# CAFs-derived Rho-associated Kinase1 Mediated EMT to Promote Laryngeal Squamous Cell Carcinoma Metastasis

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## Abstract

# Background

Interactions between tumor and tumor microenvironment is essential for tumor metastasis. Cancer-associated fibroblasts (CAFs), as the activated fibroblasts in tumor stroma, are important modifiers of tumor progression. However, the molecular mechanism underlying the tumor-promoting properties of CAFs in Laryngeal squamous cell carcinoma (LSCC) remains unclear.

# Methods

CAFs, CPFs and NFs were isolated and identified from laryngeal cancer, para-laryngeal cancer and normal tissues. Immunofluorescent staining and Western Blot were used to detect the expression of related proteins. Wound healing, migration, invasion and animal experiments were used to examine the ability of movement, migration, invasion and metastasis of LSCC.

# Results

ROCK1, not ROCK2, was highly expressed in CAFs and CAFs enhanced LSCC metastasis in vivo and vitro, and downregulation of ROCK1 in CAFs inhibited migration and invasion. Increasing ROCK1 expression in NFs possessed opposite results. Further studies revealed that epithelial-mesenchymal transition (EMT) and JAK2/STAT3/ERK1/2 pathway might play an essential role in promoting metastasis of LSCC. In addition, inhibition of ROCK1 or JAK2/STAT3/ERK1/2 signal molecules via inhibitors significantly reduced EMT and metastasis in vivo and vitro.

# Conclusions

CAFs-derived ROCK1 via JAK2/STAT3/ERK1/2 axis mediated EMT to promote LSCC metastasis and targeting ROCK1/JAK2/STAT3/ERK1/2 might provide a potential treatment strategy for LSCC.

## Background

Laryngeal squamous cell carcinoma (LSCC) is one of the most common head and neck squamous cell carcinoma (HNSCC) [1]. Surgery is the main method of LSCC treatment at present, but the 5-year survival rate after standard radical resection is not optimistic. Statistically, the 5-year survival rate was only 68%, mainly because tumors were prone to metastasis [2]. Therefore, clarifying the mechanism of metastasis is of great significance for developing effective clinical prevention programs and new targeted therapies for LSCC.

As a basic process in embryonic development, Epithelial-mesenchymal transition (EMT) is the basis of normal development including wound healing and malignant epithelial neoplasms [3]. EMT is a well characterized embryological process which epithelial cells undergo a phenotypic switch by losing their cell polarity and the epithelial marker (E-catenin, β-catenin) translate into mesenchymal phenotypic cells through acquiring the mesenchymal marker (N-catenin, Vimentin). Losing the polarity of epithelial phenotypes, such as synthesis of basement membranes, can enhance migration and progression and anti-apoptosis [4]. Researches also have found that EMT is involved in tumor metastasis, during the process, tumor cells acquire mesenchymal phenotype and invasiveness through epithelial mesenchymal transformation [5].

Recently, accumulating evidences have manifested interactions between tumor and tumor microenvironment is essential for tumor metastasis. Tumor microenvironment includes tumor and stromal cells [6]. Hence, the interactions between epithelial cells and stromal cells usually act as the regulators of EMT, and the factors creating EMT are originating from the tumor and stromal cells [7]. Normally, tumor stromal cells contain fibroblasts, endothelial, macrophages and lymphocytes cells, which secrete inflammatory factors (TNF-α, TGF-β, IGF), chemokines (IL-8, IL-6, MCP-1), matrix degrading enzyme (MMP-9, MMP-2, MMP-1) and growth factor (EGF) [8]. These factors formed the microenvironment, cancer-associated fibroblasts (CAFs) are the major cells in it which plays the key role in forming the tumor microenvironment and regulating progression and metastasis [9].

Furthermore, studies have found that rho-associated kinase1/2 (ROCK1, ROCK2) play an important role in regulating tumor progression. Overexpression or activation of ROCK1 or ROCK2 induces proliferation, while inhibition of ROCK1 or ROCK2 by interfering RNA or inhibitors markedly decreased migration [10]. Stadler et al. also found that CAFs in the tumor microenvironment could promote colorectal cancer metastasis via targeting ROCK1 and ROCK2 [11]. Although ROCK1 or ROCK2 plays a crucial role in tumor progression and metastasis, the molecule mechanism underlying the tumor-promoting properties of CAFs in LSCC remains unclear.

In our study, we first isolated CAFs from LSCC, and found ROCK1, not ROCK2, was highly expressed in CAFs and CAFs enhanced LSCC metastasis in vivo and vitro, and downregulation of ROCK1 in CAFs inhibited migration and invasion, while upregulation of ROCK1 in normal fibroblasts (NFs) possessed opposite results. Further study indicated that EMT and JAK2/STAT3/ERK1/2 pathway might play an essential role in promoting LSCC metastasis. In addition, inhibition of ROCK1 or JAK2/STAT3/ERK

1/2 signal pathway via inhibitors significantly reduced EMT and metastasis in vivo and vitro. Our study suggested that deprivation of ROCK1 or JAK2/STAT3/ERK1/2 molecules would act as an effective treatment strategy against LSCC.

# Results

### 1. CAFs were isolated from LSCC and CAFs enhanced LSCC metastasis in vitro and vivo and ROCK1, not ROCK2 was highly expressed in CAFs.

In our study, CAFs, Cancer Para-laryngeal Fibroblasts (CPFs) and NFs were isolated and identified from laryngeal cancer, para-laryngeal cancer and normal tissues. It is well known that in comparison with NFs, the phenotype of CAFs was significantly different. CAFs express specific molecules, including α-SMA, FSP1, NG2 and PDGF-β receptor, et al [12]. The specific markers were determined by Western Blot, the expressions of FAP, α-SMA, FSP1, NG2 and PDGF-βwere significantly elevated in CAFs (**$P$<0.01, Figure 1A-1B). Here, we established a co-culture system in vitro (Figure 1C), in which fibroblasts were indirectly co-cultured with laryngeal cancer Hep2 cells line, which separated by a semi-permeable membrane (pore size of 0.6 μm). At the appropriate time point, Hep2 cells were either assayed by way of the wound healing, migration, invasion and vivo metastasis. As showed in Figure 1D-1E, Hep2 co-culture with CAFs cells (Hep2/CAFs cells) enhanced movement than Hep2 co-culture with NFs (Hep2/NFs cells, **$P$<0.01). Migration assay showed that Hep2/NFs cells (105±8.6) migrated less than Hep2/CAFs cells (286±15.2). Invasion assay also showed similar results, Hep2/NFs cells (51±6.5) invaded less than Hep2/CAFs cells (101±4.0, *$P$<0.05, **$P$<0.01, Figure 1F–1G). To further illustrate CAFs played a determinant role in metastasis. Hep2/CAFs cells and Hep2/NFs cells respectively inoculated via tail vein into 4-week-old male immunodeficient mice. Six weeks after inoculation, Hep2/CAFs cells (5.8±0.7) demonstrated larger and more frequently lung metastases as compared to Hep2/NFs cells (2±1.5, *$P$<0.05, Figure 1H–1I). In our study, ROCK1, not ROCK2 considerably high expressed in CAFs in comparison with CPFs and NFs (**$P$<0.01, Figure 1J–1K). Data above indicated that CAFs were successfully isolated from LSCC and CAFs enhanced metastasis in vivo and vitro and ROCK1, not ROCK2 was highly expressed in CAFs.

**2.Deprivation of ROCK1 inhibited movement, migration and invasion and upregulation of ROCK1 possessed opposite results. CAFs with high expression of ROCK1 enhanced LSCC metastasis.**

Considering that ROCK1 was highly expressed in CAFs, and lowly expressed in NFs, it was conceivable that ROCK1 might act as a positive regulator of metastasis. To explore the effect of ROCK1 in contribution on metastasis, Y27632 [13] was used to downregulate ROCK1 in CAFs at the appropriate time point, with ROCK1 expression modification confirmed, CAFs were co-cultured with Hep2 cells. Hep2 cells were either assayed by way of the immunofluorescence (IF), Western Blot, wound healing, migration and invasion assays. IF showed that the ROCK1 level was obviously reduced in Hep2 cells co-cultured with CAFs/Y27632 (Hep2/CAFs/Y27632 cells) in comparison with Hep2 cells co-cultured with CAFs/parental (Hep2/CAFs/parental, Figure 2A). The results of Western Blot also showed that levels of ROCK1, FAP, α-SMA, FSP1, NG2 and PDGF-β were significantly reduced (*$P$<0.05, **$P$<0.01, Figure 2D-2E). For wound-healing assay, Hep2/CAFs/Y27632 cells were less motile in comparison with Hep2/CAFs/parental cells (*$P$<0.05, Figure 2B–2C). Similarly, less Hep2/CAFs/Y27632 cells migrated through trans-well chambers (62±8.4) in comparison with Hep2/CAFs/parental cells (134±14.5). Ultimately, invasion assay showed that Hep2/CAFs/Y27632 cells (22±7.3) moved across Matrigel less frequently than Hep2/CAFs/parental cells (46±6.7, *$P$<0.05, **$P$<0.01, Figure 2F–2G). All data indicated that deprivation of ROCK1 inhibited movement, migration and invasion of LSCC.

Conversely, plasmid (ROCK1, Myc-ROCK1-Delta3 (1-727), p-CAG-Myc) transfection was used to increase ROCK1 expression in NFs at the appropriate time point, the effect of transfection was showed in Figure

2H. Hep2 cells cultured with NFs/ROCK1 (Hep2/NFs/ROCK1 cells) were the experimental group, and Hep2 cells cultured with NFs/vector (Hep2/NFs/vector cells) were the control group. Hep2 cells were either assayed by way of the Western Blot, wound healing, migration and invasion assays. Western Blot results suggested the levels of ROCK1, FAP, α-SMA, FSP1, NG2 and PDGF-β were markedly enhanced (*$P$<0.05, **$P$<0.01, Figure 2K-2L). For wound-healing assay, Hep2/NFs/ROCK1 cells were more motile in comparison with Hep2/NFs/vector cells (**$P$<0.01, Figure 2I–2J). Similarly, more Hep2/NFs/ROCK1 cells migrated through trans-well chambers (162±8.8) in comparison with Hep2/NFs/vector cells (89±11.4). At last, invasion assay suggested that Hep2/NFs/ROCK1 cells (79±4.24) moved across Matrigel more frequently than Hep2/NFs/vector cells (25±9.7, *$P$<0.05, **$P$<0.01, Figure 2M–2N). All data indicated that deprivation of ROCK1 possessed opposite results. These observations suggested CAFs with high expression of ROCK1 could enhance movement, migration and invasion of LSCC.

### 3. CAFs-derived ROCK1 promoted EMT of LSCC.

Accumulating evidences suggested that EMT, a well-characterized embryological process, had been identified to play a critical role in cancer metastasis [14]. In order to examine the role of CAFs-derived ROCK1 in mediating EMT, Hep2 cells were co-cultured with NFs/ROCK1 (Hep2/NFs/ROCK1 cells) or CAFs/Y27632 (Hep2/CAFs/Y27632 cells) in a previously described co-culture system respectively. At the appropriate time point, Hep2 cells were either assayed by way of the IF and Western Blots. As Figure3A-3B and 3D showed that the expression of E-cadherin was markedly decreased, while the expressions of N-cadherin, Slug and Vimentin were observably increased in Hep2/NFs/ROCK1 cells when compared to Hep2/NFs/vector cells (*$P$<0.05). In contrast, Figure 3A, 3C and 3E showed that expression of E-cadherin was markedly increased, while the expressions of N-cadherin, Slug and Vimentin were markedly decreased in Hep2/CAFs/Y27632 cells when compared to Hep2/CAFs/parental cells (*$P$<0.05, **$P$<0.01). All data indicated CAFs with high expression of ROCK1 promoted EMT in LSCC.

### 4. Signal molecules of JAK2, STAT3 and ERK1/2 were of great importance in LSCC metastasis.

Tumor metastasis is a multifactorial process [15], signal molecules might play a crucial role in LSCC metastasis. To interrogate the role of signal molecules in CAFs-induced ROCK1 mediating EMT to promote LSCC metastasis. First and foremost, Hep2 cells were co-cultured with NFs/ROCK1 (Hep2/NFs/ROCK1 cells), NFs/vector (Hep2/NFs/vector cells) or CAFs/Y27632 (Hep2/CAFs/Y27632 cells) in a previously described co-culture system respectively. At the appropriate time point, Hep2 cells were assayed by way of the Western Blot. As Figure4A-4B showed that the expressions of p-JAK2, p-STAT3 and p-ERK1/2 were markedly increased in Hep2/NFs/ROCK1 cells when compared to Hep2/NFs/vector cells, *$P$<0.05, **$P$<0.01). Consistent with these observations, Figure4C-4D showed that the expressions of p-JAK2, p-STAT3 and p-ERK1/2 were markedly decreased in Hep2/CAFs/Y27632 cells (*$P$<0.05). All data just indicated that signal molecules of JAK2, STAT3 and ERK1/2 were of great importance in LSCC metastasis, while the upstream and downstream relationship between them were not clear.

### 5. CAFs-derived ROCK1 mediated EMT to promote movement, migration and invasion via activating ROCK1/JAK2 axis.

To further verify the upstream and downstream relationship between ROCK1 and signal molecules of JAK2, STAT3 and ERK1/2. One of JAK2 inhibitors, AG490 [16], was used to treat CAFs for 0, 12, 24h to knockdown the activation of JAK2. With JAK2 expression modification confirmed, CAFs were co-cultured with Hep2 cells (Hep2/CAFs/AG490 cells) in a previously described co-culture system. Hep2 cells were either assayed by way of the Western Blot, wound healing, migration and invasion assays. As measured by Western Blot, levels of p-JAK2, p-STAT3, p-ERK1/2 were markedly decreased, while levels of ROCK1, ROCK2, JAK2, STAT3 and ERK1/2 were not changed. This indicated that JAK2 was the downstream of ROCK1, and the upstream of STAT3 and ERK1/2 (**P*<0.01, Figure 5A-5B). We further examined whether blocking JAK2 by AG490 could also inhibit the tumor-promoting effects on LSCC. In wound-healing assay, Hep2/CAFs/AG490 cells were less motile at 48h in comparison with Hep2 cells co-cultured with CAFs/parental (Hep2/CAFs/parental cells, **P*<0.01, Figure 5C-5D). Similarly, less Hep2/CAFs/AG490 cells migrated through trans-well chambers (67±6.8) in comparison with Hep2/CAFs/parental cells (143±7.2). In the end, invasion assay showed Hep2/CAFs/AG490 cells (32±10.5) moved less frequently than Hep2/CAFs/parental cells (63±12.7, **P*<0.05, ***P*<0.01, Figure 5E-5F). These observations matched the significant increase in E-cadherin expression, while the expressions of N-cadherin, Slug and Vimentin were significantly decreased (Figure 5G-5H, **P*<0.05, ***P*<0.01). All data illustrated that CAFs-derived ROCK1 mediated EMT to reinforce movement, migration and invasion via activating ROCK1/JAK2 axis.

### 6. CAFs-derived ROCK1 mediated EMT to promote movement, migration and invasion via activating ROCK1/JAK2/ STAT3 axis.

To elucidate the role of STAT3 in LSCC metastasis. In the same way, one STAT3 inhibitor, C188-9 [17], was used to treat CAFs for 0, 6, 12h to knockdown the activation of STAT3. With STAT3 expression modification confirmed, CAFs were co-cultured with Hep2 cells (Hep2/CAFs/C188-9 cells) in a previously described co-culture system. Hep2 cells were either assayed by way of the Western Blot, wound healing, migration and invasion assays. Western Blot showed that the levels of p-STAT3, p-ERK1/2 were markedly decreased, while levels of ROCK1, ROCK2, p-JAK2, JAK2, p-STAT3, STAT3, p-ERK1/2and ERK1/2 were not changed (**P*<0.05, ***P*<0.01, Figure 6A-6B). This indicated that STAT3 was the downstream of ROCK1/JAK2, and was the upstream of ERK1/2. We further examined whether blocking STAT3 expression could restrain LSCC metastasis as well. In wound-healing assay, Hep2/CAFs/C188-9 cells were less motile at 48h in comparison with Hep2 cells co-cultured with CAFs/parental (Hep2/CAFs/parental cells, ***P*<0.01, Figure 6C-6D). Similarly, less Hep2/CAFs/C188-9 cells migrated through trans-well chambers (46±6.4) in comparison with Hep2/CAFs/parental cells (92±6.8). Finally, invasion assay indicated that Hep2/CAFs/C188-9 cells (29±6.1) moved through Matrigel less frequently than Hep2/CAFs/parental cells (44±4.3, **P*<0.05, ***P*<0.01, Figure 6E-6F). Consistent with these observations, Figure 6G-6H showed that the level of E-cadherin was markedly increased, nevertheless the levels of N-cadherin, Slug and Vimentin were markedly decreased (**P*<0.05, ***P*<0.01). Similarly, these

data indicated CAFs-derived ROCK1 mediated EMT to promote movement, migration, invasion via activating ROCK1/JAK2/STAT3 signal pathway.

### 7. CAFs-derived ROCK1 mediated EMT to promote movement, migration and invasion via activating ROCK1/JAK2/STAT3/ERK1/2 axis.

Similarly, one ERK1/2 inhibitor, U0126 [18], was used to treat CAFs for 0, 24, 48h to block activation of ERK1/2. With ERK1/2 expression modification confirmed, CAFs were co-cultured with Hep2 cells (Hep2/CAFs/U0126 cells) in a previously described co-culture system. Hep2 cells were either assayed by way of the Western Blot, wound healing, migration and invasion assays. Western Blot showed that the level of p-ERK1/2 was markedly decreased, while the levels of ROCK1, ROCK2, p-JAK2, JAK2, p-STAT3, STAT3, and ERK1/2 were not changed (*$P$<0.05, Figure 7A-7B). This indicated that ERK1/2 was the downstream of ROCK1/JAK2/STAT3. We further examined whether blocking ERK1/2 expression could also inhibit the tumor-promoting effects on LSCC. In wound-healing assay, Hep2/CAFs/U0126 cells were less motile at 48h in comparison with Hep2 cells co-cultured with CAFs/parental (Hep2/CAFs/parental cells, **$P$<0.01, Figure 7C-7D). Similarly, less Hep2/CAFs/ U0126 cells migrated through trans-well chambers (67±10.3) in comparison with Hep2/CAFs/parental cells (91±11.7). Ultimately, the results of invasion assay manifested that Hep2/CAFs/U0126 cells (23±4.8) moved through Matrigel less frequently than Hep2/CAFs/parental cells (49±6.5, **$P$<0.01, Figure 7E-7F). These observations match what the expression of E-cadherin was markedly increased, while the expressions of N-cadherin, Slug and Vimentin were markedly decreased (*$P$<0.05, **$P$<0.01, Figure 7G-7H). These data indicated CAFs-derived ROCK1 mediated EMT to aggrandize the movement, migration and invasion ability of LSCC via activating ROCK1/JAK2/STAT3/ERK1/2 axis.

### 8. Blocking ROCK1/JAK2/STAT3/ERK1/2 axis impaired LSCC metastasis induced by CAFs in vivo.

In our study, we further estimated the contribution of ROCK1/JAK2/STAT3/ ERK1/2 axis on LSCC metastasis *in vivo*. Hep2 cells, co-cultured with CAFs/Y27632, CAFs/AG490, CAFs/C188-9 and CAFs/U0126 in a previously described co-culture system respectively, were experimental groups, Hep2 cells co-cultured with CAFs were control group. Then Hep2 cells were inoculated via tail vein into 4-week-old male immunodeficient mice. Six weeks after inoculation, as showed in Figure8A-8B Hep2/CAFs (5±1.7) attested larger and more frequently lung metastases with respect to Hep2/CAFs/Y27632 (1±1), Hep2/CAFs/AG490 (0.5±0.5), Hep2/CAFs/C188-9 (0.5±0.5) and Hep2/CAFs/U0126 cells (1±0.5, *$P$<0.05). All results suggested that CAFs-derived ROCK1 via JAK2/STAT3/ERK1/2 pathway promotes LSCC metastasis *in vivo*.

# Discussion

Interactions between tumor and tumor microenvironment is essential for tumor metastasis. Microenvironment includes tumor and stromal cells [6]. CAFs, the activated fibroblasts in tumor stroma, is important modifiers of tumor progression. CAFs are known to be a heterogeneous group of cells, mainly derives from normal epithelial cells, mesenchymal fibroblasts, bone marrow mesenchymal stem cells,

vascular beds, or epithelium. In comparison with NFs, the phenotype and function were significantly different. CAFs can express specific molecules, α-SMA, FAP, FSP1/S100A4, NG2 and PDGF-receptor, et al [20]. CAFs, which act as a "stromal response", plays a prominent role in tumor microenvironment [21]. Therefore, a better understanding with the molecule mechanism of metastasis is of great significance for developing effective clinical prevention programs and new targeted therapies for LSCC.

First and foremost, we isolated CAFs, CPFs and NFs from laryngeal cancer tissues, para-laryngeal cancer tissues and normal tissues. The specific markers were determined by Western Blot, CAFs highly expressed of α-SMA, FAP, NG2 and FSP1 and enhanced LSCC metastasis in vitro and vivo. Interestingly, we found ROCK1, not ROCK2 was highly expressed in CAFs. This might indicate that CAFs with the high expression of ROCK1 played a core effect in enhancing movement, migration and invasion of LSCC.

It was well known that ROCK1/2 played a role in regulating cell cycle, proliferation and mitosis. Activation of ROCK1 could induce cell proliferation, inversely, inhibition ROCK1 could reduce migration [10]. Stadler et al. also found that CAFs could promote colorectal cancer metastasis through ROCK1 [11]. Here, we co-cultured CAFs and NFs with laryngeal cancer Hep2 cell line respectively. At the appropriate time point, Hep2 cells were assayed by way of the Western Blot. We found ROCK1, not ROCK2 was highly expressed in CAFs, and ROCK1 might promote LSCC metastasis in vivo and vitro. To further explore CAFs-derived ROCK1 promoted LSCC metastasis, we down-regulated ROCK1 expression in CAFs and up-regulated ROCK1 expression in NFs respectively, the results showed that the expressions of CAFs associative phenotypes markedly decreased, and the ability of movement, migration and invasion was also obviously reduced in Hep2 cells co-cultured with deprivation of ROCK1 in CAFs. On the contrary, the expressions of CAFs associative phenotypes markedly increased, and the ability of movement, migration and invasion was also obviously increased in Hep2/NFs/ROCK1 cells. All that indicated that CAFs-derived ROCK1 authentically promoted LSCC metastasis.

Accumulating evidence have indicated EMT, characterized by loss of polarity of epithelial cells, is important modifiers of tumor progression. The epithelial markers (E-cadherin, β-catenin) acquired the mesenchymal markers (N-cadherin, vimentin, ZEB2) to form mesenchymal cells thus these transformed epithelial cells acquire fibroblast like properties and exhibit reduced cell-cell adhesion and increased motility [22]. In order to examine the role of CAF-derived ROCK1 in contemplating EMT in LSCC, IF and Western Blot were applied to inspect the expressions of EMT associated phenotype markers in Hep2/NFs/ROCK1 and Hep2/CAFs/Y27632 cells respectively. Results showed that epithelial phenotype marker (E-cadherin) levels were markedly decreased, and mesenchymal phenotype markers (N-cadherin, Slug and Vimentin) levels were markedly increased in Hep2/NFs/ROCK1 cells. The results were reversed in Hep2/CAFs/Y27632 cells, which indicated that CAFs-derived ROCK1 mediated EMT to promote LSCC metastasis.

Furthermore, to further explore the molecular mechanism of ROCK1 mediated EMT to accelerate LSCC metastasis. Signal molecules play a crucial role in LSCC metastasis. Signal molecular pathway of JAK2 played an important role in cancer metastasis of cancer. The expression of mSTAT3 and phosphorylated

STAT3 increased in gastric cancer in comparison with normal stomach [23]. The activation of STAT3 was positive in early gastric cancer, poorly differentiated adenocarcinoma and metastatic lymph node tissues [24]. ERK1/2 was generally located in cytoplasm, but when it was activated, it could across the nuclear membrane and transfer to nucleus [25]. ERK1/2 could induce cyclin D1 expression and accelerated cell mitosis to promote cell proliferation [26].

To further interrogate the role of signal molecules in mediating CAFs-induced ROCK1 activated EMT to promote LSCC metastasis. Here, we first explored the activation of JAK2, STAT3 and ERK1/2 in Hep2/NF/ROCK1 and Hep2/CAFs/Y27632 cells respectively. Results of Western Blot showed that p-JAK2, p-STAT3 and p-ERK1/2 levels were markedly decreased in Hep2/CAFS/Y27632 cells. Inversely, p-JAK2, p-STAT3 and p-ERK1/2 levels were markedly increased in Hep2/NFs/ROCK1 cells. All data indicated that signal molecules of JAK2, STAT3 and ERK1/2 were of great importance in LSCC metastasis, while the upstream and downstream relationships were not clear. In order to explore above problem, inhibitors of JAK2, STAT3 and ERK1/2 (AG490, C188-9, U0126) were applied. All data showed that JAK2 was the downstream of ROCK1, and the upstream of STAT3 and ERK1/2. STAT3 was the downstream of ROCK1/JAK2, and the upstream of ERK1/2. ERK1/2 was the downstream of ROCK1/JAK2/ERK1/2. Ultimately, blocking ROCK1/JAK2/STAT3/ERK1/2 pathway impaired the metastasis of LSCC cells in vitro and vivo.

In summary, ROCK1, not ROCK2, was highly expressed in laryngeal CAFs, CAFs-derived ROCK1 mediated EMT via activating JAK2/STAT3/ERK1/2 signal pathway to promote LSCC metastasis. These findings exhibited that stromal ROCK1 could enhance LSCC initiation and progression, and targeting ROCK1/JAK2/STAT3**/** ERK1/2 might provide a potential treatment stratagem for LSCC.

## Materials And Methods

# Cell cultures

Human laryngeal cancer Hep2 cell lines were preserved in our laboratory. Cells were repeatedly cultured in DMEM with 10% FBS containing 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified cell incubator with an atmosphere of 5% CO2 at 37°C. CAFs and NFs were isolated from freshly resected tissues from Laryngeal cancer patients. Tumor tissues, adjacent tissues and adjacent non-tumor tissues were mechanically minced into small pieces (1–1.5 $mm^3$) and seeded onto 10 cm petri dishes in DMEM with 10% FBS containing 100 IU/ml penicillin and 100 IU/ml streptomycin. A homogeneous group of fibroblasts, produced after 7-14 days of culture, would be used in the experiments.

# Western Blot

CAFs treated with a ROCK1 inhibitor (Y27632), a JAK2 inhibitor (AG490), a STAT3 inhibitor (C188-9), or an ERK1/2 inhibitor (U0126) were lysed with RIPA buffer (Thermo Fischer Scientific). The BCA Protein Assay Kit (Pierce, Rockford, USA) was used to measure protein concentrations. 10% SDSPAGE was used to separate 100 μg proteins for 2 h and the proteins were transferred onto PVDF membranes (Millipore,

MA, USA). Blocking membranes with 5% nonfat milk in 1×TBST for 2 h and incubating with primary antibodies overnight at 4°C. Antibodies included anti-FAP, anti-SAM, anti-FSP1, anti-NG2, anti-PDGF-β, anti-ROCK1, anti-ROCK2, anti-E-cad, anti-N-cad, anti-Vimentin, anti-Slug, anti-p-JAK2, anti-JAK2, anti-p-STAT3, anti-STAT3, anti-p-ERK1/2, anti-ERK1/2, GAPDH and Secondary antibody which were purchased from Abcam, Cambridge, MA, USA. a Tanon 5200 system was used to visualize proteins.

# Immunofluorescent staining

$5\times10^4$ Hep2 co-cultured with NFs/ROCK1 or CAFs/Y27632 were seeded into slides (Millipore, MA, USA) and fixed with 4% paraformaldehyde (PFA) for 30 min. Slides were rinsed with PBS for 3 times, blocking slides with 5% BSA for 1 h at room temperature and incubating slides with primary antibodies at 4°C overnight. Next day, rinsing slides with PBS for 3 times and incubating slides with secondary antibodies in the dark at room temperature for 1 h. Antibodies included anti-ROCK1, anti-E-cad and anti-Vimentin, Alexa Fluor® 488 goat and Alexa Fluor® 555 which were purchased from Abcam, Cambridge, MA, USA. Visualizing nuclei with DAPI in the dark for 5 min. Analyzing slides by uorescent microscopy (10x).

# Transfections

Seeding $3\times10^5$ cells into 6-well plates and incubating cells overnight. Transfecting cells with ROCK1 plasmid (Myc-ROCK1-Delta3 (1-727), Gene Pharma Company, Shanghai) by lipofectamine 2000 (Invitrogen) and selecting 1200 ug/ml G418. Verifying selected clones with Western Blot and frozen.

# Wound healing assay

Inoculating $1\times10^6$ cells into 6-well plates and scratching cells by using 20μl pipette tips after overnight incubation. Then, photographing cells by using a high-powered microscope (2x).

# Cell migration and invasion assay

Seeding $2\times10^5$ Hep2 cells seeded into the upper chambers of trans-wells (Boyden trans-well chambers, Corning, MA, USA) with 200 ul of serum-free DMEM and adding $3\times10^4$ CAFs with or without inhibitors in 800ul medium containing 10% fetal calf serum into the lower chambers. Culturing cells for one day and staining filters with crystal violet at room temperature for 30 min. For invasion assay, coating upper chamber membranes in Matrigel (Becton Dickinson Labware, Bedford, MA, USA). The membranes of above layer were stained with crystal violet. Counting cells by using a high-power objective (10 x) in five random fields.

# Animal experiments

Animals were obtained from the Institute of Zoology, Chinese Academy of Sciences. Animal experiments were performed on basis of the guidelines for the care and utilization of experimental animals of the Institute of experimental animals. The following study protocol has been approved by the IRB of the medical center. Animal care and treatment were conducted on basis of the guidelines for the care and utilization of experimental animals of the NIH. At the end of the experiment, the animals were killed by

intraperitoneal injection of excess pentobarbital sodium (4%, 200 mg/kg; Sigma, Shanghai, China). The lung of each group was then collected for further analyses.

# In vivo metastasis

4-week-old male immunodeficient mice maintained by the animal resources facility of the medical school of Shanghai Jiaotong University. Animal care and experiments are performed following the "guidelines for the care and utilization of experimental animals" and "principles for the utilization and care of vertebrates", and are approved by the ethics committee of experimental animals of the Medical College of Shanghai Jiaotong University. The average volume and bodyweight of the four groups of mice were similar. The experimental animals were grouped according to the randomization formula. Researchers were not aware of the group allocation at the different stages of the experiment during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis. Mice was randomly assigned to 5 groups (five in each group): Hep2 cells, co-cultured with CAFs/Y27632, CAFs/AG490, CAFs/C188-9 and CAFs/U0126 in a previously described co-culture system respectively, were experimental groups, Hep2 cells co-cultured with CAFs were control group. Cells were inoculated via tail vein into the mice. After six months, counting pulmonary metastatic nodules by H&E staining. The error bar represents the SEM. Embed TUNEL and IH staining tumor in paraffin, section them at 6 μm, and stain them with H&E. Dewax and rehydrate paraffin-embedded tissues section. For IH staining, conduct heat-induced epitope recovery in the microwave for 6 minutes using target recovery solution. At high magnification (×400) in the field, calculate the number tumor.

# Statistical analysis

Analyze data by Graph-Pad Prism 6 software and displaying by means ± SD. Assessing differences by student's t test. $P<0.05$ was considered statistically significant.

# Abbreviations

Laryngeal squamous cell carcinoma (LSCC), Head and neck squamous cell carcinoma (HNSCC), Cancer-associated fibroblasts (CAFs), Cancer Para-laryngeal Fibroblasts (CPFs), Normal Fibroblasts (NFs), Epithelial-mesenchymal transition (EMT), Rho-associated kinase1/2 (ROCK1, ROCK2), immunofluorescence (IF).

# Declarations

### Ethics approval and consent to participate

Our study was approved by the Human Research Ethics Committee. Animals were approved by the Experimental Animal Ethics Committee based on the Institutional Animal Care and Use Committee.

### Consent for publication

All authors are consent for publication.

### Availability of data and material

The data and material during the current study were available from the corresponding author on reasonable request.

### Competing interests

There is on competing interests.

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### Authors' contributions

Performed experiments: YLY, GSQData analysis: YLY
Manuscript writing: YLY, GSQ
Revised the manuscript: AH, ZJYStudy design: AH, HSX
Data interpretation: ZJY, AH

### Acknowledgements

Not Applicable

### Data availability statement

All data during the study are available from the corresponding author by request.

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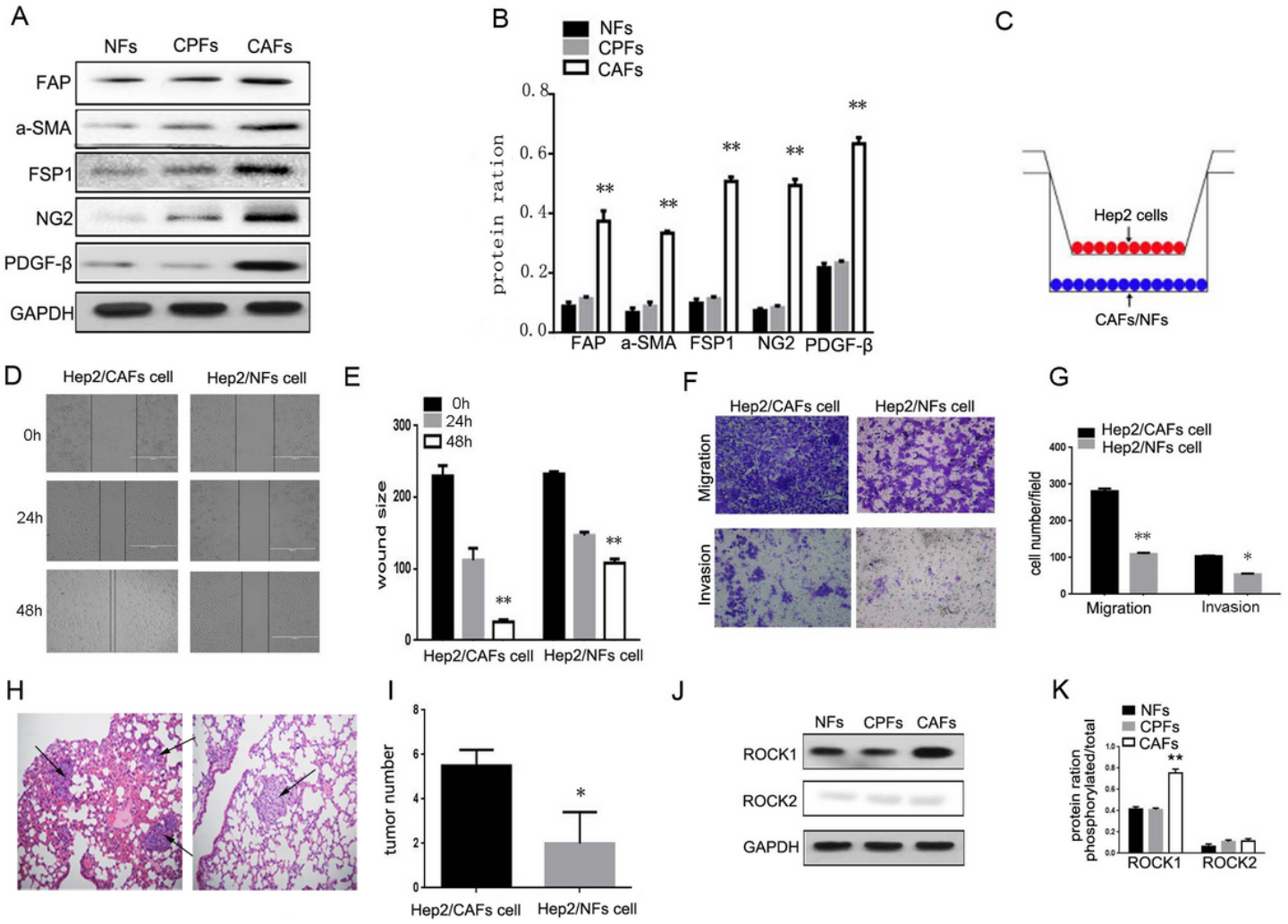
# Figures

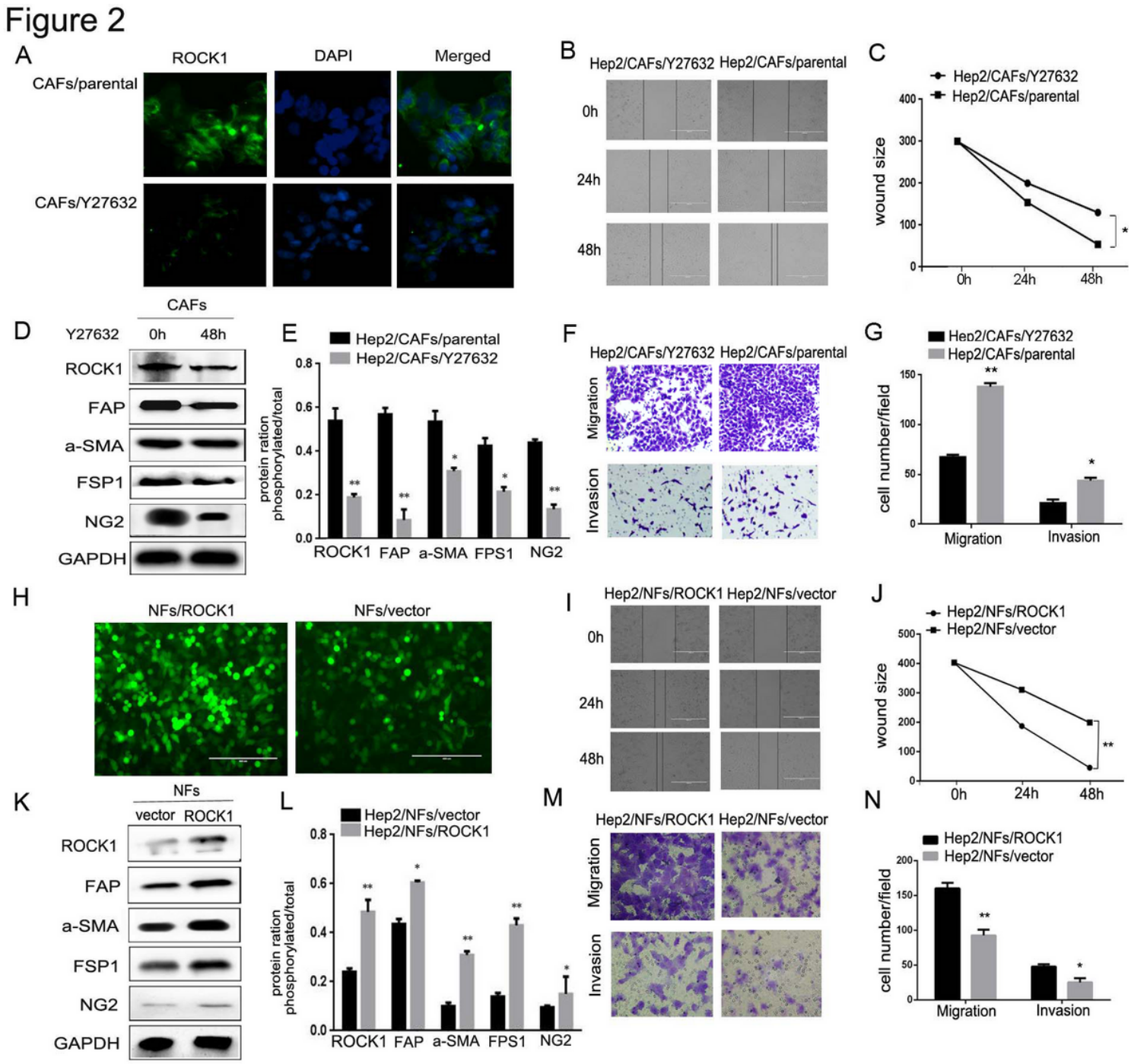
Figure 1




## Figure 1

CAFs were isolated from LSCC **and CAFs enhanced the ability of metastasis of LSCC in vitro and vivo and ROCK1, not ROCK2 was highly expressed in CAFs. A.** CAFs specific markers (FAP, a-SMA, FSP1, NG2 and PDGF-b) were determined by Western Blot. The expressions of markers were significantly elevated in CAFs. **B.** Protein ratio in NFs, CPFs and CAFs (***P*<0.01). **C.** Co-cultured model was used to separate Hep2 cells line and CAFs or NFs. **D.** Hep2 cells co-cultured with CAFs or NFs on cell mobility as assessed via wound healing assay. Hep2/ CAFs cells increased the mobility. **E.** Wound size in Hep2/CAFs or NFs cells (***P*<0.01). F. Hep2/CAFs or NFs cells on cell migration and invasion as measured via trans-well assay. Hep2/CAFs cells stimulated the ability of migration and invasion. **G.** Cells number in every field in Hep2/CAFs or NFs cells (**P*<0.05, ***P*<0.01). **H.** Hep2/CAFs or NFs cells were inoculated into nude mice and pulmonary nodules were observed after six weeks (*N*=5/group). H&E stains of pulmonary nodules (100×), Hep2/CAFs cells demonstrated larger and more frequently lung metastases as compared to Hep2/NFs cells. **I.** Pulmonary tissue and nodules were quantified by H&E staining from co-cultured with CAFs or NFs (**P*<0.05). **J.** The expressions of ROCK1 and ROCK2 were determined by Western Blot. The

expression of ROCK1, not ROCK2 was significantly elevated in CAFs. **K.** Protein ratio in NFs, CPFs and CAFs (**$P$<0.01).




## Figure 2

**CAFs enhanced the ability of movement, migration and invasion of Hep2 cells via the high expression of ROCK1. A.** ROCK1 IF staining in Hep2/CAFs/parental or Hep2/CAFs/Y27632 cells. ROCK1 level was reduced in Hep2/CAFs/Y27632 cells. **D.** The inhibition effect and CAFs associative phenotype were verified by Western Blot. ROCK1, FAP, a-SMA, FSP1, NG2 and PDGF-b levels were significantly decreased in Hep2/CAF/Y2763 cells. **E.** Protein ratio in Hep2/CAFs/parental and Hep2/NFs/ROCK1 cells (*$P$<0.05,

**$P$<0.01). **B.** Hep2/CAFs/parental or Hep2/CAFs/Y27632 cells on cell mobility as assessed via wound healing assay. Hep2/CAFs/Y2763 cells decreased the mobility. **C.** Wound size in Hep2/CAFs/parental or Hep2/CAFs/Y27632 cells (*$P$<0.05). F. Hep2 cells co-cultured with CAFs/parental or CAFs/Y2763 on cell migration and invasion as measured via trans-well assay. Hep2 cells co-cultured with CAFs/Y2763 inhibit the ability of migration and invasion. **G.** Cell number in every field in Hep2/NFs/vector and Hep2/CAFs/Y27632 cells (*$P$<0.05, **$P$<0.01). **H.** Plasmid transfection was used to up-regulate ROCK1 in NFs, the effect of transfection was showed via IF. **K.** The expression of ROCK1 and CAFs associative phenotype were verified by Western Blot. ROCK1, FAP, a-SMA, FSP1, NG2 and PDGF-b levels were significantly increased. **L.** Protein ratio in Hep2/NFs/vector or Hep2/NFs/ROCK1 cells (*$P$<0.05, **$P$<0.01). **I.** Hep2/NFs/vector or Hep2/NFs/ROCK1 cells on cell mobility as assessed via wound healing assay. Hep2/NFs/ROCK1 cells increased the mobility. **J.** Wound size in Hep2/CAFs or Hep2/NFs cells (**$P$<0.01). M. Hep2/NFs/vector or Hep2/NFs/ROCK1 cells on cell migration and invasion as measured via trans-well assay. Hep2/NFs/ROCK1 cells stimulated the ability of migration and invasion. **N.** Cell number in every field in Hep2/NFs/vector or Hep2/NFs/ROCK1 cells (*$P$<0.05, **$P$<0.01).

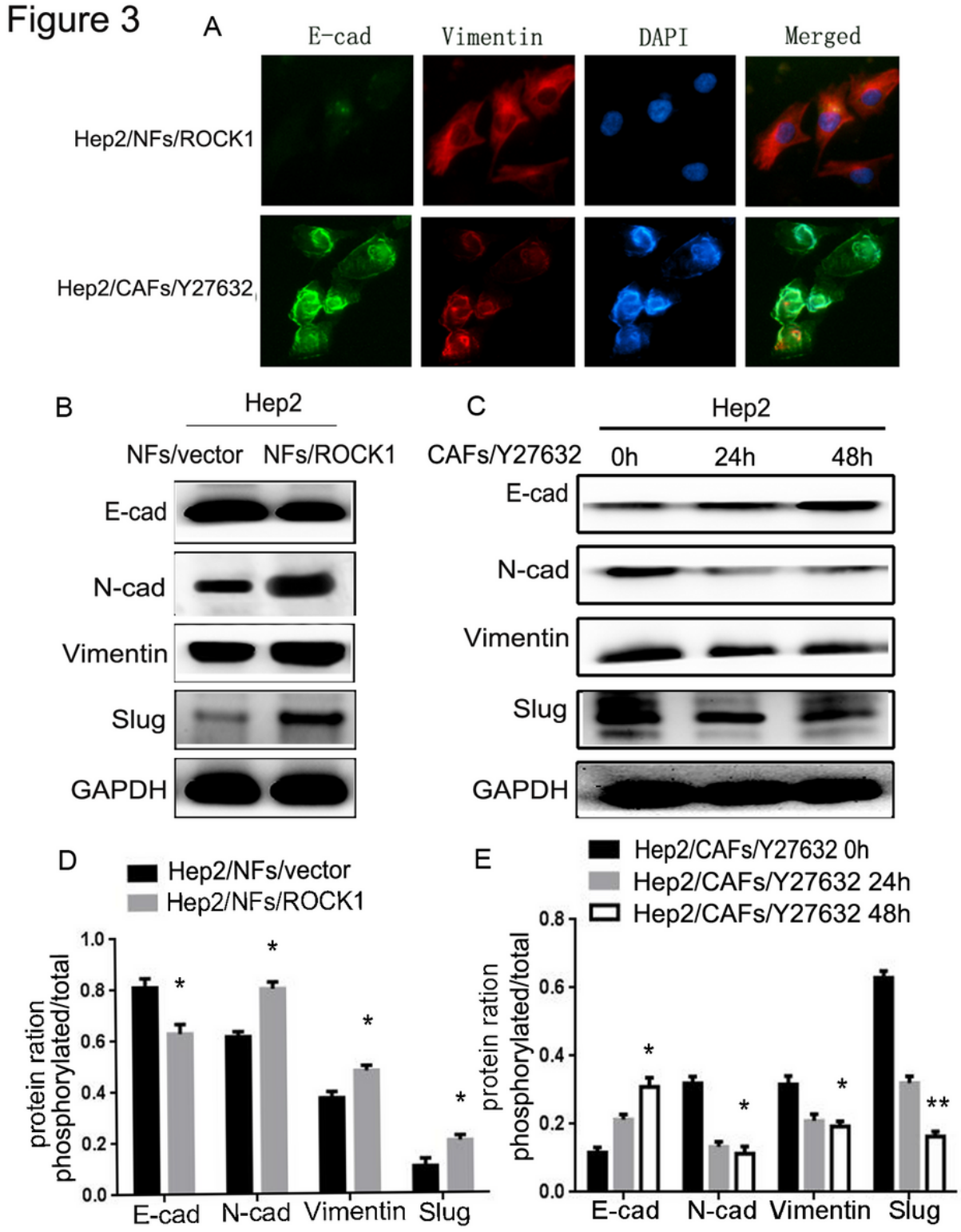



**Figure 3**

**CAFs-derived ROCK1 promoted the process of EMT of LSCC. A.** B. C. The expressions of E-cadherin and N-cadherin, Slug and Vimentin were detected via IF and Western Blot in Hep2/NFs/ROCK1 or Hep2/CAFs/Y27632 cells. E-cadherin level was reduced in Hep2/NFs/ROCK1 cells, while it was increased in Hep2/CAFs/Y27632 cells. The expressions of N-cadherin, Slug and Vimentin were increased in

Hep2/NFs/ROCK1 cells, while they were reduced in Hep2/CAFs/Y27632 cells. **D.E.** Protein ratio in Hep2/NFs/ROCK1 and Hep2/CAFs/ Y27632 cells (*$P$<0.05, **$P$<0.01).

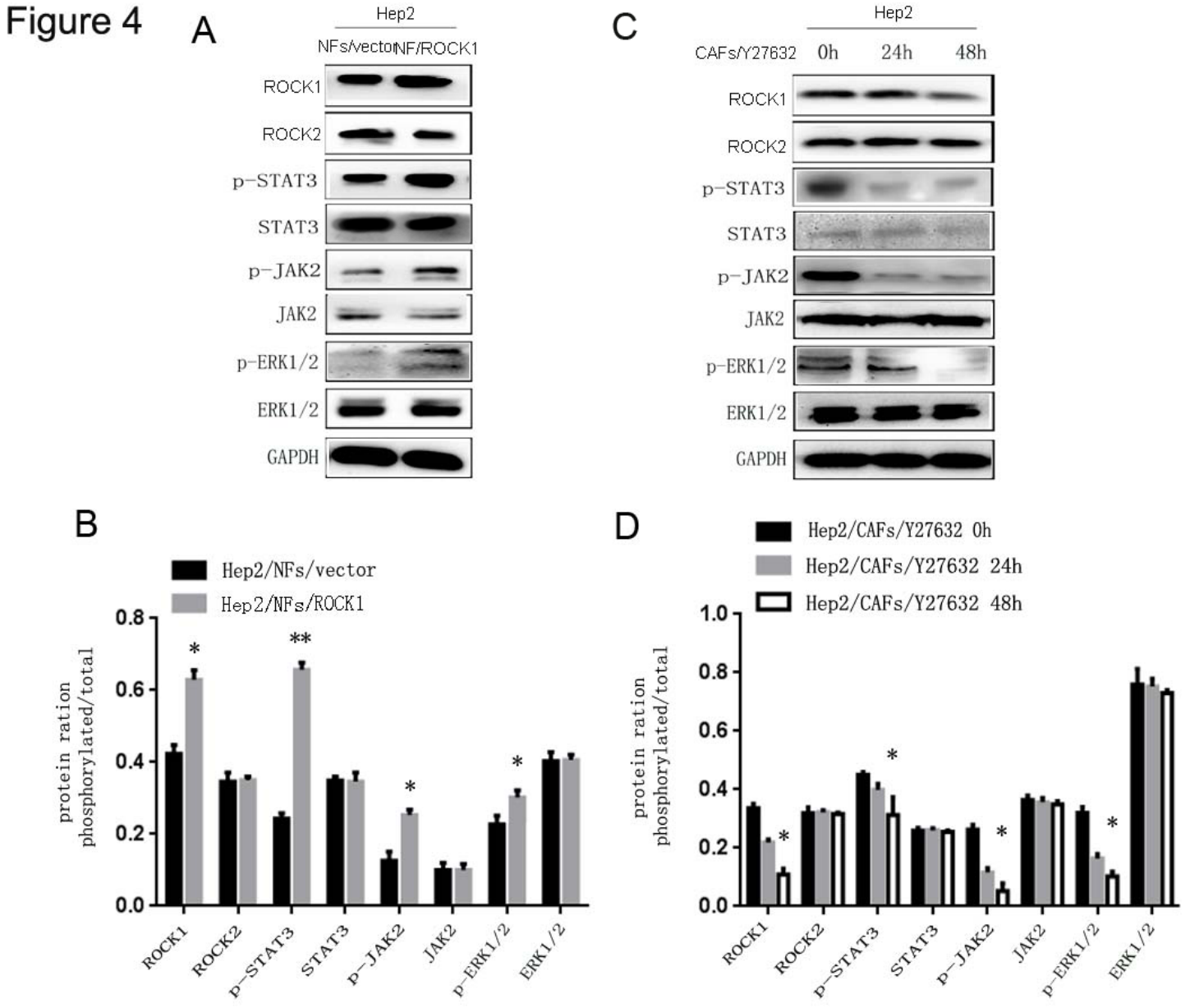
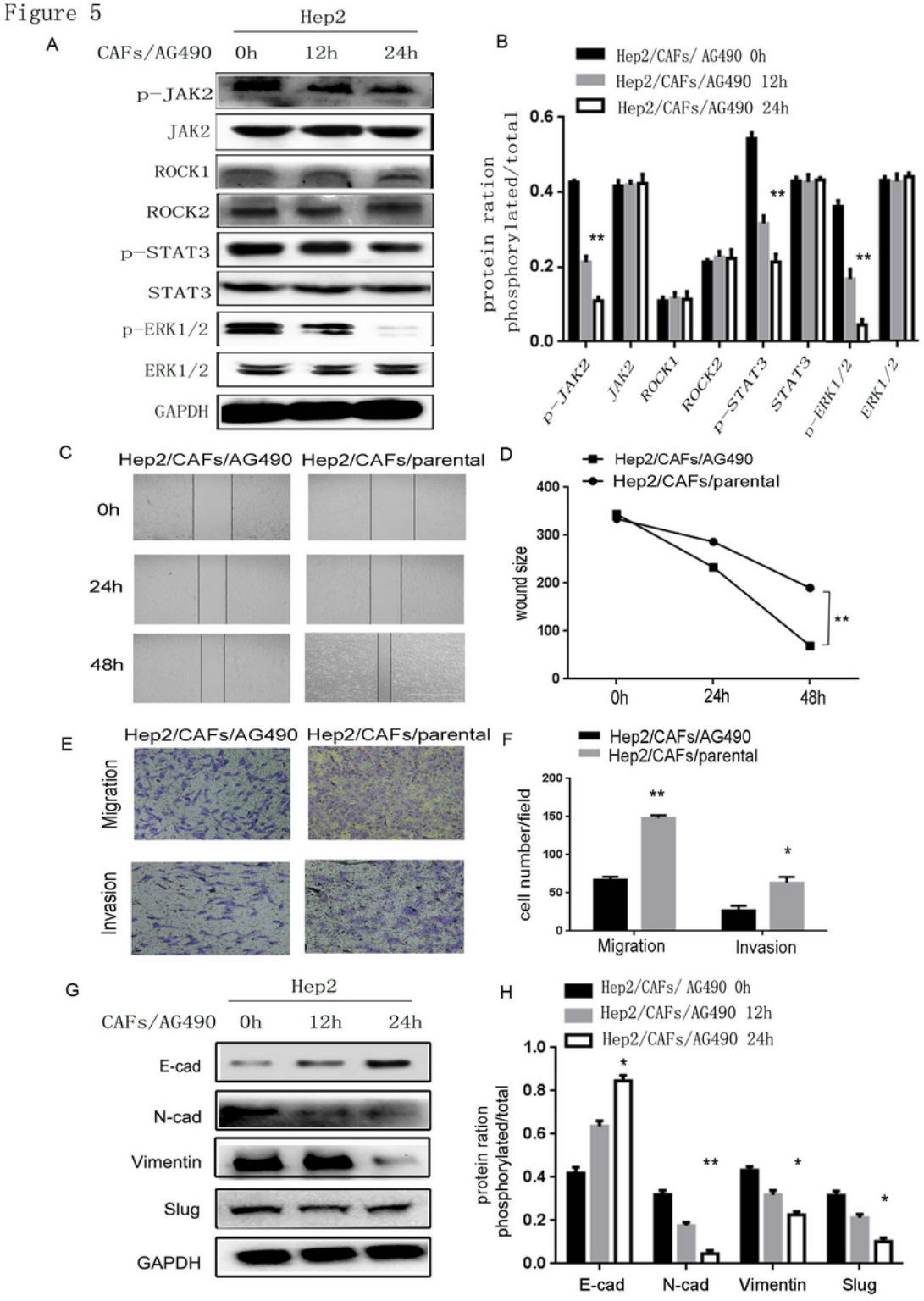



**Figure 4**

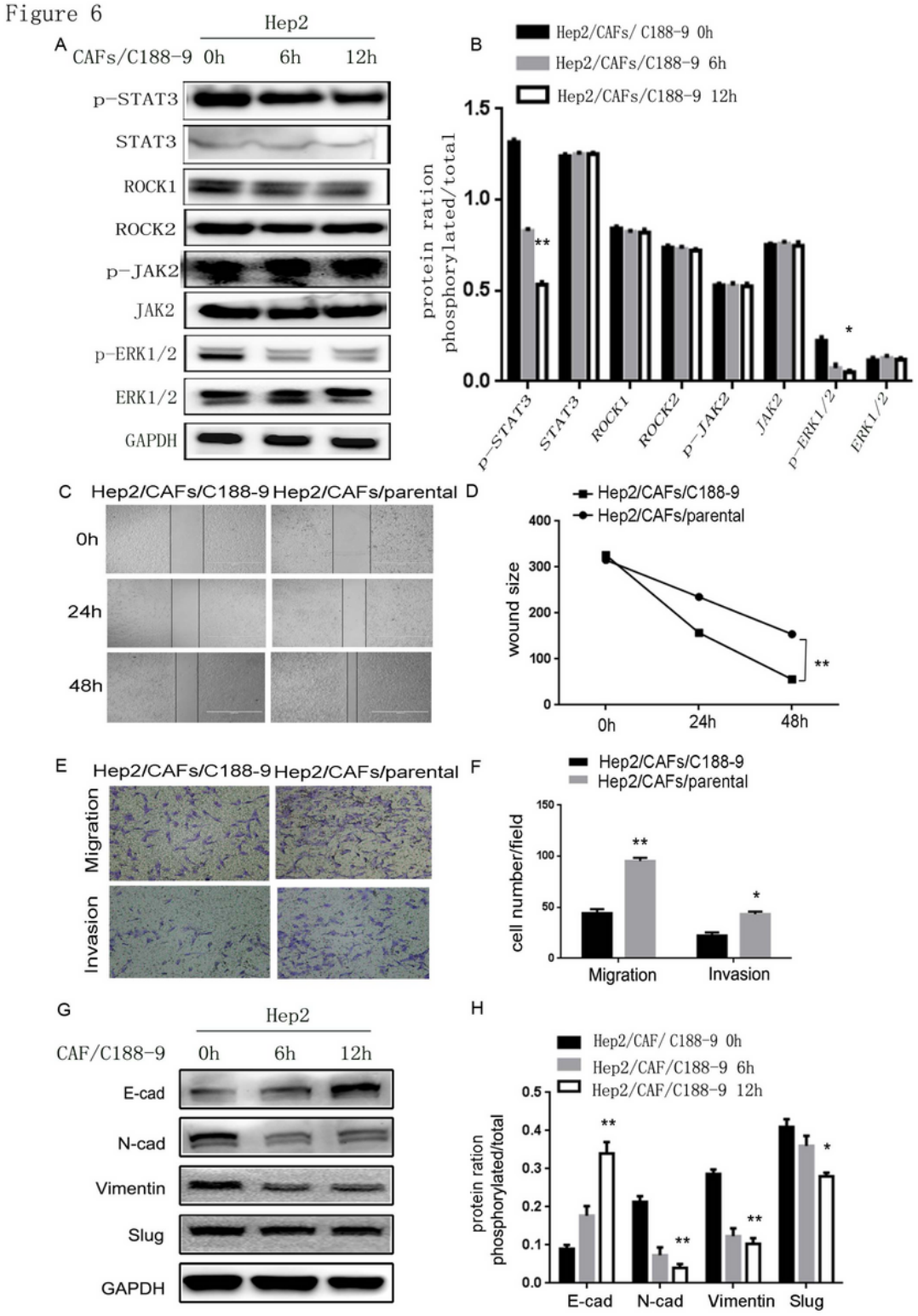
**Signal molecules of JAK2、STAT3 and ERK1/2 were of great importance in the metastasis of LSCC. A.** The expressions of ROCK1, ROCK2, JAK2, STAT3 and ERK1/2 were verified by Western Blot. The p-JAK2 p-STAT3 and p-ERK1/2 levels were markedly increased in Hep2/NFs/ROCK1 cells. **B.** Protein ratio in Hep2/NFs/vector or Hep2/NFs/ROCK1 cells (*$P$<0.05, **$P$<0.01). **C.** The expressions of ROCK1, ROCK2, JAK2 STAT3 and ERK1/2 were verified by Western Blot in Hep2/CAFs/Y27632 cells. P-JAK2 p-STAT3 and p-ERK1/22 levels were markedly decreased in Hep2/CAFs/Y27632 cells. **D.** Protein ratio in Hep2/CAFs/Y27632 cells (*$P$<0.05).




## Figure 5

**CAFs-derived ROCK1 mediated the process of EMT to promote the ability of movement, migration, invasion of LSCC via activation of ROCK1/ JAK2 axis. A.** The expressions of ROCK1, ROCK2, p-JAK2, JAK2, p-STAT3, STAT3, p-ERK1/2 and ERK1/2 were detected by Western Blot in Hep2/CAF/AG490 cells, levels of p-JAK2, p-STAT3, p-ERK1/2 were markedly decreased, while levels of ROCK1, ROCK2, JAK2, STAT3 and ERK1/2 were not changed. **B.** Protein ratio in Hep2/CAFs/AG490 cells (** $P$<0.01). **C.**

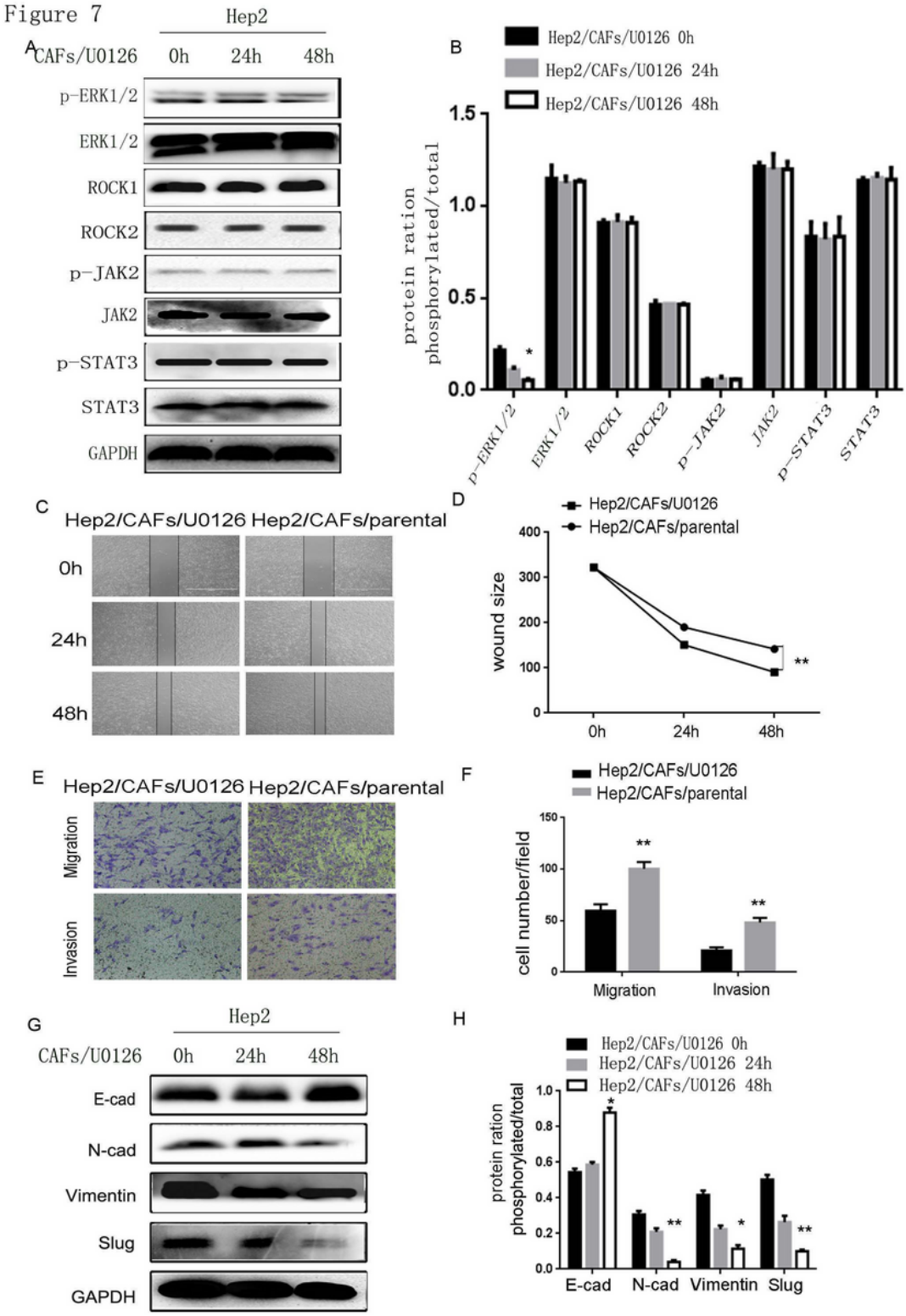
Hep2/CAFs/parental or Hep2/CAFs/AG490 on cell mobility as assessed via wound healing assay. Hep2/CAFs/AG490 cells decreased the mobility. **D.** Wound size in Hep2/CAFs/parental or Hep2/CAF/AG490 cells (** $P<0.01$). E. Hep2/CAFs/parental or Hep2/CAFs/AG490 cells on cell migration and invasion as measured via trans-well assay. Hep2/CAFs/AG490 cells inhibited the ability of migration and invasion. **F.** Cell number in every field in Hep2/CAFs/parental or Hep2/CAF/AG490 cells (* $P<0.05$, ** $P<0.01$). **G.** The expressions of E-cadherin and N-cadherin, Slug and Vimentin were detected by Western Blot in Hep2/CAFs/AG490 cells, level of E-cadherin was markedly increased, while levels of N-cadherin, Slug and Vimentin were decreased. **H.** Protein ratio in Hep2/CAFs/AG490 cells (* $P<0.05$, ** $P<0.01$).




## Figure 6

**CAFs-derived ROCK1 mediated the process of EMT and enhanced the ability of movement, migration, invasion of LSCC via the activation of ROCK1/JAK2/STAT3 signal pathway. A.** The expressions of ROCK1, ROCK2, p-JAK2, JAK2, p-STAT3, STAT3, p-ERK1/2 and ERK1/2 were detected by Western Blot in Hep2/CAFs/C188-9 cells, levels of p-STAT3, p-ERK1/2 were markedly decreased, while levels of ROCK1, ROCK2, p-JAK2, JAK2, STAT3 and ERK1/2 were not changed. **B.** Protein ratio in Hep2/CAFs/C188-9 cells

(* $P$<0.05, ** $P$<0.01). **C.** Hep2/CAFs/parental or Hep2/CAFs/C188-9 cells on cell mobility as assessed via wound healing assay. Hep2/CAFs/C188-9 cells decreased the mobility. **D.** Wound size in Hep2/CAFs/parental or Hep2/CAFs/C188-9 cells (** $P$<0.01). E. Hep2/CAFs/ parental or Hep2/CAFs/C188-9 cells on cell migration and invasion as measured via trans-well assay. Hep2/CAFs/C188-9 cells inhibited the ability of migration and invasion. **F.** Cell number in every field in Hep2/CAFs/parental or Hep2/CAFs/C188-9 cells (* $P$<0.05, ** $P$<0.01). **G.** The expressions of E-cadherin and N-cadherin, Slug and Vimentin were detected via Western Blot in Hep2/CAFs/C188-9 cells, level of E-cadherin was markedly increased, while levels of N-cadherin, Slug and Vimentin were decreased. **H.** Protein ratio in Hep2/CAFs/C188-9 cells (* $P$<0.05, ** $P$<0.01).




## Figure 7

**CAFs-derived ROCK1 mediated the process of EMT and promoted the ability of movement, migration, invasion of LSCC via the activation of ROCK1/JAK2/STAT3/ERK1/2 axis. A.** The expressions of ROCK1, ROCK2, p-JAK2, JAK2, p-STAT3, STAT3, p-ERK1/2 and ERK1/2 were detected by Western Blot in Hep2/CAFs/U0126 cells, levels of p-ERK1/2 were markedly decreased, while levels of ROCK1, ROCK2, JAK2, p-JAK2, p-STAT3, STAT3 and ERK1/2 were not changed. **B.** Protein ratio in Hep2/CAFs/U0126 cells

(* *P*<0.05). **C.** Hep2/CAFs/parental or Hep2/CAFs/U0126 cells on cell mobility as assessed via wound healing assay. Hep2/CAFs/U0126 cells decreased the mobility. **D.** Wound size in Hep2/CAFs/parental or Hep2/CAFs/U0126 cells (** *P*<0.01). E. Hep2/CAFs/parental or Hep2/CAFs/U0126 cells on cell migration and invasion as measured via trans-well assay. Hep2/CAFs/U0126 cells inhibited the ability of migration and invasion. **F.** Cell number in every field in Hep2/CAFs/vector or Hep2/CAFs/U0126 cells (** *P*<0.01). **G.** The expressions of E-cadherin and N-cadherin, Slug and Vimentin were detected via Western Blot in Hep2/CAFs/U0126 cells, level of E-cadherin was markedly increased, while levels of N-cadherin, Slug and Vimentin were decreased. **H.** Protein ratio in Hep2/CAFs/U0126 cells (* *P*<0.05, ** *P*<0.01).

Figure 8

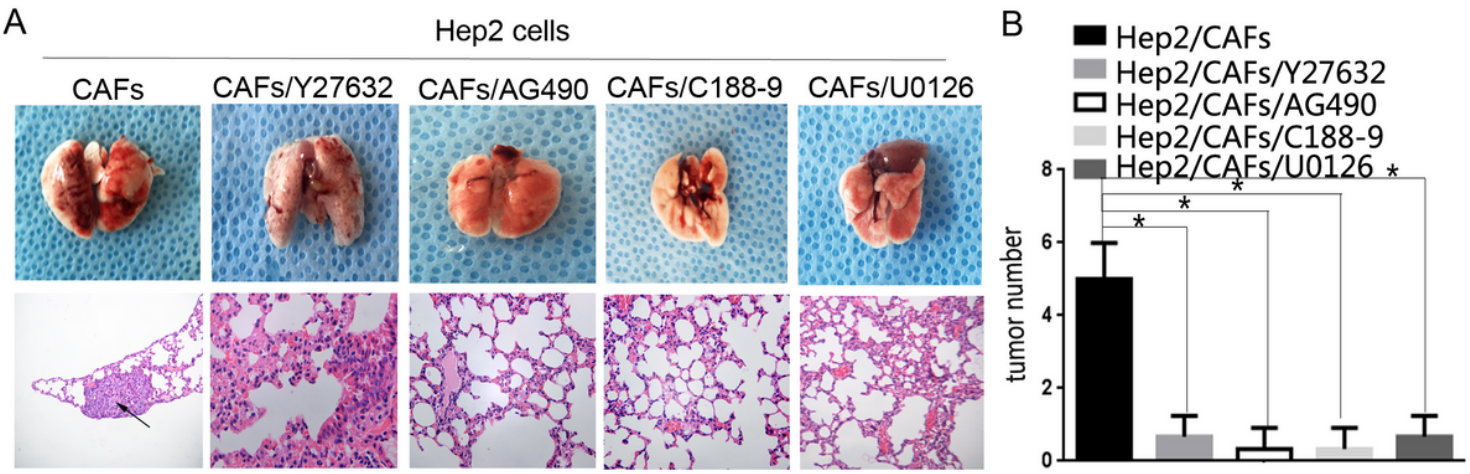



**Figure 8**

**Blocking ROCK1/JAK2/STAT3/ERK1/2 axis impaired the metastasis of LSCC induced by CAFs in vivo. A.** Hep2 cells, co-cultured with CAFs, CAFs/Y27632, CAFs/AG490, CAFs/C188-9, CAFs/U0126 respectively, were inoculated into nude mice and pulmonary nodules were observed after six weeks (*N*=5/group), Hep2 cells co-cultured with CAFs were control group. H&E stains of pulmonary nodules (100×), Hep2/CAFs/Y27632, Hep2/CAFs/Y27632, Hep2/CAFs/Y27632 and Hep2/CAFs/Y27632 cells demonstrated smaller and less frequently lung metastases as compared to Hep2/CAFs cells. B. Pulmonary tissue and nodules were quantified by H&E staining (*$P$<0.05).

# Supplementary Files

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