume, weight, cell proliferation and pulmonary function (1, 2), but the mechanism responsible for this process is unclear. Understanding lung compensatory growth requires clarification of the molecular mechanism underlying the induction of lung epithelial cell proliferation.

Rubin et al. (3) discovered keratinocyte growth factor (KGF) as a mesenchymal cell-derived epithelial cell mitogen and subsequently, the KGF receptor (KGFR; bek IIIb) was identified in various epithelial cells. KGF expression is upregulated in rodent models of lung injury and has been implicated in the induction of alveolar epithelial cell proliferation (4). Kaza et al. (5) demonstrated that exogenous KGF enhanced postpneumonectomy compensatory lung growth through alveolar proliferation. The findings of that study were significant for investigating the role of exogenous KGF on lung compensatory growth. However, it is still unclear whether endogenous KGF and KGFR play a direct role in the induction of lung epithelial proliferation and differentiation during the compensatory growth of lung following lobectomy.

We hypothesized that KGF and KGFR are involved in compensatory lung growth and that exogenous KGF administration accelerates the compensatory lung growth. In the present study, we investigated first the temporal and spatial relationships between proliferation of lung epithelial cells and expression of KGF/KGFR in regenerating rat lung after trilobectomy by using immunohistochemistry. In the second part of the study,

KGF was expressed in remnant lung tissue to examine the effect of prolonged exposure to KGF on lung growth *in vivo*. For prolonged *in situ* expression of KGF in the lung, we used electroporation in this study because, unlike other methods, it facilitates the introduction of foreign genes into a specific area of the desired tissue with minimal damage (6), and with few side effects.

Materials and Methods

Animals, surgical procedure, and tissue preparation

Pathogen-free 8-week-old male Lewis rats weighing 220–270 g used as adult rats were purchased from Charles River Japan (Kanagawa, Japan). Each rat was anesthetized with intraperitoneal injection of 25 mg/kg sodium pentobarbital. The right lung of rats consists of four lobes; the cranial, middle, accessory and caudal lobe. In the surgery group, the right three lobes (cranial, middle and accessory lobe) were excised by thoracotomy while the remnant caudal lobe was used for investigation. In the sham group, a simple right thoracotomy was performed. Each experimental group at a given time point consisted of three rats. The remnant lung lobe after trilobectomy and sham-operation were dissected out and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany). Serial 4-μm sections were cut and each specimen was stained with hematoxylin and eosin. All animal experiments were performed in accordance with NIH guidelines dictated by the Animal Care Facility at Nagasaki University Graduate School of Medicine (Approval code is 0309010318).

Cells

L2 rat lung epithelial cells (ATCC, Manassas, VA) were grown in F12K medium (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum at 37°C and 5% CO_2.

Construction of a recombinant plasmid encoding human KGF

The human KGF (hKGF) coding region was amplified by polymerase chain reaction (PCR) using pCEV9 plasmid (kindly provided by Jeffrey Rubin, National Cancer Institute, Bethesda, MD) as template. This plasmid contains the complete KGF coding sequence isolated from an M426 human embryonic lung fibroblast cDNA library (7). The sequences of forward and reverse primers were 5'-CCTAGATCTGCCACCATGCACAAATGGATACTGAC-3' and 5'-CCTCTCGAGTTAAGTTATTGCCATAGGAAG-3', respectively. The forward primer is homologous to nucleotides 1 to 20 in the hKGF open reading frame and contains a consensus (GCCACC) Kozak sequence immediately upstream of the initiation codon. In addition, a *Bgl*II site was inserted at the 5'-end to facilitate cloning. The reverse primer is homologous to nucleotides 563 to 582 and contains an *Xba*I site at the 5'-terminus. These primers allowed amplification of a 603-bp cDNA fragment. PCR was carried out in a reaction mixture containing 2 mM Tris-HCl, pH 8.0, 10 mM KCl, 2 mM $MgCl_2$, 0.01 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), 0.05% Tween 20, 0.05% nonidet P-40, 5% glycerol, 0.25 mM dNTP mixture, 100 pmol of each primer, 6 μg pCEV9 DNA, and 3 U *Taq* polymerase (Takara Mirus Bio Co, Shiga, Japan). Before cycling, the mixture was divided into four 20-μl reaction volumes. PCR assays were performed in a Gene Amp PCR system 9600 (Perkin Elmer, Foster City, CA) for 27 cycles under the following conditions: 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 15 min. Agarose gel electrophoresis using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) was used to purify the

amplified product. The recovered DNA was then cloned into the plasmid pGEM-T Easy Vector using pGEM-T Easy Vector System I (Promega, Madison, WI), resulting in the pGEM-hKGF plasmid. We then isolated the DNA insert containing the hKGF sequence from pGEM-hKGF by double digestion with *Bgl*II and *Xba*I and further subcloned into the p3xFLAG-CMV14 vector (Sigma) at the same restriction sites to produce the pKGF-FLAG plasmid. All constructions were confirmed by automated sequencing (CEQ8000; Beckman Coulter, Fullerton, CA).

Evaluation of lung cell proliferation

Lung cell proliferation was analyzed immunohistochemically using anti-proliferating cell nuclear antigen (PCNA) antibody (DAKO, Glostrup, Denmark). Briefly, the sections were autoclaved and incubated with PCNA (1:200) as the primary antibody and horseradish peroxidase (HRP)-goat anti-mouse IgG (1:200) (Chemicon International Temecula, CA) as the secondary antibody.

Western blot analysis of KGF and KGFR expression in transfected cells and remnant lung tissue

Transfected cells were lysed in 0.3 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 50 μg/ml lima bean trypsin inhibitor, and 0.5% Triton X-100). Rat lung specimens were homogenized in lysis, centrifuged, and then the supernatants were collected. Recombinant human KGF (rhKGF, Pepro Tech EC, London, UK) and recombinant human KGFR (rhKGFR, R&D Systems

Minneapolis, MN) were also processed as positive controls. Samples were separated by SDS-PAGE on a 4–20% gradient gel (Daiichi Pure Chemicals, Tokyo, Japan) for KGF or a 10–20% gradient gel (Daiichi) for KGFR and electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon, Millipore Corporation, Bedford, MA). The membranes were blocked with Blocking One (Nacalai tesque, Kyoto, Japan) and then incubated with the first antibody (1:800 anti-KGF antibody, 1:800 anti-KGFR antibody; Nichirei Co, Tokyo, Japan, and 1:6000 peroxidase-conjugated mouse monoclonal anti-FLAG antibody; Invitrogen, Tokyo, Japan). After washing, the membranes were reacted with HRP-conjugated goat anti-rabbit IgG as the secondary antibody at 1:8,000 for KGF and 1:4,000 for KGFR, and were visualized by chemiluminescence using the ECL Plus reagent (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry of KGF and KGFR in the lung

Immunohistochemistry was performed to determine the expression of KGF and KGFR in tissue sections using anti-KGF and anti-KGFR antibodies as described previously (8, 9). Sections of rat lung tissue were incubated for 2 hours with primary antibody at 1.0 μg/ml for the anti-KGF antibody and 1:600 for anti-KGFR antiserum, then incubated with HRP-conjugated goat anti-rabbit IgG (1:100) (MBL, Nagoya, Japan) for 1 hour. After washing, HRP sites were visualized by treatment with 3,3'-diaminobenzidine-4HCl (DAB; Dojindo, Kumamoto, Japan) and H_2O_2.

Transfection of KGF expression plasmid into cultured cells and assay of cell proliferation and KGF production

L2 cells (2 x 10^6 in 200 μl) were resuspended into sterile cuvettes, and plasmid DNA (10 μg) of pKGF-FLAG or pFLAG gene was added. An electric pulse (400 V, 99 μsec, five pulses) was delivered to the cells, using a BTX Transfector apparatus (BTX Inc, San Diego, CA). To determine the biological activity of KGF-FLAG fusion protein *in vitro*, cells were plated at a density of 3 x 10^3 cells/well in 96-well plates at a final volume of 100 μl/ well, and cultured in a humidified atmosphere at 37°C for analysis using the Cell Proliferation Biotrak enzyme-linked immunosorbent assay (ELISA) system (Amersham Biosciences). The optical density of each well was measured at 450 nm.

To measure KGF, the cells were plated at a density of 5 x 10^5 cells/well in 6-well plastic culture dishes (Iwaki, Tokyo) in 1 ml of F12K medium (Sigma) supplemented with 10% fetal bovine serum per well. After incubation for 24, 48, or 72 hours, the media were collected and the cells harvested with a lysate buffer (1% SDS and 1.0 mM sodium orthovanadate in 10 mM Tris-HCl, pH 7.4). The concentration of KGF was determined by ELISA (R&D Systems), according to the instructions provided by the manufacturer.

***In vivo* transfection of KGF-FLAG and FLAG expression vectors**

For tissue transfection, pulses generated with an Electro Square Porator (T820; BTX) were delivered to the organ by a pair of electrode disks (1-cm diameter) attached to the tips of tweezers (Pinsettes-Type electrode 449-10PRG; Meiwa Shoji, Tokyo). We used a

green fluorescent protein (GFP)-expressing plasmid to optimize conditions for lung transfection. The best results were obtained with six pulses of 50 V for 50 μsec each separated by 999 msec, delivered by electrode disks separated by approximately 5 mm (data not shown). After trilobectomy, a solution of 100 μg of pKGF-FLAG or pFLAG plasmid in 100 μl PBS was injected directly with a 29-gauge needle into remnant lung between the electrode disks. Immediately after the DNA injection, electric pulses were administered. Rats were sacrificed 2, 4, or 7 days after electroporation, Each experimental group consisted of five rats at each time point.

Identification of PCNA-positive and FLAG-expressing cell types

Double staining for PCNA and SP-A, an alveolar type II epithelial cell marker, was performed on sections of lung tissue to identify the types of PCNA-positive cells. The slides were stained first with anti-SP-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:30) and the HRP sites were visualized with DAB and H_2O_2. The slides were autoclaved and immersed three times with 0.1 M glycine-HCl buffer (pH 2.2) and then immunostained with anti-PCNA antibody (1:50), as described above. HRP reactivity was visualized with 4-Cl-1-naphthol (Tokyo Kasei Kogyo, Tokyo). Peroxidase-conjugated mouse monoclonal anti-FLAG antibody (Invitrogen; 1:30) was used to identify FLAG-expressing cells.

Quantitative analysis

The PCNA staining was graded as positive or negative, compared to the negative control. For each section, more than 2,000 cell nuclei were counted over randomly selected fields at x 400 magnification. The number of positive cells was expressed as the percentage of cells with positive nuclei per total number of counted nuclei (10). The signal density of KGF and KGFR per cell was measured by an image analyzer, and calculated from the sum of the gray values of all pixels corresponding to the cell.

Using a grid with lines of known length, the average air space distance [mean linear intercept (Lm)] was determined in 30 randomly selected microscopic fields per each rat in pFLAG gene- or pKGF-FLAG gene-transfected group at day 7 after operation. The observers that performed the measurements were blinded to the experimental groups.

Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using unpaired Student's *t*-test or one-way analysis of variance. A *P* value <0.05 was considered statistically significant. All analyses were performed with a statistical software package (StatView, version J5.0; Abacus Concepts, Berkeley, CA).

Results

Induction of epithelial cell proliferation in remnant lung after trilobectomy

To examine the cell kinetics of compensatory regenerating lung, proliferating cells were assessed by immunochemical staining for PCNA. A few proliferating cells were noted in the normal lung (Figure 1A and B), with a PCNA labeling index (% of proliferative cells per total cell number) of 1.74 ± 0.75 for alveolar epithelial cells. At day 4 after trilobectomy, several PCNA-positive bronchial and alveolar epithelial cells were noted (Figure 1C and D), with a PCNA labeling index of 8.49 ± 1.83 for alveolar epithelial cells. In the sham-operation lung, few PCNA-positive bronchial and alveolar epithelial cells were detected (Figure 1E and F), with a PCNA labeling index of 1.99 ± 0.61 for alveolar epithelial cells. Most of the positive cells in the alveolar regions of trilobectomy rats seemed to be alveolar type II epithelial cells (Figure 1C). As shown in Figure 1G, the percentage of PCNA-positive cells increased significantly at day 2, and reached maximum at day 4 after operation in the trilobectomy group. Many PCNA-positive cells were alveolar type II cells (Figure 1C), however, some alveolar type I cells, macrophages, fibroblasts, bronchial epithelial cells were PCNA-positive.

Western blot analysis of KGF and KGFR expression in remnant lung after trilobectomy

Western blot analysis showed specific bands corresponding to KGF and KGFR proteins at approximately 28 kDa and 130 kDa, respectively (3, 11, 12). The Approximate

molecular mass of 130 kDa is agreement with the reported molecular mass of the mature KGFR (11-13). Recombinant human KGF is an 18.9-kDa protein containing 163 amino acid residues, but has the same function as endogenous KGF. The reduced human KGFR has a calculated molecular mass of approximately 56 kDa. As a result of glycosylation, the recombinant protein migrates as an approximately 100 kDa protein in SDS-PAGE. The intensity levels of the KGF and KGFR bands in remnant lung markedly increased at day 4 after trilobectomy, compared with the sham-operation group (Figure 2).

Immunohistochemical localization of KGF and KGFR in remnant lung after trilobectomy

Normal lung collected after the sham operation showed KGF protein expression only in a few stromal cells and alveolar epithelial cells, particularly alveolar type II cells (data not shown). KGFR was also found in only a few epithelial cells. The expression of both KGF and KGFR increased following the trilobectomy, compared with the sham-operation lung (Figure 3B and D) and reached a maximum in the alveolar (Figure 3A and C) and bronchial regions (Figure 3E and F) at day 4 after operation. KGF was expressed strongly in stromal cells, alveolar type I, and type II cells, while KGFR was expressed only in epithelial cells, especially alveolar type II cells, where the signal was distributed in the cytoplasm and plasma membrane. KGF expression increased as early as day 2 after trilobectomy in remnant lung tissue (signal density in operation group; 3.92 ± 1.05-fold, sham operation group; 1.05 ± 1.11-fold, $P < 0.05$) and reached a maximum at day 4 after operation (signal density in operation group; 9.16 ± 4.38-fold, sham operation group;

0.84 ± 0.33-fold, P <0.05), as shown in Figure 3G. Similarly, KGFR expression reached a maximum at day 4 after trilobectomy (signal density in operation group; 22.96 ± 13.6-fold, sham operation group; 0.91 ± 0.30-fold, P <0.05), as shown in Figure 3H.

To clarify the relationship between PCNA-positive cells and KGF- or KGFR-positive cells, these antigens were localized in serial sections (Figure 4). KGF and KGFR were detected in both alveolar type I and type II cells, while PCNA was localized in alveolar type II cells.

Enhancement of KGF expression and proliferating activity in cultured rat lung epithelial cells (L2 cells) expressing the pKGF-FLAG gene

L2 cells were transfected with pKGF-FLAG cDNA and the concentration of KGF-FLAG protein in the lysates and culture medium was measured at various time points (Figure 5A). KGF concentration in the medium increased in a time-dependent manner, while that in the lysates was unchanged.

Next, BrdU uptake by L2 cells transfected with pKGF-FLAG or pFLAG plasmids was measured to assess the DNA synthetic activity (Figure 5B). After 24 hours of transfection, BrdU uptake increased significantly in pKGF-FLAG-transfected cells (P=0.0003), although there was no significant difference in cell numbers (Figure 5C). After 72 hours, however, the increase in cell number became significant (Figure 5C), while no significant difference was measured in the rate of BrdU uptake. These results indicated that the KGF expression vector used in the present study worked well in lung epithelial cells and that the expressed KGF was active in stimulating cell proliferation.

Effect of augmented KGF expression driven by pKGF-FLAG transfection on compensatory growth of remnant lung after trilobectomy

PCNA expression was then examined in remnant lung transfected with pKGF-FLAG or pFLAG following trilobectomy to assess the effect of prolonged exposure to KGF on compensatory lung growth process. As shown in Figure 6A and B, the number of PCNA-positive cells markedly increased in remnant lung transfected with pKGF-FLAG compared to that overexpressing pFLAG alone. In fact, immunohistochemistry of the pKGF-FLAG-transfected lung revealed a higher abundance of PCNA-positive cells (Figure 6D) in areas strongly stained for FLAG (Figure 6C) than in FLAG-negative areas (data not shown). Quantitative analysis of PCNA-positive cells also revealed a significant increase in remnant lung transfected with pKGF-FLAG (Figure 6E). Figure 6F shows Lm values measured in the two experimental groups at day 7 after operation. Rats transfected with pKGF-FLAG gene showed a significant decrease in the mean values of Lm ($P<0.001$) compared with rats transfected pFLAG gene.

Finally, PCNA-positive cells in the pKGF-FLAG-transfected lung comprised predominantly alveolar epithelial cells with some fibroblasts, endothelial cells, macrophages and bronchial epithelial cells. To correlate the PCNA-positive cells with alveolar epithelial cell-type, we double-stained for PCNA and SP-A (a marker for alveolar type II cells). SP-A-positive cells also expressed PCNA. Interestingly; however, some SP-A-negative epithelial cells were also positive for PCNA, indicating that alveolar type I cells could proliferate under these experimental conditions (Figure 7). Interestingly,

in remnant lung after trilobectomy only, a few cells excluding alveolar type II cells were proliferative (Figure1C). However, in remnant lung after trilobectomy and gene transfection, many cells, including type I cells, macrophages, fibroblasts and bronchial cells, were proliferating, especially alveolar type II cells (Figure 6B and 6D).

Discussion

The present study investigated the possible roles of KGF in the regulation of rat lung compensatory growth after right trilobectomy, and found that in parallel with more PCNA-positive alveolar type II cells, the expression levels of KGF and KGFR were increased in the remnant lung. In particular, the expression of KGFR was markedly induced after trilobectomy in alveolar epithelial cells, and such expression might be involved in the induction of proliferation of alveolar type II cells. Moreover, when the KGF-FLAG expression vector was expressed in remnant lung by electroporation, the fusion protein was effectively produced in various cells. In addition, the transfected lung showed significantly enhanced proliferation activity and possible alveolar septation. These results strongly implicate KGF in the induction of alveolar epithelial cell proliferation for compensatory growth of the lung, and that *in vivo* overexpression of KGF seems to accelerate KGF-dependent compensatory growth processes in organs.

A number of studies have documented that pulmonary alveoli are capable of regeneration. In rodents, retinoid acid enhanced lung growth after pneumonectomy in rats (1) and estradiol induced alveolar regeneration after loss of alveoli induced by ovariectomy in adult mice (14). These studies described increased cell proliferation, lung volume, lung weight, alveolar surface density and alveolar number in experimental animals. Unfortunately, it is impossible to conduct the above assessments in human, instead these tests are limited to pulmonary function tests, especially, forced vital capacity and FEV_1. Thus, there is so far no documented evidence for lung regeneration in

human. However, based on the present and previous animal experiments, further advances in medical biotechnology could perhaps allow investigation of this process in human.

The KGF-KGFR system also plays an important role in lung epithelial cell turnover in normal rodents. Many studies showed that administration of recombinant KGF could mimic the proliferation of alveolar type II cells both *in vitro* and *in vivo* (15, 16). Importantly, Kaza et al. (5) demonstrated that recombinant KGF enhances post-pneumonectomy lung growth accompanied by alveolar cell proliferation, similar to the present results. However, they did not deal with the involvement of KGFR, which is also an essential factor in the logical understanding of the role of KGF in compensatory lung growth. The present work demonstrates the involvement of both KGF and KGFR in the induction of compensatory lung growth. In fact, KGF has been implicated in various phenomena (8, 17), where a low level of constitutive KGFR expression might be sufficient for the necessary action of KGF. The present study showed that PCNA, KGF, and KGFR were simultaneously localized in the same alveolar type II cells at day 4 after trilobectomy. However, there was an apparent difference in the appearance of PCNA- and KGFR-positive cells, and it should be noted that several other factors such as basic fibroblast growth factor (18), hepatocyte growth factor (19, 20) and epidermal growth factor (21), and VEGF (22) might also play a role in lung growth.

Basic fibroblast growth factor (bFGF) induces an increase in pulmonary blood flow in the damaged lung (18). HGF is a potent mitogen for rat alveolar epithelial cells, especially alveolar type II cells (19) and may work simultaneously on various types of

cells. However, HGF alone was found to be incapable of restoring branching morphogenesis, unlike KGF and acidic FGF. HGF exhibits synergistic effects with KGF or acidic FGF in this mesenchyme-free system (20). EGF stimulates the cytodifferentiation of alveolar type II cells and biosynthesis of surfactant precursor proteins (21). Furthermore, it has been demonstrated that during pulmonary distension, the stimulus for alveolarization (capillary and alveolar growth) upregulated VEGF mRNA (22). Finally, it is unlikely that each of the above factors can singularly enhance lung growth; rather, the synergistic effects of mediators of mesenchymal-epithelial interactions are more likely to be important in lung growth.

The present study applied electroporation to maximize the transfection efficiency so that a single-dose regimen could be used and much smaller amounts of DNA would be required. One major advantage of electroporation for gene transfection into the lung is that it efficiently transfers cDNA to multiple cell types simultaneously including alveolar epithelial cells, bronchial epithelial cells, smooth muscle cells, endothelial cells, and other cells in focal or target areas of the lung. In the present study, in remnant lung after trilobectomy only, a few cells excluding alveolar type II cells were proliferative (Figure 1C), however, in remnant lung after trilobectomy and gene transfection, many cells, including type I cells, macrophages, fibroblasts and bronchial cells were proliferative, especially alveolar type II cells (Figure 6B and 6D).

The significantly elevated PCNA index in remnant lung after trilobectomy was seen by day 2 after transfection and continued until day 4, before returning to the control, untransfected level at day 7. Yew et al. (23) reported increased duration of expression of

up to 3 months with a human ubiquitin B promoter or a hybrid CMV–ubiquitin B promoter. In the present study, we are not sure that a long-term expression of KGF is beneficial or not. However, these alternative promoters might be tested for their effects on both duration and level of gene expression in our system. On the other hand, at day 7 after operation, rats transfected pKGF-FLAG gene showed a significant decrease in the mean values of Lm compared with rats transfected with pFLAG gene ($p<0.001$). In spite of the short duration of gene expression, these data support the possibility of alveolar septation and increased number of alveoli in the pulmonary parenchyma.

In conclusion, our results showed the potential involvement of endogenous KGF in compensatory tissue growth in adult rat lung. Moreover, direct injection of naked DNA coupled with electroporation seemed to significantly enhance compensatory lung growth. Although more work needs to be done, we believe this study advocates gene therapy as a potential tool to accelerate tissue growth in adult rat lung.

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Figure Legends:

Figure 1. Serial changes in PCNA-positive alveolar epithelial cell counts in remnant lung.

(A-F) Immunohistochemical staining for PCNA. Magnification: x400. Bar: 50 μm (A) Alveolar part in normal lung. (B) Bronchial part in normal lung. (C) Alveolar part at day 4 after trilobectomy. (D) Bronchial part at day 4 after trilobectomy. (E) Alveolar part at day 4 after sham-operation. (F) Bronchial part at day 4 after sham-operation. (G) Serial changes in PCNA labeling index. Open circles: trilobectomy group (n=3), closed circles: sham group (n=3) (*$p<0.05$).

Figure 2. Western blot analysis of KGF and KGFR in remnant lung.

Recombinant human KGF (rhKGF) protein (lane 1), lysates of remnant lung at day 4 after trilobectomy (lane 2) and after sham-operation (lane 3). Recombinant human KGFR (rhKGFR) protein (lane 4), lysates of remnant lung at day 4 after trilobectomy (lane 5) and after sham-operation (lane 6).

Figure 3. Immunohistochemical detection of KGF and KGFR in remnant lung after trilobectomy

The expression of KGF (A, B and E) and KGFR (C, D and F) was detected by immunohistochemistry. (A) Alveolar part at day 4 after trilobectomy. (B) Alveolar part at day 4 after sham-operation. (C) Alveolar part at day 4 after trilobectomy. (D) Alveolar

part at day 4 after sham-operation. (E) Bronchial part at day 4 after trilobectomy. (F) Bronchial part at day 4 after trilobectomy. Magnification: x400. Bar: 50 μm. (G) Serial changes in the signal density of KGF staining in trilobectomy group (n=3) (open circles) and sham-operation group (n=3) (closed circles). (H) Serial changes in the signal density of KGFR staining in trilobectomy group (n=3) (open circles) and sham-operation group (n=3) (closed circles).

Figure 4. Immunohistochemical detection of KGF, KGFR and PCNA in serial sections of remnant lung at day 4 after trilobectomy.

(A) H&E staining. (B) KGF was detected in the cytoplasm of alveolar epithelial cells. (C) PCNA staining. (D) KGFR was detected in the plasma membrane of alveolar epithelial cells. PCNA-positive alveolar type II cells are identical, at least in part, to KGF- and KGFR-double positive cells (arrows). Bar: 25 μm.

Figure 5. KGF protein production, DNA synthesis and cell proliferation in cell medium and lysate transfected with pKGF-FLAG gene and pFLAG gene.

(A) The concentrations of KGF in the medium and lysates of L2 cells transfected with pKGF-FLAG gene and pFLAG gene were measured at 24, 48 and 72 hour after transfection. KGF concentration in the medium of L2 cells transfected with pKGF-FLAG gene increased in a time-dependent manner. (B) DNA synthesis and (C) cell proliferation of L2 cells transfected with pKGF-FLAG and pFLAG. L2 cells were transfected by

electroporation, cultured for 24, 48 and 72 hours, and subsequently assessed for BrdU uptake as well as the number of cells per well. There were significant differences in BrdU uptake between pKGF-FALG gene and pFLAG gene at 24 and 48 hours after transfection. There was also a significant difference in the cell number between pKGF-FALG gene and pFLAG gene at 72 hours after transfection.

Figure 6. Effects of pKGF-FLAG gene and pFLAG gene expression on PCNA-positive cells at day 4 after electroporative transfection.

(A and B) Immunohistochemistry for PCNA in the alveolar part of remnant lung transfected with pFLAG gene (A) or pKGF-FLAG gene (B). In adjacent sections of the alveolar part transfected with pKGF-FLAG gene, one section (C) was used for the deletion of FLAG (KGF-FLAG) and another (D) was stained for PCNA expression. Magnification: x400. Bar: 50 μm. (E) Serial changes in PCNA labeling index. Open bars: group transfected with pFLAG gene (n=5), closed bars: group transfected with pKGF-FLAG gene (n=5) (*$p<0.05$). (F) Mean linear intercept values measured in remnant lung transfected with pFLAG gene or pKGF-FLAG gene. Values are mean±SEM. * $P<0.001$, for comparison of Lm of remnant lung transfected with pKGF-FLAG gene and that with pFLAG gene.

Figure 7. Double staining for PCNA and Surfactant Protein-A at day 4 after transfection in alveolar part of remnant lung transfected with pKGF-FLAG gene.

Double staining for PCNA (blue) and SP-A (brown). The majority of PCNA-positive

cells were epithelial cells. Arrows: alveolar type II cells, arrowhead: type I cells.

Magnification: x400. Bar: 25 μm.

Figure1

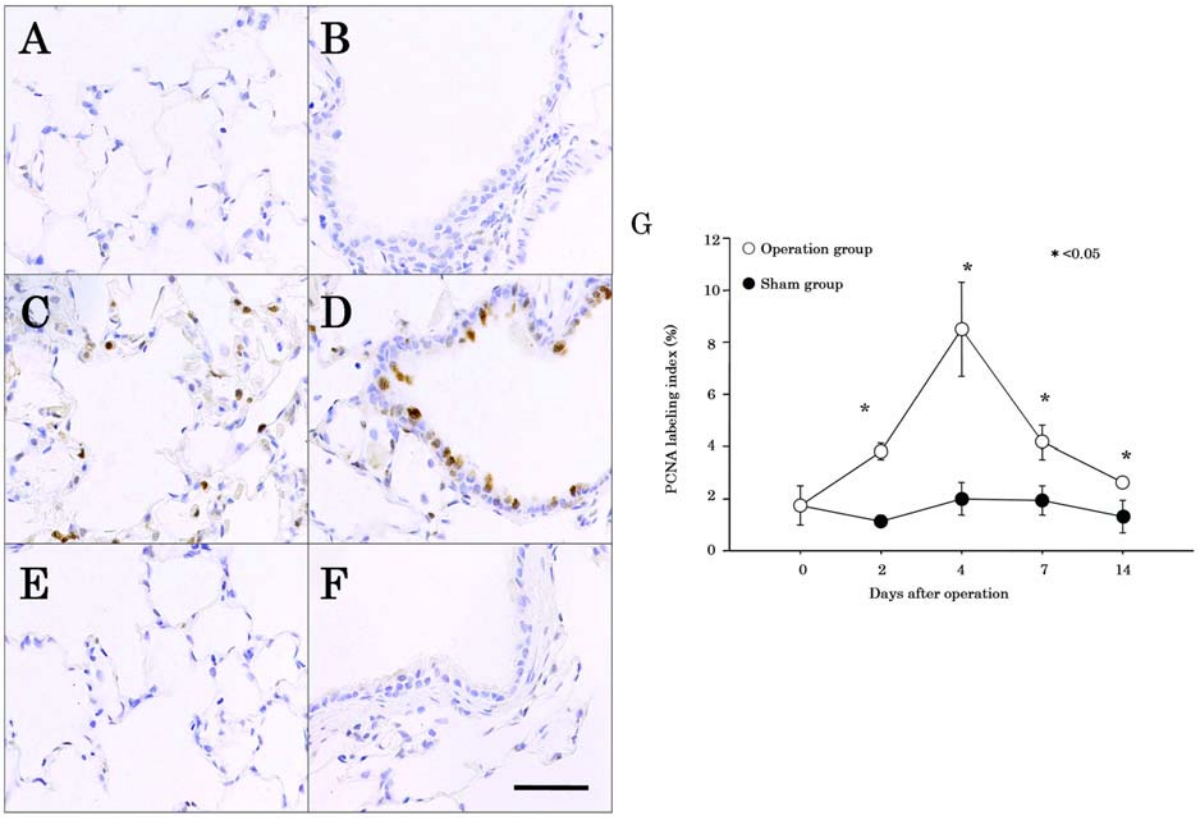

A
B
C
D
E
F
G
Operation group
Sham group
* <0.05
PCNA labeling index (%)
Days after operation
0
2
4
7
14
12
10
8
6
4
2
0

Figure2

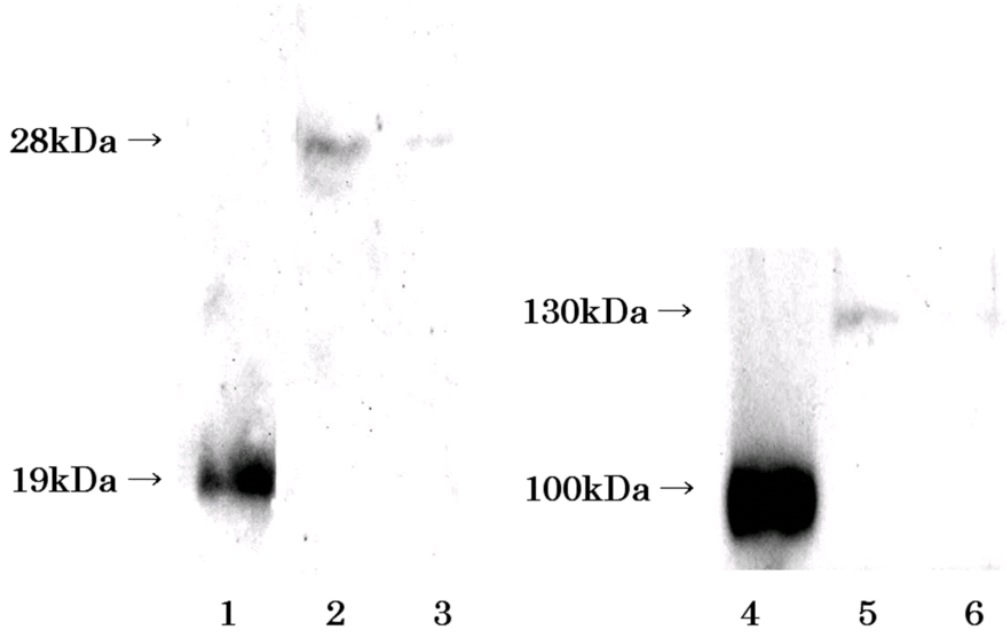


Figure3

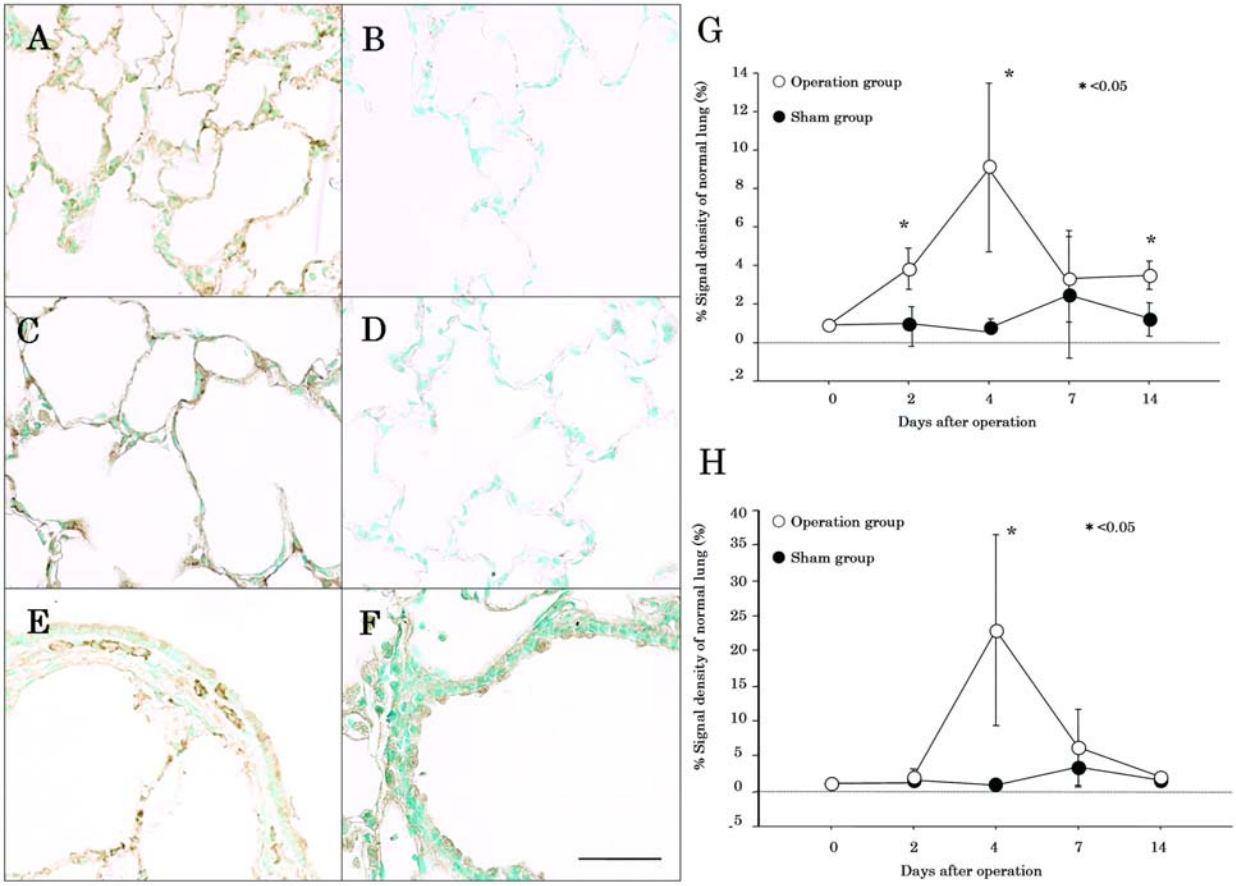

A
B
C
D
E
F
G
Operation group
Sham group
* <0.05
% Signal density of normal lung (%)
Days after operation
H
Operation group
Sham group
* <0.05
% Signal density of normal lung (%)
Days after operation

Figure4

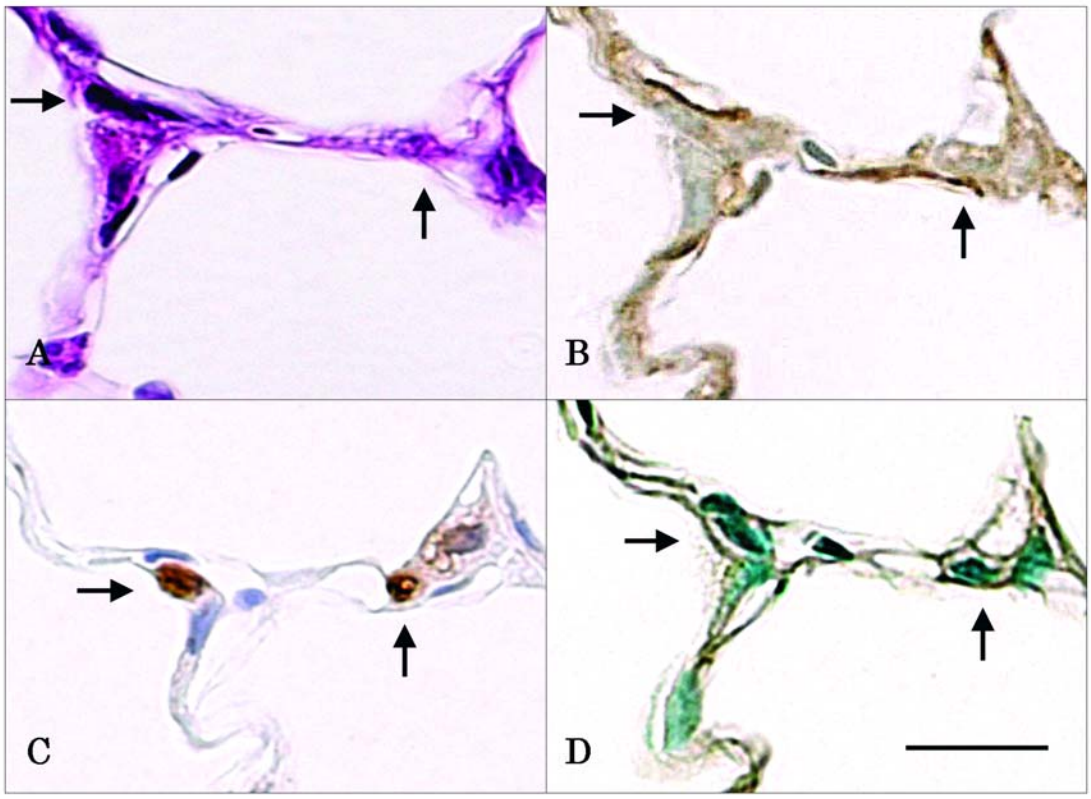
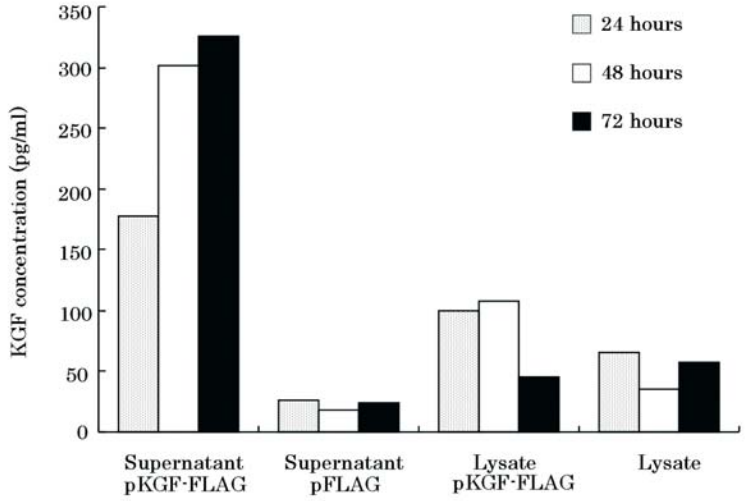
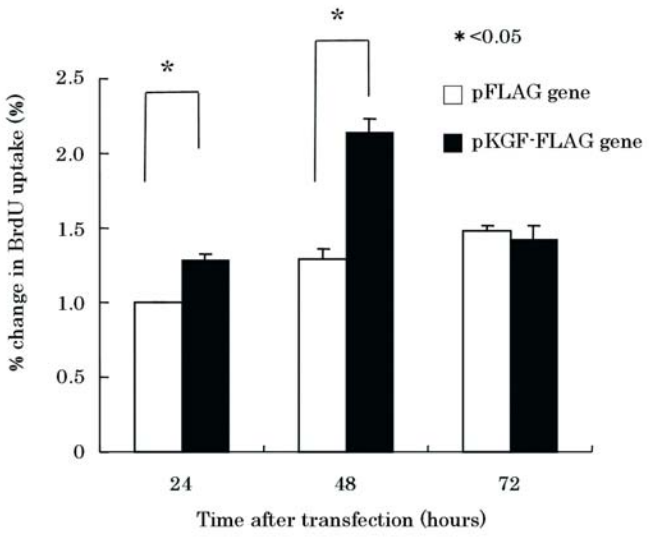


Figure5

A


KGF concentration (pg/ml)
350
300
250
200
150
100
50
0
24 hours
48 hours
72 hours
Supernatant pKGF-FLAG
Supernatant pFLAG
Lysate pKGF-FLAG
Lysate

B


*
*
* <0.05
pFLAG gene
pKGF-FLAG gene
% change in BrdU uptake (%)
2.5
2.0
1.5
1.0
0.5
0
24
48
72
Time after transfection (hours)

C

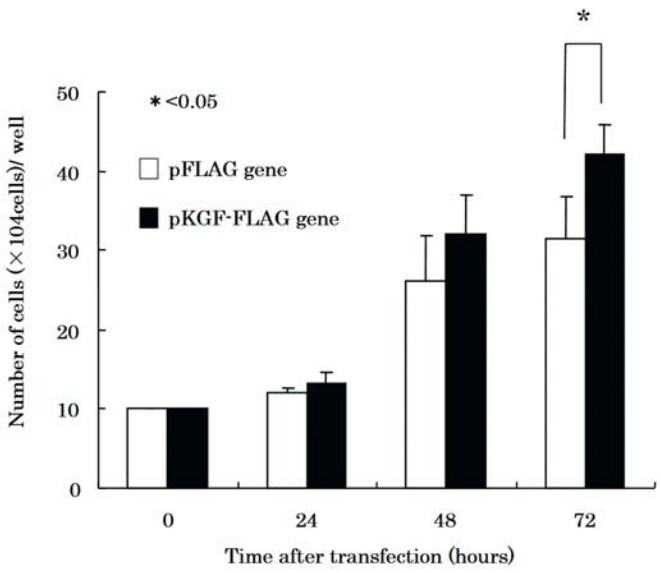
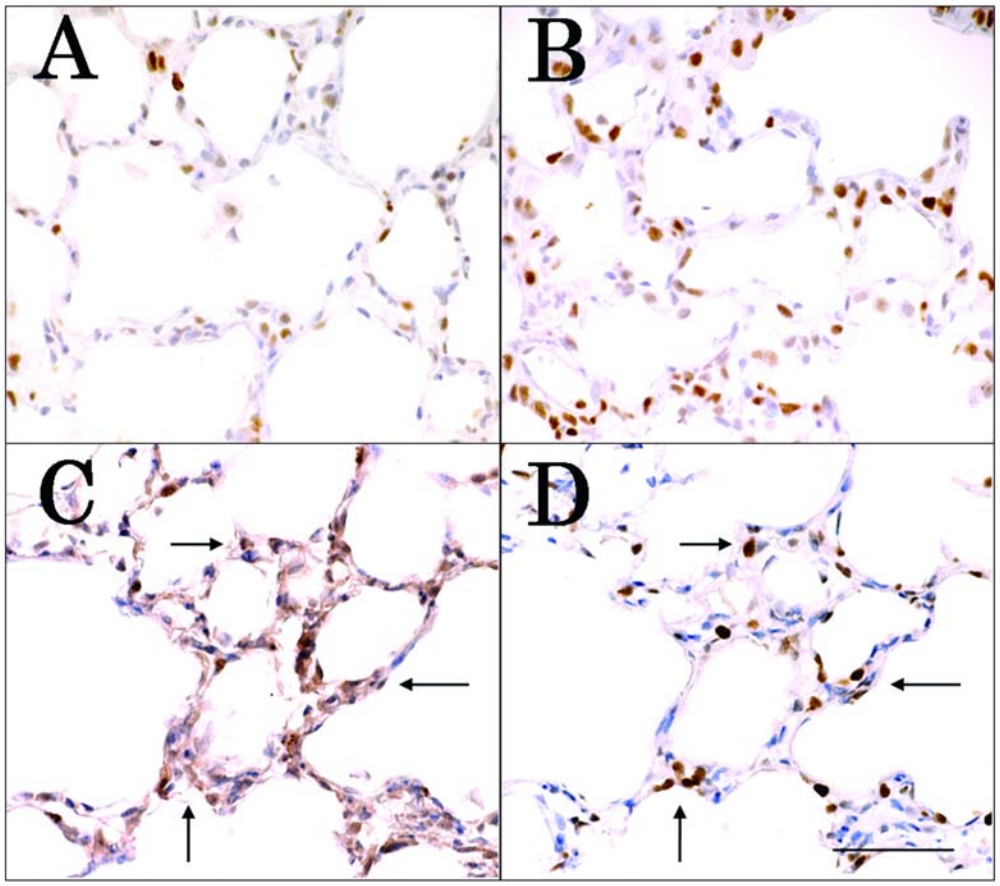
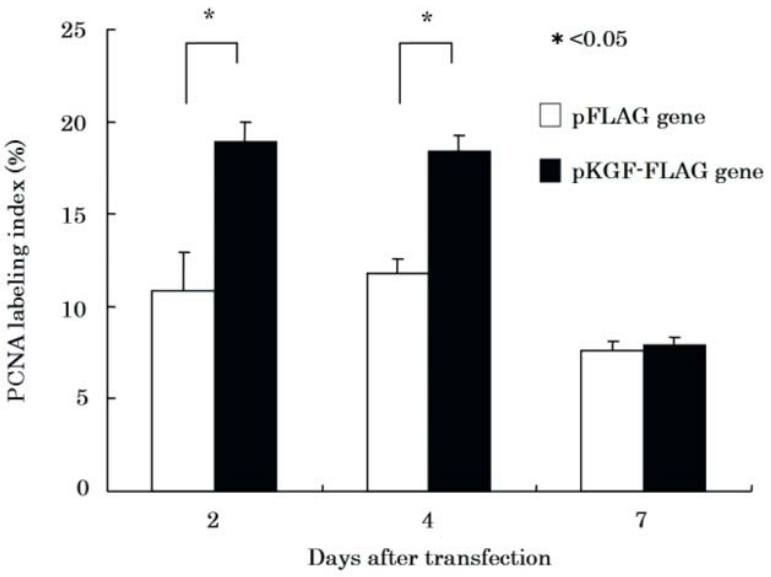

*
*<0.05
pFLAG gene
pKGF-FLAG gene
Number of cells (×104cells)/ well
50
40
30
20
10
0
0
24
48
72
Time after transfection (hours)

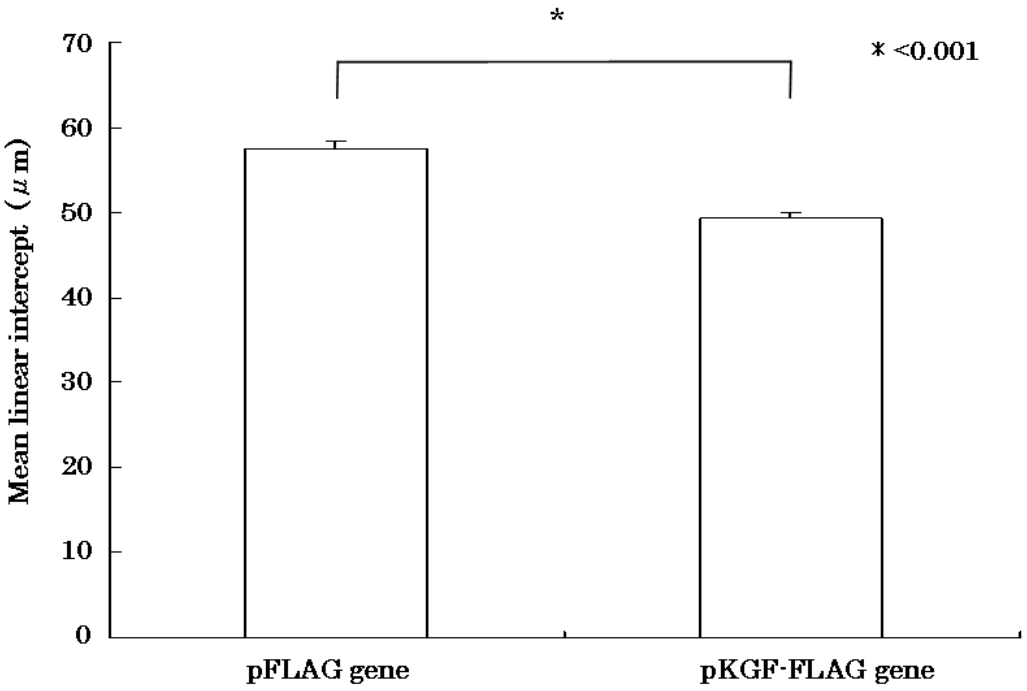
Figure6



E


*
*
* <0.05
pFLAG gene
pKGF-FLAG gene
PCNA labeling index (%)
25
20
15
10
5
0
2
4
7
Days after transfection

F


*
* <0.001
70
60
50
40
30
20
10
0
Mean linear intercept (μm)
pFLAG gene
pKGF-FLAG gene

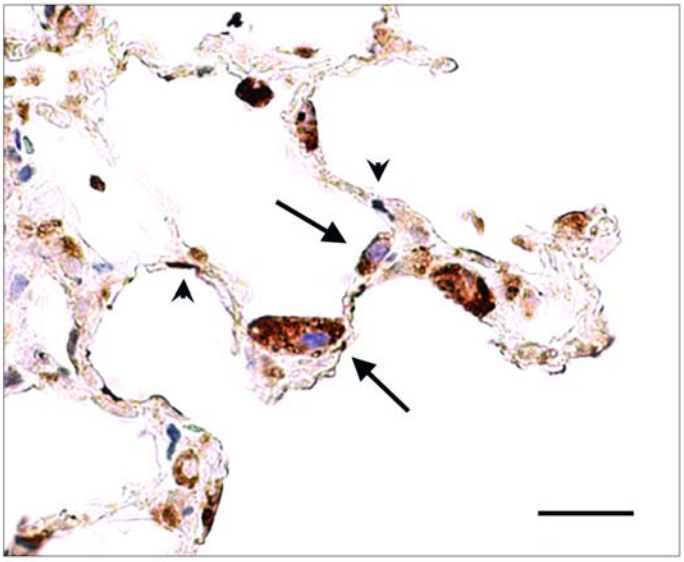



Figure7