Csk / Src / EGFR Signaling regulates migration of myofibroblasts and alveolarization

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Abstract

Bronchopulmonary dysplasia (BPD) is characterized by premature alveolar developmental arrest. Antenatal exposure to inflammation inhibits lung morphogenesis, thus increasing the risk of developing BPD. Alveolar myofibroblasts are thought to migrate into the septal tips and elongate secondary septa during alveolarization. Here we found lipopolysaccharide (LPS) disrupted the directional migration of myofibroblasts and increased actin stress fiber expression and focal adhesion formation. In addition, Csk activity was downregulated in myofibroblasts treated with LPS, while activation of Src or EGFR was upregulated by LPS treatment. Specifically, decreased Csk activity and increased activation of Src or EGFR were also observed in primary myofibroblasts isolated from newborn rat lungs with intra-amniotic LPS exposure, a model for BPD. Further investigation revealed that EGFR was involved in cell migration impairment induced by LPS, and Src inhibition blocked LPS-induced activation of EGFR or cell migration impairment. Csk silencing also resulted in EGFR activation and cell migration impairment. Besides, we found the effect of EGFR on myofibroblast migration was mediated through RhoA activation. EGFR inhibition alleviated the abnormal localization of myofibroblasts and improved alveolar development in antenatal LPS-treated rats. Taken together, our data suggest that Csk / Src / EGFR signaling pathway is critically involved in regulating directional migration of myofibroblasts and may contribute to arrested alveolar development in BPD.

Keywords: bronchopulmonary dysplasia; Csk / Src; EGFR; cell migration; myofibroblasts
Introduction

Bronchopulmonary dysplasia (BPD) is characterized by premature alveolar developmental arrest, resulting in fewer and larger alveoli. Antenatal exposure to bacterial infections and inflammation may inhibit lung morphogenesis, thus increasing the risk of developing BPD (22, 44). Better understanding of how inflammation disrupts lung development may provide insight into BPD pathogenesis and therapies.

During alveolar development, the growth of secondary septa leads to saccule subdivision and enlargement of the respiratory space. Alveolar myofibroblasts are found at the tips of secondary septa and are associated with extracellular collagen and elastic fiber deposition (36, 40). Complete loss of myofibroblasts resulted in lack of secondary septa, leading to defects in alveolarization at birth (5). We initially speculated that lipopolysaccharide (LPS) may reduce the number of myofibroblasts and cause developmental defects of secondary septa observed in BPD. However, contradictory to that hypothesis, the number of myofibroblasts was increased in lungs of LPS-treated rats, consistent with lung pathology of infants with BPD (50). Indeed, the topographical localization of myofibroblasts in alveolar septa is crucial to their specific functions (5). Previous study demonstrated that LPS led to abnormal localization of myofibroblasts in the lung of newborn rats, mostly in primary septa with only a few in secondary septa (30). In addition, alveolar myofibroblasts, which are contractile cells and express alpha-smooth muscle actin (α-SMA), are thought to migrate into the crests and elongate secondary septa during alveolarization (40, 55). Therefore, it seems conceivable that LPS may disrupt the directed migration of alveolar myofibroblasts, preventing correct localization of the cells and their ability to promote alveolar septation and maturation. The details of how the cells fail to migrate into the correct location remains to be elucidated.

Reorganization of the actin cytoskeleton is both essential and central for cell migration (17). The epidermal growth factor receptor (EGFR) is a key regulator of the actin cytoskeleton and cell migration in various cell types. In addition to being activated by its ligands such as EGF and transforming growth factor (TGF)-α, EGFR has also been shown to act as a mediator of diverse signaling systems. Studies show that EGFR signaling guides dorsal migration of border cells during oogenesis and regulates directed migration of breast cancer cells (10, 41). In particular, EGFR has been implicated in lung development and shown
to be elevated in lungs of infants with BPD (14). It is also recognized that EGFR signaling is
downstream of proinflammatory pathways, reactive oxygen species and hyperoxia, which are
believed to be major contributors to the etiology of BPD (25, 39). Similarly, we also found
that LPS induced activation of EGFR in cultured myofibroblasts (30). Nonetheless, it remains
unclear how over-activation of EGFR contributes to the BPD phenotype.

Several studies have suggested that C-terminal Src kinase (Csk), which is a negative
regulator of c-Src (Src), plays a role during cell motility. For example, Csk mediates G protein
signals for actin cytoskeletal reorganization and Csk-deficiency blocked cell migration
induced by various stimuli (32, 34). Timpson et al (49) reported that Src acted in concert with
the Rho GTPases to regulate cell polarity and migration. Intriguingly, a complex relationship
exists between c-Src and EGFR. c-Src can be activated by the receptor tyrosine kinases EGFR
while EGFR can also be phosphorylated by Src at several phosphorylation sites (2, 18).
Furthermore, Src is required for EGFR transactivation by cytokines and other stimuli (1, 19).
In this signaling cascade, membrane-bound EGFR proligands are cleaved by proteases, such
as tumor necrosis factor-α-converting enzyme (TACE), and bind to EGFR, enabling them to
activate downstream signaling pathway (46).

In the present study, we report that LPS induces EGFR activation through Csk / Src,
leads to RhoA / Rho-kinase activation and disrupts directional migration of myofibroblast.
Furthermore, EGFR inhibition partially restored alveolar development impaired by LPS,
indicating that this signaling pathway may serve as a key pathophysiological mechanism of
BPD.
Materials and methods

Experimental animals. Timed-pregnancy Sprague-Dawley (SD) rats were provided by Shanghai Laboratory Animal Center (SLAC) and the experimental protocol was approved by the ethics committee of Xinhua Hospital, Shanghai Jiaotong University School of Medicine.

Reagents and antibodies. LPS (Escherichia coli 055:B5), rhTGF-α, AG1478, Y27632 dihydrochloride, DNase I and anti-α-SMA were from Sigma-Aldrich (St. Louis, MO). anti-phospho-Src (Tyr 416), anti-Src, anti-phospho-EGFR (Tyr 845) and anti-EGFR antibodies were purchased from Cell Signaling Technology (Beverly, MA). Other reagents used were: anti-RhoA mAb and Acti-stain 488 phalloidin (Cytoskeleton, Denver, CO), erlotinib (Santa Cruz Biotechnology, Santa Cruz, CA), PP1 (Calbiochem, Billerica, MA), anti-GM130 antibody and anti-Paxillin antibody (Abcam, Cambridge, MA) and anti-Csk (Epitomics, Burlingame, CA).

Drug administration in vivo. LPS and erlotinib were injected into the amniotic sac in accordance with previous methods (30). Briefly, LPS (1μg, 0.2μg/μl) with or without erlotinib (50μg) was injected into the amniotic sac of pregnant rats at 16.5 days post coitum (E16.5). Rats injected with saline (sterile, endotoxin-free) were used as control. The day pups were born was counted as postnatal day (P0) and newborn rats were euthanized at P7 for further investigation.

Lung histology. Left upper lobes were formaldehyde-fixed, and paraffin-embedded. Serial 5mm-thick sections were stained with hematoxylin and eosin (H&E) or immunostained with α-SMA for myofibroblast identification. Images were captured from randomly chosen non-overlapping fields (including similar representations of large conducting airways, small airways, and distal lung parenchyma) using an inverted microscope (Leica DMI-3000B). For morphometric analysis, five random fields per pup and 3 pups per experimental group were analyzed. Terminal airspace, secondary septa and myofibroblasts were counted manually in each field. Mean linear intercept (MLI) was determined by superimposing a predetermined grid on the image, with set randomly placed lines totaling 1 mm in actual length, and the number of times the lines cross an air-tissue interface counted. The actual MLI was calculated as the inverse of the number of air-tissue interfaces per millimeter (1,000 μm) and used to estimate mean distal airspace size.
Lung myofibroblast isolation. Rat lung myofibroblasts were isolated as described elsewhere (16). Briefly, Newborn rats with intra-amniotic LPS exposure were euthanized at P7, myofibroblasts were obtained by enzymatic digestion of finely divided lung tissue obtained after vascular perfusion with PBS and bronchoalveolar lavage. Tissue fragments were digested in DMEM containing 1 mg/ml collagenase I, 2.5 mg/ml trypsin, and 2 mg/ml DNase I at 37°C in 5% CO₂. Liberated cells were filtered through sterile gauze, centrifuged 400 × g for 10 min to pellet cells, and resuspended in 10% FBS-DMEM. The cells were plated at ~1 × 10⁵/cm², cultured for 24h, and then collected for Western blot analysis. The expression of α-SMA was analyzed by immunofluorescence for identification of myofibroblasts.

Cell culture. Human lung fibroblasts MRC-5 cells were purchased from Cell Bank, Chinese Academy of Sciences. Cells were cultured in minimum essential medium (MEM), supplemented with heat-inactivated 10% fetal bovine serum (GIBCO, Gaithersburg, MD) at 37°C with 5% CO₂ in air. For inducing myofibroblasts, cells were counted after trypsinization and passaged at 10³ cells/ml (33). Myofibroblasts were identified and distinguished from fibroblasts by expression of α-SMA. Cell culture population consisted of nearly 80% myofibroblasts, 4-5 days after seeding.

Transient transfection. Csk shRNA (Genechem, Shanghai, China) or a plasmid encoding a dominant negative mutant c-Src (K295R/Y527F) (gift from Joan Brugge & Peter Howley, Addgene plasmid #13657) were transiently transfected into myofibroblasts using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were serum starved and used for further experiments.

Transwell migration assay. Cells (3×10⁴) were resuspended in 100µl MEM containing 0.5% FBS with indicated drugs, then seeded into the upper chamber of each insert (8 µm pore size, Corning, NY), while 600µl MEM containing 10% FBS was added into the lower chamber. After incubation for 24h, Cells in the top compartment were removed with a cotton swab, and the cells in the lower compartment were fixed and stained with crystal violet (Beyotime, Shanghai). The number of cells that had migrated through the pores was quantified by manually counting five non-overlapping independent fields.

Golgi Polarization assay. The polarization of Golgi apparatus was assessed as described
previously (4, 37). Briefly, cells were grown onto glass coverslips in 24-well plates, cultured
to reach confluency and serum starved overnight. Then cells were wounded with a pipette tip
and incubated in fresh MEM containing 10% FBS with indicated drugs for 6h before cell
immunofluorescence was performed. Golgi apparatus was stained using anti-GM130 antibody
and cells on the wound edge were counted as polarized when the majority of the stained Golgi
were orientated within a 120° angle facing the wound. Golgi angle was also calculated as
described elsewhere (3). The centers of mass of the nucleus and Golgi apparatus fluorescence
were measured sequentially in Image-pro Plus and used to determine coordinates of the
nucleus-to-Golgi vector. Golgi angle is calculated according to the formula provide by Blaine
et al (3). A minimum of 100 cells were counted per treatment per experiment.

**Cell morphology.** Myofibroblasts were grown onto glass coverslips in 24-well plates,
cultured to reach semi-confluency and serum starved overnight. Then cells were incubated in
fresh MEM containing 10% FBS with indicated drugs for 2h before cell immunofluorescence
was performed. After fixation, cells were blocked, incubated overnight with anti-paxillin
rabbit polyclonal (1:100), then incubated with the Alexa Fluor-555 conjugated secondary
antibodies for 1h followed by incubation with Acti-stain 488 phalloidin (100 nM).

**RhoA pull-down assay and Csk activity assay.** To assess RhoA activation, RhoA pull-down
assays were performed to isolate the active GTP-bound form using RhoA activation assay kits
(Cytoskeleton, Denver, CO) following the manufacturer’s protocol. To assess Csk activity,
myofibroblasts were collected and equal amounts of protein extracts were subjected to Csk
activity assay with a spectrophotometric determination kit (Genmed, Shanghai, China). Csk
activity was calculated according to a formula provided by the manufacturer.

**TACE activity assay.** Cultured myofibroblasts were analyzed for TACE activity using
SensoLyte520 TACE Activity Assay Kit (AnaSpec Inc. CA), following the manufacturer’s
instructions. Enzymatic activity was measured using a fluorescence resonance energy transfer
peptide containing the fluorescent probe 5-FAM quenched by QXL520. TACE cleaves the
peptide and releases 5-FAM, leading to increased fluorescence. Active TACE cleaves FRET
substrate into two separate fragments resulting in an increase of 5-FAM fluorescence which
can be monitored at excitation/emission = 490 nm/520 nm.

**Immunostaining.** For immunohistochemistry, paraffin-embedded lung sections were
dewaxed and rehydrated before antigen recovery was performed. Then sections were blocked with 10% goat serum for 1h at room temperature, followed by incubation overnight at 4°C with anti-SMA (1:200). After rinsing in PBS, sections were incubated with fluorescently conjugated secondary antibody (1:1,000, Beyotime, shanghai, china) for 1h. Nuclei were stained with 4’6-diamidino-2-phenylindole (DAPI, Beyotime, shanghai, china). For immunocytochemistry, cells were fixed with 4% paraformaldehyde and 0.3% Triton-100 in PBS. After that, they were blocked, overnight incubated with anti-GM130 (1:200), followed by incubation with secondary antibody for 1h.

**Western blot analysis.** To obtain whole cell protein extract, cells were washed, scraped and lysed. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with T-TBS containing 5% nonfat milk and probed with indicated primary antibodies (anti-EGFR: 1:1,000; anti-phospho-EGFR (Tyr 845): 1:500; anti-Src: 1:1,000; anti-phospho-Src (Tyr 416): 1:500; anti-Csk: 1:1,000). The membranes were rinsed and then incubated for 2h with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Chemiluminescence performed with the ECL kit (Millipore, Billerica, MA) was detected using Bio-Rad Chemi DocTM XRS+ imaging system (Hercules, CA) and densitometric analysis was conducted by Imagelab 2.0 software.

**Statistical analysis.** Results were given as mean ± SEM. Student’s t-test and one-way ANOVA analysis were performed to analyze the statistical significance using the software SPSS 18.0. Cumulative distributions of Golgi angle data were analyzed with Kolmogorov-Smirnov non-parametric tests. Significance is defined as $P<0.05$. 
Results

LPS disrupted directional migration of myofibroblasts

To investigate the effect of LPS on directional migration of myofibroblasts, Golgi polarization assays and transwell migration assays were applied. No obvious wound closure was observed after 6 hours following wounding in each group of Golgi polarization assays. Cultured myofibroblasts treated with LPS exhibited significantly reduced Golgi reorientation toward the wound compared with PBS treatment (Fig. 1B). Consistent with the Golgi polarization assay, LPS substantially inhibited the migratory capacity of myofibroblasts, as indicated by a marked decrease in the number of migrated cells (Fig. 1C). These data suggest LPS interrupted directional migration of myofibroblasts. As cell morphology and dynamics are important in cell migration, we further observed the effect of LPS on cell cytoskeleton and focal contacts. Results show that myofibroblasts treated with LPS exhibited a marked increase of actin stress fiber expression and focal adhesion formation (identified by paxillin staining) (Fig. 1D).

Csk, Src and EGFR activities were altered by LPS.

To delineate the mechanisms of defective myofibroblast migration after LPS treatment, we examined Csk activity and activation of Src or EGFR. Compared with PBS group, Csk activity was decreased in myofibroblasts treated with LPS after 30 min (Fig. 2A). By contrast, phosphorylation levels of Src (Tyr 416) and EGFR (Tyr 845) were significantly elevated by LPS treatment, suggesting increased activation of Src and EGFR (Fig. 2B). Significant changes were observed after 30 min of LPS exposure. Moreover, similar results were also observed in myofibroblasts isolated from antenatal LPS-treated rat lungs. Although their expression demonstrated no significant difference, Csk activity was decreased after LPS exposure while Src and EGFR activation were increased (Fig. 2C, D). These results imply that LPS-induced impairment of myofibroblast migration may be mediated through Csk / Src and EGFR.

Inhibition of EGFR activation enhanced directional migration of myofibroblast.

In accordance with our earlier studies (30), LPS induced EGFR activation in myofibroblasts. As TGF-α is a ligand of EGFR, we applied exogenous rhTGF-α in a bath to mimic the effects of LPS. With additional application of rhTGF-α, myofibroblasts also
exhibited significantly reduced directional migration (Fig. 3A). Similar to myofibroblasts treated with LPS, cells treated with rhTGF-α also exhibited significantly increase of actin stress fibers and focal adhesions (Fig. 3B). To further test the role of EGFR, cells were treated with the EGFR-specific inhibitor AG1478. As shown in Fig. 3C, AG1478 rescued the myofibroblast migration deficiency induced by LPS treatment. Similarly, the inhibitor also abolished the effects of rhTGF-α on cell migration (Fig. 3D). However, AG1478 treatment had no marked effects on increased actin stress fibers and focal adhesions induced by LPS or rhTGF-α (data not shown). This phenomenon may occur due to diverse signaling pathways controlling cell polarization and cytoskeleton reorganization (15).

Csk / Src act upstream of the EGFR.

We next asked whether the Src activation observed in LPS-stimulated cells is upstream or downstream of EGFR. Results show the EGFR inhibitor AG1478 failed to prevent Src activation in myofibroblasts induced by LPS (Fig. 4A). However, the Src inhibitor PP1 prevented EGFR phosphorylation induced by LPS (Fig. 4B, C). PP1 also attenuated the impaired directional migration caused by LPS (Fig. 4E). To further confirm the role of Src, a plasmid encoding dominant negative Src (DN-Src) was applied. Expression of DN-Src in myofibroblasts abrogated LPS-induced EGFR phosphorylation (Fig. 4D) and migration impairment (Fig. 4F), suggesting Src was required for LPS-induced EGFR transactivation in regulating cell migration. As Csk is a negative regulator of Src, we next examined the role of Csk in LPS-induced EGFR activation. Silencing of Csk by shRNA significantly up-regulated EGFR phosphorylation (Fig. 4G, H) and also reduced directional migration of myofibroblast (Fig. 4I). Taken together, these data imply that Csk / Src act upstream of EGFR. To further explore the possible mechanism of Src-mediated EGFR transactivation, we focused on TACE because it has been documented to be involved in Src-mediated EGFR transactivation (46). Results show that LPS stimulated a twofold increase of TACE activity, which was blocked by the Src inhibitor PP1 (Fig. 4J).

The effect of EGFR on myofibroblast migration was mediated through RhoA activation.

Considering the established role of RhoA in cell migration (12), we investigated the contribution of RhoA to cell migration in our cell system. RhoA activation was detected using a pull-down assay that specifically recognizes the active, GTP-bound form. As shown in Fig.
5A, LPS treatment increased RhoA activation. Similarly, rhTGF-α also stimulated RhoA activation in cultured myofibroblasts (Fig. 5B). In addition, EGFR-antagonism with AG1478 blocked LPS- or TGF-α-induced RhoA activation (Fig. 5A, B). Furthermore, LPS or TGF-α treated cells showed a significant reduction in directional migration, which was restored by the Rho-associated kinase (ROCK) inhibitor Y-27632 (Fig. 5C, D). Interestingly, we also found that treatment with Y-27632 alone could enhance cell migration. Altogether, these results indicate that RhoA is a downstream effector of EGFR-mediated cell migration.

**Inhibition of EGFR prevented abnormal localization of myofibroblasts and improved alveolar development in antenatal LPS-treated rats.**

We further tested whether EGFR inhibition with erlotinib could rescue alveolar development following LPS exposure. LPS with or without erlotinib was injected into the amniotic sac of pregnant rats at E16.5. No fetal death occurred in the control group, and almost all rats survived to the end of the experiment. Pups treated with LPS showed a survival ratio of 80% of the fetal rats. Most fatalities happened before birth. Lung tissue samples from LPS-injected group displayed alveolar simplification, as indicated by enlarged alveoli with decreased terminal airspace, decreased secondary septa and increased mean linear intercept (MLI). Compared with LPS treatment alone, co-treatment with erlotinib and LPS increased terminal airspace and secondary septa counts while decreased MLI (Fig. 6A). We next examined the effect of erlotinib on LPS-induced abnormal localization of myofibroblasts. For quantitative analyses, lung alveolar architecture was divided into three parts: 1) primary septa (1), 2) junction of secondary septa and primary septa (2) and 3) secondary septa (3) (Fig. 6C). Compared with the saline group, LPS treatment resulted in fewer myofibroblasts in secondary septa and more accumulation in the primary septa, while treatment with erlotinib effectively alleviated the abnormal localization of myofibroblasts at P7 (Fig. 6B). Our results indicate that alveolar developmental arrest induced by LPS is partially rescued by erlotinib.
Discussion

Human lung development is a complex process, which is characterized by five stages of development based on histological appearance, including: embryonic, pseudoglandular, canicular, saccular and alveolar stages. Lung development in BPD appears to go awry during the saccular stage of development. Exposure of the saccular-stage lung to bacteria has been shown to increase the risk of developing BPD and may therefore inhibit lung morphogenesis (6). Several studies have reported the relationship between antenatal LPS administration and postnatal lung structural changes in animal models (6, 45, 51). In previous study (30), we examined the effects of LPS exposure on prenatal lung development. Intra-amniotic injection of LPS was administered to pregnant SD rats at E16.5, a transitional period from the canalicular to saccular stage. Results show that less alveoli and runtish secondary septa, a phenotype similar to BPD, were observed in LPS-treated newborn rats.

The definitive alveoli are established during lung development by the outgrowth of secondary septa from the primary septa present in newborns. At the beginning of alveolarization, small ridges appear along the primary septa. These ridges increase in height to become the secondary septa which subdivide the air spaces into smaller units, the alveoli. The growth of secondary septa leads to saccule subdivision and enlargement of the gas exchange area (30, 36, 40). Although the role of myofibroblasts during alveolarization is not completely understood, it is possible that they migrate into the septal tips from the base of the septa or primary septa, where they secrete the precursor of elastin and later drive alveolar septation (5, 36, 55). On the other hand, myofibroblasts have been identified to play an important role in remodeling after inflammation or injury, including cutaneous wound repair and liver fibrosis. During these processes, the directed migration of myofibroblasts leads to their accumulation at the wound site (28, 29). Here, we found that LPS disrupted the directional migration of myofibroblasts, consistent with previous report (30). Thus we speculate that LPS may disrupt directional migration of myofibroblasts, leading to abnormal localization of myofibroblasts and decreased secondary septa formation. Further studies are necessary to understand the molecular mechanisms underlying the signaling cascade induced by LPS.

Erlotinib, an EGFR tyrosine kinase inhibitor, has been widely used to treat non-small cell lung cancer (21). Not limited to cancer cells, studies have also shown beneficial effects of
erlotinib in animals with abdominal aortic aneurysm or pulmonary hypertension (8, 38). Our recent studies revealed the involvement of EGFR in LPS-induced defective myofibroblast migration (30). In the current study, we have extended this observation and demonstrated that erlotinib administration partially restored myofibroblast localization in lungs and improved alveolar development in antenatal LPS-treated rats, suggesting that EGFR signaling is important in maintaining the stability of myofibroblast polarized migration and may contribute to arrested alveolar development in BPD. In addition, TGF-α and EGFR are also widely expressed on other cells and have been reported to be involved in several aspects of BPD development, such as inflammation and disrupted vascular development (26, 27). Therefore, it is possible that erlotinib improves lung development following antenatal LPS exposure through other mechanisms besides influencing myofibroblast migration. In this study, we focus on the role of EGFR in regulating directional migration of myofibroblasts and try to elucidate how EGFR regulates this process.

Activation of the EGFR, either through interaction with its ligands or via a ligand-independent process, results in dimerization and phosphorylation of tyrosine residues (10). In previous study, we reported LPS increased TGF-α expression and activated EGFR (30). Here we found another signaling pathway was also important in LPS-induced EGFR activation. Src is a multifunctional protein involved in the regulation of a variety of normal processes, including proliferation, differentiation, migration and adhesion (19, 47). To carry out these functions, Src interacts with numerous cellular factors, including cell surface receptors such as EGFR. EGFR can be phosphorylated at multiple sites by Src, most notably Tyr 845, suggesting that Src can modulate lateral activation of the EGFR by extracellular stimuli (24, 48). Indeed, multiple extracellular factors can transactivate the EGFR. In many cases, this transactivation requires Src. For instance, c-Src is necessary for integrins to associate with and transactivate the EGFR (19, 35). Conversely, Src has also been shown to act downstream of EGFR activation (18). The relationship between Src and EGFR is likely to vary depending on the stimulus. In this work, we assessed whether EGFR was activated upstream of Src activation by LPS treatment or the other way around. Our results revealed that inhibition of Src activity significantly blocked LPS-induced transactivation of EGFR and increased the directional migration of myofibroblasts. As Csk is an important negative
regulator of Src, we also demonstrated that Csk silencing induced EGFR activation in myofibroblasts, further confirming the role of Csk / Src in LPS-induced EGFR transactivation. Besides phosphorylating EGFR directly to regulate the receptor function (2), Src has also been reported to influence EGFR signaling in several aspects. For example, Src may interact with the endosomal machinery and influences the endosomal recycling or degradation of EGFR (9, 23). In this study, we observed the cell-surface metalloproteinase TACE activity is regulated by Src following LPS treatment. As TACE has been implicated as a sheddase for EGFR ligands, TACE activation cleave membrane bound EGFR proligands, such as EGF or TGF-α, enabling them to bind to EGFR and activate downstream kinases (46). Therefore, Src may regulate EGFR activation in a direct or indirect way.

Cell migration is a highly integrated, multi-step process, including formation of stable adhesions at the leading edge, reorientation of the Golgi toward the leading edge, translocation of the cell body in the direction of cell movement and focal adhesion (FA) release and retraction at the trailing edge (53). In this process, the Rho family of small GTPases is a widely known pivotal regulator controlling cytoskeleton remodeling and FA formation as well as turnover. Among the family members, RhoA regulates assembly of actin stress fibers and associated focal adhesions through activation of its down-stream effectors, the ROCKs (42, 54). In the current study, we observed a marked increase in focal adhesions and actin stress fibers in myofibroblasts following LPS or rhTGF-α treatment. Activation of RhoA appears to impair myofibroblast migration partly through increasing stress fiber-dependent adhesions to the substrate (7, 52). For migrated cells, vesicles are transported to the leading edge and new membranes are formed continually to complete migration. Thus, it is important to polarize Golgi toward the leading edge (20). The ROCK inhibitor Y27632 abolished the decreased Golgi orientation caused by LPS treatment, possibly due to Cdc42 activation following RhoA inhibition as RhoA has been reported to inhibit cell polarization through decreased Cdc42 activity (12). Activation of Cdc42 induces the reorganization of the actin and microtubule cytoskeletons, which causes the nucleus to move to the back of the cell and the microtubule organizing center, together with the Golgi, to orient facing the leading edge (11, 13). Interestingly, we also observed that cell migration was significantly increased by Y27632 treatment alone, which was in agreement with other studies (43). However,
several studies have shown that ROCK inhibition reduces cytoskeleton assembly and focal adhesion, resulting in loss of cell motility (31). Such variations may be one of the reasons for cell type-specific responses to the ROCK inhibitors in cell migration. The underlying mechanisms, however, remain unclear. This may be due to varied roles of RhoA / ROCK in different processes of cell migration.

In summary, our results establish a new molecular mechanism pertaining to disrupted lung morphogenesis following intrauterine inflammation. We demonstrate that the Csk / Src / EGFR signaling pathway is critically involved in regulating directional migration of alveolar myofibroblasts and contributes to arrested alveolar development as observed in BPD. Most importantly, the EGFR inhibitor erlotinib provides therapeutic benefit in LPS-injected rats. These results suggest that this pathway may be a promising target for BPD treatment.
Acknowledgments

We are grateful to Dr. Craig Bolte (Department of Pediatrics, Cincinnati Children’s Hospital Research Foundation, Cincinnati, Ohio) for critical revision of the manuscript.

Grants

This work was supported by National Natural Science Foundation of China (grant No. 81470201 and No. 81270729 to Y. Zhang), and the Natural Science Foundation of Shanghai (No.12ZR1419600)

Disclosures

No conflicts of interest, financial or otherwise are declared by the author(s).

Author contributions

J.L., Y. L., H.H., C.L. and W.L. performed experiments; J.L., Y.L. H.H. and L.X. analyzed data; J.L., Y.L. and H.H. interpreted results of experiments; J.L., Y.L. and H.H. prepared figures; Y.L. drafted the manuscript; Y.Z. conception and design of research; Y.Z. edited and revised the manuscript; Y.Z. approved the final version of the manuscript.
References


**Figure legends**

**Figure 1. Impaired directional migration of myofibroblasts by LPS treatment.** (A) Diagram shows quantification method for Golgi apparatus polarization. Golgi stained with GM130 antibody is shown in red and nuclei are stained with DAPI (blue). Golgi was counted as polarized (+) if the majority lay within a 120° angle facing the wound edge. (B) Cultured myofibroblasts were seeded on glass coverslips, serum starved, wounded and incubated with LPS (1μg/ml) for 6h before Golgi polarization was determined. Golgi angle were also calculated to assess Golgi orientation. Representative images were presented with quantification analysis. Bars, 20μm. (C) Cultured myofibroblasts (3×10^4 cells) resuspended in medium containing 0.5% FBS with LPS (1μg/ml) were added into the upper chamber of transwell and allowed to migrate toward 10% FBS for 24h. Then cells were fixed and stained with crystal violet. Representative images were presented with quantification analysis. Bars, 100μm. (D) Cultured myofibroblasts were immunostained for actin and paxillin following LPS treatment for 2h. Representative pictures were presented. Data are shown as mean ± SEM. *p < 0.05, each experiment was repeated at least three times.

**Figure 2. Effect of LPS on the activation of Csk, Src and EGFR.** (A) Cultured myofibroblasts were serum starved and treated with LPS (1μg/ml) for indicated times before Csk activity was examined by a spectrophotometric-based kit. Results were normalized to PBS group. (B) Cultured myofibroblasts treated with LPS (1μg/ml) were harvested and protein was utilized for Western blot analysis of indicated molecules. The activation of EGFR or Src was determined by the ratio of phospho-EGFR (Tyr 845) or phospho-Src (Tyr 416) vs total protein expressions respectively. (C) Activation of EGFR and Src together with expressions of Csk, Src and EGFR in primary myofibroblasts isolated from antenatal LPS-treated rat lungs were investigated. Results were normalized to Saline group. (D) Csk activity in primary lung myofibroblasts was analyzed and expressed per microgram of the total cellular protein. Data are shown as mean ± SEM. *p < 0.05, each experiment was repeated at least three times.

**Figure 3. Disruption of directional migration by EGFR activation.** (A) Myofibroblasts were treated with rhTGF-α (1ng/ml) for 6h before Golgi polarization was determined and myofibroblasts were allowed to migrate for 24h in the presence of rhTGF-α prior to
evaluation of cell migration. Representative images were shown with quantification analysis. Scale bars in upper panels indicate 20μm and in lower panels indicate 100μm. (B) Cultured myofibroblasts were immunostained for actin and paxillin following rhTGF-α treatment for 2h. Representative images were shown. (C, D) Effect of EGFR-blockade with AG1478 (1μm) on directional migration impairment of myofibroblasts induced by LPS (C) or rhTGF-α (D) treatment was assessed. Data are shown as mean ± SEM. *p < 0.05, each experiment was repeated at least three times.

Figure 4. Role of Csk/Src in EGFR activation and directional migration impairment. (A) Myofibroblasts were pretreated with the EGFR inhibitor AG1478 (1μm) before treatment with LPS for 30min and Src phosphorylation was assessed. (B, C) Myofibroblasts were pretreated with the Src inhibitor PP1 (1μm) before treatment with LPS for 30min and phosphorylation of Src (B) or EGFR (C) was analyzed. (D) Myofibroblasts were transfected with dominant negative Src (DN-Src) plasmid or control vector before treatment with LPS for 30min and EGFR phosphorylation was examined. (E) Myofibroblasts were treated with LPS in the presence or absence of PP1 before Golgi polarization was determined and migration of cells in the presence of LPS with or without PP1 was assessed. Representative images were shown with quantification analysis. Scale bars in upper panels indicate 20μm and in lower panels indicate 100μm. (F) The effect of LPS on directional migration of DN-Src transfected myofibroblasts was assessed. (G) Expression of Csk in myofibroblasts transfected with Csk shRNA (1.6 μg/ml) was analyzed by western blotting. (H) Myofibroblasts were transfected with Csk shRNA or control shRNA and allowed to recover for 24h before EGFR phosphorylation was examined. Cell transfection only with lipofectamine 2000 was set up as a mock control. (I) Golgi polarization and cell migration were assessed after myofibroblasts were transfected with Csk shRNA. (J) Myofibroblasts were pretreated with the Src inhibitor PP1 (1μm) before treatment with LPS for 30min and TACE activity was examined using SensoLyte520 TACE Activity Assay Kit. Data are shown as mean ± SEM. *p < 0.05, each experiment was repeated at least three times.

Figure 5. Involvement of RhoA in impaired directional migration. Myofibroblasts were pretreated with AG1478 (1μm) before stimulation by LPS (1μg/ml) (A) or rhTGF-α (1ng/ml) (B) and RhoA activation was evaluated. The effect of RhoA-blockade with Y27632 (10μm)
on directional migration impairment induced by LPS (C) or rhTGF-α (D) was assessed. Representative images were shown with quantification analysis. Scale bars in upper panels indicate 20μm and in lower panels indicate 100μm. Data are shown as mean ± SEM. *p < 0.05, each experiment was repeated at least three times.

**Figure 6. Effect of EGFR-blockade with erlotinib on lung morphology.** LPS (1μg) with or without erlotinib (50μg) was injected into the amniotic sac of timed-pregnancy rats (E16.5). Lungs of surviving pups were removed and fixed at P7. (A) Representative lung sections stained with H&E were shown with quantification of terminal airspace, secondary septa and mean linear intercept (MLI). Bars, 100μm. (B) Immunofluorescence images show the location of myofibroblasts (α-SMA-positive stained cells). Arrows indicate the presence of myofibroblasts at the tips of secondary septa and arrowheads show myofibroblasts in the primary septa. Myofibroblasts were counted for quantitative analysis. Bars, 50μm. (C) Diagram depicting secondary septa (1), junction of secondary septa and primary septa (2) and primary septa (3). Data are shown as mean ± SEM. * p < 0.05, each experiment was repeated at least three times.