Effects of c-Src kinase on lens diseases associated with EMT of human lens epithelial cells

CURRENT STATUS: ACCEPTED

Xing-Yu Li
Xijing Hospital, Fourth Military Medical University

Fang Wang
Xijing Hospital, Fourth Military Medical University

Mei-Xia Ren
Xijing Hospital, Fourth Military Medical University

Min-Juan Du
Xijing Hospital, Fourth Military Medical University

Jian Zhou
Xijing Hospital, Fourth Military Medical University

zhoujian@fmmu.edu.cn Corresponding Author

DOI: 10.21203/rs.2.9375/v1

SUBJECT AREAS
  Internal Medicine Specialties

KEYWORDS
  c-Src kinase, lens epithelial cells, epithelial-mesenchymal transition, cataract, fibrosis
Abstract

Background
c-Src kinase regulated the signaling pathway of epithelial to mesenchymal transition (EMT) in many cells. The purpose of this study was to investigate the effects of c-Src kinase on EMT of human lens epithelial cells in vivo stimulated by different factors.

Methods

Human lens epithelial cells, HLE-B3 were exposure to inflammatory factors including IL-1α, IL-6, TNF-α, and IL-1β at 10 ng/mL, and high glucose (35.5 mM) respectively, for 30 mins. Activity of c-Src kinase was evaluated by expression of p-Src418 with western blot assay. To investigate activation of c-Src on EMT, HLE-B3 cells were transfected with pCDNA3.1-SrcY530F to up regulate c-Src, and pSilence4.1-ShSrc to knock down it. The expressions of c-Src kinase and molecular markers of EMT such as E-cadherin, ZO-1, α-SMA and vimentine were examined at 48 hours by RT-PCR and western blot. At 48 hours and 72 hours of transfection, cell proliferation was detected by MTT, cell mobility and migration were determined by scratch and transwell assay.

Results

Activity of c-Src kinase, expression of p-Src418 was upregulated by different inflammatory factors and high glucose in HLE-B3 cells. When HLE-B3 cells were transfected with pCDNA3.1-SrcY530F, the expression of c-Src kinase was upregulated on both mRNA and protein level, and activity of c-Src, p-Src418 increased. The expressions of E-cadherin and ZO-1 suppressed, while the expressions of vimentin and αSMA elevated on both mRNA and protein level at the same time. Cell proliferation, mobility and migration increased along with activation of c-Src kinase. Conversely, when HLE-B3 cells were transfected with pSilence4.1-ShSrc, both c-Src kinase and p-Src418 were knocked down. The expressions of E-cadherin and ZO-1 increased, but the expressions of vimentin and α-SMA decreased, meanwhile cell proliferation, mobility and migration reduced.

Conclusions
c-Src kinase in lens epithelial cells is easily activated by external stimuli, resulting in inducing of cell proliferation, mobility, migration and EMT.
Background
Previous studies have shown that lens fibrotic disorders, such as anterior subcapsular cataract (ASC) and posterior capsular opacification (PCO), are common types of cataract and visual impairment. ASC is a primary cataract, which is characterized by dense fibrotic regions underneath the anterior capsule and is mainly caused by inflammation, ocular trauma and irritation [1]. PCO is known as secondary cataract, occurs in 30 % to 50 % of adults and almost 100 % of children who receive cataract surgery [2], which is associated with fibrosis and contraction of posterior lens capsule [2-4]. ASC and PCO share many molecular features such as aberrant proliferation, migration and epithelial to mesenchymal transition (EMT) of lens epithelial cells (LECs) [5]. Accumulating evidences have shown that anti-inflammation after cataract surgery could reduce migration and fibrosis of epithelial cells [6-8]. It has been reported that fibrosis of epithelial cell in diabetes mellitus was significantly higher than those without diabetes at 6 and 12 months after cataract extraction [9]. These evidences suggest that inflammatory factors and high glucose are the stimulating factors, which have induced the fibrosis of LECs.

EMT is associated with many molecular and morphologic changes to epithelial cells that enable them to lose their cell polarity and cell-cell adhesion, gain properties in migration and invasion, and become mesenchymal cells [10, 11]. The most marked characteristics of EMT are loss of epithelial markers, such as E-cadherin and ZO-1, and acquisition of a spindle shape cell that is accompanied by accumulation of Vimentin and a-smooth muscle actin (a-SMA) [12]. This specific process is present in embryonic development, wound healing and tissue repairment, and tumor metastasis. In organ fibrosis such as renal fibrosis, pulmonary fibrosis, hepatic fibrosis and ocular fibrosis, EMT is triggered by various biomolecules and signaling pathway, such as transforming growth factor-β (TGF-β) [13], insulin-like growth factor-1 (IGF-1) [14], transcription factor snail [15] and PI3K/Akt/mTOR/NF-κB signaling [16].

c-Src, one of the Src family kinases (SFKs), is activated by many stimulators, such as epidermal growth factor receptor (EGFR) [17], P2RY2 (a purinergic GPCR receptor) and reactive oxygen species (ROS) [18], high glucose [19], heterotrimeric G protein-coupled receptors [20], PKA signaling [21] and
the pathways of IL-1 and EGFR/integrin signaling [22]. Activation of c-Src kinase is required for cell differentiation, migration and changes of intercellular junction, including cadherin-based intercellular adhesions and integrin-mediated cell-matrix adhesions of epithelial cells, particularly during EMT [23, 24]. Inhibition of SFKs with their specific inhibitors attenuates fibrosis in lung, pancreas, and skin, which suggest that activation of Src kinase is an attractive trigger point of organ fibrosis [25, 26]. In lens epithelial cells, activation of Src kinase induced by serum increased cell migration, weakened cell-cell junctions, and made lens epithelial cell acquire the phenotype of mesenchymal cells [27].

c-Src is comprised of lipophilic N-terminus, followed by the regulatory SH3 and SH2 domains, catalytic protein tyrosine kinase (PTK) core and c-terminus regulatory tail [28-30]. The PTK domain contains the kinase domain and a conserved tyrosine residue involved in autophosphorylation. Phosphorylation of the Tyr 418 residue of the PTK domain is required for maximum kinase activity [31]. A negative regulatory domain is adjacent to the PTK domain. Phosphorylated Tyr 530 interacts and binds with the SH2 domain to keep the SFK in the inactive conformation. In the other words, c-Src kinase is activated by phosphorylation at Tyr 418 or dephosphorylation at Tyr 530 [32].

In the present study we hypothesized that activation of c-Src kinase stimulated by a variety of factors, such as inflammatory factors or high glucose could be a trigger for EMT of lens epithelial cells. By transfecting HLE-B3 cells with c-Src activated vector or Sh RNA vector, the effects of c-Src kinase on cell proliferation, mobility, migration, and EMT were observed.

Methods

**Plasmid construction**

Construction of pCDNA3.1-c-Src<sub>Y530F</sub> recombination vector was described previously [33-35]. Mutant c-Src<sub>Y530F</sub> cDNA generated by RT-PCR from total mRNA of HLE-B3 cells with the primers: sense 5'-GGCAAGCTTATGGGTAGCAACAAGAGCAAGCCCAAG-3';

antisense 5'-GCTCTAGACTAGAGGTTCTCCCCGGGGCTGGAAG-3';

(underlined sequences were the mutation site) was cloned into the expression vector pCDNA3.1 (Invitrogen, Carlsbad, CA, USA), creating pCDNA3.1-c-Src<sub>Y530F</sub> recombination vector. ShRNA expression vectors were generated by annealing single-stranded oligonucleotides and inserting them
into the BamHI and HindIII enzyme sites of pSilencer4.1-CMVneo vector (Ambion, Austin, TX, USA).

The target sequences were as follows: ShSrc (c-Src, NM_005417, 1682-1700 bp): 5'-TCGGCTCATTGAAGACAAT-3' (provided by Genepharma Inc, Shanghai, China) and a scrambled sequence was used as a negative control (ShNC): 5'-TTCTCCGAACGTGTCACGT-3' (Ambion, Austin, TX, USA). The recombinant ShRNA vectors were named as pSilence4.1-ShSrc and pSilence4.1-ShNC.

Cell culture

HLE-B3 cells were grown adherently in Dulbecco's modified Eagle's medium (DMEM, with 5.5 mM glucose) with 10% fetal bovine serum and 2mM L-glutamine, and incubated at 37°C with 5% CO₂. All cells used in the experiments were taken in logarithmic phase.

Groups and treatment

Groups of stimulation with inflammatory factors: in the treatment groups, HLE-B3 cells were treated with IL-1α, IL-6, TNF-α, and IL-1β at 10 ng/mL, respectively, for 30 mins. In the control group, HLE-B3 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 0.5% fetal bovine serum for 30 mins.

Groups of stimulation with high glucose: in the control group (5.5 mM), HLE-B3 cells were cultured in DMEM with 5.5 mM glucose in the medium, in the osmotic control group (mannitol) the cultured cells were treated with 30 mM mannitol, while in high glucose group (35.5 mM), the cells were treated with 30 mM glucose for 30 mins, respectively.

Groups of c-Src kinase activation: in c-Src activation group (pCDNA3.1-c-Src<sub>Y530F</sub>), HLE-B3 cells were transfected with pCDNA3.1-c-Src<sub>Y530F</sub> recombination vector. In blank control group (pCDNA3.1), cells were transfected with blank vector, pCDNA3.1 vector, and in negative control group (control), cells were transfected with transfection reagent Lipofectamine 2000.

Groups of c-Src kinase inhibition: in c-Src silence group (ShSrc), HLE-B3 cells were transfected with pSilence4.1-ShSrc vector. In blank control group (ShNC), the cells were transfected with pSilence4.1-ShNC vector, and in negative control group (control), cells were transfected with transfection reagent Lipofectamine 2000.
**Transfection**

HLE-B3 cells (2×10^5 cells per well) were seeded in 6-well plates and grown overnight to 80% confluence prior to transfection. All transfections for plasmids were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, plasmid DNA-lipid complexes (4μg plasmids in 8μl Lipofectamine 2000 per well) were prepared, and then incubated for 20 minutes at room temperature. The DNA-lipid complexes were added to HLE-B3 cells in 6-well plates and were cultured for 4-6 hours. Lastly, DNA-lipid complexes were discarded and 2 ml complete medium were added. 400μg G418 (Invitrogen, Carlsbad, CA, USA) was applied to select neomycin-resistant cells.

**Quantitative real-time RT-PCR**

Total RNA was extracted from cells using Trizol reagent (Takara, Dalian, China), and 1 μg total RNA was used as the template for cDNA synthesis in a reverse transcription kit (Takara, Dalian, China). RT-PCR was performed in the SYBR Green kit (Takara, Dalian, China) using specific primers for c-Src, E-cadherin, ZO-1, vimentin and αSMA (Table 1). The relative expression levels of genes were normalized to the endogenous housekeeping gene GAPDH.

**Western blot**

Cells were harvested and lysis in RIRA cell lysate (20 mM Tris-HCl, 1% NP-40, 0.5 mM PMSF), and extracted for total proteins. The proteins were quantified by BCA assay kit (Beyotime Biotechnology, Beijing, China) and added with 5×loading buffer (250mM Tris-HCl (pH6.8), 10% SDS, 0.5% BPB, 50% Glycerine, 5% 2-Mercaptoethanol). 35μg total proteins from each sample were uploaded and separated in 12% SDS-PAGE at 120V voltage, then transferred onto PVDF membranes at 40 mA for 2.5 hours at room temperature. According to the standard protein marker, the PVDF membrane was cut according to the molecular weight of the target protein. After blocking by 5% defatted milk powder for 2 hours at room temperature, PVDF membrane bands were incubated with primary antibodies overnight at 4°C, such as, c-Src (1:1000), p-Src^{418} (1:500), E-Cadherin (1:200), ZO-1(1:1000), Vimentin (1:1000), α-SMA (1:1000), and GAPDH (1:2000), respectively. After washed with
TBST for three times (each for 15 mins) next day, PVDF membrane bands were incubated with secondary antibodies (1:4000) for 4 hours at 4 °C, respectively. After washed with TBST, proteins in PVDF membrane bands were detected with Western blotting luminol reagent (Santa Cruz, USA). GAPDH expression was used as the internal standard. The protein bands were observed and captured under scanner (HP Deskjet F2288, China). The images were analyzed by Quantity One software.

**Cell proliferation assay**

Cell proliferation was evaluated by MTT (Sigma, USA) assay at indicated time points [36]. HLE-B3 cells (2×10^5 cells/well) were seeded in 6-well plates. The next day, cells were transfected with pCDNA3.1-c-SrcY530F, pSilence4.1-ShSrc and control vectors. After transfection for 24 hours, cells in activation of c-Src kinase group, inhibition of c-Src kinase group and control group were trypsinized and seeded in 96-well plate (6×10^3 cells/well). At different time points (after stuck for 0, 12, 24, 48 and 72 hours), the medium was replaced with 100 μl MTT (5 mg/ml), and the plate was incubated at 37 °C for another 4 hours. After incubation, the culture medium was removed gently, and 100 μl DMSO was added. Finally, the absorbance was determined on a microreader (Bio-Rad) at 570 nm. All experiments were performed 3 times independently. The cell proliferation diagram was plotted using the absorbance at each time point.

**Scratch assay**

HLE-B3 cells (2×10^5 cells/well) were seeded in 6-well plates. On the second day, cells were transfected with pCDNA3.1-c-SrcY530F, pSilence4.1-ShSrc and control vectors. After 24 hours, cells in each well were scratched with 200μl pipette tip. Once scratch was made, the plates were gently washed with PBS for 3 times, and then added 2 ml serum-free medium. Cell mobility was examined after 24 hours and 48 hours, respectively. The images just after scratching 0 hour (T0), 24 hours (T24) and 48 hours(T48) were taken with a digital camera (Olympus DP71, Japan) connected to an inverted microscope (Olympus IX71, Japan). Ten fields of each plate were picked randomly and marked. Measurements of the width of gap were repeated 3 times at the same field. Gap closure (%) = [Gap in width (T0- T24/48)/Gap in width T0] × 100%.
**Transwell assay**

Cell migration was determined using transwell assay (Corning incorporated, NY, USA). HLE-B3 cells (2×10^5 cells/well) were seeded in 6-well plates. On the following day, cells were transfected with pCDNA3.1-c-Src^{Y530F}, pSlience4.1-ShSrc vector and control vectors. After transfection for 24 hours, cells in each group were trypsinized and seeded in matrigel coated filters (2x10^4 cells/per well) and cultured with 100 μl serum-free medium. 600 μl completed medium was added into the lower compartment of chamber. After incubation for 24 hours and 48 hours, cells on the upper surface of the filter were wiped off with a swab, while cells invaded through the filter were fixed with 95% ethanol, stained with crystal violet and counted under the microscope. Relative migration was based on the average number of cells on the underside of the membrane in ten random images generated at 4 × magnification under the microscope.

**Data analysis and statistics**

Each experiment was repeated 3 times independently and all results were presented as mean ± standard deviation (SD). All data were analyzed using SPSS 19.0 software. Multiple-group comparison was performed by analysis of variance (ANOVA), followed by LSD test for between-group comparison. Values of P < 0.05 were considered as significant and indicated by asterisks in the figures.

**Results**

**Inflammatory factors and high glucose activated c-Src kinase**

Using Western blot assay, we found that after treatment with inflammatory factors IL-1α, IL-6, TNF-α and IL-1β for 30 mins, the activity of c-Src kinase (gray ratio of p-Src^{418}/c-Src) in HLE-B3 cells was enhanced significantly, compared with the control group. The effect of TNF-α on activation of c-Src kinase was the strongest one (Fig. 1A). The expression of p-Src^{418} in 35.5 mM glucose group was significantly higher than that in 5.5 mM glucose group and mannitol group, while in mannitol group it was almost the same as in 5.5 mM glucose group (Fig. 1B). These results suggested that both inflammatory factors and high glucose stimulated activity of c-Src kinase in HLE-B3 cells.

**Alteration of c-Src kinase activity in HLE-B3 transfected with pCDNA3.1-c-Src^{Y530F} vector**
First, pCDNA3.1-c-SrcY530F and pSilence4.1-ShSrc vectors were constructed and transfected into HLE-B3 cells for 48 hours. To evaluate the active and inhibitive effects on c-Src kinase, respectively, RT-PCR and Western blot assay were applied to examine the expressions of c-Src kinase on mRNA and protein levels. In HLE-B3 cells transfected with pCDNA3.1-c-SrcY530F (group pCDNA3.1-c-SrcY530F), the expressions of c-Src mRNA and protein were higher than two control groups (group pCDNA3.1 and group control) significantly. Furthermore, active c-Src kinase, the expression of p-Src418 was much higher than controls (Fig. 2A&B), suggesting that c-Src kinase was activated by transfected with pCDNA3.1-SrcY530F vectors in the cells. In HLE-B3 cells transfected with pSilence4.1-ShSrc vector (group ShSrc), obvious suppressions of c-Src in mRNA and protein level were demonstrated, and p-Src418 protein expression were also decreased (Fig. 2C&D), implying the silencing of endogenous c-Src expression by ShRNA.

**Activation of c-Src kinase promoted EMT of HLE-B3 cells**

To explore the biological roles of activation of c-Src kinase in EMT of LECs, we further examined the expression of the epithelial cell proteins such as E-cadherin and ZO-1, and the mesenchymal cell proteins such as vimentin and αSMA in cells of group pCDNA3.1-c-SrcY530F, which c-Src kinase was activated. It was shown that expressions of E-cadherin and ZO-1 reduced significantly, and vimentin and αSMA increased dramatically compared with cells in control groups (group pCDNA3.1 and group control) by RT-PCR and Western blot, respectively (Fig. 3A&B). In pSilence4.1-ShSrc vector transfected cells, which c-Src was knocked down, the expressions of E-cadherin and ZO-1 increased, while vimentin and αSMA reduced significantly compared with control cells (group ShNC and group control), respectively (Fig. 3C&D). Altogether, activation of c-Src kinase could induce EMT process in HLE-B3 cells.

**Activation of c-Src kinase stimulated cell proliferation**

MTT assay showed that proliferation of cells in group pCDNA3.1-c-SrcY530F increased by 9%, 9%, 25% and 39% compared with that in group pCDNA3.1 at 12 hours, 24 hours, 48 hours and 72 hours,
respectively. And the proliferation of cells in group pCDNA3.1- c-Src\textsuperscript{Y530F} increased by 4%, 9%, 21% and 37% compared with that in group control at 12 hours, 24 hours, 48 hours and 72 hours, respectively (Fig. 4A). While in cells of group ShSrc, the proliferation did not change at 12 hours compared with two control groups, but reduced by 2%, 7% and 13% compared to group ShNC and 3%, 8% and 14% compared to group control at 24 hours, 48 hours and 72 hours, respectively (Fig. 4B). It indicated that activation of c-Src kinase stimulated cell proliferation.

**Activation of c-Src kinase increased cell mobility and migration**

By scratch assay, gap closure in cells of group pCDNA3.1- c-Src\textsuperscript{Y530F} increased by 84%, 60% compared with group pCDNA3.1 and 137%, 65% compared with group control at 24 hours and 48 hours, respectively, which suggested the enhancing of migration ability after activating c-Src kinase in HLE-B3 cells (Fig. 5A). While in HLE-B3 cells transfected with pSilence4.1-ShSrc vector (group ShRNA), gap closure reduced by 63%, 62% compared with group ShNC and 65%, 65% compared with group control at 24 hours and 48 hours, respectively (Fig. 5B).

In transwell assay, the migrating cell number in group pCDNA3.1- c-Src\textsuperscript{Y530F} increased by 71%, 152.8% compared with group pCDNA3.1 and 83.9%, 177.9% compared with group control at 24 hours and 48 hours, respectively (Fig. 6A), and vice versa, in cells of group ShSrc, this number reduced by 31.5%, 41.6% compared to group ShNC and 29.6%, 40.3% compared with group control at 24 hours and 48 hours, respectively (Fig. 6B). It suggested that activation of c-Src could induce cell mobility and migration.

**Discussion**

EMT is a conserved and essential process shared by developmental morphogenesis and carcinogenesis as well as physiological response to injury, operation, and tissue fibrotic diseases. In lens fibrotic diseases, EMT is an important pathological process, for instance, in ASC and PCO. Our present study showed that inflammatory factors and high glucose stimulated the activity of c-Src in HLE-B3 cells, and activation of c-Src kinase promoted EMT process, cell migration and proliferation in HLE-B3 cells, and vice versa, when c-Src kinase was inhibited. All these data suggested that c-Src may be a key regulator in the lens diseases associated with EMT.
Activation of c-Src kinase could promote EMT process, cell migration and proliferation in HLE-B3 cells, which is consistent with the role of c-Src kinase in tumor. Activation of c-Src kinase affected EMT process and enhanced cell migration and proliferation in lots of cancer cells [37, 38]. In various cancers, such as breast cancer, pancreatic cancer and castration-resistant prostate cancer, activation of c-Src increased cell invasiveness through altering activity of cadherins, adhesion proteins and integrins [39].

Src activation stimulates downstream kinase such as extracellular signal-regulated kinase (ERK) [40] and GSK3 [41, 42], which involved in the regulation of cell survival, proliferation, and promotion of EMT. Activated Src interaction with p120-catenin may cause dissociation of cell-cell junctions facilitating cell mobility [43]. Similarly, LECs in EMT process underwent losing of cell adhesion from the epithelial cells and gaining ability of proliferation and migration, transformed towards the mesenchymal cells [44]. In fibroblasts, the binding of integrins to their ligands leaded to activation of focal adhesion plaques adhesion kinase (FAK), which, in turn, recruits and activates c-Src [45]. Furthermore, activation of c-Src is required to disrupt cadherin-dependent cell-cell contacts [46].

Our results showed that activation of c-Src reduced the expression of E-cadherin at the protein and mRNA levels in HLE-B3 cells. This may be one of the mechanisms in which activation of c-Src induced EMT of lens epithelial cells. E-cadherin is the major cadherin molecule expressed in epithelial cells and is down-regulated in mesenchymal cells [47]. Loss of E-cadherin is the characteristic associated with increasing potential to invade surrounding tissues and disseminate to distant sites and is hallmark of EMT [48-50]. E-cadherin is a single-span transmembrane glycoprotein that maintains intercellular contacts and cellular polarity in epithelial tissues. In tumor cells, loss of E-cadherin is associated with cell invasion and metastasis [51]. In pancreatic ductal adenocarcinoma (PDAC) cell lines, overexpression of activated c-Src induced down-regulation of E-cadherin [52]. c-Src binding to E-cadherin, disrupted cell-cell interaction, enabled cancer cells to detach from their original site [53].

These supported our findings that activation of Src kinase increased cell motility and induced EMT.

Conclusions

c-Src kinase could be activated by inflammatory factors and high glucose. Activation of c-Src kinase
elevated EMT, cell proliferation, mobility and migration of LECs, and vice versa when silencing of c-Src gene in HLE-B3. Activation of c-Src was a trigger for EMT of lens epithelial cells associated with fibrosis of lens diseases.

Abbreviations
ASC: anterior subcapsular cataract; DMEM: Dulbecco's modified Eagle's medium; EGFR: epidermal growth factor receptor; EMT: epithelial-mesenchymal transition; ERK: extracellular signal-regulated kinase; FAK: focal adhesion plaques adhesion kinase; IGF-1: insulin-like growth factor-1; LECs: lens epithelial cells; PCO: posterior capsular opacification; PDAC: pancreatic ductal adenocarcinoma; PTK: protein tyrosine kinase; ROS: reactive oxygen species; SFKs: Src family kinases; a-SMA: a-smooth muscle actin; TGF-β: transforming growth factor-β.

Declarations

Acknowledgment
HLE-B3 cells were kindly provided by Dr. Wei Zhang (Beijing Tongren Eye Center, China).

Funding
The work was supported by National Natural Science Foundation of China (No.81370998), the Innovation Science and Technology Project of Shaanxi Province (No.2012KTCQ03-03), the New Century Excellent Talents Program of China (NCET-06-932).

Availability of data and materials
All data generated or analysed during this study are included in this published article. More details are available from the corresponding author on reasonable request.

Authors' contributions
Conceived and designed the experiments: J. Z.
Performed the experiments: X-Y. L., M-X. R., M-J. D.
Analyzed the data: X-Y. L., F.W., J. Z.
Wrote the paper: X-Y. L., J. Z.
All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

References
1. Eldred JA, Dawes LJ, Wormstone IM. The lens as a model for fibrotic disease. Philos Trans R Soc Lond B Biol Sci. 2011;366(1568):1301-1319. http://doi.org/10.1098/rstb.2010.0341.

2. Awasthi N, Guo S, Wagner BJ. Posterior capsular opacification: a problem reduced but not yet eradicated. Arch Ophthalmol. 2009;127(4):555-562. http://doi.org/10.1001/archophthalmol.2009.3.

3. Raj SM, Vasavada AR, Kaid Johar SR, Vasavada VA, Vasavada VA. Post-operative capsular opacification: a review. Int J Biomed Sci. 2007;3(4):237-250.

4. Moreno-Montanes J, Alvarez A, Maldonado MJ. Objective quantification of posterior capsule opacification after cataract surgery, with optical coherence tomography. Invest Ophthalmol Vis Sci. 2005;46(11):3999-4006. http://doi.org/10.1167/iovs.04-1531.

5. Su Y, Wang F, Yan Q, Teng Y, Cui H. Inhibition of proliferation of rabbit lens epithelial cells by S-phase kinase-interacting protein 2 targeting small interfering RNA. Mol Vis. 2010;16:907-915.

6. Zaczek A, Laurell CG, Zetterstrom C. Posterior capsule opacification after phacoemulsification in patients with postoperative steroidal and nonsteroidal treatment. J Cataract Refract Surg. 2004;30(2):316-320. http://doi.org/10.1016/j.jcrs.2003.07.006.

7. Cortina P, Gomez-Lechon MJ, Navea A, Menezo JL, Terencio MC, Diaz-Llopis M.
Diclofenac sodium and cyclosporin A inhibit human lens epithelial cell proliferation in culture. Graefes Arch Clin Exp Ophthalmol. 1997;235(3):180-185.

8. Nishi O, Nishi K, Fujiwara T, Shirasawa E. Effects of diclofenac sodium and indomethacin on proliferation and collagen synthesis of lens epithelial cells in vitro. J Cataract Refract Surg. 1995;21(4):461-465.

9. Ebihara Y, Kato S, Oshika T, Yoshizaki M, Sugita G. Posterior capsule opacification after cataract surgery in patients with diabetes mellitus. J Cataract Refract Surg. 2006;32(7):1184-1187. http://doi.org/10.1016/j.jcrs.2006.01.100.

10. De Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer. 2013;13(2):97-110. http://doi.org/10.1038/nrc3447.

11. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-1428. http://doi.org/10.1172/JCI39104.

12. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. J Clin Invest. 2009;119(6):1429-1437. http://doi.org/10.1172/JCI36183.

13. Lu L, Zhu J, Zhang Y, Wang Y, Zhang S, Xia A. Febuxostat inhibits TGFbeta1 induced epithelial-mesenchymal transition via downregulation of USAG1 expression in MadinDarby canine kidney cells in vitro. Mol Med Rep. 2019;19(3):1694-1704. http://doi.org/10.3892/mmr.2019.9806.

14. Graham TR, Zhau HE, Odero-Marah VA, Osunkoya AO, Kimbro KS, Tighiouart M, et al. Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res. 2008;68(7):2479-2488. http://doi.org/10.1158/0008-5472. CAN-07-2559.

15. Brzozowa M, Michalski M, Wyrobiec G, Piecuch A, Dittfeld A, Harabin-Slowinska M, et al. The role of Snail1 transcription factor in colorectal cancer progression and metastasis. Contemp Oncol (Pozn). 2015;19(4):265-270.
16. Ahmad A, Biersack B, Li Y, Kong D, Bao B, Schobert R, et al. Targeted regulation of PI3K/Akt/mTOR/NF-kappaB signaling by indole compounds and their derivatives: mechanistic details and biological implications for cancer therapy. Anticancer Agents Med Chem. 2013;13(7):1002-1013.

17. Singh S, Trevino J, Bora-Singhal N, Coppola D, Haura E, Altiok S, et al. EGFR/Src/Akt signaling modulates Sox2 expression and self-renewal of stem-like side-population cells in non-small cell lung cancer. Mol Cancer. 2012;11:73-87. http://doi.org/10.1186/1476-4598-11-73.

18. Giannoni E, Chiarugi P. Redox circuitries driving Src regulation. Antioxid Redox Signal. 2014;20(13):2011-2025. http://doi.org/10.1089/ars.2013.5525.

19. Han ZH, Wang F, Wang FL, Liu Q, Zhou J. Regulation of transforming growth factor beta-mediated epithelial-mesenchymal transition of lens epithelial cells by c-Src kinase under high glucose conditions. Exp Ther Med. 2018;16(2):1520-1528. http://doi.org/10.3892/etm.2018.6348.

20. Malarkey K, Belham CM, Paul A, Graham A, McLees A, Scott PH, et al. The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. Biochem J. 1995;309 (Pt 2):361-375.

21. Beristain AG, Molyneux SD, Joshi PA, Pomroy NC, Di Grappa MA, Chang MC, et al. PKA signaling drives mammary tumorigenesis through Src. Oncogene. 2015;34(9):1160-1173. http://doi.org/10.1038/onc.2014.41.

22. Bjonge JD, Jakymiw A, Fujita DJ. Selected glimpses into the activation and function of Src kinase. Oncogene. 2000;19(49):5620-5635.

23. Avizienyte E, Frame MC. Src and FAK signalling controls adhesion fate and the epithelial-to-mesenchymal transition. Curr Opin Cell Biol. 2005;17(5):542-547.
24. Avizienyte E, Fincham VJ, Brunton VG, Frame MC. Src SH3/2 domain-mediated peripheral accumulation of Src and phospho-myosin is linked to deregulation of E-cadherin and the epithelial-mesenchymal transition. Mol Biol Cell. 2004;15(6):2794-2803. http://doi.org/10.1091/mbc.e03-12-0879.

25. Hu M, Che P, Han X, Cai GQ, Liu G, Antony V, et al. Therapeutic targeting of SRC kinase in myofibroblast differentiation and pulmonary fibrosis. J Pharmacol Exp Ther. 2014;351(1):87-95. http://doi.org/10.1124/jpet.114.216044.

26. Skhirtladze C, Distler O, Dees C, Akhmetshina A, Busch N, Venalis P, et al. Src kinases in systemic sclerosis: central roles in fibroblast activation and in skin fibrosis. Arthritis Rheum. 2008;58(5):1475-1484. http://doi.org/10.1002/art.23436.

27. Zhou J, Leonard M, Van Bockstaele E, Menko AS. Mechanism of Src kinase induction of cortical cataract following exposure to stress: destabilization of cell-cell junctions. Mol Vis. 2007;13:1298-1310.

28. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev. 2003;22(4):337-358.

29. Kopetz S, Shah AN, Gallick GE. Src continues aging: current and future clinical directions. Clin Cancer Res. 2007;13(24):7232-7236. http://doi.org/10.1158/1078-0432.CCR-07-1902.

30. Boggon TJ, Eck MJ. Structure and regulation of Src family kinases. Oncogene. 2004;23(48):7918-7927. http://doi.org/10.1038/sj.onc.1208081.

31. Williams JC, Wierenga RK, Saraste M. Insights into Src kinase functions: structural comparisons. Trends Biochem Sci. 1998;23(5):179-184.

32. Schlessinger J. New roles for Src kinases in control of cell survival and angiogenesis. Cell. 2000;100(3):293-296.
33. Kmiecik TE, Shalloway D. Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell. 1987;49(1):65-73.

34. Cooper JA, Gould KL, Cartwright CA, Hunter T. Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science. 1986;231(4744):1431-1434.

35. Boerner RJ, Kassel DB, Barker SC, Ellis B, DeLacy P, Knight WB. Correlation of the phosphorylation states of pp60c-src with tyrosine kinase activity: the intramolecular pY530-SH2 complex retains significant activity if Y419 is phosphorylated. Biochemistry-Us. 1996;35(29):9519-9525. http://doi.org/10.1021/bi960248u.

36. Chen X, Shen J, Li X, Wang X, Long M, Lin F, et al. Rlim, an E3 ubiquitin ligase, influences the stability of Stathmin protein in human osteosarcoma cells. Cell Signal. 2014;26(7):1532-1538. http://doi.org/10.1016/j.cellsig.2014.03.018.

37. Patel A, Sabbineni H, Clarke A, Somanath PR. Novel roles of Src in cancer cell epithelial-to-mesenchymal transition, vascular permeability, microinvasion and metastasis. Life Sci. 2016;157:52-61. http://doi.org/10.1016/j.lfs.2016.05.036.

38. Bartscht T, Rosien B, Rades D, Kaufmann R, Biersack H, Lehnerta H, et al. Inhibition of TGF-beta Signaling in Tumor Cells by Small Molecule Src Family Kinase Inhibitors. Anticancer Agents Med Chem. 2017;17(10):1351-1356. http://doi.org/10.2174//1871520617666170103094946.

39. Hamaguchi M, Matsuyoshi N, Ohnishi Y, Gotoh B, Takeichi M, Nagai Y. p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. Embo J. 1993;12(1): 307-314.

40. Zhang S, Yu D. Targeting Src family kinases in anti-cancer therapis: turning promise into triumph. Trends Pharmacol Sci. 2012;33(3):122-128. http://doi.org/10.1016/j.tips.2011.11.002.
41. Gao F, Al-Azayzih A, Somanath PR. Discrete functions of GSK3alpha and GSK3beta isoforms in prostate tumor growth and micrometastasis. Oncotarget. 2015;6(8):5947-5962. http://doi.org/doi:10.18632/oncotarget.3335.

42. Goc A, Al-Husein B, Katsanevas K, Steinbach A, Lou U, Sabbineni H, et al. Targeting Src-mediated Tyr216 phosphorylation and activation of GSK-3 in prostate cancer cells inhibit prostate cancer progression in vitro and in vivo. Oncotarget. 2014;5(3):775-787. http://doi.org/10.18632/oncotarget.1770.

43. Reynolds AB, Rocznik-Ferguson A. Emerging roles for p120-catenin in cell adhesion and cancer. Oncogene. 2004;23(48):7947-7956. http://doi.org/10.1038/sj.onc.1208161.

44. Wu X, Ruan J, Ma B, Luo M. Bit1-a potential positive regulator of epithelial-mesenchymal transition in lens epithelial cells. Graefes Arch Clin Exp Ophthalmol. 2016;254(7):1311-1318. http://doi.org/10.1007/s00417-016-3357-3.

45. Matsuoka H, Nada S, Okada M. Mechanism of Csk-mediated down-regulation of Src family tyrosine kinases in epidermal growth factor signaling. J Biol Chem. 2004;279(7):5975-5983. http://doi.org/10.1074/jbc.M311278200.

46. Owens DW, McLean GW, Wyke AW, Paraskeva C, Parkinson EK, Frame MC, et al. The catalytic activity of the Src family kinases is required to disrupt cadherin-dependent cell-cell contacts. Mol Biol Cell. 2000;11(1):51-64. http://doi.org/10.1091/mbc.11.1.51.

47. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science. 1991;251(5000):1451-1455.

48. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol. 2006;7(2):131-142. http://doi.org/10.1038/nrm1835.
49. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell. 2008;14(6):818-829. http://doi.org/10.1016/j.devcel.2008.05.009.

50. Kang Y, Massague J. Epithelial-mesenchymal transitions: twist in development and metastasis. Cell. 2004;118(3):277-279. http://doi.org/10.1016/j.cell.2004.07.011.

51. Christofori G, Semb H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci. 1999;24(2):73-76.

52. Menke A, Philippi C, Vogelmann R, Seidel B, Lutz MP, Adler G, et al. Down-regulation of E-cadherin gene expression by collagen type I and type III in pancreatic cancer cell lines. Cancer Res. 2001;61(8):3508-3517.

53. Chen J. Is Src the key to understanding metastasis and developing new treatments for colon cancer? Nat Clin Pract Gastroenterol Hepatol. 2008;5(6):306-307.

Tables

Table 1 Quantitative real-time PCR primers

| Gene   | GenBank ID | Primer pairs (5’→3’)         |
|--------|------------|------------------------------|
| c-Src  | NM_005417  | F: AAGCCTGGCAGCATGTCT        |
|        |            | R: CGATGTAAATGGGGCTCCTCT     |
| E-Cadherin | AB025106  | F: CCCCAGCTTTATGATTCTC      |
|        |            | R: GCCCCATTCCGTCAAGTA        |
| ZO-1   | NM_003257  | F: CTGCTTGACCTCCCTAAA        |
|        |            | R: ATCCAAACACGGAACAC         |
| Vimentin | NM_003380 | F: AGCTCCAGCCGGAGCTAC        |
|        |            | R: CGCTGCTCCGACGGCGCA        |
| αSMA   | NM_001141945 | F: TGCTCCAGGGCTGTATT      |
|        |            | R: GCCATGTCTTATCGGTTACCTTC  |
| GAPDH  | NG 007073.2 | F: CCACATCGCTCAACACCAT      |
|        |            | R: GGCAACAATATCCACTTACCAGAT |

Figures
1. c-Src kinase was activated by inflammatory factors and high glucose. (A) Left, the expressions of c-Src and p-Src418 (active c-Src kinase) were examined by western blot at 30 mins after treatment with inflammatory factors in HLE-B3 cells. Right, the relative expression of p-Src418, radio of p-Src418/c-Src. (B) Left, the expressions of c-Src and p-Src418 were examined by western blot in different concentrations of glucose. Right, the relative expression of p-Src418, radio of p-Src418/c-Src. (**P < 0.01 compared with control, n = 3).
Alterations of c-Src kinase in HLE-B3 transfected with pCDNA3.1-c-SrcY530F vector or pSlence4.1-ShSrc vector. (A) The expressions of mRNA of c-Src kinase examined by RT-PCR in HLE-B3 cells transfected with pCDNA3.1-c-SrcY530F vector (group pCDNA3.1-c-SrcY530F), pCDNA3.1 vector (group pCDNA3.1), and Lipofectamine 2000 (group control) at 48 hours.

(B) Left, the expressions of c-Src kinase examined by western blot at 48 hours after transfection. Right, relative expression of protein in left figure. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group pCDNA3.1, n = 3). (C)

The expression of c-Src was examined by RT-PCR in HLE-B3 cells transfected with pSlence4.1-ShSrc vector (group ShSrc), pSlence4.1-ShSrc (group ShNC), and Lipofectamine 2000 (group control) at 48 hours after transfection. (D) Left, the expressions of c-Src kinase (c-Src) and activated c-Src kinase (p-Src 418) examined by western blot at 48 hours after transfection. Right, relative expression of protein in left figure. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group ShNC, n = 3).
Figure 3

Effects of c-Src on the expressions of EMT marker proteins in HLE-B3 cells. (A) The expressions of EMT marker proteins were examined by RT-PCR in group pCDNA3.1-c-SrcY530F, group pCDNA3.1 and group control at 48 hours after transfection. (B) Left, the expressions of EMT marker proteins were examined by western blot in group pCDNA3.1-c-SrcY530F, group pCDNA3.1 and group control at 48 hours after transfection. Right, relative expression of protein in left figure. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group pCDNA3.1, n = 3). (C) The expression of EMT marker proteins was examined by RT-PCR in group ShSrc, group ShNC and group control at 48 hours after transfection. (D) Left, the expressions of EMT marker proteins were examined by western blot in group ShSrc, group ShNC and group control at 48 hours after transfection. Right, relative expression of protein in left figure. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group ShNC, n = 3).
Effect of c-Src on cell proliferation by MTT assay. (A) The effect of c-Src on cell proliferation examined by MTT assay in group pCDNA3.1-c-SrcY530F, group pCDNA3.1 and group control. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group pCDNA3.1, n = 3). (B) The effect of c-Src on cell proliferation examined by MTT assay in group ShSrc, group ShNC and group control. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group ShNC, n = 3).
Effect of c-Src on cell migration. (A) The effect of c-Src on cell migration examined by scratch assay in group pCDNA3.1-c-SrcY530F, group pCDNA3.1 and control. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group pCDNA3.1, n = 3). (B) The effect of c-Src on cell migration examined by scratch assay in group ShSrc, group ShNC and control. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group ShNC, n = 3).
Effect of c-Src on cell mobility. (A) The effect of c-Src on cell mobility examined by transwell assay in group pCDNA3.1-c-SrcY530F, group pCDNA3.1 and control. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group pCDNA3.1, n = 3). (B) The effect of c-Src on cell mobility examined by transwell assay in group ShSrc, group ShNC and group control. (*P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with group ShNC, n = 3).