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Fibroblast growth factor-10 prevents H_2O_2-induced cell cycle arrest by regulation of G1 cyclins and cyclin dependent kinases

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Abstract **We studied the effects of fibroblast growth factor (FGF-10) on H_2O_2-induced alveolar epithelial cell (AEC) G1 arrest and the role of G1 cyclins. FGF-10 prevented H_2O_2-induced AEC G1 arrest. FGF-10 induced 2–4-fold increase in cyclin E, cyclin A and CDKs (2, 4) alone and in AEC treated with H_2O_2. H_2O_2 downregulated cyclin D1; FGF-10 blocked these effects. FGF-10 prevented H_2O_2-induced upregulation of CDK inhibitor, p21. SiRNAp21 blocked H_2O_2-induced downregulation of cyclins, CDKs and AEC G1 arrest. Accordingly, we provide first evidence that FGF-10 regulates G1 cyclins and CDKs, and prevents H_2O_2-induced AEC G1 arrest.**

Keywords: Fibroblast growth factor-10; G1 Cyclins; H_2O_2; p21

1. Introduction

Alveolar epithelial cell (AEC) injury and repair are important in the pathogenesis of oxidant-induced lung damage and fibrosis [1–7]. Exposure to oxidants may trigger an intense inflammation and loss of alveolar epithelium. DNA-damaging agents such as H_2O_2 may induce damage to cellular DNA, lipids, and proteins. Oxidants generate signals that converge to cause wide range of cellular responses ranging from transient growth arrest, to permanent growth arrest, apoptosis and ultimately necrosis, depending on the level of oxidative stress experienced [8–10]. H_2O_2, in particular, is known to induce multiphase cell cycle arrest [9]. Control of cell cycle progression in response to oxidative stress is linked to activation of a checkpoint mechanism operating before entry into the S phase of the cell cycle [11–15]. Progression through the G1 phase and the G1–S transition involves sequential assembly and activation of G1 cyclins and cyclin dependent kinases (CDKs) [11–15]. After oxidant injury, the rapidity of initiation of type II cell proliferation is crucial for a proper healing, as delay in the reepithelialization process has been implicated in the development of pulmonary fibrosis [1,3–7]. Therefore, characterization of the mechanisms involved in the block of type II cell replication by oxidants; and the internal and external stimuli such as antimitogens and growth factors that regulate the repair mechanisms appear to be critical for the understanding and management of many lung diseases that are associated with oxidative stress.

Fibroblast growth factor-10 (FGF-10) a 19.3 kDa heparin-binding protein, is a potent AEC mitogen that promotes epithelial cell differentiation, migration, wound healing; prevents oxidant (H_2O_2, asbestos, cyclic stretch)-induced AEC DNA damage and apoptosis; and is required for lung development [3–7]. The mechanisms underlying the protective effects of FGF-10 are multiple and not well defined. It is known that growth factors and antimitogens regulate cell cycle in order to integrate recovery from oxidant injury leading to DNA repair and cell division [12–16]. Particularly, bFGF and FGF-2 are shown to modulate cell cycle arrest by regulation of cyclins; however, there is no information on regulation of cell cycle by FGF-10 [12–14]. In this study, we sought to determine whether FGF-10 regulates the cell cycle by restoring the progression of the cell cycle after oxidative stress. We demonstrate that FGF-10 upregulates cyclins and CDKs, downregulates CDK inhibitor, p21 and prevents H_2O_2-induced AEC arrest.

2. Materials and methods

2.1. Materials

FGF-10 was purchased from R&D systems. The antibodies against cyclin E, cyclin A, CDK2, CDK4, cyclin D1, p21, c-Myc and pRb were purchased from Santa Cruz Biotechnology. The anti-α-tubulin monoclonal antibody was purchased from Molecular Probes. All other chemicals were purchased from Sigma Chemicals.

2.2. Cell culture

A549 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (0.3 μg/ml), non-essential amino acids, penicillin (100 U/ml), streptomycin (200 μg/ml), and 10% fetal bovine serum (FBS; GIBCO) in a humidified 95% air–5% CO_2 incubator at 37 °C.

2.3. Cell synchronization by serum starvation

Cells were synchronized at G0/G1 phase by serum starvation and then incubated in DMEM with 0.5% bovine calf serum for 48 h, then 10% serum was added to induce the cells to re-enter the cell cycle.

2.4. Cell cycle analysis

Cells were synchronized as above, exposed to FGF-10 (10 ng) for 1 h followed by H_2O_2 (50 μM) for 4 h, then trypsinized, harvested, washed with phosphate buffer (PBS), resuspended gently in 5 ml of 90% ethanol and fixed at 25 °C for 1 h. After washing with PBS, the cells were incubated with DNase-free RNase A (200 μg/ml) at 37 °C for 1 h,

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Abbreviations: AEC, alveolar epithelial cells; CDK, cyclin dependent kinases; CKI, cyclin dependent kinase inhibitor; FGF-10, fibroblast growth factor-10; siRNA, small interfering RNA p21

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washed with PBS and then incubation with Propidium iodide (10 μg/ml) at 37 °C for 5 min. Cells were separated by sonicating at 20% output level for 15 s using a VirSonic 50 sonicator (Vitis), sorted by fluorescence-activated cell sorter (FACS) and analyzed using FlowJo (version 3.0.3) (Tree Start).

2.5. [3H]Thymidine incorporation assay

Cells were treated with FGF-10 followed by H_2O_2, incubated with [3H]Thymidine (1 μCi/ml [3H]-TdR) for 6 h and washed once with PBS. Then cells were incubated with 10% trichloroacetic acid (TCA) solution twice for 5 min each at 4 °C followed by 10% SDS for 2 min at room temperature. The amount of radioactivity was quantified in a scintillation counter.

2.6. MTT cell viability assay

Cells were treated with FGF-10 followed by H_2O_2 and incubated with MTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt) (20 μl) for 3 h. An absorbance at 490 nm was measured to quantify the amount of formazan product.

2.7. Western blot analysis

Cells were treated, washed and lysed. Proteins were size fractionated by 10% gel electrophoresis and transferred to nitrocellulose membranes using a semi-dry transfer (Bio-Rad). Blots were incubated with cyclin- and CDK-specific antibodies overnight at 4 °C and developed with an enhanced chemiluminescence detection kit (Amersham). The bands were quantified by densitometric scan NIH Image 1.62.

2.8. Kinase assay

Two hundred micrograms of protein lysate was immunoprecipitated with 1–2 μg of anti-cyclin E antibody or anti-CDK2 antibody and incubated at 4 °C overnight. Cyclin-CDKs were isolated by incubation at 4 °C for 1 h with 50 μl of GSH-agarose beads (Pharmacia) and beads were washed. Five hundred nanograms of histone H1, 20 μM ATP and 0.1 μCi of [γ-32P] ATP (3000 Ci/mmol; Amersham) were added in 20 μl of assay buffer and incubated for 30 min at 37 °C. The reaction was terminated by sample buffer (50 μl) and boiling for 3 min. The samples were resolved on 12% gel, fixed, Coomassie-stained, dried and then exposed to a PhosphorImager screen.

2.9. ELISA

p21 ELISA was performed by using TiterZyme ELISA Kits from Assay Designs as per the manufactures protocol.

2.10. Statistics

Data are reported as means ± S.E.M. Statistical analysis was done by one-way ANOVA and Tukey tests. Results were considered significant when $P < 0.05$.

3. Results and discussion

Given the important role of growth factors in the regulation of cell cycle, and the role of FGF-10 in the prevention of oxidant-induced AEC DNA damage and apoptosis, we determined the effects of FGF-10, a potent AEC mitogen, on the regulation of G1 cyclins and CDKs on H_2O_2-induced AEC arrest [1–7]. Barnouin et al. recently described that a sublethal dose of H_2O_2 induces transient multi-phase cell cycle arrest [9]. Using this study model, we observed that FGF-10 attenuates H_2O_2-induced AEC arrest in G1 by FACS with propidium iodide staining (Fig. 1a). In this study, we focused on examining the effect of FGF-10 in G1 phase. Cell cycle analysis after starvation of AEC for 48 h revealed 92.2% cells were in G0/G1, with 5.43% in S and 2.37% in G2/M (Fig. 1a). H_2O_2 (50 μM) for 4 h did not affect the cell cycle distribution of starved AEC but prevented serum-induced G1 to S progression; however, pretreatment with FGF-10 (10 ng) for 1 h

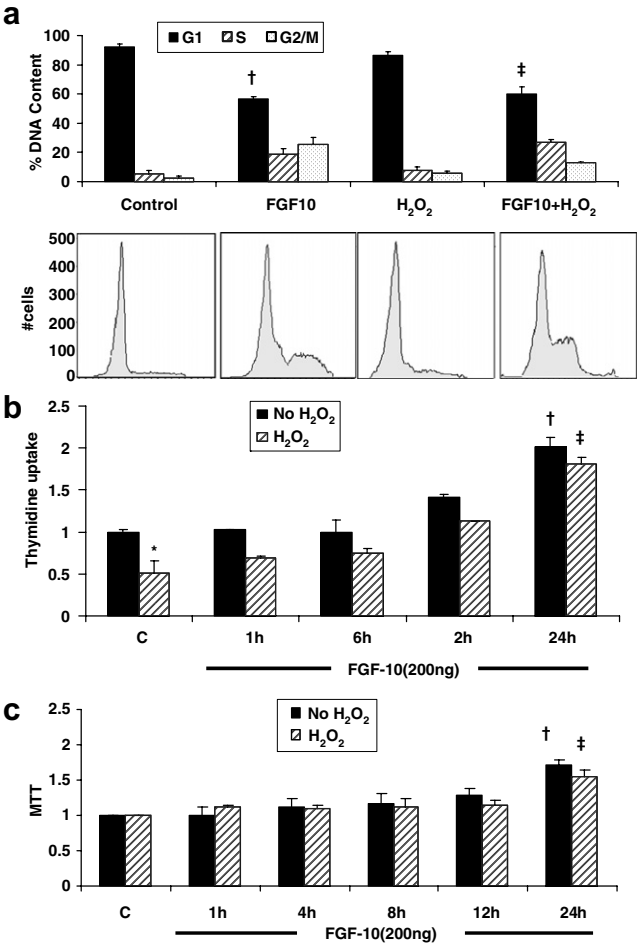

Fig. 1. (a) FGF-10 attenuates H_2O_2-induced AEC cycle G1 arrest: cell cycle analysis was performed by FACS with propidium iodide. Upon starvation for 48 h, 92.3% cells were in G0/G1, with 5.43% in S and 2.37% in G2/M. Addition of H_2O_2 (50 μM) for 4 h alone after serum stimulation did not change this distribution. However, pretreatment with FGF-10 (10 ng) for 1 h prevented H_2O_2-induced AEC arrest in G1 phase. Means ± S.E.M., $n = 3$. $^{\dagger}P < 0.05$ control vs. H_2O_2, $^{\ddagger}P < 0.05$ H_2O_2 vs. FGF-10 + H_2O_2. (b and c): Effect of FGF-10 on AEC DNA synthesis and proliferation: although FGF-10, at 24 h induced 1/2–1-fold increase in DNA synthesis and cell proliferation in cells exposed to H_2O_2; it had no effect on [3H]Thymidine uptake or MTT counts at 1 h. Means ± S.E.M., $^{*}P < 0.05$ control vs. H_2O_2, $^{\dagger}P < 0.05$ FGF-10 1 h vs. 24 h, $^{\ddagger}P < 0.05$ H_2O_2 vs. FGF-10 + H_2O_2, $n = 3$.

moved 27 ± 2.7% cells to S, 12.8% to G2/M while 60.2 ± 4.6% cells remained in G0/G1. Following exposure to FGF-10 alone, 44.1 ± 2.9% cells moved to S/G2/M while 56.9 ± 2.1% cells remained in G0/G1. These data suggest that H_2O_2 prevents progression of cell cycle by causing arrest in G0/G1 and FGF-10 attenuates these effects. Further, we found that FGF-10 did not affect [3H]Thymidine uptake or AEC proliferation after 1 h exposure; however, augmented DNA synthesis and improved cell viability by MTT assays was observed at 24 h in AEC exposed to FGF-10 alone and with H_2O_2 (Fig. 1b, c).

The cell cycle progression is controlled by the cooperative activity of the cyclins and CDKs (Fig. 4) [17–20]. G1–S transition is regulated by cyclin E in association with its catalytic partner CDK2; this is a rate limiting step for entry into the

S phase [16–20]. bFGF and FGF-2, both, upregulate cyclins and CDKs [12,13]. Although growth factors are known inhibit G1 arrest and apoptosis via mitogen activated protein kinases (MAPK), sustained protection against G1 arrest and apoptosis by bFGF was shown to occur by inhibition of CDK inhibitor [4,6,12,13]. We previously showed that MAPK mediate the

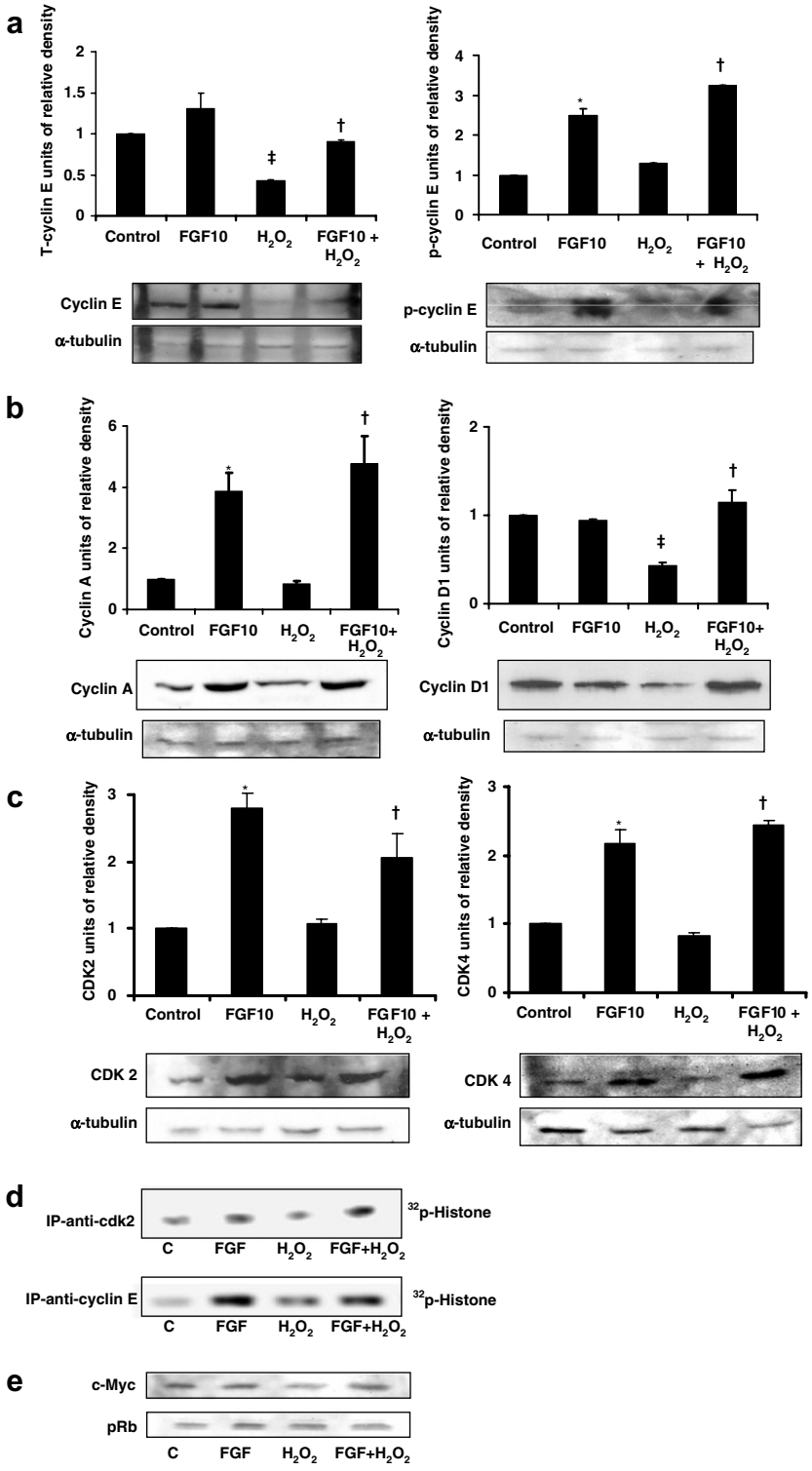

Fig. 2. FGF-10 attenuates H_2O_2-induced AEC G1 arrest by upregulation of positive cell cycle regulatory cyclins and CDKs: we analyzed the effect of FGF-10 and H_2O_2 on G1 cyclins and CDKs by Western blot. H_2O_2 decreased total-and phosphorylated-cyclin E in AEC. Pretreatment of AEC with FGF-10 increased phosphorylated-cyclin E (a), cyclin A (b), CDK2 and CDK4 (c) alone as well as in AEC exposed to H_2O_2, while H_2O_2 did not. H_2O_2 caused down regulation of cyclin D1 in AEC and FGF-10 prevented these effects (b). FGF-10 increased CDK2 and cyclin E kinase activity in AEC alone and in the cells exposed to H_2O_2, while H_2O_2 did not, cmyc and pRb were unchanged (e). Means ± S.E.M., $n = 3$. $^{*}P < 0.05$ control vs. FGF-10; $^{\ddagger}P < 0.05$ control vs. H_2O_2; $^{\dagger}P < 0.05$ H_2O_2 vs. FGF-10 + H_2O_2.

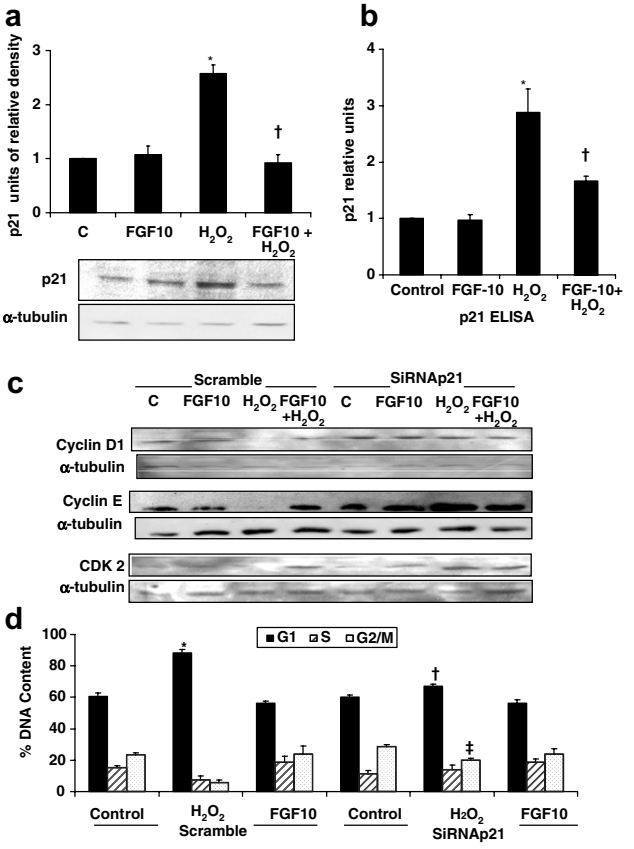

Fig. 3. FGF-10 prevents H_2O_2-induced upregulation of CDK inhibitor, p21 in AEC: H_2O_2 (50 μM) induces 11/2–2-fold increase in p21 while FGF-10 blocks H_2O_2-induced increase in p21 as assessed by Western blot (a) and ELISA (b, assay designs). $^*P < 0.05$ control vs. H_2O_2; $^\dagger P < 0.05$ H_2O_2 vs. FGF-10 + H_2O_2, $n = 3$. SiRNAp21 prevents H_2O_2-induced downregulation of cyclin E, D1, CDK2 (c) and AEC arrest (d); however, dose not affect effects of FGF-10. $^*P < 0.05$ control vs. H_2O_2 (Scramble); $^\dagger P < 0.05$ H_2O_2 (Scramble) vs. H_2O_2(SiRNAp21) G1 Phase; $^\ddagger P < 0.05$ H_2O_2 (Scramble) vs. H_2O_2 (SiRNAp21) G2/M Phase; $n = 3$.

protective effect of FGF-10 against AEC apoptosis [4,6]. In this study, we show that, FGF-10 prevents H_2O_2-induced decrease in total- and phosphorylated cyclin E, induces 21/2-fold increase in phosphorylated cyclin E and 31/2-fold increase in cyclin A in AEC alone and in cells exposed to H_2O_2, while, H_2O_2 alone does not affect either (Fig. 2a, b). H_2O_2 causes downregulation of cyclin D1, while pretreatment of AEC with FGF-10 prevents these effects (Fig. 2b). FGF-10 induces 2–21/2-fold increase in CDK2 and CDK4 as assessed by Western blot (Fig. 2c) and increases CDK2 and cyclin E kinase activity in AEC alone as well as in the cells exposed to H_2O_2, while H_2O_2 does not (Fig. 2d); cmyc and pRb remain unchanged (Fig. 2e). These data suggest that FGF-10 upregulates forward cell cycle regulatory cyclins (E, D1, A) and CDKs (2, 4), prevents downregulation of cyclin D1 in AEC exposed to H_2O_2 and promotes cell cycle progression after oxidative stress.

p21 is a G1-specific CDK inhibitor (CKI) that prevents cell cycle progression by causing inhibition of cyclins, CDKs, the cyclin-CDK2 complex activity and transcription factors (E2F, retinoblastoma protein and proliferator cell nuclear antigen) [20–22]. Increased expression of CKIs attenuates

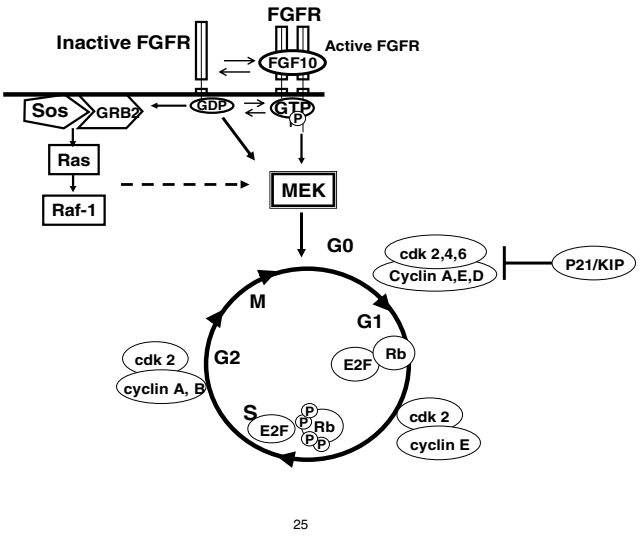

Fig. 4. Schematic diagram of FGF signaling pathways involved in the regulation of cell cycle, cyclins and CDKs.

FGF-2-stimulated cell proliferation [12]. Oxidative stress is known to induce p21; therefore we examined role of p21 in H_2O_2-induced AEC arrest [22]. We found that H_2O_2 causes 11/2–2-fold increase in p21 and pretreatment of AEC with FGF-10 blocks these effects (Fig. 3a, b). Further, abrogation of p21 expression by small interfering RNA (siRNA) targeting in AEC prevents H_2O_2-induced downregulation of cyclins and CDKs, and prevents H_2O_2-induced AEC arrest; however, dose not change FGF-10-induced regulation of cyclins and CDKs (Fig. 3c, d). Collectively, these data suggest that the protective effects of FGF-10 against H_2O_2-induced G1 arrest, in part, are mediated by the inhibition of H_2O_2-induced p21 in AEC.

In summary, we show for the first time that FGF-10 regulates AEC G1 cyclins and CDKs. The induction of forward cell cycle regulatory cyclins and CDKs and inhibition of p21 by FGF-10 may have a role in mediating the protective effects of FGF-10 against H_2O_2-induced AEC arrest. We recently showed that FGF-10 protects against oxidant-induced DNA damage and apoptosis in AEC and these data coupled with our findings here show that FGF-10 regulates oxidant-induced lung injury both by inhibiting oxidant-induced growth arrest and by inhibiting apoptosis.

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