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AMPK inhibits the stimulatory effects of TGF- $\beta$  on Smad2/3 activity, cell migration and epithelial to mesenchymal transition

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**Running title:** AMPK inhibits TGF- $\beta$  signaling

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**Non-standard Abbreviations:**

AICAR, 5-amino-4-imidazolecarboxamide riboside; AMP, adenosine monophosphate; AMPK, 5' AMP-activated protein kinase; CXCR4, chemokine (C-X-C motif) receptor 4; CTGF, connective tissue growth factor; FN, fibronectin; HMGA2, high-mobility group AT-hook 2; IL-6, interleukin-6, IL-11, interleukin-11; LKB1, liver kinase 1; PAI-1, plasminogen activator inhibitor-1; SNAIL1/2, transcription repressors for E-cadherin, ZEB1/2, zinc finger E-box binding homeobox 1; ZMP, 5-amino-4-imidazolecarboxamide ribotide

**Abstract**

AMP-activated protein kinase (AMPK), an important downstream effector of the tumor suppressor LKB1 and pharmacological target of metformin, is well-known to exert a preventive and inhibitory effect on tumorigenesis. However, its role in cancer progression and metastasis has not been well characterized. The present study investigates the potential roles of AMPK in inhibiting cancer cell migration and epithelial-to-mesenchymal transition (EMT) by regulating the canonical TGF- $\beta$  signaling pathway, an important promoting factor for cancer progression. Our results showed that activation of AMPK by metformin inhibited TGF- $\beta$ -induced Smad2/3 phosphorylation in cancer cells in a dose-dependent manner. The effect of metformin is dependent upon the presence of LKB1. A similar effect was obtained by expressing a constitutive active mutant of AMPK $\alpha$ 1 subunit, whereas expression of a dominant negative mutant of AMPK $\alpha$ 1 or ablation of AMPK $\alpha$  subunits greatly enhanced TGF- $\beta$  stimulation of Smad2/3 phosphorylation. As a consequence, expression of genes downstream of Smad2/3, including plasminogen activator inhibitor-1, fibronectin, and connective tissue growth factor, was suppressed by metformin in a LKB1-dependent fashion. In addition, metformin blocked TGF- $\beta$ -induced interleukin-6 expression through both LKB1-dependent and independent mechanisms. Our results also indicate that activation of LKB1/AMPK inhibits TGF- $\beta$ -stimulated cancer cell migration. Finally, TGF- $\beta$  induction of EMT was inhibited by metformin and enhanced by knockdown of LKB1 expression with shRNA. Together, our data suggest that AMPK could be a drug target for controlling cancer progression and metastasis.

## **Introduction**

AMP-activated protein kinase (AMPK) acts downstream of the tumor suppressor LKB1 to regulate metabolism and growth of cancer cells (Luo et al. 2005; Luo et al. 2010; Shackelford and Shawe 2009). Loss-of-function mutation of LKB1 accounts for the etiology and pathogenesis of Peutz-Jeghers syndrome, an autosomal dominant genetic disorder, featured by multiple hamartomatous polyps in the gastrointestinal tract (Alessi et al. 2006). Somatic mutations of the *LKB1* gene have been found in approximately 34% of lung adenocarcinomas, 19% of squamous cell carcinomas, 20% cervical carcinomas, and sporadically in other cancers (Luo et al. 2010). In addition, ablation of LKB1 on the context of the oncogenic *ras* transgene promotes cancer metastasis in animals (Carretero et al. 2010). Evidence has shown that tumor with a hypomorphic mutation of LKB1 becomes resistant to the AMPK activator metformin, supporting the notion that the tumor suppressive function of LKB1 is mediated by AMPK (Huang et al. 2008). Consistent with this notion, a retrospective investigation has reported that the incidence of cancer is significantly reduced in patients with type 2 diabetes taking metformin (Evans et al. 2005). Furthermore, clinical studies have demonstrated that AMPK activity is reduced in advanced breast cancer (Hadad et al. 2009; Shen et al. 2002). Intriguingly, it has been reported that breast cancer patients concurrently having type 2 diabetes with metformin as a neoadjuvant exhibit a higher pathologic complete response rate than those with other glucose lowering medicine and that the latter have a trend to distant metastasis (Bayraktar et al. 2012; Jiralerspong et al. 2009).

Transforming growth factor beta (TGF- $\beta$ ), which inhibits tumor growth in early stages of tumorigenesis, promotes tumor progression in late stages (Drabsch and ten Dijke. 2012). The mechanism of promoting cancer progression by TGF- $\beta$  is rather complex. First, TGF- $\beta$  plays an important role in regulating epithelial to mesenchymal transition (EMT), a critical step for cancer

stem cell (CSC) formation and cancer metastasis (Singh and Settleman.2010) in which TGF- $\beta$  downregulates claudins, occludins, and ZO1, followed by degradation of tight junction (Moustakas and Heldin.2007). Phosphorylated R-SMADS, associating with EMT-related transcription factors such as SNAIL1/2, HMGA2, ZEB1/2, upregulate EMT modulators (Fuxe et al.2010 ; Xu et al. 2009). In addition, TGF- $\beta$  regulates many genes involved in metastasis. In estrogen receptor-negative primary breast tumors, for instance, TGF- $\beta$  stimulates angiopoietin-like 4, which is associated with metastasis specifically to the lung (Padua et al. 2008). The TGF- $\beta$ /SMAD signaling pathway also promotes osteoclastogenesis and bone metastasis by inducing pro-osteolytic factors, such as parathyroid hormone-related protein, interleukin 11 (IL-11), chemokine (CXC) receptor 4 (CXCR4), and connective tissue growth factor (CTGF)(Guise et al. 1996; Kang et al. 2003; Muller et al. 2001; Yin et al. 1999).

Recent studies have shown that AMPK inhibits the TGF- $\beta$  signaling pathway (Cufi et al, 2010; Fisslthaler and Fleming 2009; Lim et al.2012; Mishra et al. 2008; Xiao et al.2010). In addition, AMPK inhibits cancer cell migration and EMT via bothTGF- $\beta$  dependent and independent mechanisms (Chou et al. 2014; Cufi et al. 2010; Lim et al. 2012; Thakur et al. 2015; Wang et al. 2014; Xiao et al2010; Yan et al. 2015; Zhang et al. 2014). With regards to AMPK regulation of the TGF- $\beta$  signaling pathway, the mechanism is not clear. In the present study, we interrogated the action of AMPK on the canonical TGF- $\beta$  signaling pathway in cancer cells as well as its impact on malignant behavior and EMT. Our results showed that AMPK activation inhibited Smad2/3 phosphorylation as well as the expression of target genes involved in EMT and metastasis. Accordingly, AMPK activation also suppressed tumor cell migration and EMT. These finidngs thus indicate that AMPK can be a therapeutic target for cancer metastasis.

## **Materials and Methods**

**Materials** Metformin, phenformin, doxycycline, and 5-amino-4-imidazolecarboxamide riboside (AICAR) were purchased from Sigma-Aldrich (St Louise, MO). A769962 was from LC laboratories (Woburn, MA). Human TGF- $\beta$ 1 and antibodies against phospho-smad2/3 (Ser423/425), total smad3, phospho-AMPK $\alpha$  (T172), total AMPK $\alpha$ , EMT markers, and  $\beta$ -actin were from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibodies against LKB1 and  $\beta$ -actin were from EMD Millipore Corporation (Billerica, MA). Dual luciferase assay kit was from Promega (Madison, WI). Lentiviral construct for LKB1 ShRNA was a gift from Dr. Bin Zheng of Harvard Medical School. Luciferase reporter plasmid for Smad2/3 binding elements was a gift from Dr. Ye-Guang Chen of Tsinghua University.

**Cell Culture** Lung adenocarcinoma A549 cells and mouse embryonic fibroblast cells (MEF) were cultured in 10% FBS-DMEM and human bronchial epithelial BEAS-2B cells in BEGM medium (Lonza, Allendale, NJ, USA). Prostate cancer C4-2 cells were cultured in 10% FBS-RPMI1640 at 37<sup>o</sup>C and 5% CO<sub>2</sub>. BEAS-2B cells stably expressing LKB1 shRNA or GFP shRNA were established by infecting lentivirus and selected with puromycin. A549 cells stably expressing LKB1 and C4-2 cells stably expressing a dominant negative mutant of AMPK $\alpha$ 1 were established as described previously (Zhou et al. 2009).

**Western Blot** Cell extracts were prepared in lysis buffer (25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 25 mM  $\beta$ -glycerol-phosphate, 1 mM DTT, 1% NP-40 and protease inhibitors). Cell debris was removed by centrifugation at 14,000xg at 4<sup>o</sup>C for 15 min. Protein concentrations were determined using Bio-Rad Protein Assay kit. Protein samples (25  $\mu$ g) were subjected to SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The membranes were sequentially blotted with the first and second antibodies, and signals were visualized by the enhanced chemiluminescence (ECL) method (Luo et al. 2013).

**Real Time PCR** The expression of mRNA was examined by real-time PCR with the ABI Step One Plus PCR System using SYBRGREEN PCR Master Mix 2xreagent in 20  $\mu$ l reaction volume according to protocol provided by manufacture (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 1. Each sample was amplified in triplicates and normalized with the expression level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Results were evaluated by the comparative threshold cycle value method ( $2^{-\Delta\Delta Ct}$ ) for relative quantification of gene expression (Zhou et al. 2009).

**Wound Healing Assay** Cells were seeded in 6-well plates and the wells marked with a straight black line on the bottom. When cell density reached confluence, cells were starved in 0.1 % FBS for 8 hours before three scratches across the black line were made in each well with a 200  $\mu$ l pipette tip. Loose cells were washed off with medium. TGF- $\beta$ 1 (5 ng/ml) and/or metformin (10 mM) in fresh medium (0.1% FBS), or fresh medium alone, were added to the wells. and the cells were incubated for a given period of time (indicated in figure legends). Microphotographs were taken on an inverted microscope immediately at scratch (0 h) and the endpoints of experiment. Images were aligned using the orientation line to ensure that the identical spots were followed over time. Experiments were conducted in triplicates.

**Boyden Chamber Migration Assay** Cell migration was measured using Transwell chambers with 8  $\mu$ m pore size membranes (6.5 mm diameter inserted into 24 well plates), according to the protocol provided by manufacture (Corning, New York, USA). The lower chamber was filled with 600  $\mu$ l DMEM containing 10% FBS. Cells ( $1 \times 10^5$ ) suspended in 100  $\mu$ l serum-free DMEM were added and evenly distributed onto the upper chamber. After 16h of culture, cells remaining on the upper surface of the filters were removed with cotton-tipped applicators and those on the lower surface were fixed with 100% methanol and stained with crystal violet. The membranes containing stained cells were cut out and mounted on a glass

slide. Microphotographs were taken under fluorescent microscope and cell numbers counted. The average number of cells from triplicates represents the number of migrated cells.

**Adenovirus Preparation** cDNA encoding the constitutively active mutant of human AMPK $\alpha$ 1 subunit was made by deleting the segment of wildtype cDNA encoding amino acids 313 to 390, tagged with flag epitope, and subcloned to pAdTrack-CMV vector (He et al. 1998). Recombinant adenovirus was prepared and purified using AdEasy Adenoviral Vector System kit and AdEasy virus purification kit (Agilent Technologies, Santa Clara, CA).

**Statistical Analysis** Significance between groups was tested using two tailed student t test.



## Results

**AMPK inhibits phosphorylation and transcriptional activity of Smad2/3** The effects of AMPK activation on the canonical TGF- $\beta$  signaling pathway were first studied using A549 cells, a human lung adenocarcinoma cell line with a loss-of-function mutation of LKB1, and A549-LKB1 cells in which LKB1 was stably expressed. The cells were treated with TGF- $\beta$ 1 for different periods of time and the levels of phosphorylation of Smad2/3 and AMPK were examined. As shown in Figure 1A, while TGF- $\beta$ 1 did not affect Smad2/3 or AMPK expression, it significantly stimulated Smad2/3 phosphorylation in A549 cells. In contrast, the induction of Smad2/3 phosphorylation was barely detectable in A549-LKB1 cells. In A549 cells, metformin failed to activate AMPK, while TGF- $\beta$ 1 potently induced Smad2/3 phosphorylation (Fig. 1B). Ectopic expression of LKB1 (labeled A549-LKB1 cells) restored AMPK activation by metformin, concomitantly with a marked inhibition of TGF- $\beta$  signaling (Fig. 1B). Inhibition of Smad2/3 phosphorylation by metformin was dose-dependent, as shown in A549-LKB1 cells where the inhibition of Smad2/3 phosphorylation paralleled with degree of AMPK activation by metformin (Figure 1C).

To ascertain if the effect of metformin was mediated by AMPK, we used a fag-tagged active mutant of AMPK $\alpha$ 1 subunit where the autoinhibitory domain encompassing amino acids 313 to 390 was deleted (Crute et al. 1998). Expression of active AMPK $\alpha$ 1 subunit in A549 cells via adenoviral vector inhibited TGF- $\beta$ 1-induced Smad2/3 phosphorylation in a dose-dependent fashion, while no effect was observed with adenovirus encoding GFP (Fig. 1D).

We next examined the effects of AMPK on TGF- $\beta$ 1 signaling in C4-2 cells, a prostate cancer cell line, and mouse embryonic fibroblast (MEF) cells with deletion of both *AMPK $\alpha$ 1* and  *$\alpha$ 2* alleles (Figure 2). When treated with different doses of metformin, the level of AMPK activation in C4-2 cells was inversely correlated with the levels of TGF- $\beta$ 1-induced Smad2/3

phosphorylation (Figure 2A). The influence of AMPK on TGF- $\beta$ 1 signaling was further investigated in C4-2 cells by expressing a dominant negative mutant of AMPK  $\alpha$ 1 (DN-AMPK). Stable expression of DN-AMPK was made by lentiviral vector, and the expression was under the control of the tet-off system (Zhou et al. 2009). When DN-AMPK was expressed in the absence of doxycycline, TGF- $\beta$ 1 significantly induced Smad2/3 phosphorylation (Fig. 2B). Addition of doxycycline, which abrogated DN-AMPK expression and allowed endogenous wild type AMPK to function, blocked the TGF- $\beta$ 1-induced Smad2/3 phosphorylation (Fig. 2B). Apparently, while phospho-signals of AMPK in the presence or absence of doxycycline appeared to be similar, the ratio of p-AMPK to total AMPK, which included DN-AMPK, was much greater after doxycycline treatment, suggesting that the dominant negative AMPK was not phosphorylated, but it effectively inhibited the function of endogenous AMPK. Finally, we compared the ability of TGF- $\beta$ 1 to stimulate Smad2/3 phosphorylation in MEF cells with and without AMPK. TGF- $\beta$ -induced Smad2/3 phosphorylation was significantly higher in the cells with no AMPK (Figure 2C). These results provide evidence that TGF- $\beta$  signaling is regulated by AMPK.

**AMPK inhibits expression of TGF- $\beta$  downstream effectors** To examine how the expression of genes downstream of Smad2/3 is influenced by AMPK activators in the A549 cells, we performed quantitative PCR to analyze the expression of PAI-1 (Lund et al. 1987), fibronectin (FN) (Ignatz and Massague 1986), CTGF (Igarashi et al. 1993) and IL6 (Elias et al. 1991) (primer sequences are listed in Table 1). A549 and A549-LKB1 cells were treated with metformin with or without TGF- $\beta$ 1 for 8 hours, and the expression of a given gene was determined by real time qPCR. As shown in Figure 3, TGF- $\beta$ 1 remarkably upregulated transcription of all 4 target genes in A549 cells and metformin did not show significant inhibition of PAI-1, FN and CTGF. Ectopic expression of LKB1 inhibited TGF- $\beta$ -induced expression in all 4 genes (Fig. 3). Remarkably,

metformin further suppressed the TGF- $\beta$ -induced expression in all 4 genes in A549-LKB1 cells (Fig. 3). These results clearly demonstrate that metformin suppresses gene expression of these TGF- $\beta$  targets through a LKB1/AMPK-dependent mechanism. Interestingly, metformin was able to inhibit TGF- $\beta$ -induced IL6 expression in A549 cells, suggesting that metformin regulates TGF- $\beta$ -induced expression of IL6 through both LKB1-dependent and independent mechanisms.

**AMPK inhibits cell migration in response to TGF- $\beta$**  To examine the effect of metformin on cell mobility in response to TGF- $\beta$ , we first conducted wound healing analysis in which cells were serum starved for 8 hours and plates scratched to form cell-free paths. Remaining cells were then incubated in medium for 24 hrs, with or without metformin, and with or without TGF- $\beta$ 1. As shown in Figure 4A-B, in response to TGF- $\beta$ 1, A549 cells migrated faster than than A549-LKB1 cells. And metformin elicited greater inhibition of TGF- $\beta$ 1-triggered closure of scratch in A549-LKB1 cells than A549 cells. Next, we carried out cell migration assay using Boyden chambers. Migration of both A549 and A549-LKB1 cells was stimulated by TGF- $\beta$ 1, which was suppressed by metformin or A769962 (Figure 4C). The inhibitory effect of AMPK activators appeared greater in A549-LKB1 cells.

The wound healing assay was repeated with C4-2 cells expressing DN-AMPK. Cell migration was faster under basal and TGF- $\beta$ 1-treated conditions in the absence of doxycycline than in the presence of doxycycline (Figure 5). Hence, LKB1/AMPK counteracted the stimulatory effect of TGF- $\beta$  on migration of the cancer cells.

**AMPK inhibits TGF- $\beta$ -induced epithelial mesenchymal transition (EMT)** TGF- $\beta$  is known to play an important role in EMT, a critical step for cancer metastasis as well as fibrosis. To explore the potential inhibitory effect of LKB1/AMPK on TGF- $\beta$ -induced EMT, we used BEAS-2B cells, a human bronchial epithelial cell line transformed with adenovirus-12 and SV40 hybrid virus but are not tumorigenic. E-cadherin was downregulated whereas N-cadherin and

Slug were upregulated in BEAS-2B cells after prolonged incubation with TGF- $\beta$ . (Figure 6A). Since BEAS-2B cells require special culture medium and are sensitive to addition of metformin in large volume during chronic incubation, we decided to use phenformin instead. The latter activates AMPK by increasing intracellular AMP as metformin, but with more potency. In fact, our data indicate that phenformin is at least 10 times more potent in activation of AMPK than metformin (Figure 6B). Smad2/3 phosphorylation was found to be progressively inhibited, which paralleled to activation of AMPK after cells were incubated with phenformin for a given time period (Figure 6C). Then we treated the cells with TGF- $\beta$ 1 for 48 hours and added phenformin in the last 18 hours. Phenformin restored the expression of E-cadherin and suppressed the TGF- $\beta$ -induced expression of N-cadherin and Slug and thus effectively blocked the ability of TGF- $\beta$  to induce EMT (Figure 6D).

To ascertain that the effect of phenformin is mediated by LKB1/AMPK, the expression of LKB1 was knocked down in the cells stably expressing LKB1 shRNA. The cells stably expressing GFP shRNA were used as control. Our results showed that expression of E-cadherin was significantly reduced by LKB1 shRNA (Fig. 7A). In contrast, the TGF- $\beta$ 1-induced expression of N-cadherin, vimentin, Slug, and  $\beta$ -catenin was markedly enhanced (Figure 7A), and the efficacy of phenformin in inhibiting TGF- $\beta$ -induced changes of EMT markers was markedly suppressed (Figure 7B). Furthermore, the expression levels of these EMT markers correlated with degrees of AMPK activation, which strongly suggests that the effect of phenformin is mediated by AMPK.

Morphologically, the TGF- $\beta$ -induced EMT change is less affected by A769962 in the cells with LKB1 knockdown than the cells with GFP shRNA. When treated with TGF- $\beta$ , the majority of the cells containing GFP shRNA remained epithelium shape in the presence of A769962 (Figure 8A) or detached. Cell detachment, a sign of cell death, was more severe upon

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treatment with phenformin or metformin (data not shown). In contrast, the cells bearing LKB1 shRNA displayed mesenchymal shape, which was not altered by A769962. These results indicate that AMPK activation prevents TGF- $\beta$ -induced EMT or even induces cell apoptosis. In addition, we found that with LKB1 knockdown, BEAS-2B cells migrated faster than control cells on wound healing (Figure 8B), an indication of mesenchymal transition.

## Discussion

AMPK is a well-received drug target for type 2 diabetes and other metabolic disorders inasmuch as its activation enhances insulin sensitivity, lowers blood glucose levels and improves lipid profiles. In the last decade, mounting evidence has indicated that AMPK is implicated in control of tumor cell growth and tumorigenesis. However, less is known about its effect on cancer progression and metastasis. In present study, we attempted to explore the effect of AMPK on the canonical TGF- $\beta$  signaling pathway, which is often activated in the advanced stage of cancer and plays an important role in EMT and cancer metastasis. Our results showed that AMPK activators, including metformin, phenformin or A769962, suppressed Smad2/3 phosphorylation and expression of target genes including PAI-1, CTGF, FN and IL6 in response to TGF- $\beta$ , concurrently with a decrease in the ability of cancer cells to migrate on wound healing and transwell assays. Furthermore, we found that AMPK activation counteracted the stimulatory effect of TGF- $\beta$  on expression of EMT markers and morphologic changes of BEAS-2B cells. All these effects of metformin were dependent on functional LKB1, although the inhibitory effect of metformin on TGF- $\beta$ -induced IL6 expression can also be mediated by a putative LKB1-independent pathway. Consistently, expression of dominant negative mutant of AMPK or deletion of AMPK  $\alpha$  subunits augmented TGF- $\beta$ -stimulated phosphorylation of Smad2/3, whereas expression of the active mutant of AMPK diminished the effect of TGF- $\beta$ . Therefore, our results indicate that AMPK activation inhibits TGF- $\beta$ -modulated EMT and cancer metastasis.

AMPK consists of three subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and is activated by both allosteric activators and phosphorylation. A physiological allosteric activator is 5' AMP that is elevated under stresses such as hypoxia, ischemia, and glucose starvation. Other allosteric activators include AICAR (a cell permeable molecule that is phosphorylated by nucleoside kinase and

converted to ZMP, an analog of AMP), as well as salicylate and A769662, both of which directly bind to the autoinhibitory domain of AMPK (Hawley et al. 2012). Metformin, a clinically used anti-diabetic drug, along with phenformin which had been in clinic use but withdrawn due to toxicity, belong to biguanides family that activate AMPK by inhibiting mitochondrial respiratory chain and thus increasing intracellular AMP. Steady state concentration of metformin in plasma of type 2 diabetes is in the range of 20~40  $\mu\text{M}$ , and can reach 50  $\mu\text{M}$  in portal vein under oral administration at regularly prescribed doses (He et al. 2015; Owen et al. 2000). Metformin and phenformin are positively charged and require transporters to enter cells and mitochondria. The expression level of transporters vary on cells (Viollet et al. 2012). Phenformin is relatively hydrophobic and easy to be delivered to cells. Thus, for all most all *in vitro* studies, metformin is used in the range of 1~10 mM, as compared to 100  $\mu\text{M}$  ~1 mM of phenformin to reach optimal doses in mitochondria (Janzer et al. 2014; Zhou et al. 2001).

Binding of AMP to AMPK  $\gamma$  subunit facilitates phosphorylation of T172 on the activation loop of the  $\alpha$  catalytic subunit and also protects the phosphorylated T172 against dephosphorylation by phosphatases. Several protein kinases have been reported to phosphorylate T172, including LKB1 and calmodulin-dependent protein kinase kinase beta (CamKK $\beta$ ). While CamKK $\beta$  is activated by  $\text{Ca}^{2+}$ , LKB1 is constitutively active. Maximal activation of AMPK is achieved by both AMP binding and phosphorylation of T172 (Hardie 2011). In addition to AMPK, LKB1 also regulates 13 other protein kinases and some of the tumor suppressive functions of LKB1 are mediated by these other AMPK-related kinases (Luo et al. 2010). One way to distinguish them from AMPK is the fact that the activation of the latter depends on both AMP and LKB1, while others are independent of AMP.

Recent studies have illustrated an inhibitory effect of AMPK on the canonical TGF- $\beta$  signaling pathway, but data published are not consistent with regard to the underlying

mechanisms. Mishra et al (Mishra et al. 2008) have shown that AMPK suppresses transdifferentiation of myofibroblast induced by TGF- $\beta$ , an event that is not impinged upon phosphorylation of Smad2/3, but through inhibition of a downstream event. A later study has reported that AMPK could induce degradation of the transcription coactivator p300, reduce acetylation of Smad3, and disrupt TGF- $\beta$ -elicited interaction between p300 and Smad3 but without an effect on Smad phosphorylation and nuclear translocation (Lim et al. 2012). Another mechanism involves metformin-induced inhibition of smad2/3 phosphorylation (Park et al. 2014). The differences among these studies might reflect the cell context. Our present study provides solid evidence that AMPK attenuates the canonical TGF- $\beta$  signaling pathway by inhibiting Smad2/3 phosphorylation and transcriptional events. We have tested both cancer cells and precancerous cells with consistent results that lead to the same conclusion.

Intriguingly, our data suggest that AMPK could attenuate the pro-metastatic effect of TGF- $\beta$  through regulation of transcriptional targets of Smad2/3. Our previous study has found that AMPK activation downregulates fibronectin in prostate cancer cells (Zhou et al. 2009). In the present study using a lung adenocarcinoma cell line, our results underpinned the negative effect of AMPK on fibronectin. Furthermore, our data demonstrate that AMPK activation can offset TGF- $\beta$  in the regulation of CTGF and IL6, two important molecules in cancer metastasis and EMT (Chu et al. 2008; Kang et al. 2003; Sullivan et al. 2009; Zhao et al. 2014). Interestingly, we found that IL6 is regulated via both LKB1/AMPK dependent and independent mechanisms. It has been reported that metformin can regulate events independent of AMPK. For instance, metformin was found to suppress SNARK, an AMPK-related kinase, that can enhance TGF- $\beta$  signaling (Goto et al. 2013). It will be interesting to test if SNARK is an additional target of metformin in our setting.

AMPK has been recently documented to be involved in EMT (Chou et al. 2014; Cufi et al. 2010; Lee et al. 2013; Vazquez-Martin et al. 2010 ; Wang et al. 2010; Wang et al. 2014; Zhang et al.



2014). Its effect was first described in tissue fibrosis, where opposite effects were reported (Lee et al. 2013; Wang et al. 2010). Several studies have shown that AMPK inhibits cancer EMT, which could occur through different mechanisms (Chou et al. 2014; Zhang et al. 2014; Zhao et al. 2014). Our study using BEAS-2B cells clearly demonstrates that inhibition of TGF- $\beta$  action is one of the mechanisms.

In summary, we have shown that AMPK activation inhibits TGF- $\beta$ -induced phosphorylation and transcriptional activity of Smad2/3. As such, AMPK attenuates cancer cell migration and invasion. In addition, our results have revealed that AMPK inhibits TGF- $\beta$ -evoked EMT. Therefore, our present study delineates the role of AMPK in tumor cell migration, progression and EMT and strongly supports the notion that AMPK could serve as a preventive and therapeutic target for cancer metastasis. This study may help to tune the focus of current clinical trials with metformin to cancer metastasis (He et al.2015).

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### **Authors' Contributions**

*Participated in research design:* HL, HH, D-Q H, ZL

*Conducted experiments:* HL, N-S L, HH, YY, SS, L-Y L

*Performed data analysis:* HL, D-Q H, N-H L, ZL

*Writing and contributed to writing of the manuscript:* ZL, DH.

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**Footnotes**

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**Competing Financial Interests**

We declare no competing interests.

## Figure Legends

**Figure 1. LKB1 mediates the effect of metformin on Smad2/3 phosphorylation** (A) Time course of TGF- $\beta$ -induced phosphorylation of Smad2/3. A549 and A549-LKB1 cells were incubated with TGF- $\beta$ 1 (5 ng/ml) for different time, (B) Time course effect of metformin on TGF- $\beta$  signaling. The cells as in (A) were treated with metformin (10 mM) for different time, followed by TGF- $\beta$ 1 for 30 min. (C) Dose effect of metformin on TGF- $\beta$  signaling. A549-LKB1 cells were treated with metformin at different doses for 2 hours, followed by TGF- $\beta$ 1 for 30 min, (D) Effect of active AMPK mutant on TGF- $\beta$  signaling. A549 cells were infected with different volumes ( $\mu$ l) of adenovirus encoding an active mutant of AMPK $\alpha$ 1 subunit tagged with flag epitope (AMPK-CA) or GFP as a control for 2 days and then treated with TGF- $\beta$ 1 for 30 min. Equal amounts of cell extracts (25  $\mu$ g) for all panels were subjected to SDS-PAGE and immunot blots with antibodies, as indicated.

**Figure 2. AMPK inhibits Smad2/3 phosphorylation by TGF- $\beta$**  (A) Dose-dependent inhibition of TGF- $\beta$  signaling by metformin. C4-2 cells were treated with metformin at different doses, followed by TGF- $\beta$ 1 for 30 min. (B) Effect of dominant negative mutant of AMPK on TGF- $\beta$  signaling. C4-2 cells bearing flag-tagged dominant negative mutant of AMPK $\alpha$ 1 under the control of Tet-off system were treated with or without doxycycline (2  $\mu$ g/ml) for two days and then with TGF- $\beta$ 1 for indicated periods of time. (C) Effect of AMPK ablation on TGF- $\beta$  signaling. MEF cells, wild type and  $\alpha$ 1 $\alpha$ 2 knockout, were treated with TGF $\beta$ 1 for different times. Equal amounts of cell extracts (25  $\mu$ g) were blotted with antibodies, as indicated.

**Figure 3. AMPK activation suppresses expression of TGF- $\beta$  target genes** A549 and A549-LKB1 cells were treated with metformin and/or TGF- $\beta$ 1 for 8 hours. Total RNA was prepared and quantitative real time PCR conducted on (A) PAI-1, (B) FN, (C) CTGF, and (D) IL6. The results were normalized with values of GAPDH and expressed as fold of A549 control (mean $\pm$ SD, n=3). Significance was tested by student t test (\*p>0.05, \*\*p<0.05).

**Figure 4. AMPK activation inhibits migration of A549 cancer cells** Assays were performed according to Materials and Methods. (A) Wound healing assay. Confluent A549 and A549-LKB1 cells were starved for 8 hours, scratched and grown in the presence of metformin (10 mM) and/or TGF- $\beta$ 1 (5 ng/ml), as indicated. Photos were taken at the beginning (0H) and end of experiments (24H). Wound closure was calculated by the equation (R1-R2)/R1, as indicated. The graph represents averages of 3 independent experiments (mean $\pm$ SD, n=3). \*P>0.05, \*\*P<0.05. (B) Migration on transwells. The cells were loaded into membranes of chambers and treated with or without metformin or A769962 (1  $\mu$ M)  $\pm$  TGF- $\beta$ 1, as indicated, and migration was terminated after 20 hours. The number of cells migrated to the lower side of the membranes were counted and expressed as percentage of control cells (mean $\pm$ SD, n=3). Each assay was repeated at least four times. Significance (P<0.05) has been found between (1) control and TGF- $\beta$ , and (2) TGF- $\beta$  and TGF- $\beta$ +metformin or TGF- $\beta$ +A769962 in A549 and A549-LKB1 cells, respectively.

**Figure 5. AMPK inhibits TGF- $\beta$  enhancement of C4-2 cancer cell migration** (A)

Wound healing assays. C4-2 cells expressing the dominant negative mutant of AMPK were treated with or without doxycycline for two days, as described in Figure 2B, would healing assay conducted, and photo taken at the beginning (0H) and end of experiments (24H). (B) The graph

represents averages of a triplicate experiment (mean $\pm$ SD, n=3). Significance was tested with student t test (\*\*P<0.05).

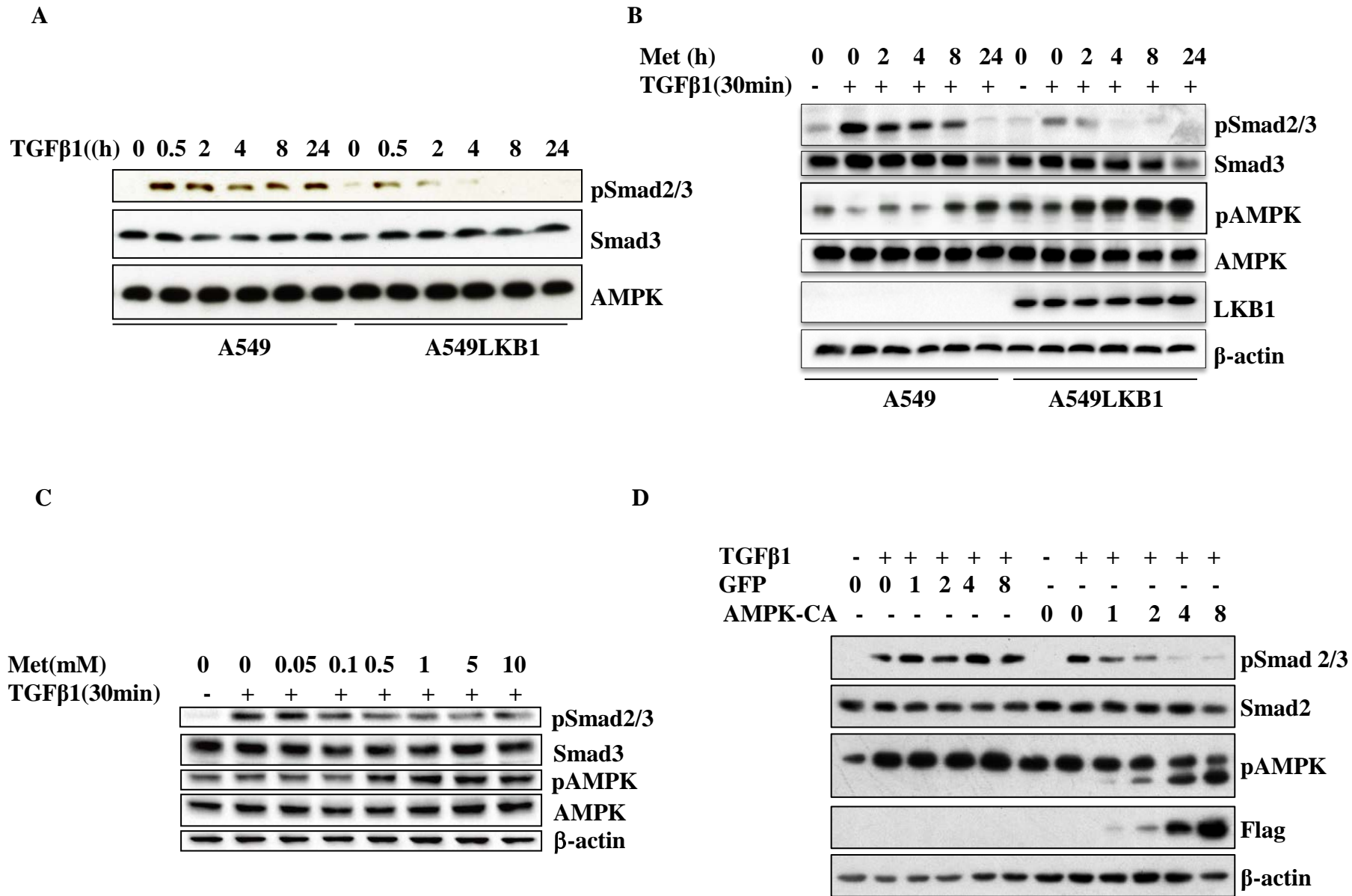
**Figure 6. Regulation of EMT marker expression by phenformin and TGF- $\beta$**  (A) TGF- $\beta$  regulation of EMT markers. BEAS-2B cells were treated with TGF- $\beta$ 1 for different time. (B) Dose-dependent activation of AMPK by biguanides. BEAS-2B cells were treated with metformin or phenformin at different doses, as indicated, to assess the efficiency of AMPK activation. (C) Inhibition of TGF- $\beta$  signaling by phenformin. The cells were treated with phenformin (1 mM) for indicated time and then with TGF- $\beta$  (5ng/ml) for 30 min. (D) AMPK inhibition of TGF- $\beta$ -induced expression of EMT markers. The cells were treated with TGF- $\beta$ 1 for 48 hours and phenformin was added in the last 18 hours. Cell extracts (25  $\mu$ g) were blotted with antibodies, as indicated.

**Figure 7. LKB1 inhibits the regulatory effect of TGF- $\beta$  on EMT marker expression** (A) Effect of silencing LKB1 on TGF- $\beta$ -induced changes of EMT markers. BEAS-2B cells containing shRNA for LKB1 or GFP as a control were treated with TGF- $\beta$ 1 for different time. (B) Silencing LKB1 offsets the inhibitory effect of phenformin on TGF- $\beta$ -induced changes of EMT markers. The cells were treated with TGF- $\beta$ 1 for 48 hours and phenformin was added in the last 18 hours. The cell extracts (25  $\mu$ g) were blotted with antibodies, as indicated.

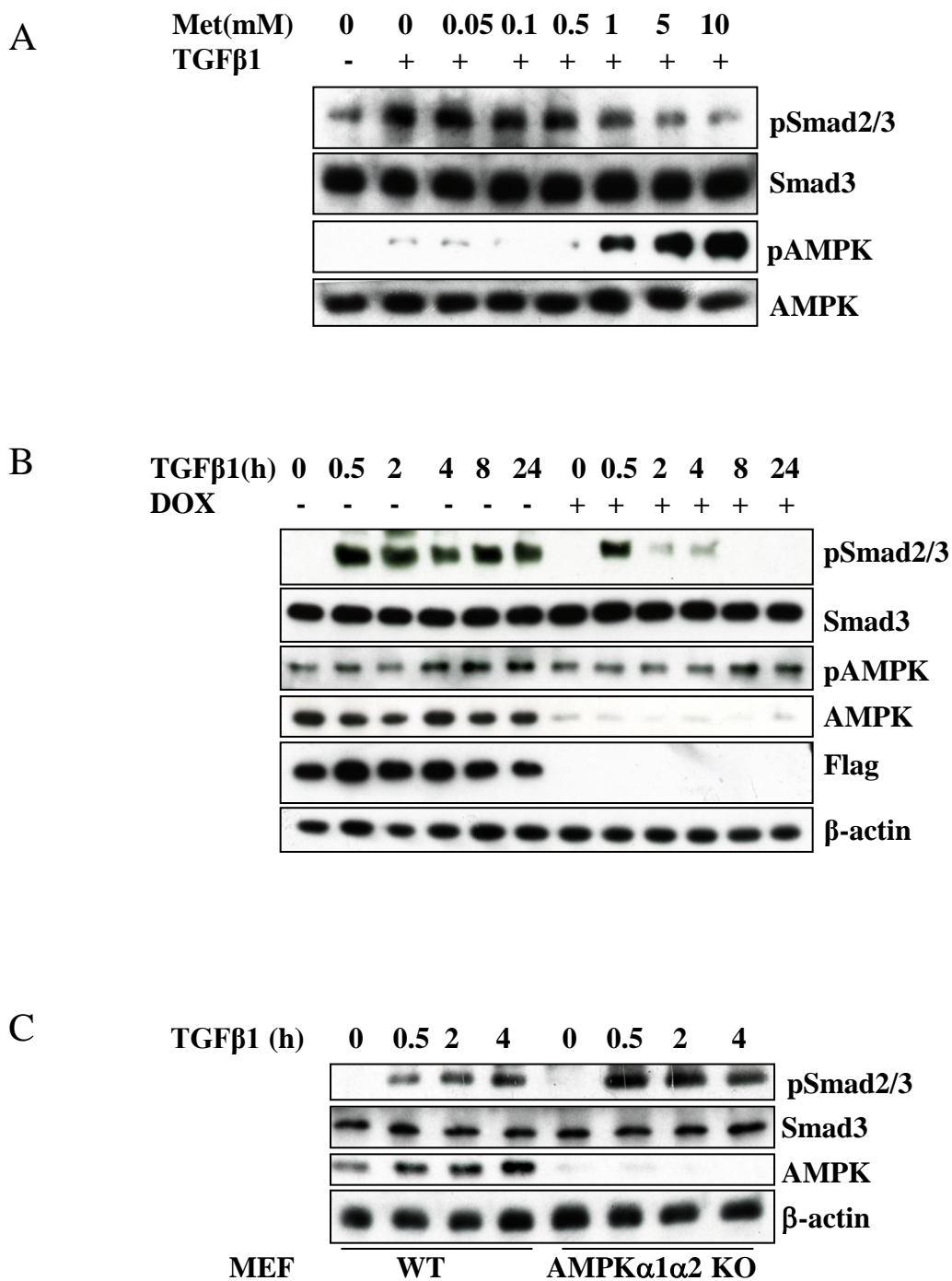
**Figure 8. Knockdown of LKB1 enhances migration of epithelial cells and confers resistance to phenformin-induced inhibition of EMT** Behaviors of the BEAS-2B cells with LKB1 shRNA and GFP shRNA were compared. (A) Knockdown of LKB1 changes morphology of the cells toward mesenchymal-like shape. The cells were treated with or without TGF- $\beta$ 1 for 30 hours and cell culture was continued in the presence of TGF- $\beta$ 1 $\pm$  A769962 (1  $\mu$ M) for additional 18 hours. (B) Wound healing assay showed that knockdown of LKB1 enabled the cells to migrate faster. Photos were taken under phase contrast microscope.

Table 1

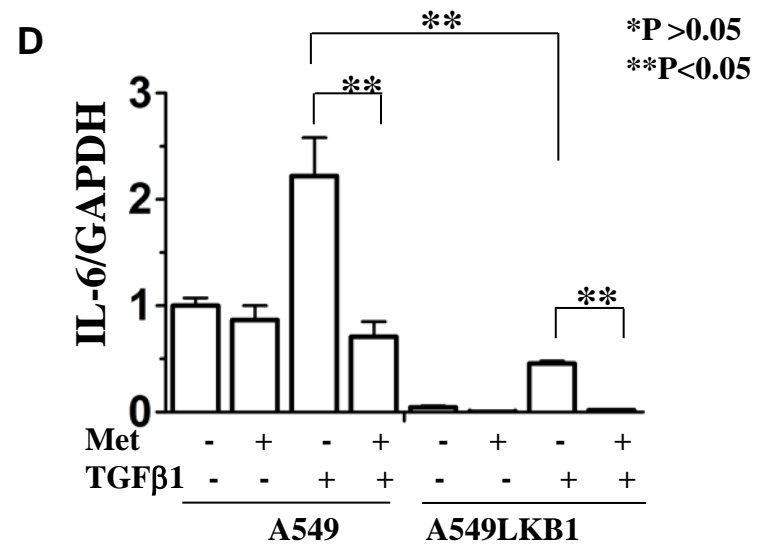
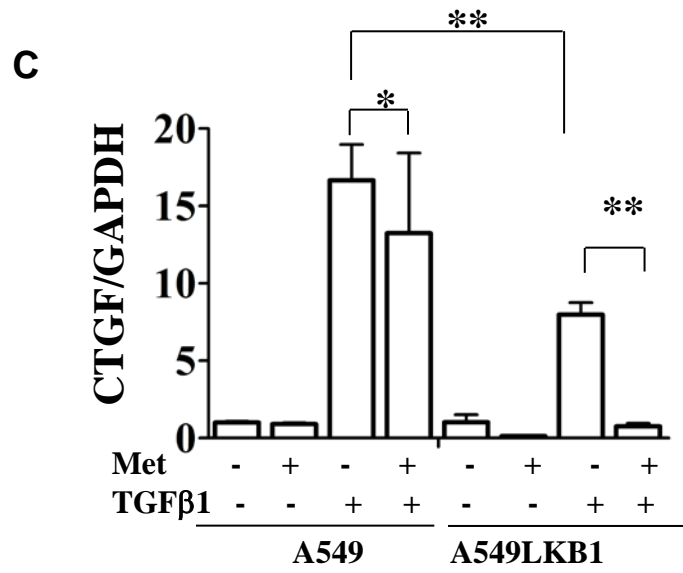
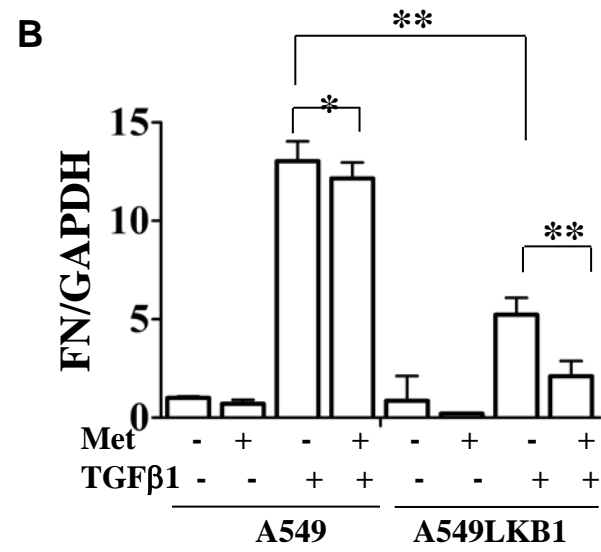
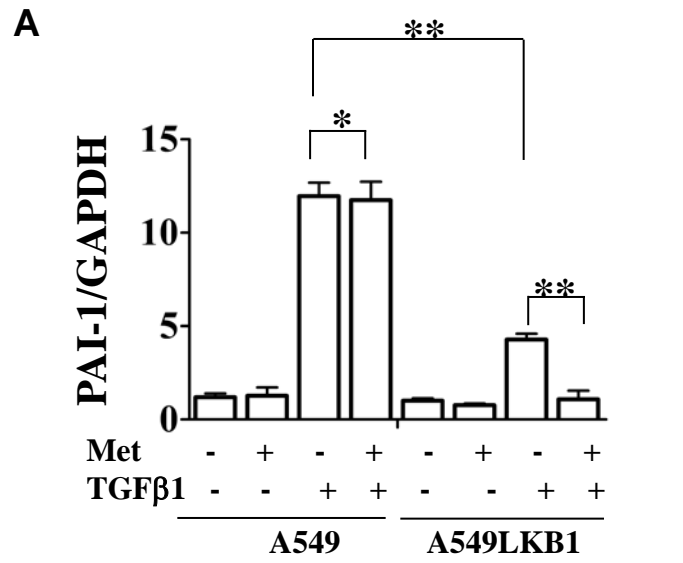
qPCR primers	Sequence (5'→3')
PAI-1	
Forward primer	GGCCATGGAACAAGGATGA
Reverse primer	CTCCTTTCCCAAGCAAGTTG
IL-6	
Forward primer	GGT ACA TCC TCG ACG GCA TCT
Reverse primer	GTG CCT CTT TGC TGC TTT CAC
FN	
Forward primer	CAG GAT CAC TTA CGG AGA AAC AG
Reverse primer	GCC AGT GAC AGC ATA CAC AGT G
CTGF	
Forward primer	GCTGGAGAAGCAGAGTCGTC
Reverse primer	CCACAGAACTTAGCCCGGTA
GAPDH	
Forward primer	CAGGGCTGCTTTTAACTCTGGT
Reverse primer	GATTTTGGAGGGATCTCGCT



**Figure 1**

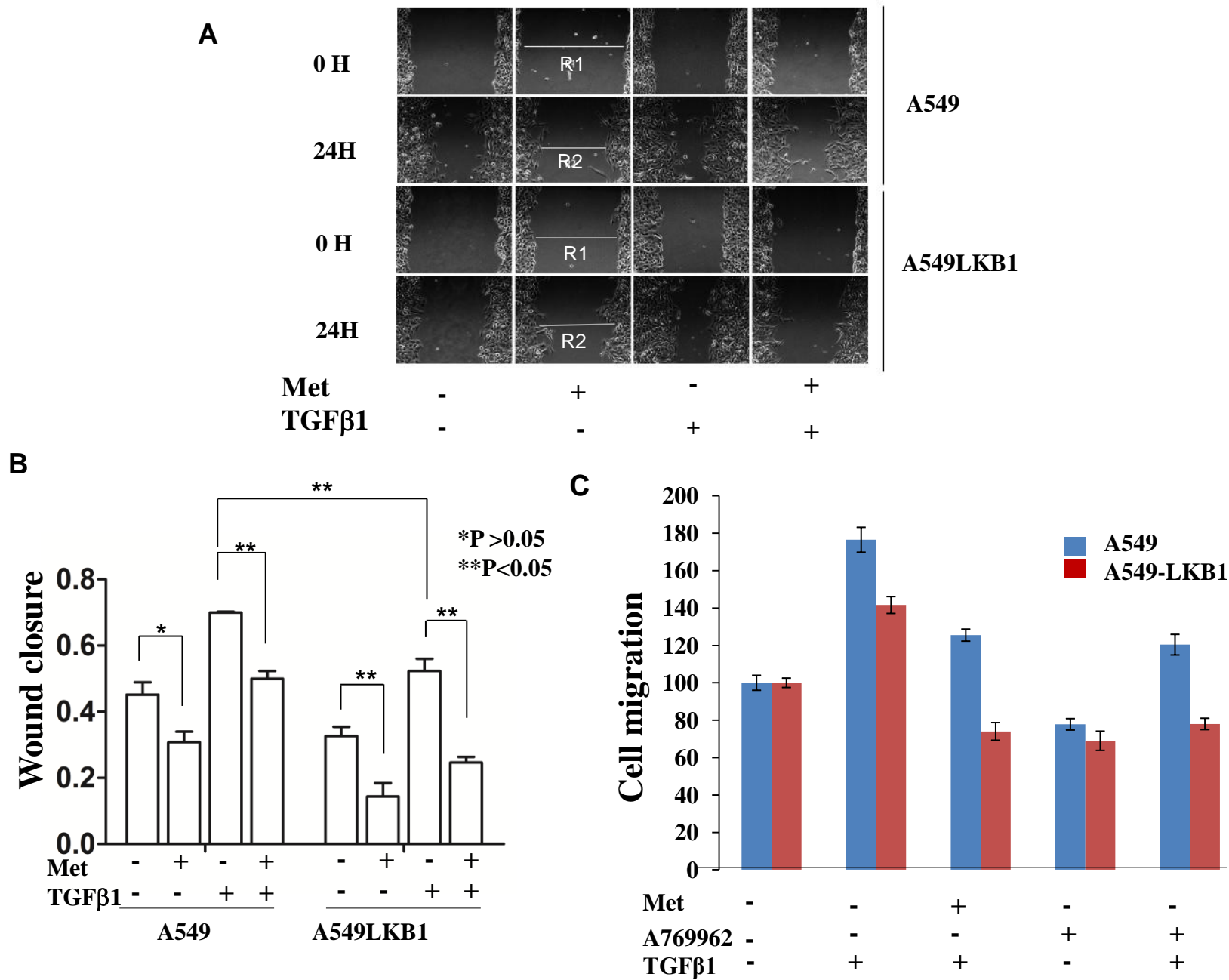


**Figure 2**

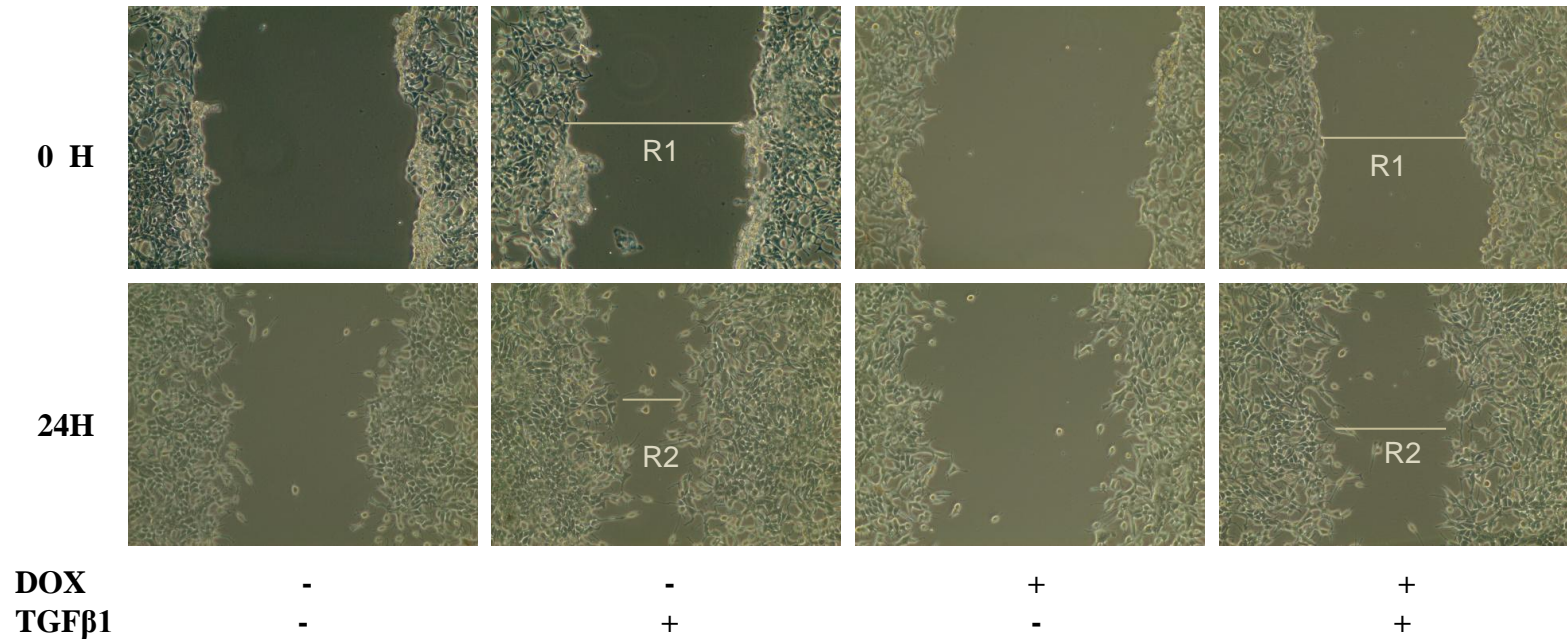
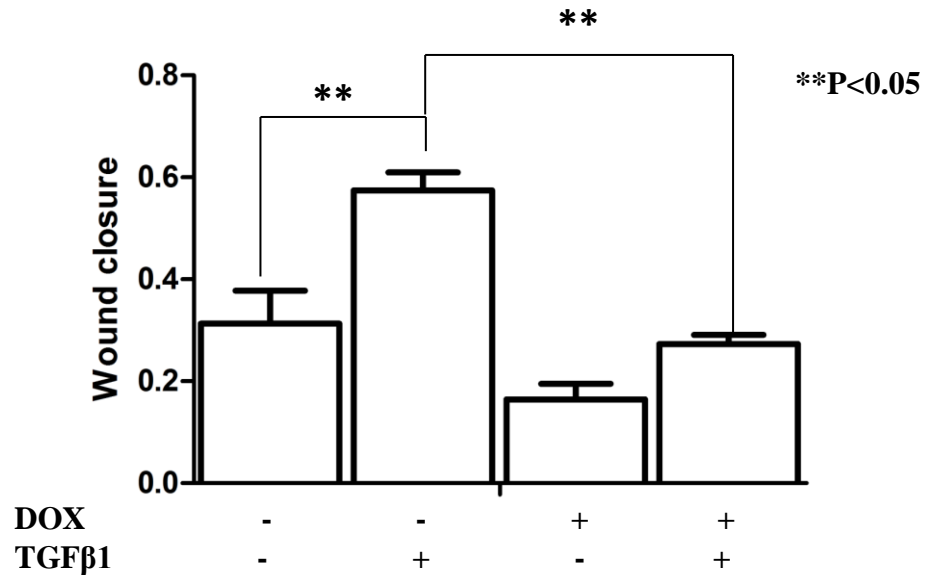


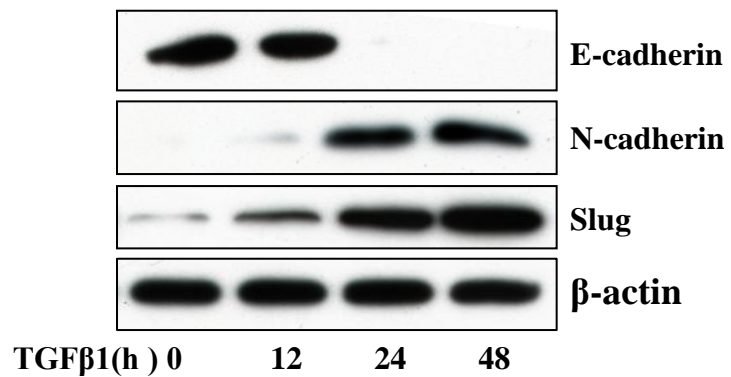
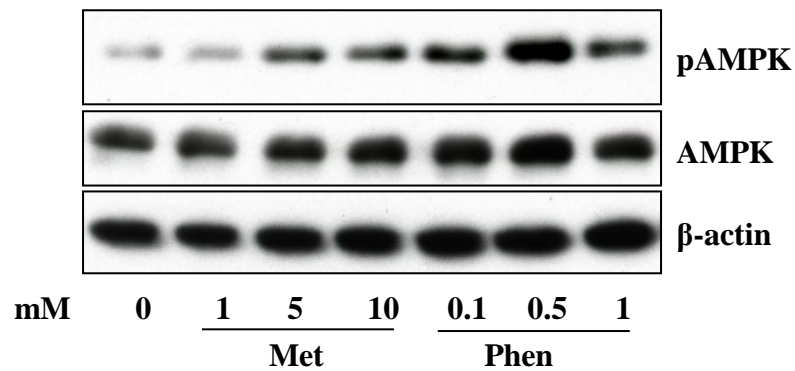
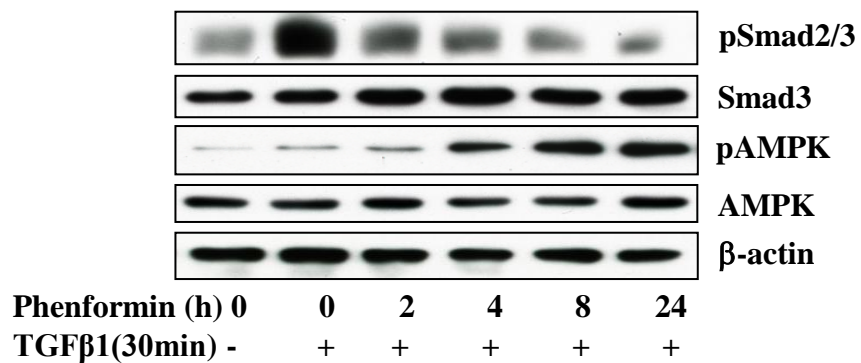
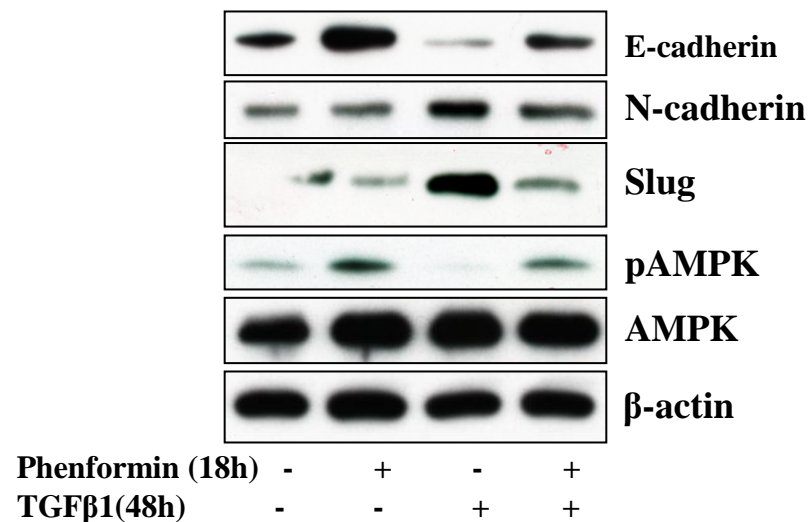
**Figure 3**

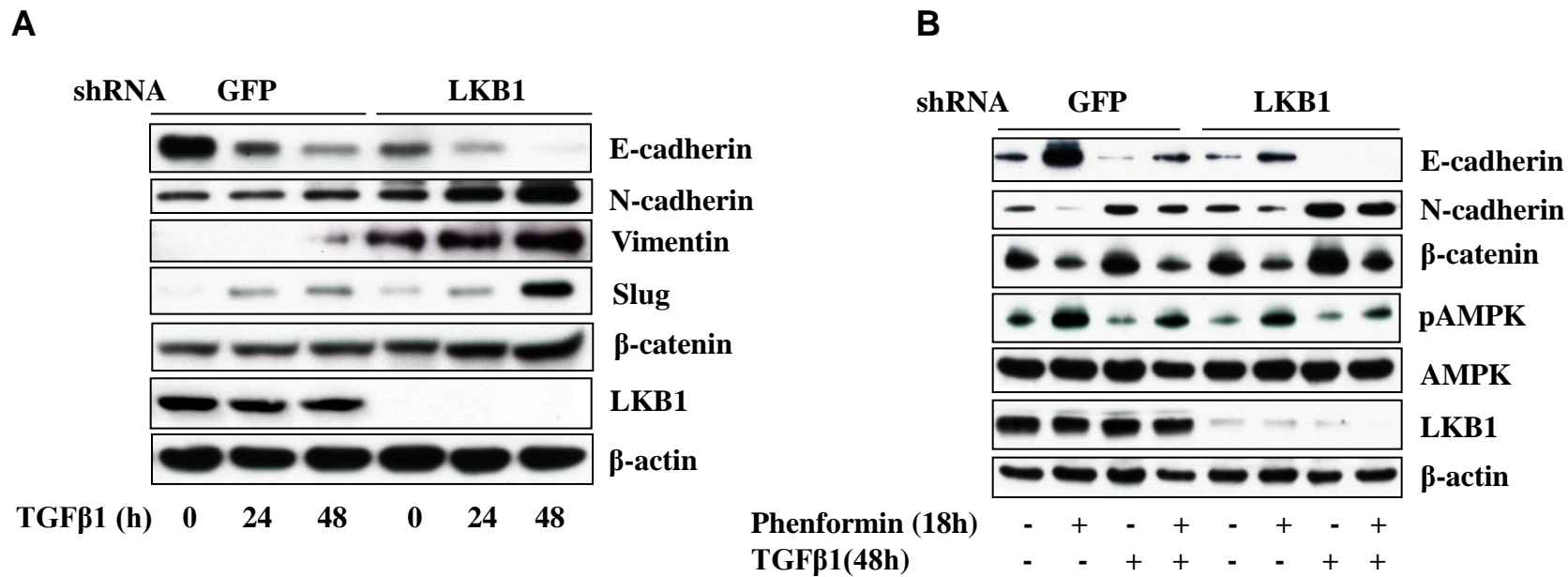




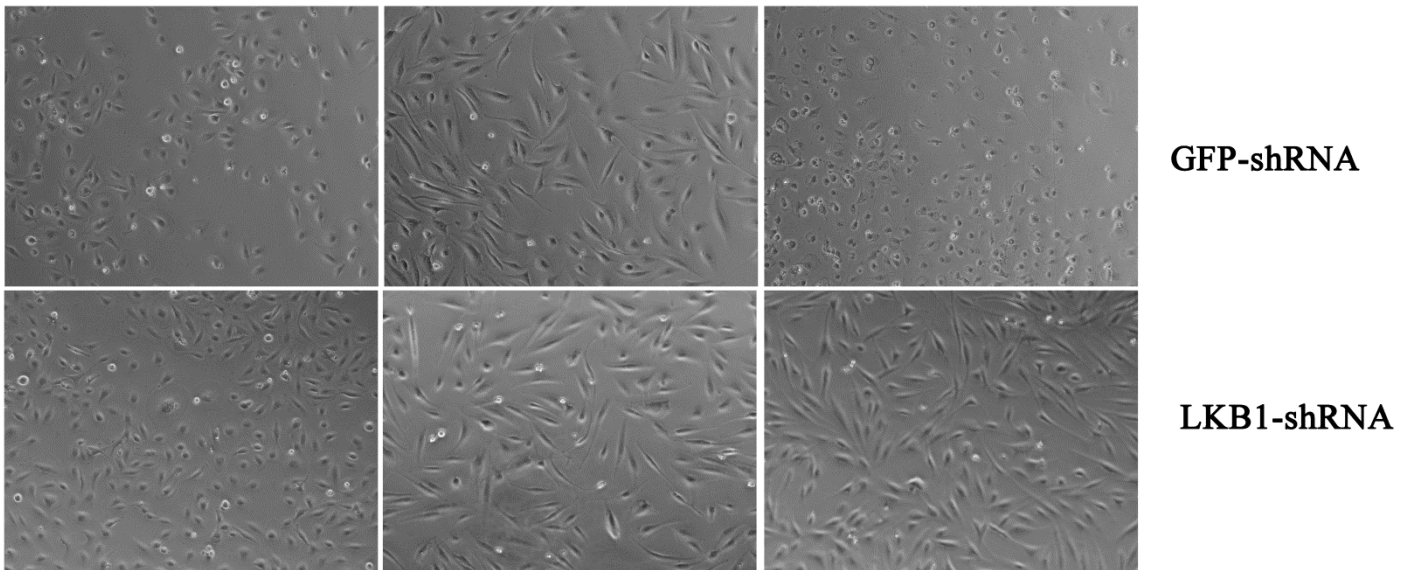
**Figure 4**

**A****B****Figure 5**

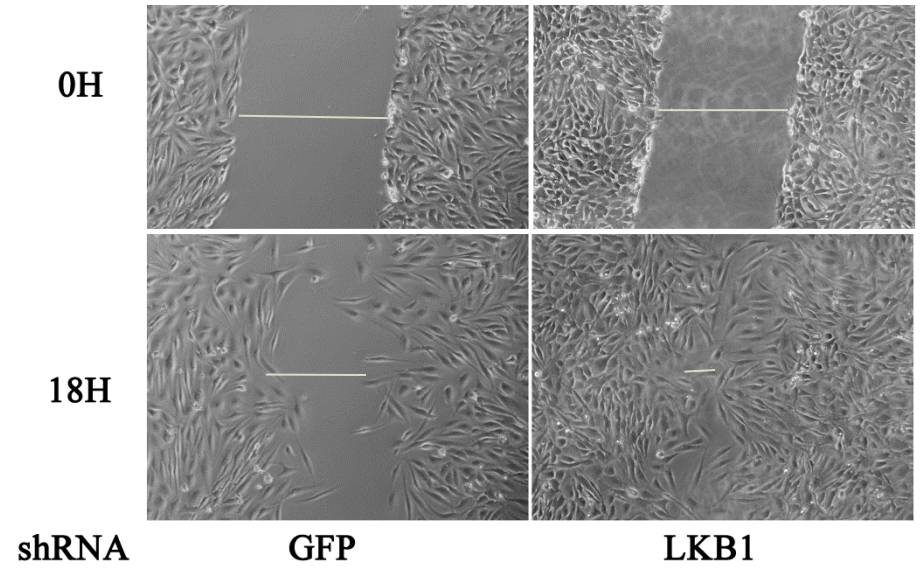
**A****B****C****D****Figure 6**



**Figure 7**

**A**

A769962	-	-	+
TGFβ1	-	+	+

**B****Figure 8**