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Fn1 Promotes Pediatric Postoperative Peritoneal Adhesion Formation via the PI3K/Akt Pathway

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Abstract:

Background/Aims: Peritoneal adhesions represent a frequent postoperative complication following abdominal surgery. In pediatric patients, these adhesions may lead to severe clinical consequences, including adhesive small bowel obstruction, intestinal strangulation, and bowel necrosis, which can adversely affect children's physical growth, development, and psychological well-being.

Materials and Methods: Bioinformatics analysis was performed to identify core genes and key signaling pathways associated with peritoneal adhesions. Clinical adhesion tissue samples were examined using histological staining and immunohistochemistry. The correlation between Fn1 expression and the PI3K/Akt pathway was assessed through quantitative real-time PCR and western blotting. Functional assays—including cell viability analysis, flow cytometry, and migration assays—were conducted to evaluate the role of Fn1 in human fibroblast cell lines.

Results: Analysis of clinical samples revealed elevated Fn1 expression in postoperative peritoneal adhesion tissues. In human peritoneal mesothelial cells, Fn1 knockdown resulted in no significant change in total PI3K and Akt protein expression; however, the phosphorylation levels of both proteins were notably decreased ($p < 0.05$). Furthermore, downregulation of Fn1 was found to inhibit fibroblast proliferation and migration, and to induce apoptosis.

Conclusion: These findings suggest that Fn1 may promote the function of mesothelial cells during peritoneal adhesion formation by modulating the phosphorylation of the PI3K/Akt pathway, and may also enhance the activity of fibroblasts. This dual role underscores its potential as a therapeutic target for the prevention of peritoneal adhesions.

Keywords: Abdominal adhesion, Bioinformatics, Fn1, PI3K, Akt

Introduction

Peritoneal adhesions (PA) refer to the pathological formation of fibrous tissue between the abdominal wall and visceral organs following abdominal surgery [1]. PA represent one of the most frequent complications following abdominal surgery, with a spectrum of contributing factors including the underlying disease type, the surgical approach employed, and the use of drainage tubes [2, 3]. It is widely accepted that postoperative peritoneal adhesions arise from surgical trauma-induced disruption of the normal peritoneal structure, which serves as the critical initiating event in their formation [4]. This disruption initiates a cascade that includes the release of chemotactic factors by peritoneal mesothelial cells, the recruitment of inflammatory cells, and a subsequent imbalance between peritoneal fibrogenesis and fibrinolysis. This cascade ultimately leads to excessive fibrous tissue proliferation and fibrosis [5]. Furthermore, mesothelial cells may undergo mesothelial-mesenchymal transition (MMT), giving rise to a subset of fibroblasts that contribute to fibrous tissue formation. Bioinformatics is defined as the interdisciplinary field that develops and applies computational methods to store, retrieve, and analyze biological data, with the goal of extracting meaningful knowledge. Its primary focus is on analyzing and extracting meaningful biological information from nucleic acid and protein sequences [6, 7]. In this study, core genes involved in peritoneal adhesion were identified through screening of the GEO database. These findings were further validated using clinical samples and in vitro cellular assays, thereby establishing a foundation for future investigations.

Materials and Methods

Data Collection and Identification of Differentially Expressed Genes

The GEO database (<https://www.ncbi.nlm.nih.gov/geo>) was searched using the keyword "Peritoneal adhesions". The results were subsequently refined by applying the filters "mRNA" and "Mus musculus". Through this screening, the dataset GSE123413, based on the GPL21163 platform, was selected for further analysis. This dataset comprises transcriptome data obtained via microarray analysis of cecal tissue from C57BL/6 mice. Samples were collected at three time points during adhesion formation—3, 12, and 72 hours—following either burn injury or sham surgery [8].

Differential expression analysis was performed using the limma package in R software (version 4.0.3) to identify differentially expressed genes (DEGs) [9]. The fold change (FC) and P-value for each gene were calculated. Genes with a P-value < 0.05 and $|\log FC| > 1$ were considered significantly differentially expressed. Volcano plots and heatmaps were constructed to visualize the identified DEGs and their expression patterns [10, 11].

Weighted Gene Co-expression Network Analysis (WGCNA) and Venn Analysis

Weighted gene co-expression network analysis (WGCNA) was performed on the GSE123413 dataset using the WGCNA package in R software (version 4.0.3). First, an appropriate soft-thresholding power was selected to approximate a scale-free network. Subsequently, the adjacency matrix was transformed into a Topological Overlap Matrix (TOM), which was then used to cluster and assign genes into distinct modules [12]. Module-trait relationships were calculated to identify modules significantly associated with clinical features. The module showing the strongest correlation with postoperative peritoneal adhesions was selected for subsequent analysis.

To identify candidate genes for further investigation, the intersection between differentially expressed genes (DEGs) and genes from the selected module was calculated using the "VennDiagram" package in R software (version 4.0.3) [13].

Function and Pathway Enrichment Analysis and Protein-Protein Interaction (PPI) Network Construction

Functional enrichment analysis, including GO and KEGG pathway annotations, was conducted using R software (version 4.0.3). Significantly enriched terms were identified using the criteria of $P < 0.05$, with the top 5 terms displayed for each category [14]. The STRING database was used to construct a Protein-Protein Interaction (PPI) network. The resulting PPI network was visualized using Cytoscape software. The top 10 proteins with the highest connectivity, representing the most central nodes in the interaction network, were subsequently identified.

Clinical Sample Collection and Processing

Three samples of adhesion bands and their corresponding normal peritoneal tissues were collected from patients undergoing peritoneal adhesion (PA) surgery at the Children's Hospital affiliated with Soochow University from September 2021 to September 2022. Normal peritoneal tissues were located 5 cm away from the adhesion bands. All samples used in this study were collected following approval from the Ethics Committee of the Children's Hospital affiliated with Soochow University and after obtaining informed consent from the patients. (Ethics No: 2022CS129). Subsequently, the collected clinical samples were subjected to Hematoxylin and Eosin (HE) staining, Masson staining, and immunohistochemistry.

Cell Transfection

The MET-5A cells (Wuhan Punosai Biotechnology Co., Ltd. CL-0666) and HFF-1 cells (Shanghai Qida Biotechnology Co., Ltd. CD0147) were cultured in M199 complete medium (Wuhan Punosai Biotechnology Co., Ltd. CM-0666) and DMEM medium (Hyclone SH3022.01), respectively, to adjust the cell status. Cells were trypsinized for 1 minute, harvested by centrifugation, and the supernatant was removed. The cell pellet was resuspended in fresh medium, and an aliquot was mixed with trypan blue for counting. Following concentration adjustment to 2×10^5 cells/mL, the cells were incubated overnight in a cell culture incubator.

Transfection was performed when the seeded cells reached over 70% confluency. The transfection complexes were prepared by mixing siRNA with OPTI-MEM at a ratio of 1 nmol : 250 μ L, and Lipofectamine 2000 with OPTI-MEM at a ratio of 5 μ L : 250 μ L. The mixtures were combined and incubated at room temperature for 15 minutes. Following transfection, the cells

were cultured in an incubator for 6 hours, after which cell morphology was observed. Cells were collected 24 hours post-transfection.

qRT-PCR and WB

Total RNA was extracted from MET-5A cells using TRIzol reagent (Suzhou Ruijie Biotechnology). Complementary DNA (cDNA) was synthesized by reverse transcription using a cDNA synthesis kit (Beijing TsingKe Biotech). Real-time quantitative PCR (RT-qPCR) was performed using the UltraSYBR One-Step RT-qPCR kit (Beijing TsingKe Biotech) according to the manufacturer's instructions. The expression levels were determined using the $2^{-\Delta\Delta CT}$ method with GAPDH as the reference gene.

Protein extraction was performed using the RIPA protein extraction kit (Shanghai Yisheng Biotechnology Co., Ltd.). The extracted proteins were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. The membrane was incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 hour at 37°C. Protein bands were visualized using a chemiluminescence imaging system (Shanghai QinXiang).

Cell growth assay

After successful transfection, si-NC and si-FN1 transfected HFF-1 cells were cultured for 48 hours. Subsequently, 10 μ L of CCK8 solution was added to each well, and the cells were further incubated at 37°C in a 5% CO₂ incubator in the dark for 1 hour. The absorbance at 450 nm wavelength was measured using a microplate reader at the same time point. The obtained OD values were used for the analysis of cell proliferation effects.

Cell migration

After collecting and processing the cells cultured for 48 hours, the cell density was adjusted to 10,000 cells per well, and they were seeded in the upper chamber of a 24-well culture plate with 200 μ L of medium per well. Subsequently, 800 μ L of medium containing 20% serum was added to the lower chamber. The plate was then incubated overnight at 37°C in a 5% CO₂ incubator. After 24 hours of cell culture, the insert was removed, and the upper chamber liquid was aspirated. The cells that had not migrated on the membrane were carefully wiped off using a cotton swab. The membrane was then washed twice with pre-warmed PBS at 37°C and fixed with 4% paraformaldehyde pre-cooled on ice for 30 minutes. After fixation, the membrane was stained with crystal violet for 10 minutes.

Carefully remove the polycarbonate membrane from the insert base, mount it on a slide, and count the cells that have infiltrated the back of the insert under a microscope. Random fields of view were captured under a light microscope.

Flow cytometry

After treating the cells for 48 hours, collect them by digesting with EDTA-free trypsin, followed by centrifugation at 1500 rpm for 5 minutes. Discard the supernatant. Resuspend the cells once with pre-chilled 1×PBS (4°C), then centrifuge again at 1500 rpm for 5 minutes to wash the cells. Suspend the cells in 300 μ L of 1×Binding Buffer. Add 10 μ L of Annexin V-APC (Kechuangda KCD-T1004) and 5 μ L of PI (Kechuangda KCD-T1004) to the mixture, and incubate in the dark at room

temperature for 15 minutes. Analyze the cells using a flow cytometer (BD FACSVerse), and process the data with the software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.0 software. In all analyses, $p < 0.05$ was considered statistically significant. All data were derived from three independent replicates and are presented as mean $\pm$ standard deviation (Mean $\pm$ SD).

Results

DEG Analysis

A total of 1,375 differentially expressed genes (DEGs) were identified. Among them, 923 genes were upregulated, and 452 genes were downregulated. The volcano plot and heatmap of these differentially expressed genes are shown in Figures 1 .

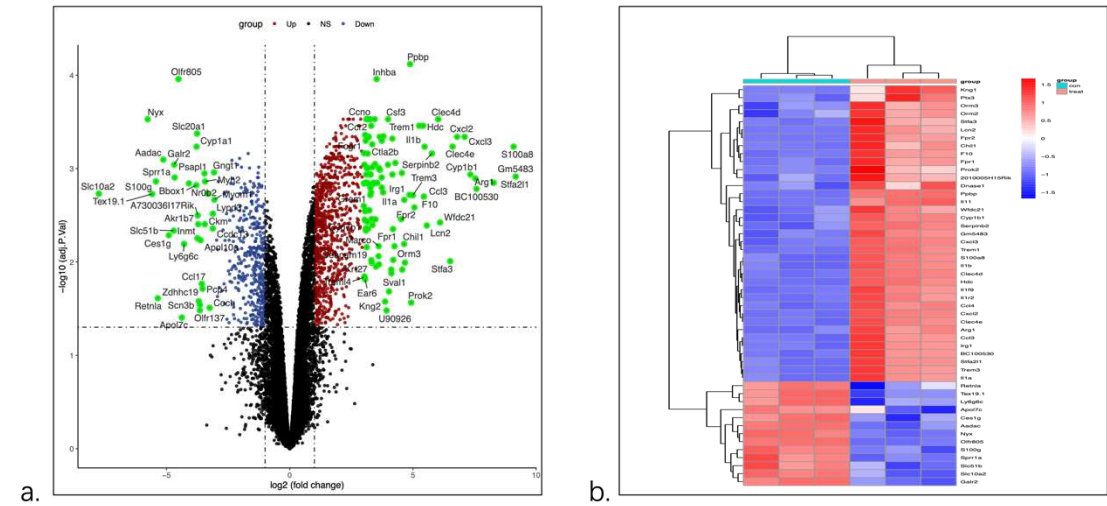


Figure 1.(a) Volcano plot of differential analysis from the GSE123413 dataset (Blue indicates downregulated genes, Red indicates upregulated genes, Black indicates genes with no significant differential expression (NS)).(b) Heatmap of the top 50 differential analysis results (Red indicates high expression, Blue indicates low expression).

WGCNA

The soft threshold (β) value was determined to be 25 (Figure 2a). Based on this value, a co-expression network was constructed, and modules were generated through expression similarity (Figure 2b). Further analysis was conducted to explore the relationship between sample characteristics and the modules (Figure 2c). Ultimately, the green module ($cor = 0.96$, $P < 0.05$) was identified as the module most highly associated with postoperative peritoneal adhesions (Figure 2d).

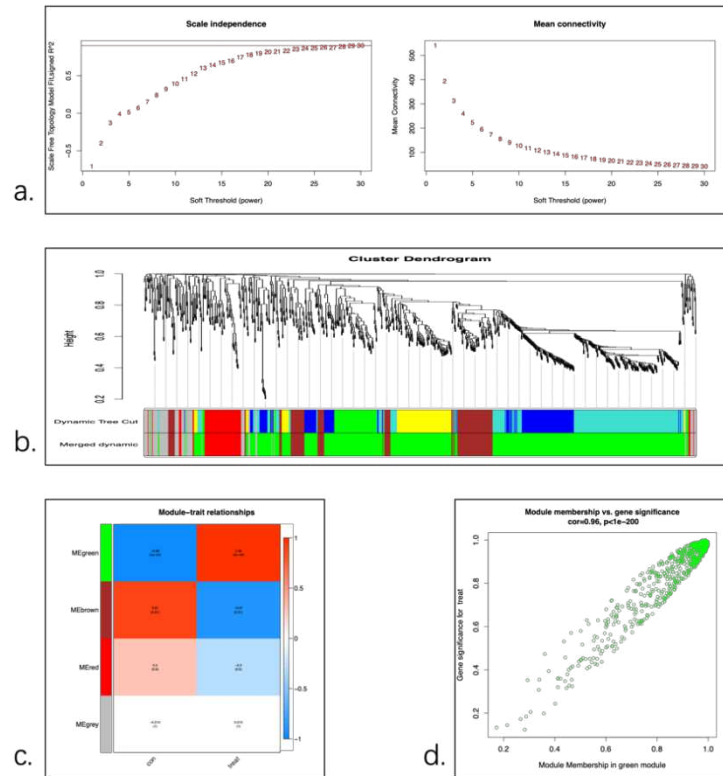


Figure 2.(a)Determination of the soft threshold in WGCNA analysis. (b)Results of co-expression module data analysis in WGCNA. (c)Pearson correlation coefficients between module feature genes and sample trait vectors, with numbers in parentheses indicating the corresponding P-values. (d)Correlation between module membership and gene significance, with "cor" representing the absolute correlation coefficient between GS and MM.

GO and KEGG Pathway Analysis Results

Using Venn analysis, the intersection of DEGs and genes from the green module was identified, resulting in 368 key genes related to postoperative peritoneal adhesions (Figure 3a). GO revealed that the most enriched processes were extracellular matrix organization, extracellular structure organization, collagen fibril organization, and collagen metabolic process. These enrichment results were filtered with $P < 0.05$ and visualized using the ggplot2 package in R software, displaying the top 5 enriched results in a bar chart (Figure 3b).

The KEGG pathway analysis indicated that the key genes were primarily enriched in pathways related to protein digestion and absorption, complement and coagulation cascades, ECM-receptor interaction, and focal adhesion signaling pathways. These results were also visualized in a bar chart (Figure 3c).

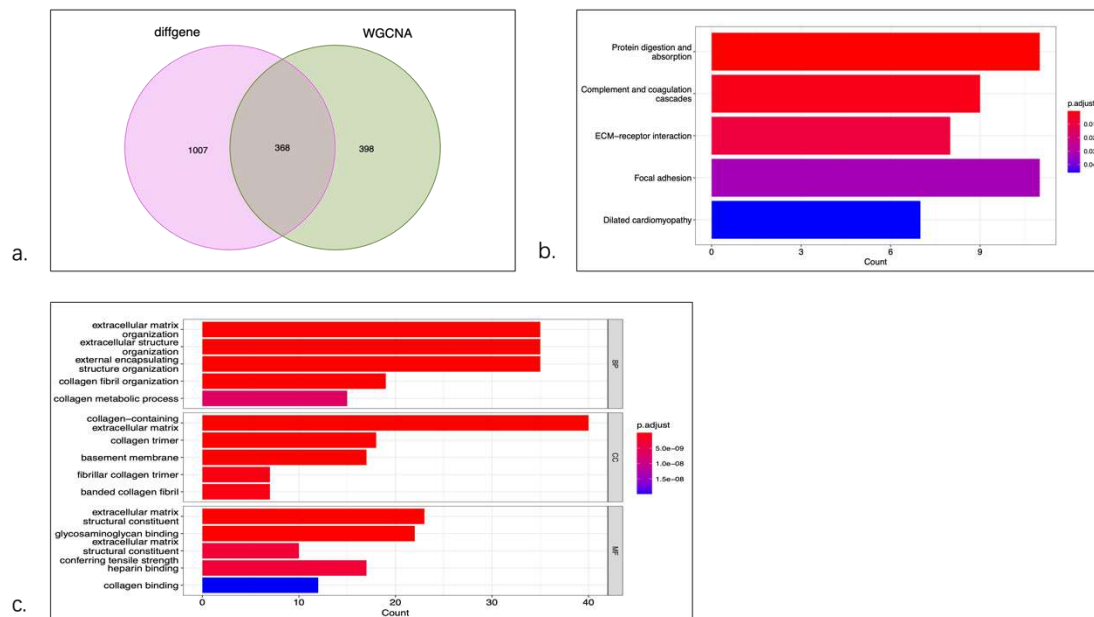


Figure 3.(a) Key gene analysis (Intersection of differentially expressed genes and the green module from weighted co-expression, resulting in 368 key genes).(b) Bar chart of key gene enrichment (from top to bottom: Biological Process (BP), Cellular Component (CC), Molecular Function (MF)).(c) Bar chart of key gene pathway enrichment.

Construction of PPI Network and Screening of Hub Genes

Using the 368 key genes related to postoperative peritoneal adhesions, identified by intersecting the DEGs and genes from the green module, a PPI (Protein-Protein Interaction) network was constructed using the STRING database (Figure 4a). This network provided information on the interactions between the corresponding proteins of these genes. The top 10 proteins with the most interactions were selected as hub proteins, and their corresponding genes were included in the study (Figure 4b). The KEGG pathway analysis of these hub genes showed that they were involved in pathways such as Focal adhesion and ECM-receptor interaction. Based on literature reports and the analysis, Fn1 was identified as the core gene [15].

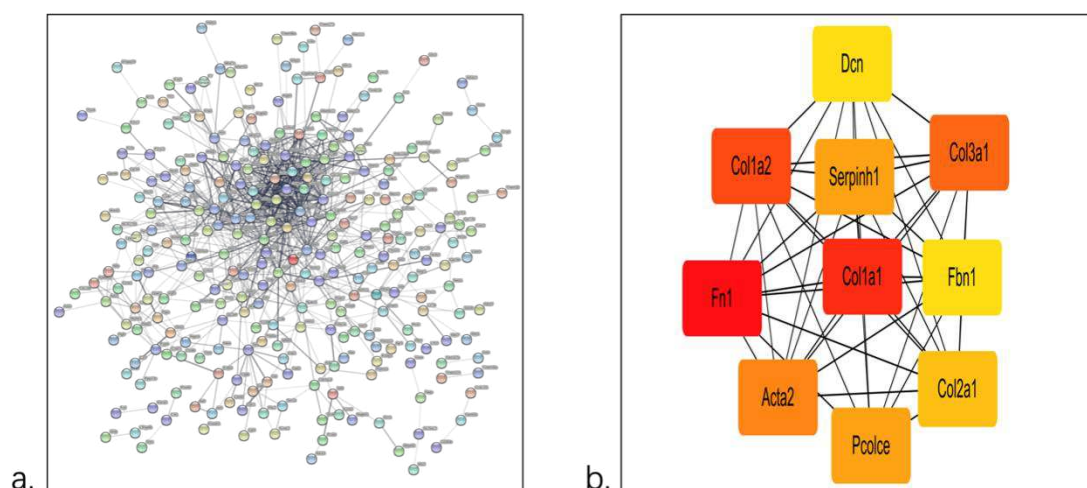


Figure 4.(a) PPI network of key genes.(b) Top 10 protein-protein interaction relationships.

HE Staining, Masson Staining, and Immunohistochemistry

HE staining was performed on abdominal adhesion band tissue. As shown in the figure, the continuity of the peritoneal tissue is disrupted, with varying degrees of thickening and denser fibrous tissue, resulting in darker staining. Inflammatory cell infiltration and neovascularization can be observed within the tissue (Figure 5). Masson staining results, as shown in Figure 6, indicate that compared to normal peritoneal tissue, the morphology of the abdominal adhesion tissue is disordered, with significant deposition of collagen fibers.

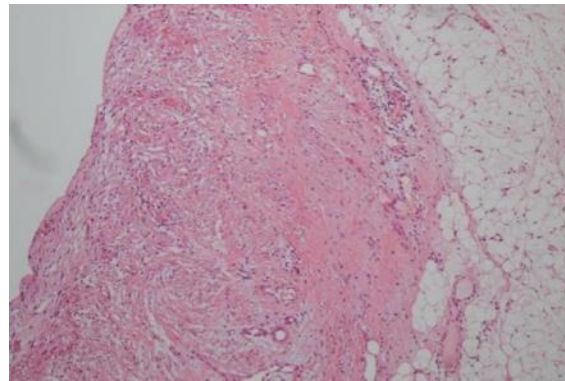


Figure 5. HE staining results of abdominal adhesion tissue (40×)

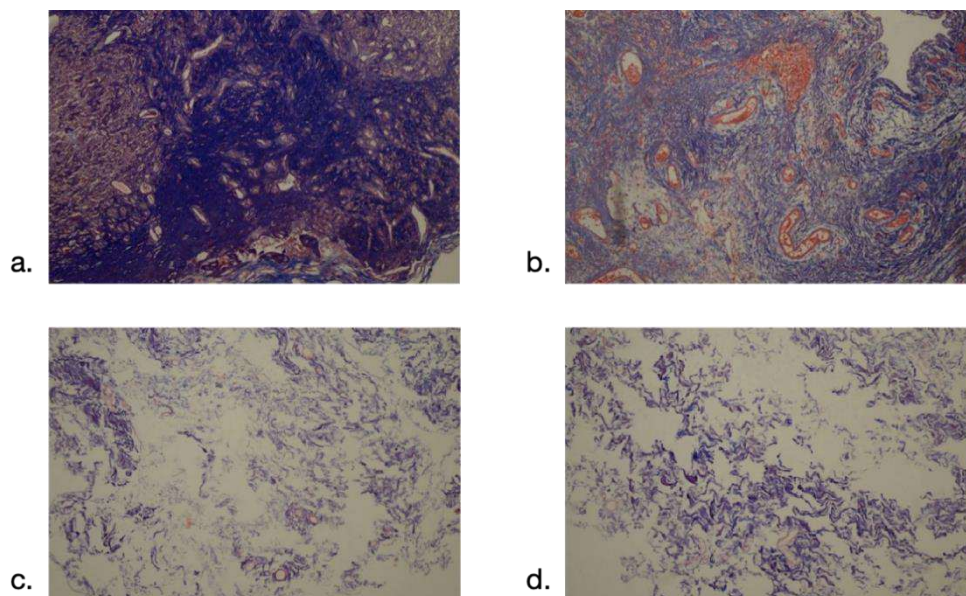


Figure 6. Masson staining results (a, b: abdominal adhesion tissue; c, d: normal peritoneal tissue. Pinkish-purple represents smooth muscle components, and blue-green represents collagen fibers. 40×)

Immunohistochemistry was used to assess the protein expression level of FN1 in the adhesion tissue (Figure 7). Compared with normal peritoneal tissue, the expression level of FN1 in the adhesion tissue was significantly elevated, and the difference was statistically significant ($P < 0.01$), which was consistent with the results of the bioinformatics analysis.

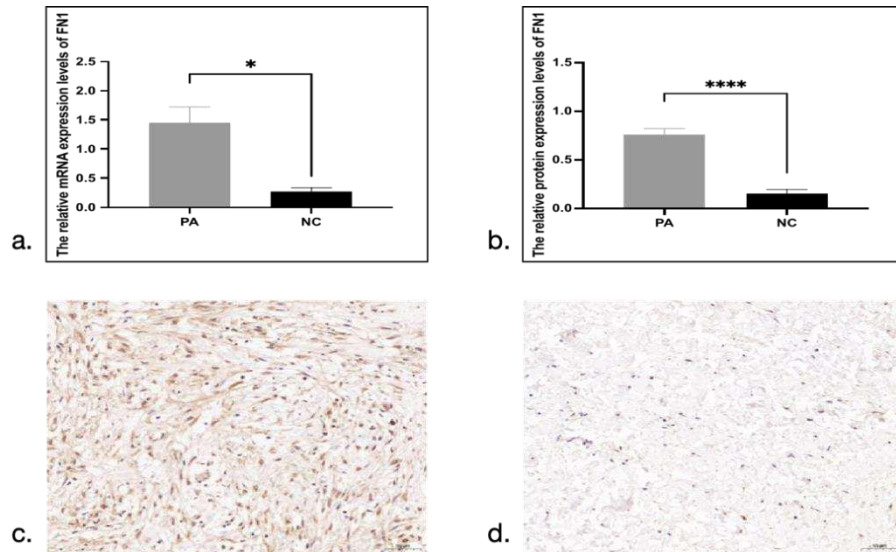


Figure 7. Expression of Fn1 in adhesion tissue and normal peritoneal tissue (Figure a shows the expression of Fn1 at the RNA level; Figure b shows the expression of Fn1 at the protein level in adhesion bands; Figure c shows the immunohistochemistry results of adhesion tissue; Figure d shows the immunohistochemistry results of normal peritoneal tissue).

Changes in PI3K and Akt Expression Levels After si-Fn1 Transfection in MET-5A Cells

The results (Figure 8a) show that the expression level of Fn1 in MET-5A cells was significantly downregulated after si-RNA transfection, with statistical significance ($P < 0.01$). Following transfection in MET-5A cells, Western blot analysis was used to detect the expression levels of PI3K, p-PI3K, Akt, p-Akt, and GAPDH. As shown in the figure (Figure 8b), the relative expression levels of PI3K, p-PI3K, Akt, and p-Akt were calculated based on protein content and grayscale values, with the results presented in Figures 8c and 8d.

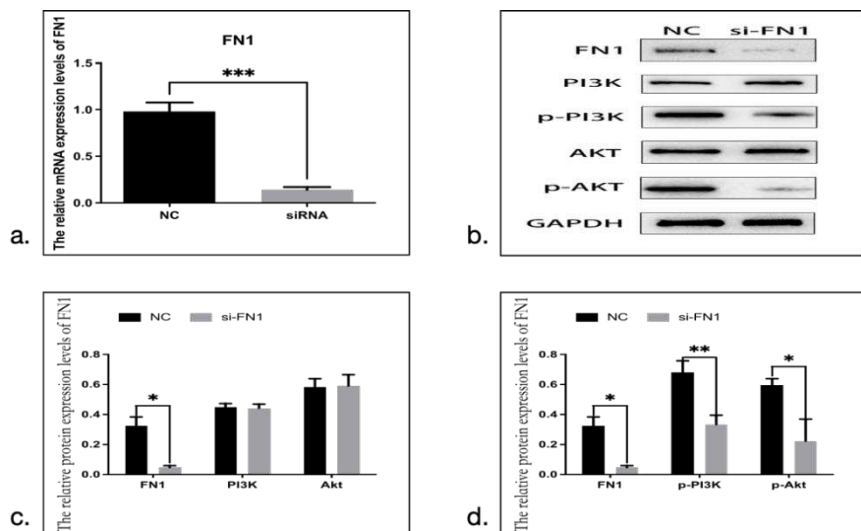


Figure 8. a. Results of si-RNA transfection in MET-5A cells, b. Western Blot results showing the bands for PI3K, p-PI3K, Akt, p-Akt, and GAPDH, c, d. Relative expression levels of PI3K, p-PI3K, Akt, p-Akt, and si-Fn1.

In MET-5A cells transfected with si-FN1, the FN1 protein band was markedly lighter than that in the control group. As shown in Figure 8, the difference in relative expression levels between the

two groups was statistically significant ($P < 0.05$). Grayscale analysis of total PI3K revealed no notable color change between the untreated group and the si-FN1 group. Accordingly, the relative expression analysis showed no statistically significant difference. Similar results were obtained for total Akt. However, for phosphorylated PI3K (p-PI3K) and phosphorylated Akt (p-Akt), the grayscale values were significantly lighter in the si-FN1 group, particularly for p-Akt. Statistical analysis confirmed significant differences between the two groups for both p-PI3K and p-Akt ($P < 0.05$). Therefore, we speculate that FN1 acts on the downstream PI3K/Akt pathway by promoting the activation (phosphorylation) of these proteins.

Effect of si-Fn1 Transfection on HFF-1 Cell Function

The results (Figure 9) show that FN1 expression was significantly downregulated in HFF-1 cells following siRNA transfection ($P < 0.01$). After transfection, cell proliferation, apoptosis, and migration were assessed using CCK-8 assay, flow cytometry, and migration assays, respectively. The results demonstrated that si-FN1 significantly inhibited proliferation and migration of HFF-1 cells, while enhancing apoptosis, compared to the si-NC group.

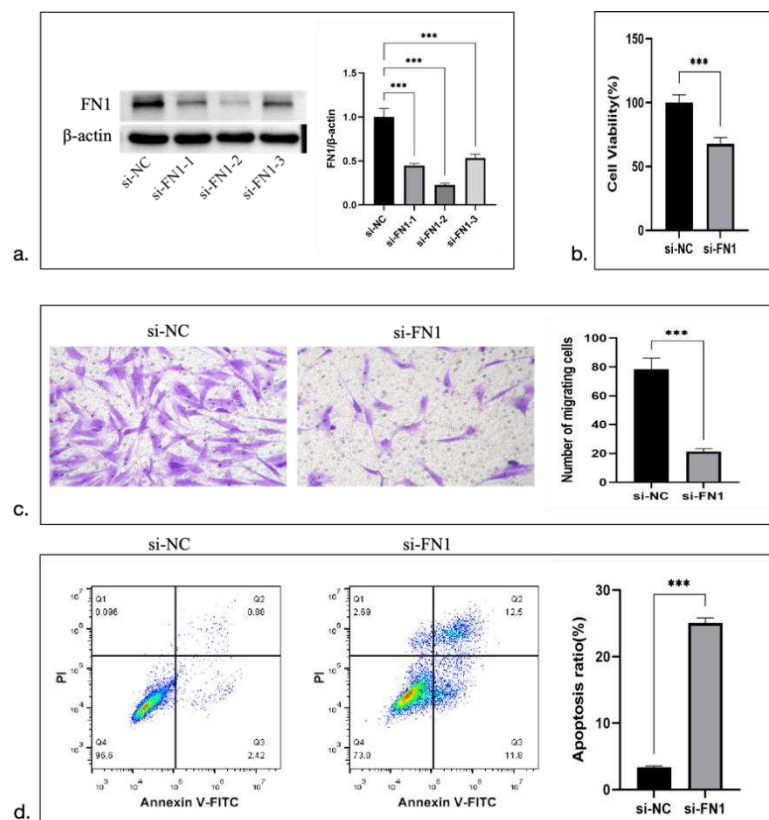


Figure 9.a. Results of si-RNA transfection in HFF-1 cells, b. CCK8 assay results, c. Transwell migration assay results, d. Flow cytometry apoptosis analysis

Discussion

Abdominal adhesion is a pathological phenomenon in which fibrous connective tissue abnormally links the peritoneum to the abdominal organs, or directly connects different abdominal organs. It can be caused by abdominal trauma, intra-abdominal infection, or abdominal surgery [16]. The formation of abdominal adhesions can lead to severe complications, with mechanical bowel obstruction being a common issue in pediatric cases. The essence of the adhesive process is an excessive response of the body's immune and wound

repair mechanisms. Following surgical trauma, normal peritoneal tissue experiences ischemia and hypoxia, leading to increased vascular permeability accompanied by the migration and infiltration of inflammatory cells. Reactive oxygen species (ROS) play a significant role in exacerbating tissue damage, resulting in the formation of fibrous aggregates through the actions of mesothelial cells, neutrophils, and macrophages. Concurrently, the transformation of mesothelial cells and the migration of fibroblasts promote excessive collagen deposition, ultimately leading to fibrous adhesion [17, 18]. Analysis of adhesion tissue samples reveals compromised mesothelial integrity, characterized by peritoneal loss, increased infiltration of inflammatory cells, and significant fibrous tissue proliferation, which is consistent with previously published findings [19, 20].

Numerous cells are involved in the formation of adhesions following abdominal surgery, among which mesothelial cells and fibroblasts play particularly significant roles. Mesothelial cells (MCs) contribute to the process through transdifferentiation, secretion of adhesion molecules, and production of inflammatory chemotactic factors. Fibroblasts, in contrast, promote tissue repair by depositing collagen within the fibrin matrix, thereby leading to permanent adhesion formation. The activation of fibroblasts is a critical step in peritoneal fibrosis [21]. Early during peritoneal injury, mesothelial cells can acquire a myofibroblast phenotype through transdifferentiation [22], thereafter, they migrate from the peritoneal surface into the underlying matrix, wherein they secrete collagen as well as other constituents of the extracellular matrix [23, 24]. Sandoval et al. found that the mesenchymal transformation of mesothelial cells is involved in the development of adhesion formation [22]. The transition of mesothelial cells to myofibroblasts has also been described in pleural tissue. Consistent with this finding, Uyama et al. demonstrated that adhesion-associated myofibroblasts primarily originate from mesothelial cells and are responsible for producing adhesion bands [8]. In addition to the MMT mechanism, mesothelial cells can also activate resident fibroblasts through ILK-rich extracellular vesicles, thereby promoting peritoneal fibrosis. Therefore, we selected mesothelial cells and fibroblasts as the tool cells for this study.

Fibronectin 1 (Fn1), an important member of the FN family, is composed of high-molecular-weight glycoproteins. Its biological roles primarily involve participation in cell matrix remodeling, cell adhesion, cell proliferation, and migration, which has made it a focus of extensive research in cancer and fibrotic diseases. Previous studies have shown that in diabetic cardiomyopathy mouse models, the severity of cardiac fibrosis is positively correlated with Fn1 expression levels [25]. Wang W. et al. examined kidney tissue from mouse models of diabetic kidney fibrosis and found that high expression of Fn1 exacerbates the degree of renal fibrosis [26]. Additionally, studies on neurogenic bladder fibrosis have shown that Fn1 is one of the key molecules in bladder fibrosis, and miR-219c-5p can target and inhibit Fn1, thereby alleviating the progression of bladder fibrosis [27]. The high expression of FN1 in abdominal adhesion tissue observed in this study is consistent with findings reported in the relevant literature.

The PI3K/Akt pathway plays a crucial role in fibrotic diseases. Studies have shown that this pathway significantly contributes to pulmonary fibrosis by promoting collagen expression and cell proliferation. In addition, it also plays a key role in the progression of fibrosis in the heart, kidneys, and liver. Qin W et al. have proposed that PI3K/Akt is of great importance in the

occurrence and development of cardiac fibrosis[28]. The role of Fn1 in the PI3K/Akt pathway is also reflected in its involvement in cartilage calcification and osteogenic differentiation [15]. To date, no studies have elucidated the mechanism by which Fn1 acts on the PI3K/Akt pathway during abdominal adhesion formation. In this study, we speculate that Fn1 may contribute to abdominal adhesion formation by affecting the phosphorylation of PI3K and Akt. Furthermore, we found that downregulating FN1 promotes fibroblast apoptosis while inhibiting cell proliferation and migration, leading us to further propose that Fn1 enhances the role of fibroblasts in the process of abdominal adhesion.

In summary, our data indicate that Fn1 may contribute to the formation of abdominal adhesions by affecting the phosphorylation of PI3K and Akt, offering new insights for future targeted prevention of abdominal adhesions.

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