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RESEARCH PAPER

Bit1 is involved in regulation between integrin and TGFβ signaling in lens epithelial cells

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ABSTRACT

Bit1, as an integrin-specific effector, is specifically expressed in lens epithelial cells (LECs) and may be essential to maintain the normal function of LECs. The present study investigated the function of Bit1 and its regulatory mechanism in LECs. Knockdown of Bit1 was mediated by a lentivirus with a specific short-hairpin RNA against Bit1 in SRA01/04 cells. Cell proliferation ability was measured by CCK-8 assay. Cell migration was examined by transwell and wound-healing assays. The effect of Bit1 knockdown on genome-wide expression patterns was studied via a GeneChip® PrimeView™ Human Gene Expression Array. Based on the ingenuity pathway analysis (IPA), Bit1's regulation of target pathways and genes was verified by real-time qPCR and Western blotting. Bit1 knockdown inhibited proliferation, migration, and regulated cell cycle and apoptosis of LECs. Microarray gene expression analysis and IPA assays revealed that integrin and TGFβ signaling pathways were remarkably impacted by Bit1 expression. FAK, PAK2, ITGA5, and ITGB1 were identified as core node molecules under the control of Bit1. Bit1 participates in integrin and TGFβ signaling via regulating downstream FAK and PAK2 and subsequently affecting EMT-related gene expression including ITGA5, ITGB1, and αSMA. In conclusion, Bit1 plays as an important role in the regulation between integrin and TGFβ signaling, which affects cell survival, migration, and EMT of LECs.

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Introduction

The ocular lens is an important part of the eye that aids in focusing light rays onto the retina. In anatomy, the lens is a transparent elliptic structure suspended between the cornea and vitreous. The ocular lens contains a surrounding capsule, lens epithelium, and lens fibers. The epithelium is a single layer of lens epithelial cells (LECs) on its anterior surface, which is essential to maintain the normal development and function of lens tissue. Therefore, a large number of studies have been performed to explore the function of the lens epithelium both in ocular health and disease [1]. In lens development, LECs migrate and elongate from the equatorial region to form lens fiber cells at the posterior. In adulthood, the lens fiber cells are terminally differentiated and lose their organelles, while the LECs retain the restricted capacity to proliferate and differentiate. Any insults or damages to the lens can alter the biological function of LECs through various factors or signalings. Under such circumstances, lens epithelial cell may be activated to proliferate, migrate, and undergo epithelial–mesenchymal transition (EMT), which leads to cataract formation or "after-cataract" that is also known as posterior capsule opacification (PCO) following cataract removal surgery [2,3].

The transforming growth factor β (TGFβ) has been identified as an important growth factor that mediates various functional events in cataract and PCO formation [2–4]. Particularly, TGFβ2 is well known as a potent inducer of EMT in LECs [4,5]. The TGFβ2 in aqueous humor can be upregulated in conditions of anterior subcapsular cataract or PCO, and is believed to play a central role in regulating lens epithelial cell proliferation, migration, and EMT [2,3,6].

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On the other hand, the lens capsule membrane separates lens epithelial cell from the ocular media and may have dynamic effects on lens epithelium functions associated with the disease of anterior subcapsular cataract and PCO [7]. The lens capsule membrane is composed of different collagens and mainly extracellular matrix (ECM) proteins including laminin, fibronectin, and others. Interaction signals between lens epithelial cells and the capsular ECM are mediated by integrins, which are heterodimeric transmembrane proteins consisting of one α subunit and one β subunit [8]. Interestingly, crosstalk between integrin and TGFβ signaling has been proven during EMT process [9], but the underlying mechanism remains to be further elucidated.

B-cell lymphoma 2 (Bcl-2) inhibitor of transcription 1 (Bit1, also known as PTRH2) is a mitochondrial protein that is evolutionarily conserved from bacteria to humans. Bit1 is a key effector controlling cell-death fate in response to cell–ECM interactions [10]. When released from mitochondria into the cytoplasm following loss of cell attachment, Bit1 interacts with the Groucho/TLE family transcription factor, AES, and subsequently induces caspase-independent apoptosis (anoikis) [11]. However, further studies have revealed that Bit1 mediates integrin-dependent cellular survival in substrate-attached cells and some tumor cells in culture [12–14]. Thus, Bit1 may function differently in various cell types. We have previously reported that Bit1 retains its expression only in lens epithelial cell once lens development was complete [15]. In another study, we revealed that Bit1 levels were dramatically higher in the lens capsule membrane from PCO tissues and knockdown of Bit1 by small-interfering RNA (siRNA) depressed lens epithelial cell proliferation and TGFβ2-induced EMT process [16]. All of these findings indicate that Bit1, as an integrin-specific effector, may be involved in the TGFβ signaling pathway in LECs. However, the Bit1′s role in integrin or TGFβ signaling needs to be further explored.

In the present study, firstly, we verified that the downregulation of Bit1 mediated by shRNA lentivirus-inhibited growth and migration of LECs, and regulated the cell cycle and apoptosis of LECs. Secondly, we examined differentially expressed genes between wild-type and Bit1-knockdown LECs via microarray analysis and ingenuity pathway analysis (IPA). We found that Bit1 was involved in both integrin and TGFβ signaling and validated downstream core molecules of Bit1. Finally, we further investigated the potential mechanism of Bit1 regulation on these molecules involved between integrin and TGFβ signaling pathways.

Materials and methods

Cell culture and transduction

The human lens epithelial cell line, SRA01/04, was kindly provided by Dr. X.J. Zhu of Eye and ENT hospital affiliated with Fudan University. SRA01/04 cells were cultured in 5% fetal bovine serum (FBS; Gibco/Brl, USA) containing Dulbecco's modified Eagle's medium (DMEM; Gibco/Brl, USA) with 5% CO_2 in air at 37°C. Cells were digested with 0.25% trypsin-0.02% EDTA solution and passaged at approximately 80% confluency. Moreover, the cells were transferred to non-supplemented DMEM for further 24 hours culture before experimental application.

A Bit1-shRNA (LV-Bit1-shRNA-EGFP) lentivirus and a non-targeted negative control lentivirus (LV-control-EGFP) were purchased from Shanghai Genechem Co., Ltd. (China). SRA01/04 cells were infected with LV-Bit1-shRNA-EGFP to knockdown Bit1 (KD), and LV-control-EGFP was used to produce negative-control cells (NC). The Bit1-shRNA sequences were as follows: TGGAGCCTTCGAGTATGCTT (KD1), GCATCCCAAAGCATACTCGAA (KD2), and AACAGCCAAGCCGAGTGTAC (KD3). The non-targeted negative-control shRNA sequence was TTCTCCGAACGTGTCACGT (NC). For cellular infection of lentiviruses, 1×10^5 cells/well were seeded in six-well plates. The next day, cells were transduced with corresponding lentiviruses at appropriate MOI. The infection efficiency was evaluated by EGFP at 72 h after infection.

For TGFβ2 induction, SRA01/04 cells were cultured in six-well plates and were then treated with 10 ng/ml of recombinant human TGFβ2 (Sigma-Aldrich, USA). For integrin intervention, SRA01/

04 cells were treated with 5 ng/ml of GLPG0187 that is a broad-spectrum integrin receptor antagonist. Cell grouping information was abbreviated as NC (negative control group), KD (Bit1-knockdown group), and G (GLPG0187 intervention group).

Immunofluorescent staining

In this study, adult Sprague-Dawley (SD) rats (8-week old) were used. Rat lens tissue was dissected and fixed prior to freezing. Immunofluorescence was performed on frozen sections of rat tissue and cultured SRA01/04 cells. Briefly, the samples were first incubated in 5% goat serum in PBS buffer for 30 min and were then incubated with primary antibodies overnight at 4°C. After this incubation, samples were washed with PBS buffer and incubated with secondary antibodies for 1 h at room temperature followed by washing with PBS. Finally, the samples were mounted with fluorescent mounting medium (DAKO Corporation, CA). The primary antibody used in this study was Bit1 polyclonal rabbit antibody (Abcam, MA). Goat anti-Rabbit IgG conjugated with Cy3 (Jackson, PA) was used as the secondary antibody.

Cell proliferation assay

For cell proliferation assay, LV-shBit1 (KD) or LV-shControl (NC) cells were seeded into 96-well plates at a density of 2,000 cells per 150 µl per well. Subsequently, the numbers of viable cells were examined at 24, 48, 72, 108, and 120 h after seeding with a CCK-8 assay (Sigma, USA). Specifically, 10 µl of CCK-8 solution was added into 100 µl of culture media and incubated at 37°C for 0.5 h. The absorption at 450 nm was measured using a microplate reader (Tecan Infinite, USA).

Cell cycle analysis

For cell cycle analysis, cells plated in six-well plates were collected at 3 days after LV-shBit1 or LV-shControl cells were seeded. Cells were fixed with cold 70% ethanol for 1 hour. After PBS washing, cells were stained with propidium iodide (PI) (Sigma) and RNase (Fermentas) solution. Stained cells were then loaded for flow cytometry (Millipore).

Detection of apoptosis

Cells plated in six-well plates were collected for apoptotic detection at 3 days after LV-shBit1 or LV-shControl cells were seeded. Annexin V-APC apoptosis detection kit (eBioscience) and flow cytometry (Millipore) were used to analyze the level of apoptosis according to the manufacturer's instructions. In short, the collected cells were suspended after trypsin digestion, and stained with Annexin V-APC solution. Stained cells were then loaded for flow cytometry.

Transwell migration assay

Cell migration was tested by transwell assays (Corning, UK). Briefly, 5×10^5 cells/ml were seeded into the upper polycarbonate-membrane insert, which allows the cell to migrate through 8-µm pores. In the lower well, 300 µl of 10% FBS containing DMEM as a chemoattractant was incubated for 48 h at 37°C with 5% CO_2 in air. After the incubation, the membranes were fixed in 10% formaldehyde and were then stained with hematoxylin. The cells migrating to the bottom of the insert were counted in four random microscope fields (100x).

Wound-healing migration assay

Cell migration was also detected through wound-healing assays. SRA01/04 cells were seeded into wells in a 24-well plate (1×10^5 cells/well). Approximately 24 h after seeding, the cell density nearly reached 90% confluency and the confluent cell monolayers were scratched with a sterile, 20 ml pipette tip. The wounded monolayers were washed with PBS buffer several times to remove detached floating cells and debris, and adequate fresh medium was supplemented into each well. The wounds were photographed at 0, 8, and 24 h. The breadth of the remaining wound in each image was measured five times. The final quantification was expressed as the percentage of the average distance.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from approximately 10^6 lentiviral-transduced cells using Trizol reagent (Invitrogen, USA) and was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Specifically, 2 μg of isolated total RNA was reverse-transcribed using the M-MLV reverse transcription kit (Promega, USA) according to the manufacturer's protocol. RNA and cDNA were quantified with a NanoDrop. Next, 1 μg of reverse-transcribed cDNA was used as the qPCR template for the SYBR® Green PCR Master mix (TaKaRa, China). Target genes were amplified by qPCR on the ABI 7500 Real-time PCR System (ABI, USA) with specific primers. The primer sequences are listed in Table S1.

Protein extraction and western blotting

Collected cells were washed twice with ice-cold PBS buffer and were then lysed in RIPA lysis buffer (Beyotime, China). The concentration of extracted protein was determined with a BCA Protein Assay Kit (Beyotime, China) according to the manufacturer's protocol. Then, 30 μg of extracted total protein was added into 5× protein sample buffer and heated at 100°C for 2 min. SDS-PAGE electrophoresis was performed with 3 μl of pre-stained protein molecular weight markers (Thermo, USA) as a standard. After electrophoresis, the protein bands in SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane via Bio-rad Trans-Blot (Bio-rad, USA). The membranes were pre-incubated with blocking solution (TBS buffer containing 5% non-fat-dried milk) for 2 h and incubated overnight at 4°C with corresponding primary antibodies including anti-Bit1 (1:10000 diluted with blocking solution), anti-FAK (1:1000 diluted with blocking solution), anti-ITGA5 (1:500 diluted with blocking solution), anti-ITGB1 (1:200 diluted with blocking solution), anti-PAK2 (1:500 diluted with blocking solution), anti-αSMA (1:500 diluted with blocking solution), and control anti-β-actin (1:500 diluted with blocking solution) overnight at 4°C, followed by several washes and incubation with corresponding secondary antibodies (goat anti mouse, 1:5000 dilution; goat anti rabbit, 1:2000 dilution) conjugated with horseradish peroxidase. After sufficient washing, membranes were finally developed with the ECL detection system (Thermo, USA).

Gene microarrays

The effect of Bit1 knockdown on genome-wide expression was studied via a GeneChip® PrimeView™ Human Gene Expression Array (Affymetrix, USA). Three biological replicates of SRA01/04 cells infected by shBit1 (KD) or shCtrl (NC) lentiviruses were microarrayed at the transcriptional level. RNA isolation refers to the method described above, in the subsection on RNA isolation and reverse transcription-polymerase chain reaction. The quality of isolated RNA was determined using the NanoDrop and Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Each sample used an individual microarray for gene expression profiling. In brief, 500 ng of total RNA was reverse-transcribed, and the reverse-transcribed cDNA was labeled with biotin, in accordance with the manufacturer's protocol of the GeneChip® 3' IVT labeling kit (Agilent Technologies, USA). Next, labeled cDNA was hybridized onto the GeneChip® PrimeView™ Human Gene Expression Array (Agilent Technologies, USA) overnight at 60°C. Microarrays were performed with the GeneChip®Hybridization Wash and Stain kit (Agilent Technologies, USA) on GeneChip® Fluidics Station 450 (Agilent Technologies, USA). Post-hybridization, the array was scanned directly by using a GeneChip® Scanner 3000 (Agilent Technologies, USA). Microarray chip data were analyzed with GeneSpring software (version 11; Agilent Technologies, USA). Normalization of data was performed by using the GeneSpring normalization algorithm. Finally, genes that were differentially expressed >2-fold between NC and knockdown samples and that had differential scores of false discovery rate (FDR)-adjusted

P-value < 0.05 were identified from normalized data sets.

Ingenuity pathway analysis (IPA)

Data of differentially expressed genes derived from gene microarray assays were uploaded into the IPA tool (http://www.ingenuity.com; Ingenuity® Systems, USA). The "core analysis" function was applied to interpret the differential expression. The core analyses included "disease and functions", "pathway", and "molecular network". Genes differentially expressed in NC and knockdown samples were mapped onto genetic networks of the IPA database and were then ranked based on the P-value to determine the enrichment degree of diseases, functions, pathways, and networks in the present dataset, as well as the activation z-score algorithms, which were computed by IPA software and used to assess the regulatory effects (upregulation or downregulation).

Statistical analysis

All experimental data are reported as the mean ± standard deviation (SD), and comparisons between the two groups were evaluated with Student's *t*-tests using SPSS 13.0. A P < 0.05 was considered to indicate a statistically significant difference.

Results

Bit1 expression in rat lens tissue and the human lens epithelial cell line, SRA01/04

We have previously demonstrated the Bit1 expression in lens tissue and cell line [15,16]. For consistency of our manuscript, we here show again the immunostaining pattern of Bit1. As shown in Figure 1(a), Bit1 was expressed specifically in LECs of lens tissue from adult rats. Bit1 staining showed a basal cytoplasmic pattern in lens epithelial cells and was negative in lens fiber cells. For Figure 1(b), cell immunofluorescent staining confirmed that Bit1 expression was prominent in the cytoplasm of SRA01/04 cells, which was consistent with lens tissue staining. These results indicate a high-level endogenous expression of Bit1 in both LECs of lens tissue and the cultured SRA01/04 cell line.

Bit1 knockdown suppresses cell proliferation and migration, promotes apoptosis, and influences cell cycle in SRA01/04 cells

To investigate the biological function of Bit1 in LECs, the human lens epithelial cell line, SRA01/04, which exhibits high expression of endogenous Bit1, was used to construct a Bit1-knockdown cell model. Knockdown of Bit1 was achieved by a lentivirus with a specific short-hairpin RNA (shRNA) against Bit1. We detected Bit1 expression in three knockdown groups via real-time quantitative polymerase chain reaction (RT-qPCR). As shown in Figure 2(a), the second knockdown (KD2 group) showed the most striking reduction of Bit1 expression. The mRNA expression was reduced by approximately 70%, compared to that of the control group (which was infected with a lentivirus using non-targeted shRNA). The specific shRNA virus against Bit1 stably reduced Bit1 protein levels in SRA01/04 cells over time. Bit1 knockdown markedly suppressed LEC proliferation in adherent cultures for 48–120 h (Figure 2(b); p-values for day 4 and day 5 were 4.2E-7 and 0.00015, respectively). Additionally, apoptosis of LECs was elevated significantly in the shBit1 group. The proportion of apoptotic LECs in the shBit1 group increased to approximately 8.73% that was almost two-fold of that of the control group (Figure 2(c), p = 6.9E-6). Additionally, the cell cycle of LECs was also regulated by the expression of Bit1. In the Bit1 knockdown group, the proportion of cells in the G1 or G2/M phase was reduced, whereas the proportion of cells in the S phase was concomitantly increased (Figure 2(d), p-values for G1, S, G2/M phases were 9.2E-6, 5.3E-5, and 0.0035, respectively). These findings are consistent with our results of apoptosis. Next, transwell migration assay and wound-healing migration assay performed in SRA01/04 cells both revealed that Bit1 knockdown inhibited migration of LECs (Figure 3(a–b)), p-value = 1.4E-7 [Figure 3(a)] and 1.4E-7 [Figure 3(b), 24 h]). The functional results verified that

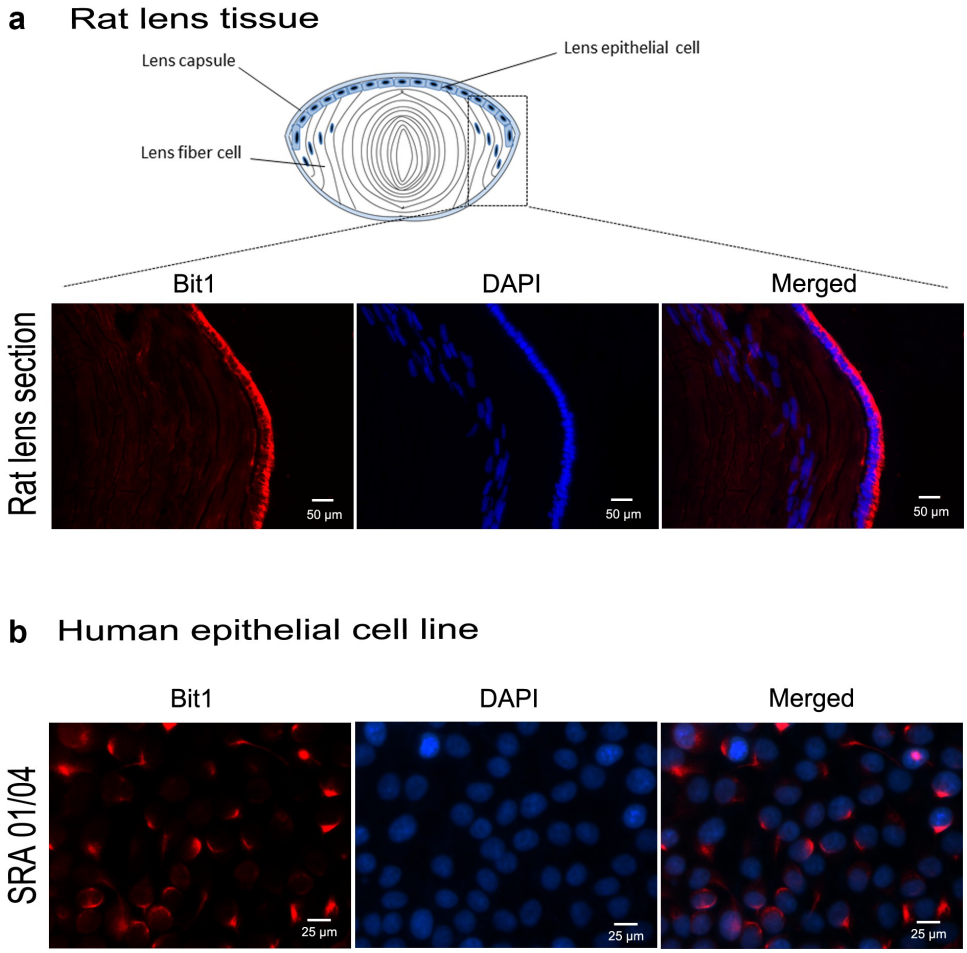

Figure 1. Bit1 expression in rat lens tissue and in the human lens epithelial cell line, SRA01/04. (a) Immunofluorescent staining in frozen sections of lens tissue from adult Sprague-Dawley rats. Bit1 staining (red) showed a basal cytoplasmic pattern in lens epithelial cells and was negative in lens fiber cells. (b) Cellular immunofluorescent staining in human lens epithelial cell line SRA01/04 cells. Bit1 was significantly accumulated in the cytoplasm of SRA01/04 cells, which is consistent with lens tissue staining. Nuclei were stained with DAPI (blue). Merged images are shown in the right panel.

suppressing the expression of Bit1 inhibited LEC vitality and mobility, both of which are also important for the EMT of LECs.

Microarray gene expression analysis and IPA assays in NC and Bit1-knockdown SRA01/04 cells

To further explore the potential molecular mechanism of how Bit1 influences the function of LECs, we first carried out microarray analysis to compare gene expression of control NC versus Bit1-knockdown SRA01/04 cells. In total, 524 differentially expressed genes with at least a twofold change and with adjusted P-value < 0.05 were identified. Among these, expression levels of 113 genes were up-regulated; the levels of the other 411 genes were down-regulated in KD SRA01/04 cells (Figure 4(a)). The 524 differentially expressed genes were imported into the IPA tool and analyzed. Through gene annotations, we investigated disease/function enrichment, pathway enrichment, and functional changes of these differently expressed genes, as well as their speculative Bit1-based regulatory network. Enriched pathways in analysis, of our particular interest, were integrin signaling, FAK/paxillin signaling, and ERK/MARK signaling, all of which were significantly suppressed in shBit1 LECs (Figure 4(b)). Additionally, TGFβ signaling was also distinctly enriched (-Log (P-value) was 1.82) as seen in supplementary data. Disease and functional enrichment analysis based on these differentially expressed genes

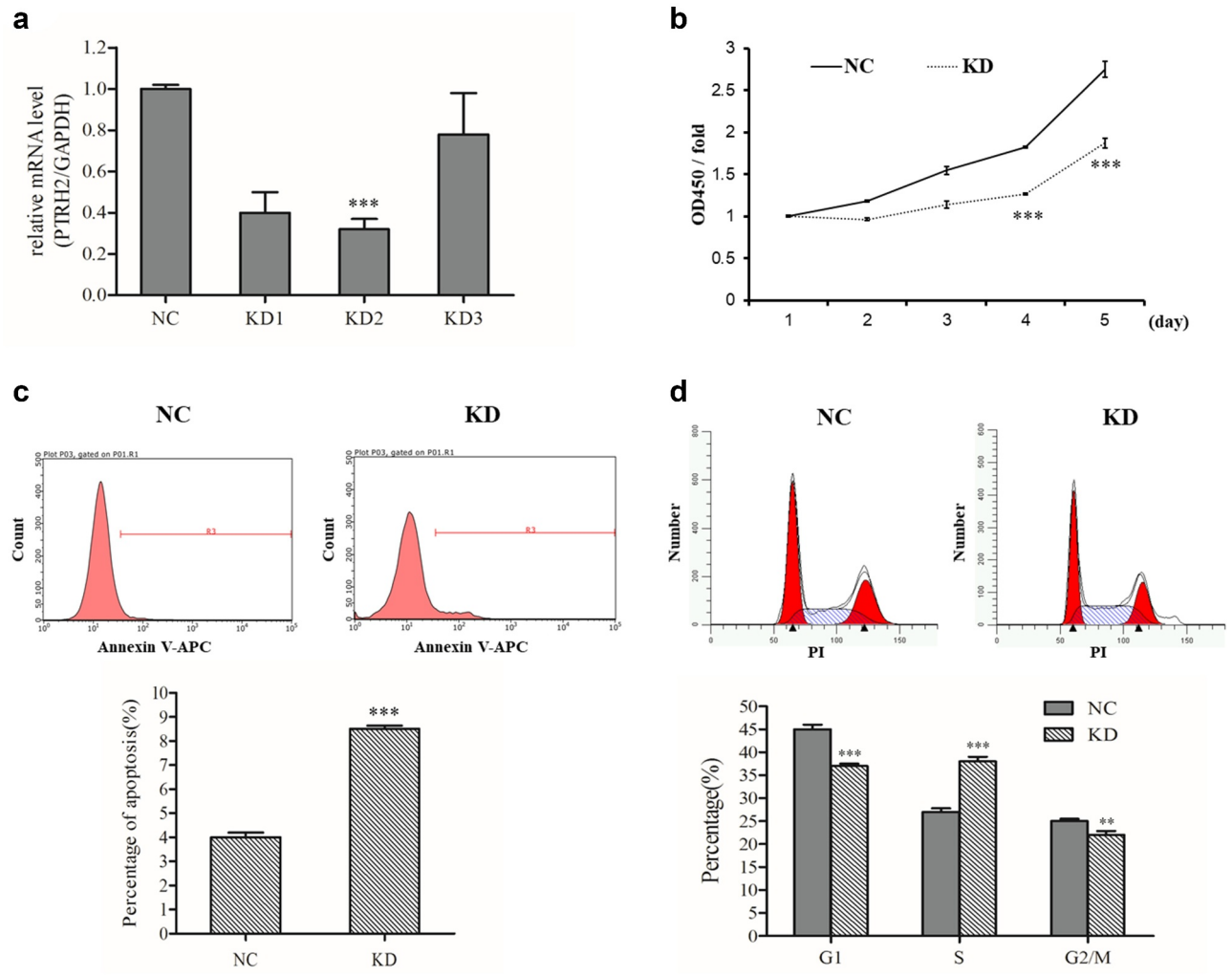

Figure 2. The effects of Bit1 knockdown on cell proliferation, cell cycle and apoptosis in SRA01/04 cells. (a) SRA01/04 cells were infected with LV-Bit1-shRNA-EGFP to knockdown Bit1 (KD), and LV-control-EGFP was used to produce negative-control cells (NC). Bit1 mRNA levels in LV-shBit1 SRA01/04 cells (KD1, KD2, and KD3) and LV-shControl (NC) SRA01/04 cells were examined by RT-qPCR. The KD2 group showed the most striking reduction of Bit1 expression (by approximately 70%), which was used for following experiments. (b) Bit1-shRNA significantly suppressed cellular growth on days 2, 3, 4, and 5 in SRA01/04 cells. The numbers of metabolically active cells were assessed by CCK-8 assay at 1, 2, 3, 4, and 5 d after equal cells were plated for KD and NC groups. (c) Bit1 knockdown reduced cells in the G1 and G2/M period, and increased cells in the S period. Flow cytometry assays were carried out to analyze cell cycle. (d) Knockdown of Bit1 expression promoted SRA01/04 apoptosis. Three days after infection by LV-shBit1 or LV-shControl, cells were processed with the Annexin V-APC apoptosis detection kit and counted with flow cytometry. For all assays, three independent experiments were performed. (** $P < 0.01$; *** $P < 0.001$.).

indicated that cellular movement, growth, and proliferation were significantly inhibited in the Bit1-downregulated group (Figure 4(c)). Besides, organismal death and morbidity/mortality were greatly increased in KD cells (z-scores of 6.654 and 6.389, respectively) as seen in supplementary data. These IPA results illuminated the impact of Bit1 knockdown on cellular growth, migration, and apoptosis in LECs. These findings inspired us to further investigate the potential regulatory network and key node molecules that Bit1 interacts with in LECs.

Bit1 is involved in integrin and TGFβ signaling regulatory networks

Via IPA assay-based microarrays, Bit1 was inferred to regulate transcription of genes involved in integrin signaling, FAK/paxillin signaling, TGFβ signaling, and ERK/MARK signaling, which relate

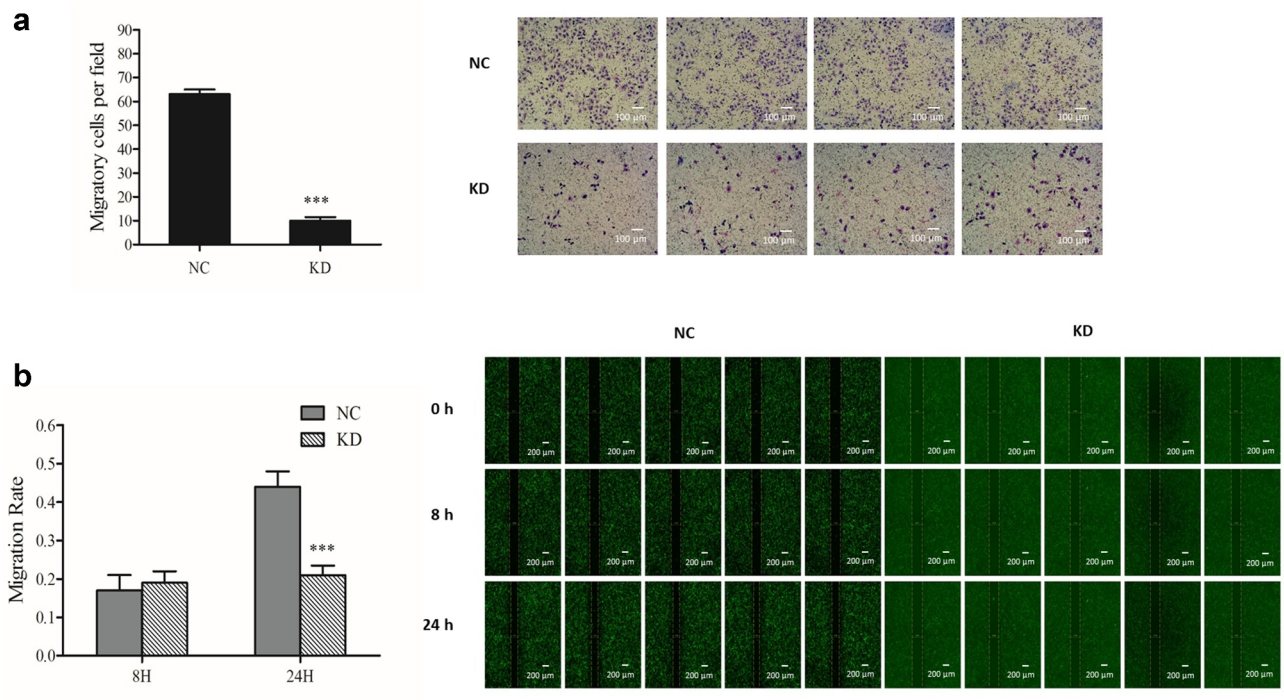

Figure 3. The effects of Bit1 knockdown on cell migration ability in SRA01/04 cells. (a) Transwell invasion assay was performed by counting cells that had migrated from the upper polycarbonate-membrane insert to the bottom wells in four random microscope fields (100x). Bit1 knockdown markedly impeded cell migration. (b) Wound-healing migration assay was carried out by measuring the length of the remaining wound scratched out with a tip. The wounds were photographed at 0, 8, and 24 h. The wound-healing ability was markedly suppressed in Bit1-knockdown cells. Histogram shown here represents the mean ± standard deviation (SD) of three independent experiments. (*** $P < 0.001$.).

to cell survival, proliferation, migration, and EMT in LECs. In microarrays, 30 genes related to these pathways exhibited reduced expression that correlated with reduced Bit1 expression. For further verification, transcription levels of these genes were determined by RT-qPCR. Details and expression levels of these genes are shown in Table S1-S2. The predicted regulatory network surrounding Bit1 indicated that Bit1 may participate in regulation between integrin signaling and TGFβ signaling via downstream FAK and/or p21-activated kinase 2 (PAK2) (Figure 5(a)). Therefore, FAK, PAK2, integrin subunit α5 (ITGA5), and β1 (ITGB1) were identified as core node molecules according to the results of RT-qPCR (Figure 5(b)). Transcription levels of these four genes were greatly suppressed via knockdown of Bit1. Furthermore, protein levels of FAK, PAK2, ITGA5, and ITGB1 were examined by Western blotting. As shown (Figure 5(c)), FAK, PAK2, ITGA5, and ITGB1 expression levels were all distinctly reduced in shBit1 LECs ($P < 0.001$). These data suggest that Bit1 may play a bridge role in integrin and TGFβ signaling network through the regulation of FAK, PAK2, ITGA5, and ITGB1.

Molecular mechanism of Bit1 regulation in both integrin and TGFβ signaling

To further investigate the molecular mechanism of Bit1 regulation, we designed *in vitro* pathway-stimulator and pathway-inhibitor experiments. Specifically, we activated TGFβ signaling or inhibited integrin signaling to explore the role of Bit1′s role in integrin and TGFβ signaling pathways.

Although Bit1 is an integrin-specific effector, we showed that TGFβ2 modulates Bit1 in a dose-dependent manner, with a peak effect at a concentration of 10 ng/ml TGFβ2 (Figure 6(a)). This finding indicates that Bit1 acts downstream of both integrin and TGFβ2 activation. Subsequently, Bit1 regulation of downstream FAK and PAK2 was investigated by TGFβ2

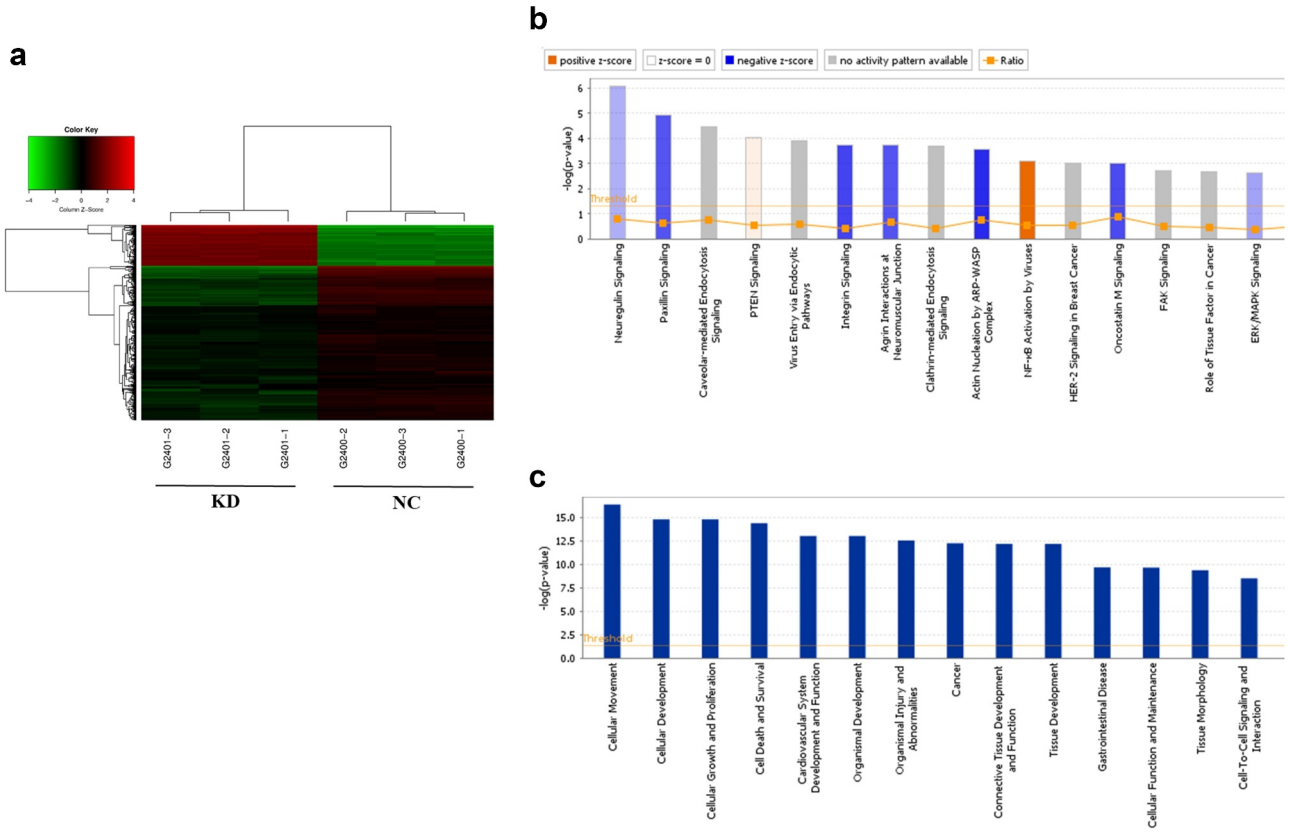

Figure 4. Microarray gene expression analysis and IPA assays in Bit1-knockdown SRA01/04 cells. (a) Heat map of differentially expressed genes derived from microarrays. Red indicates that the signal values of genes that were up-regulated. Green indicates relative down-regulation. Among 524 differentially expressed genes, expression levels of 113 genes were up-regulated; the other 411 genes were down-regulated in KD SRA01/04 cells. (b) The histogram of signaling pathways enrichment shows the enrichment of differentially expressed genes in classical signaling pathways. All the pathways were ranked by -Log (P-value). The Orange represents the pathway was significantly activated (z-score > 0). Contrarily, the blue represents the pathway was significantly suppressed (z-score < 0). Of our particular interest, Integrin signaling, FAK/paxillin signaling, ERK/MARK signaling were significantly suppressed in shBit1 LECs (Figure 4b). (c) The histogram of disease and function derived from the enrichment of differential genes in this project. All the diseases and functions were ranked by -Log (P-value).The results indicated that cellular movement, growth and proliferation were significantly inhibited in the Bit1-downregulated group.

induction. FAK and PAK2 increased in response to TGFβ2 induction, but these elevations were suppressed via Bit1 knockdown (Figure 6(b-c)). These results confirm that Bit1 could regulate integrin and TGFβ2 signaling via downstream FAK and PAK2.

Bit1 is activated by cellular attachment to the ECM through fibronectin-α5β1-integrin interactions [17]. Additionally, gene expression of integrin is positively regulated by TGFβ signaling [18]. Therefore, ITGA5 and ITGB1 – as core node molecules involved in Bit1 regulation – were examined via TGFβ2 induction and blocking of integrin. GLPG0187 is a potent integrin receptor antagonist that specifically inhibits integrin α5β1. As expected, TGFβ2 induction promoted the production of ITGA5 and ITGB1. However, treatment with GLPG0187 did not show a significant inhibitory effect on TGFβ2-induced elevations of ITGA5 and ITGB1. Further, the ITGA5 and ITGB1 levels were reduced in GLPG0187 treated LECs when combined with knockdown of Bit1 (Figure 6 (d-e)). These findings indicate that TGFβ2-induced expression levels of ITGA5 and ITGB1 were affected by Bit1. We further investigated changes in the expression of the cytoskeletal protein, αSMA, which is a representative EMT marker. Consistently, integrin inhibition by GLPG0187 led to a slight decrease in αSMA; with the combination of GLPG0187 treatment and Bit1

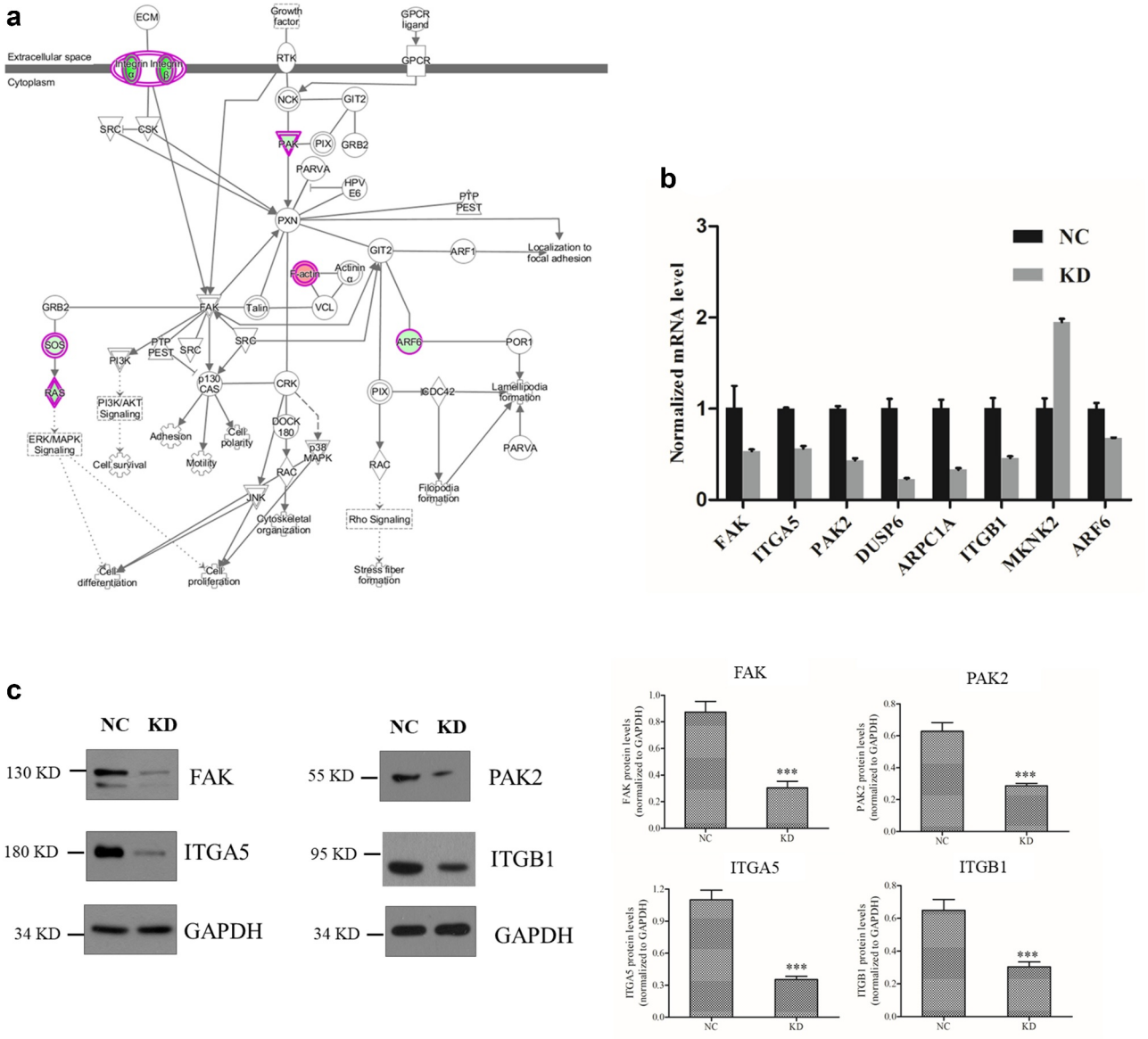

Figure 5. Critical pathway and key node molecules in modulation of integrin and TGFβ signaling network with Bit1 knockdown in SRA01/04 cells. (a) The summarized genetic network by IPA. Potentially upregulated genes in Bit1-knockdown lens cells are depicted in red, whereas downregulated genes are depicted in green. The predicted regulatory network surrounding Bit1 indicated that Bit1 may participate in regulation between integrin signaling and TGFβ signaling via downstream FAK and/or p21-activated kinase 2 (PAK2). (b) Expression changes of selected genes from IPA analysis data were confirmed by qPCR. FAK, PAK2, integrin subunit α5 (ITGA5), and β1 (ITGB1) were identified as core node molecules according to the results. (c) The regulation of Bit1 on FAK, PAK2, ITGA5, and ITGB1, as indicated by Western blotting. GAPDH was used as a loading control for densitometric analysis. FAK, PAK2, ITGA5, and ITGB1 expression levels were all distinctly reduced in shBit1 LECs ($P < 0.001$). These data suggest that Bit1 may be involved in the regulation network in integrin and TGFβ signaling through downstream FAK, PAK2, ITGA5, and ITGB1.

knockdown, αSMA expression decreased markedly in LECs (Figure 6(f)). The results further reveal that Bit1 regulates EMT through both integrin and TGFβ signaling in LECs.

Discussion

Bit1 acts as a pivotal molecular switch between cellular survival and apoptosis. Initially, Bit1 is known as an effector regulating anoikis, which is

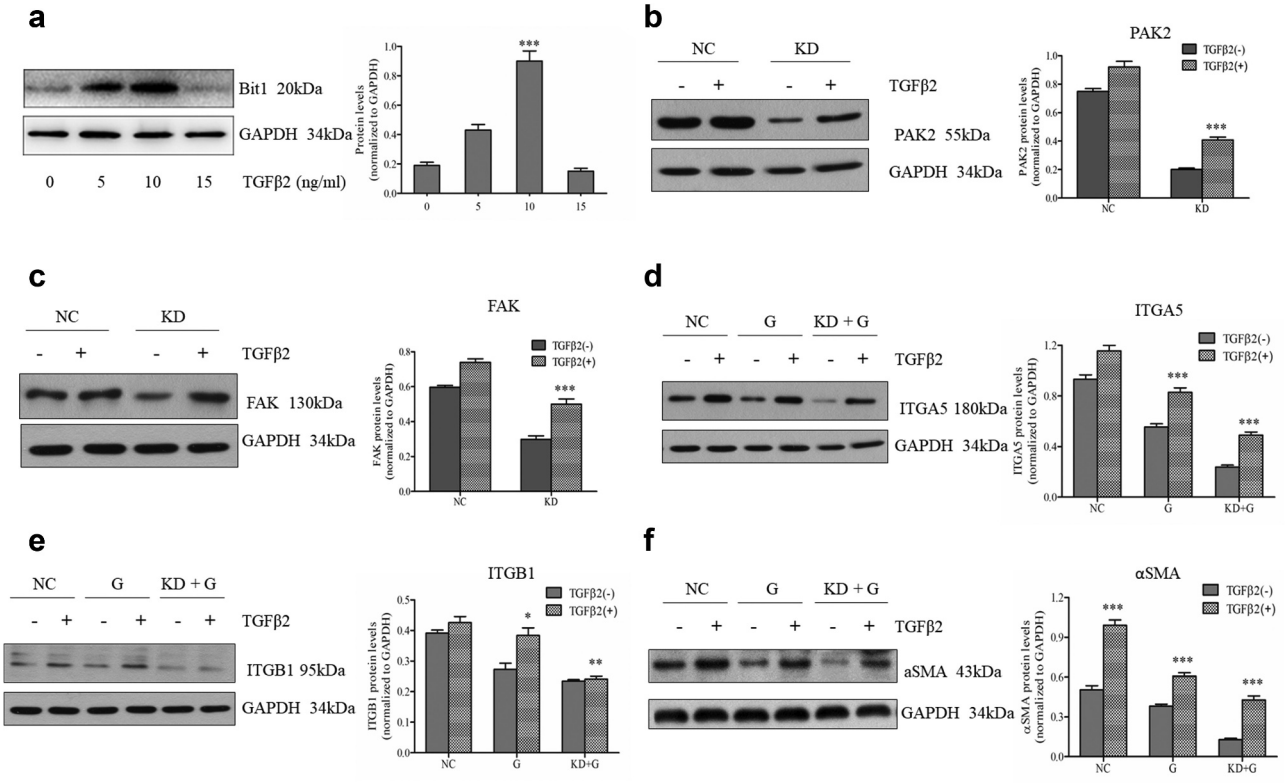

Figure 6. Verification of molecular mechanism of Bit1's interaction with integrin and TGFβ signaling network. (a) Dose-response examination of TGFβ2 inducement in SRA01/04 cells. Bit1 protein expression level was improved by TGFβ2 inducement in a dose-dependent manner, with a peak effect at a concentration of 10 ng/ml. (b–f) TGFβ2 and GLPG0187 were correspondingly used to activate TGFβ signaling and block integrin signaling. Cell grouping information was abbreviated as NC (negative control group), KD (Bit1-knockdown group) and G (GLPG0187 intervention group). Concentrations of TGFβ2 and GLPG0187 were 10 ng/ml and 5 ng/ml, respectively. Additionally, "+" represents addition of TGFβ2, whereas "–" represents no addition of TGFβ2. (b-c) Expression of PAK2 and FAK in response to TGFβ2 inducement and Bit1 knockdown. The expression level of FAK and PAK2 increased in response to TGFβ2 induction, but these elevations could be suppressed by Bit1 knockdown. (d-f) Expression of ITGA5, ITGB1 and αSMA in response to TGFβ2 inducement and Bit1 knockdown combined with GLPG0187 intervention. TGFβ2 inducement promoted production of ITGA5 and ITGB1 as well as EMT marker αSMA. TGFβ2-induced elevations of ITGA5, ITGB1 and αSMA could be inhibited only when GLPG0187 treatment combined with Bit1 knockdown in LECs. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.).

a type of programmed cell death that is induced upon cellular detachment from the ECM [11,19]. However, Bit1 is reported to protect cells from serum deprivation-mediated apoptosis in attached cells by up-regulating NFκB activity and subsequent bcl-2 gene transcription [14]. When cells are attached to fibronectin, Bit1 may activate FAK/PI3K/NFκB signaling pathway and elevate bcl-2 transcription to promote cellular survival. We have shown that Bit1 was continuously and specifically expressed in LECs. The human lens epithelial cell line, SRA01/04, was also shown to have a high expression of Bit1. Therefore, the function of Bit1 in LECs and its regulatory mechanism were explored. Our study employed lentiviral transduction for stable knockdown of Bit1 and confirmed that Bit1 knockdown inhibited LEC proliferation, retarded cells in the S phase, and promoted apoptosis. These findings reveal a conserved function of Bit1 as a mediator of integrin-mediated cell survival. Moreover, previous studies have shown that Bit1 may enhance cell motility and migratory ability in some tumor cells [12,13]. Our recent finding showed that knockdown of Bit1 led to decreased cell migration and wound healing in astrocytes from the developing retina [20]. Consistently, in the present study, we found that suppressing the expression of Bit1 inhibited cell mobility and migration of LECs. During PCO development, resilient LECs divide and migrate to the posterior capsule, and undergo EMT process, leading to capsular fibrosis.

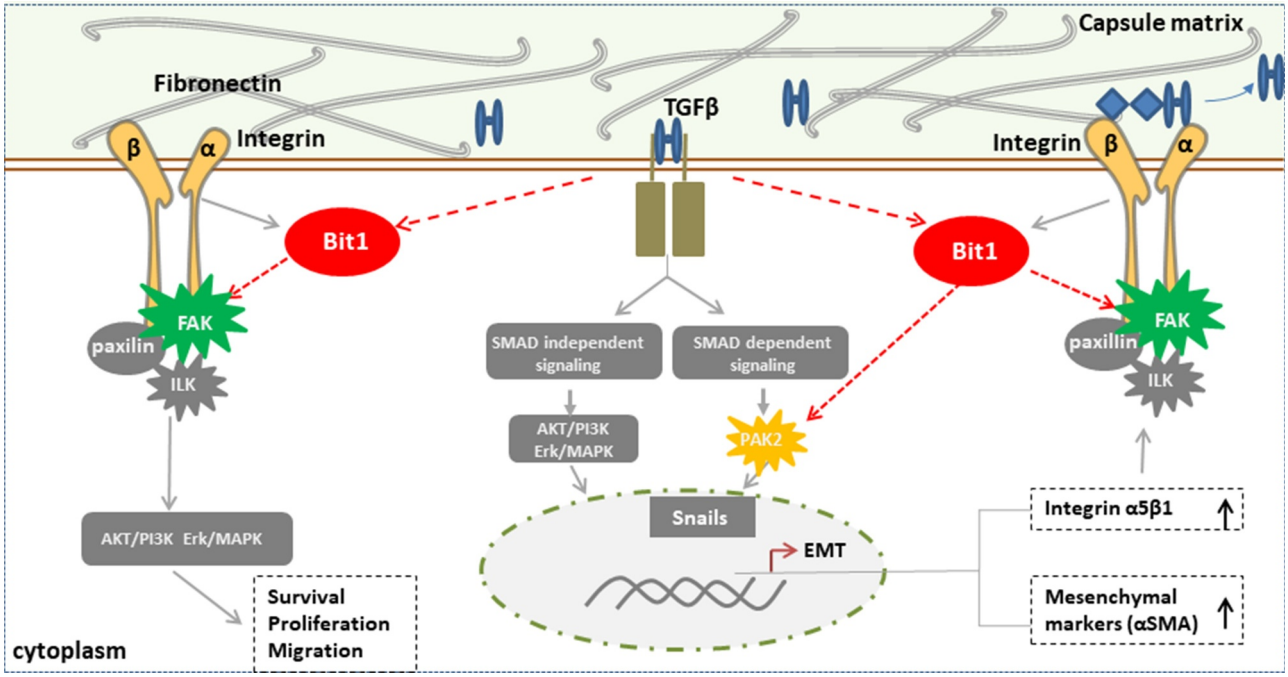

Figure.7 A summary model proposing the molecular mechanism of Bit1 involvement in integrin and TGFβ signaling pathways. Integrin–α5β1 interaction with extracellular fibronectin activates cytoplasmic expression of Bit1. Bit1 interacts with and regulates FAK, which activates subsequent adhesion-initiated and integrin-dependent EMT signal transduction pathways to promote cellular survival and migration. Additionally, by regulating the TGFβ2-induced mediator, PAK2, Bit1 positively participates in the TGFβ signaling pathway and further promotes EMT-related gene expression including ITGA5, ITGB1, and αSMA. This collectively forms a feedforward circuit and Bit1 may serve as a pivotal role in keeping balance between integrin and TGFβ signaling. Inhibition of Bit1 may lead to reduced cell proliferation and migration. Overexpression of Bit1 may accelerate the EMT induced by TGFβ signaling which provokes production of integrin α5β1 and amplifies both signaling pathways. Gray solid arrows denote previously reported positive regulation, red solid arrows denote positive regulation of Bit1 in the present study. Other icons are described by explanatory notes.

Additionally, TGFβ2-induced expression of αSMA in LECs has been commonly related to imitate EMT. We have shown that Bit1 levels are dramatically higher in lens-capsule membranes from PCO tissues [16]. Here, we found that Bit1 levels increased in TGFβ2-treated LECs and that down-regulating Bit1 inhibited expression of TGFβ2-induced αSMA. The results indicate that high expression of Bit1 contributes to the EMT process of LECs.

Previous studies show that cell apoptosis and EMT are linked with various pathological processes including PCO or after cataract [21–23]. Cell apoptosis and EMT, which are opposite cell fates, can be concurrent in the same type of cells. It is proposed that cultured cells are heterogeneous in terms of cell cycle phases, and could respond differentially to certain stimuli leading to different cell fates. Researchers found that TGFβ treatment could induce not only apoptosis but also EMT, which is closely related to cell cycle stages. Cell apoptosis was induced mostly in cells at G2/M phase, whereas EMT was only induced in cells at G1/S phase [24]. In our study, cell cycle analysis showed that about 75% of cultured LECs were at G1/S phase and 25% at G2/M phase. The fact regarding the cell cycle distribution tends to support the possibility that the dominant G1/S phase stage predisposes the cultured cells to undergo EMT. Nevertheless, a recent study showed that EMT and apoptosis markers were detected in the same single cell. Their results indicated that apoptosis might contribute to TGF2-induced EMT during PCO [25]. Therefore, more functional studies are needed to clarify the relationship between cell apoptosis and EMT during PCO. Here, as our data showed, knockdown of Bit1 in LECs could inhibit cell migration and TGFβ2-induced EMT. Meanwhile, the proportion of apoptotic LECs in Bit1 knockdown group increased. This is

consistent with a previous study of an ex vivo model that showed that COX-2 inhibitors suppressed PCO formation through decreased migration and proliferation, and increased apoptosis [23].

Much evidence shows that proliferation and the EMT of lens epithelial cells represent major pathologic changes in the development of anterior subcapsular cataracts (ASCs) [4] and PCO [26]. As we expected, disease and function enrichment analysis from IPA indicated that cell survival, cell viability, proliferation of cells, and cell movement were all significantly inhibited via down-regulation of Bit1. Furthermore, we found that integrin and TGFβ signaling were remarkably inhibited via Bit1 knockdown, as indicated by pathway enrichment analysis. The predicted regulatory network surrounding Bit1 provided clues for potential Bit1-regulated downstream target molecules involved in integrin and TGFβ signaling. ITGA5, ITGB1, FAK, and PAK2 were identified as target molecules for their coincident behaviors in microarrays verified by RT-qPCR and Western blotting.

Integrin binds extracellularly to the ECM and intracellularly to the cytoskeleton, and thereby integrates the extracellular environment with the cellular interior [27,28]. Upon its binding to the ECM, integrin signaling is propagated to intracellular integrin-associated adaptor proteins such as integrin-linked kinase (ILK) and focal adhesion kinase (FAK); this sequence subsequently activates other downstream players such as paxillin, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and AKT [29]. Bit1 in attached cells has been reported to co-localize with FAK, activate PI3K, and elevate bcl-2 transcription to promote cellular survival [14]. Reduced cell motility and enhanced focal-adhesion contact formation have been observed in cells from FAK-deficient mice [30]. A recent study also showed that Bit1/FAK interactions – detected by immunoprecipitation – contributes to increased migration and invasion in carcinomas [13]. Our data are in agreement with these previous studies and indicate that α5β1-Bit1-FAK signaling is activated through the integrin signaling, which affects cell proliferation and migratory ability in LECs.

TGFβ isoforms – TGFβ1, TGFβ2, and TGFβ3 – are pleiotropic cytokines that induce many aspects of effects on a wide range of cell types, and subsequently affect Smad-dependent and Smad-independent pathways to initiate gene expression promoting the EMT [31]. PAK2 is a member of TGFβ2 Smad-independent signaling and is a critical effector that links Rho GTPases to regulate EMT-related gene expression. In our study, the level of PAK2 expression was suppressed in Bit1-KD LECs. Furhtermore, TGFβ2-induced upregulation of PAK2 was diminished by Bit1 knockdown. The results suggest that Bit1 participates in TGFβ signaling via regulating downstream PAK2 and subsequently affecting EMT-related gene expression including ITGA5, ITGB1, and αSMA.

Previous studies have demonstrated that there is a cycle of regulation between integrin and TGFβ signaling [9,18,32]. Integrins modulate the signaling cascades elicited by several growth factors, including TGFβ. A vast subset of transcriptional targets controlled by TGFβ are integrins and their ligands. Therefore, our present study revealed that Bit1 may serve as a bridge involved in both integrin and TGFβ signaling pathways in LECs. A possible molecular mechanism of Bit1′s involvement in these two signalings is depicted in Figure 7. We propose that integrinα5β1 interaction with extracellular fibronectin activates cytoplasmic expression of Bit1. Then, Bit1 interacts with and regulates FAK, which activates subsequent anti-apoptosis and adhesion-initiated signaling pathways to promote cellular survival and migration. Additionally, by regulating the TGFβ2-induced mediator, PAK2, Bit1 contributes to the TGFβ signaling pathway and promotes EMT-related gene expression including ITGA5, ITGB1, and αSMA. This collectively forms a feedforward circuit, and Bit1 may serve as a pivotal role in maintaining the balance between integrin and TGFβ signaling. Inhibition of Bit1 may lead to reduced cell proliferation and migration. Overexpression of Bit1 may accelerate the EMT induced by TGFβ signaling that provokes the production of integrin α5β1 and amplifies both signaling pathways.

In conclusion, Bit1 plays an important role in the regulation of both integrin and TGFβ signaling, which affects cell survival and migration of LECs and promotes the development of EMT. This work will help understanding the Bit1′s

biological function in LECs and the pathological process of anterior subcapsular cataract or PCO as a result of LECs dysfunction. The limitation of our work is that most of the conclusions were built based on results from human lens epithelial cell line SRA01/04. Further studies of the experimental system of lens *in vivo* are needed to verify the Bit1 regulation effects in LECs to show the treatment prospects.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

MB and LM conceived and designed the research. JJL, XJY, and SWY collected samples. MB, NN, and SWY conducted experiments. MB, XJY, and NN analyzed the data. MB wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional animal care and use committee of Shanghai Ninth People's Hospital affiliated Shanghai Jiao Tong University School of Medicine (No. HKDL2017131).

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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References

[1] Martinez G, de Iongh RU. The lens epithelium in ocular health and disease. Int J Biochem Cell Biol. 2010;42(12):1945–1963.
[2] Wormstone IM, Wang L, Liu CS. Posterior capsule opacification. Exp Eye Res. 2009;88(2):257–269.
[3] Nibourg LM, Gelens E, Kuijer R, et al. Prevention of posterior capsular opacification. *Exp Eye Res.* 2015;136:100–115.
[4] de Iongh Ru, Wederell E, Lovicu FJ, et al. Transforming growth factor-beta-induced epithelial-mesenchymal transition in the lens: a model for cataract formation. Cells Tissues Organs. 2005;179(1–2):43–55.
[5] Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene. 2005;24 (37):5764–5774.
[6] Lovicu FJ, Shin EH, McAvoy JW. Fibrosis in the lens. sprouty regulation of TGFbeta-signaling prevents lens EMT leading to cataract. Exp Eye Res. 2016;142:92–101.
[7] Walker J, Menko AS. Integrins in lens development and disease. Exp Eye Res. 2009;88(2):216–225.
[8] Hynes RO. Integrins: a family of cell surface receptors. Cell. 1987;48(4):549–554.
[9] Margadant C, Sonnenberg A. Integrin-TGF-beta cross-talk in fibrosis, cancer and wound healing. EMBO Rep. 2010;11(2):97–105.
[10] Biliran H, Jan Y, Chen R, et al. Protein kinase D is a positive regulator of Bit1 apoptotic function. J Biol Chem. 2008;283(42):28029–28037.
[11] Jan Y, Matter M, Pai J-T, et al. A mitochondrial protein, Bit1, mediates apoptosis regulated by integrins and Groucho/TLE corepressors. Cell. 2004;116 (5):751–762.
[12] Hua W, Miao S, Zou W, et al. Pathological implication and function of Bcl2-inhibitor of transcription in ovarian serous papillary adenocarcinomas. Neoplasma. 2013;60(2):143–150.
[13] Fan T, Chen J, Zhang L, et al. Bit1 knockdown contributes to growth suppression as well as the decreases of migration and invasion abilities in esophageal squamous cell carcinoma via suppressing FAK-paxillin pathway. Mol Cancer. 2016;15:23.
[14] Griffiths GS, Grundl M, Leychenko A, et al. Bit-1 mediates integrin-dependent cell survival through activation of the NFkappaB pathway. J Biol Chem. 2011;286(16):14713–14723.
[15] Ma B, Sen T, Asnaghi L, et al. betaA3/A1-Crystallin controls anoikis-mediated cell death in astrocytes by modulating PI3K/AKT/mTOR and ERK survival pathways through the PKD/Bit1-signaling axis. Cell Death Dis. 2011;2:e217.
[16] Wu X, Ruan J, Ma B, et al. Bit1-a potential positive regulator of epithelial-mesenchymal transition in lens epithelial cells. Graefes Arch Clin Exp Ophthalmol. 2016;254(7):1311–1318.

[17] Stupack DG, Cheresh DA. A Bit-role for integrins in apoptosis. Nat Cell Biol. 2004;6(5):388–389.
[18] Pechkovsky DV, Scaffidi AK, Hackett TL, et al. Transforming growth factor beta1 induces alphav-beta3 integrin expression in human lung fibroblasts via a beta3 integrin-, c-Src-, and p38 MAPK-dependent pathway. J Biol Chem. 2008;283 (19):12898–12908.
[19] Kairouz-Wahbe R, Biliran H, Luo X, et al. Anoikis effector Bit1 negatively regulates Erk activity. Proc Natl Acad Sci U S A. 2008;105(5):1528–1532.
[20] Liu Y, Ji J, Shao W, et al. Bit1-a novel regulator of astrocyte function during retinal development: proliferation, migration, and paracrine effects on vascular endothelial cell. Hum Cell. 2019;32(4):418–427.
[21] Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest. 2003;112(12):1776–1784.
[22] Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol. 2005;17(5):548–558.
[23] Chandler HL, Barden CA, Lu P, et al. Prevention of posterior capsular opacification through cyclooxygenase-2 inhibition. *Mol Vis.* 2007;13:677–691.
[24] Yang Y, Pan X, Lei W, et al. Transforming growth factor-beta1 induces epithelial-to-mesenchymal transition and apoptosis via a cell cycle-dependent mechanism. Oncogene. 2006;25(55):7235–7244.
[25] Jing R, Hu C, Qi T, et al. FILIP1L-mediated cell apoptosis, epithelial-mesenchymal transition and extracellular matrix synthesis aggravate posterior capsular opacification. *Life Sci.* 2021;286:120061.
[26] Meacock WR, Spalton DJ, Stanford MR. Role of cytokines in the pathogenesis of posterior capsule opacification. Br J Ophthalmol. 2000;84(3):332–336.
[27] Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673–687.
[28] Legate KR, Wickstrom SA, Fassler R. Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev. 2009;23(4):397–418.
[29] Dedhar S. Integrins and signal transduction. Curr Opin Hematol. 1999;6(1):37–43.
[30] Ilic D, Furuta Y, Kanazawa S, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature. 1995;377 (6549):539–544.
[31] Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. Cell Res. 2009;19(2):156–172.
[32] Mamuya FA, Duncan MK. aV integrins and TGF-beta-induced EMT: a circle of regulation. J Cell Mol Med. 2012;16(3):445–455.