EFFECT OF FIBULIN-2 ON PROLIFERATION AND APOPTOSIS OF GASTRIC CANCER CELLS BY INHIBITING TGF-β1/SMAD4 SIGNAL PATHWAY

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ABSTRACT

***Objective**: To investigate the effect of fibulin-2 on the proliferation and apoptosis of gastric cancer cells and its mechanism.*

***Methods**: This study analysed the expression of fibulin-2 and TGF-β1 in 36 gastric cancer samples and 36 normal gastric epithelial samples with immunohistochemistry and RT-PCR, respectively. The expression of fibulin-2 and pathological features of these clinical cases were also studied. In addition, the expression levels of fibulin-2, TGF-β1, Smad4 and pSmad4 in various gastric cancer cell lines were determined using RT-PCR and Western blot. Through transfecting a fibulin-2 mimic, we observed TGF-β1 expression at different times. In order to validate that fibulin-2 impacts the proliferation and apoptosis of gastric cancer cells through the TGF-β1/Smad4 signal pathway, we used MTT and flow cytometry to analyse the proliferation and apoptosis of cells in different groups, successively.*

***Results**: The expression of fibulin-2 was much lower in the gastric cancer samples than in the control group, but the expression of TGF-β1 was much higher in the gastric cancer samples. The expression and differentiation degree of fibulin-2 were significantly related to TNM staging. Thereinto, the expression of fibulin was positively associated with differentiation degree, and the positive rate of fibulin-2 declined from stage I to stage IV, successively. Besides, the fibulin-2 mimic greatly restrained the proliferation of gastric cancer cells and promoted their apoptosis compared to the control group.*

***Conclusions**: Fibulin-2 promotes the apoptosis of gastric cancer cells and restrains their proliferation through inhibiting the TGF-β1/Smad4 signal pathway.*

***Keywords**: Fibulin-2, TGF-β1, gastric cancer, proliferation and apoptosis.*

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Introduction

Fibulin-2, a new parent protein, does not participant in the integrity of tissue structure, but mainly involves the activation of regulatory enzymes, proteases and growth factors[1]. Spatially, fibulin-2 greatly up-regulates the site of epithelial-mesenchymal transition during embryogenesis, but its expression is restricted to the vascular endothelial basement membrane, heart valves, skin basal layer and perimembranous membrane[2]. It is up-regulated and healed during skin tissue repair[3]. Besides, its deletion can prevent cardiac rupture and ventricular dysfunction, and it significantly improves postoperative survival by abating TGF-β signalling[4].

TGF-β family members play a complex role in carcinogenesis. TGF-β was originally named for its transformation activity in in vitro assays, and now, it is clearly confirmed that TGF-β is a tumour suppressor with carcinogenic activity. In the current model, the inhibitory activity predominates in normal tissues, but changes in TGF-β expression and cellular responses contribute to its balance of carcinogenic activity during tumourigenesis[5]. In epithelia, this process usually refers to the responsiveness of tumour cells to TGF-β, the increasing secretion and/or weakening activation or quality changes of TGF-β ligands. Almost all cells produce and respond to ligands, so to understand the role of TGF-β in tumourigenesis requires some insight into the changing and response patterns of many interacting cell types, including stromal cells. As a multifunctional peptide growth factor, TGF-β plays an essential role in regulating cell proliferation, differ-

entiation, inflammation, angiogenesis and wound healing[6-8]. Excessive TGF-β plays an important role in the pathogenesis of incompatible remodelling and heart failure[9-11]. TGF-β acts as a downstream gonadotropin and pro-fibrotic growth factor induced by Ang II[12]. Even though excessive TGF-β is harmful to stress myocardia, baseline TGF-β is vital for protecting the integrity and structure of myocardia[13]. Thoroughly eliminating this important growth factor may not be completely benign, and TGF-β activation is regulated at several different sites, including intracellular signalling pathways and extracellular interactions[14].

We investigate the expression of fibulin-2 and TGF-β1 in gastric cancer samples, analyse the relationship between fibulin-2 and TGF-β1 expression and clinical manifestations in this study. What's more, using gastric cancer cell lines as models, we explore the effect of fibulin-2 on the proliferation and apoptosis of gastric cancer cells through the TGF-β1/Smad4 signalling pathway.

Materials and methods

Methods

Case and cell line source

Normal, low, medium and highly differentiated cell lines (GES-1, MKN45, SGC7901 and MKN28) were purchased from Shanghai Huiying Biological Co., Ltd. Thirty-six clinical specimens showed clear gastric cancer specimens and 36 normal gastric epithelial tissue specimens, which were divided into two groups: gastric cancer group and control group. A total of 36 gastric cancer samples and 36 normal gastric epithelial tissue samples from Changzhou Second People's Hospital affiliated to Nanjing Medical University from 2016 to 2017 were involved in this study and assigned into the gastric cancer group and control group, respectively.

Main reagents and instruments

The main reagents and instruments were as follows: BD LSR flow cytometry (BD Biosciences); Apoptosis detection kit (Bestbio, China); Thermomax microplate reader (Bio-Tek EL, USA); MTT (Sigma, USA); β-actin (Santa Cruz, USA); PVDF membrane (Millipore Corp, Billerica, MA, USA); mouse polyclonal anti-fibulin-2 (Proteintech, USA); BCA protein assay kit (Boster, China); RIPA lysis buffer (Beyotime, China).

Methods

Immunohistochemistry

After fixed, embedded, stained, dewaxed and dehydrated, antigen retrieval was conducted on the stomach tissue specimens by microwaving them in citrated saline for 15 min. The sections were deparaffinised and treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. They were further blocked with 2% bovine serum albumin and then reacted with primary antibody for 12 h at 4 °C. After being washed, the biotin-labelled secondary antibody was added and gently shaken at room temperature for 1 h. Finally, the DAB reaction was used to observe the results. The fibulin-2 positive region in the fibrotic region was then observed. Quantitative analysis was used to calculate five fields from each stomach slice.

Cell culture

In order to prevent the influence of TGF-β1 in FBS, cells must be cultured in serum-free DMEM containing double antibody, glutamine and amphotericin at 37 °C in a humidified incubator with 5% CO2. The cells were detected for each test when their density reached 70%-80%.

RT-PCR

RNA extracted using magnetic bead cell sorting was reverse transcribed into Cdna by RT-PCR reverse transcription kit, and fibulin-2 and TGF-β1 mRNA levels were analysed by quantitative real-time PCR. RT-PCR was performed based on the standard protocol using the following primers: fibulin-2: (forward direction: 5'-GCCTGCTTTCCT GGCTTCTCA-3'; reverse direction: 5'-TGGCAGCGGGATGGTGTTAG-3') and TGF-β1: (forward direction: 5'- GCTGCTGACCCCCACT-GAT-3', reverse direction: 5'-TGCCGGA-CAACTCCAGTGA-3'). The PCR was carried out at 94 °C for 5 min, followed by amplification of 30–35 cycles using the ABI 9700 at 94 °C for 40 s, 51 °C for 40 s and 72 °C for 1 min. Band intensity was measured by a densitometer, whose results were normalised with β-actin. The results were independently repeated at least three times with three different templates.

Western blot

Gastric tissues and cells were lysed with RIPA lysis buffer. The protein concentration in whole extracts prepared was analysed using the BCA pro-

tein assay kit. After being centrifuged at 12000 g for 10 min at 4 °C, the supernatant was collected and total protein was extracted. The protein concentration was determined by BCA method, and the whole protein extract was mixed with 5 × protein loading buffer (1:5), boiled in water for 15 min and cooled at 4 °C. The corresponding SDS-PAGE gel was prepared according to the molecular weight of the target protein. A protein sample denatured at 4 °C was added to the wells and loaded according to the protein concentration, so as to make sure that each well had the same volume of the total protein. Electrophoresis was carried out at a constant voltage of 220 V until the bromophenol blue reached the bottom of the gel and stopped. According to the molecular weight of the target protein, the gel was cut and then put into the transfer buffer. A PVDF film and six layers of filter paper were tailored according to the size of the gel. After being soaked in methanol for 10 s, the PVDF film and filter papers were placed into the transfer buffer. In accordance with the order of positive electrodes, three layers of filter paper PVDF film gel and three layers of filter paper in accordance with the order of negative electrodes were put into a transmembrane apparatus, and their edges were aligned to prevent foaming. They were transferred at a constant pressure of 60 V for 1 h. The protein-attached PVDF film was blocked overnight in 5% skim milk powder at 4 °C on a shaker. The blocked film was washed with TBST for 10 min, mixed with the corresponding ratio of primary antibody and incubated overnight at 4 °C. Afterwards, it was washed with TBST three times, 10 min per time, mixed with the corresponding secondary antibody and incubated for 3 h at room temperature on a shaker. After that, the mixture was re-washed with TBST three times, 10 min per time. Isometric A and B reagents in the ECL luminescence kit were symmetrically mixed, dropped on the PVDF film, fully contacted and exposed to light for 1 min. The excess liquid around the film was blotted with filter paper and placed in a gel imager and photographed with a dynamic integration mode to observe the results. The images were analysed using Lab Works 4.6 professional image analysis software.

Determination of cell proliferation

Live cell uptake and the ability to convert soluble MTT to formazan crystals were measured by MTT. The exponentially growing cells were seeded in 96-well plates at an initial density of 5 x 103/well, treated with different concentrations of fibulin-2 mimics and negative controls and maintained during the indicated time of culture. Then MTT (0.2 mg/mL) was added to the medium for culturing for an additional 2 h. The medium was replaced with acidified isopropanol (isopropanol solution with 0.04 N HCl), and the plate was incubated for 1 h at room temperature. The colorimetric absorbance of the samples was determined by a Spectramax M5 plate reader (Molecular Devices, CA). Cell proliferation was expressed as the percentage of cell viability of matrine-treated cells relative to the control cells untreated. All experiments were performed in triplicate and repeated at least three times.

***Analysis of apoptos*is**

For apoptosis analysis, the quantification of apoptotic cells was performed using the Annexin-V-FITC apoptosis detection kit according to the instructions of the manufacturer. Early apoptotic cells, defined as annexin-V-FIT, PI-negative cells, were analysed on a BD LSR flow cytometer. The experiment was repeated three times.

***Statistical analysi*s**

All data were expressed as mean ± SE. The statistical significance was analysed by Student's t test for means comparison or one-way ANOVA and post Dunne's test. The difference was statistically significant with $P < 0.05$.

Results

Expression of fibulin-2 and TGF-β1 in clinical cases

The immunohistochemical results on gastric cancer samples and normal gastric epithelial tissues showed that the positive rate of fibulin-2 in gastric cancer samples was only about 1/3 of that of normal tissues, which was significantly lower than that in the normal control group ($P < 0.05$). The expression of TGF-β1 mRNA in tissue samples was detected by RT-PCR, with findings that revealed the expression of TGF-β1 mRNA in gastric cancer tissues was significantly higher than that in the normal control group ($P < 0.01$) (Figure 1).

Relationship between fibulin-2 expression and pathological features in clinical cases

In order to seek the relationship between fibulin-2 expression and pathological features in clinical

cases, with statistical analysis, it was found that fibulin-2 expression was correlated with the degree of differentiation and TNM staging. Also, the positive rate of fibulin-2 was positively correlated with the degree of differentiation.

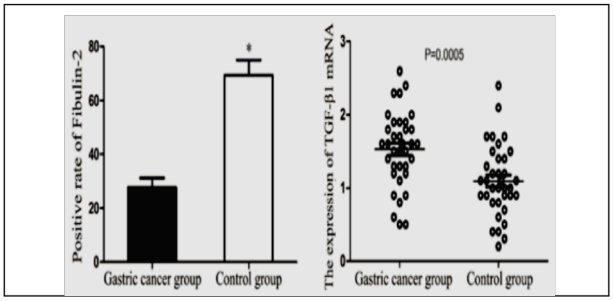

Figure 1. Expression of fibulin-2 and TGF-β1 in clinical cases. *represents $P < 0.05$, with a significant difference.

In addition, fibulin-2 expression was definitely associated with cancer TNM staging, and the positive rate of fibulin-2 declined in turn according to the order of stage I, II, III and IV (stage I: 40%; stage II: 33.3%; stage III: 27.2% and stage IV: 15.4%) (Figure 2).

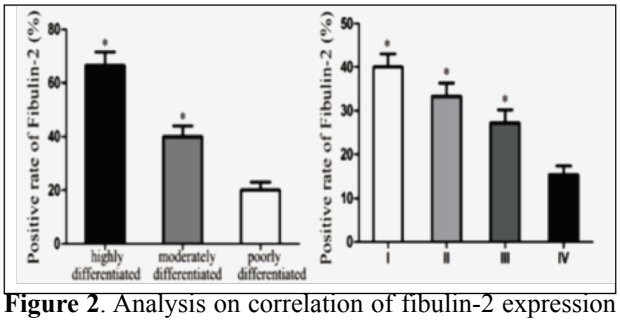

Figure 2. Analysis on correlation of fibulin-2 expression and the pathological features of gastric cancer. *represents $P < 0.05$, with a significant difference; **represents $P < 0.01$, with an extremely significant difference.

Fibulin-2 and TGF-β1 expression in gastric cancer cell lines

To clarify the intrinsic relationship between fibulin-2 and TGF-β1 in gastric cancer cells, their expression in different cell lines was detected by RT-PCR and Western blot, respectively. The results revealed that fibulin-2 expression was lower in low, medium and highly differentiated gastric cancer cell lines than in the normal gastric epithelial cell lines. What's more, with the increase in degree of differentiation, its expression level was also elevated.

However, TGF-β1 expression was significantly higher in various differentiated gastric cancer cell lines than in the normal gastric epithelial cell lines. And with the increase of the degree of differentiation, its expression level was gradually reduced. Consequently, it was suggested that the degree of differentiation of gastric cancer cells was negatively correlated with fibulin-2 and TGF-β1 (Figure 3).

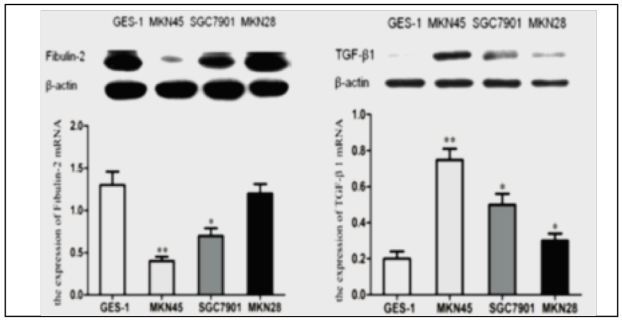

Figure 3. Fibulin-2 and TGF-β expression in various cell lines were detected by RT-PCR and Western blot. *represents $P < 0.05$, with a significant difference; **represents $P < 0.01$, with an extremely significant difference.

Effect of fibulin-2 mimics on TGF-β1 and Smad4 expression in gastric cancer cell lines

In order to further investigate whether fibulin-2 affects the TGF-β1/Smad4 signalling pathway, the expression levels of TGF-β1, Smad4 and pSmad4 in the fibulin-2 mimic group and the control group at different times were detected using Western blot. As shown in Fig. 4, the expression of TGF-β1 mRNA was gradually decreased in the fibulin-2 mimic group as time went on and fell after 48 h.

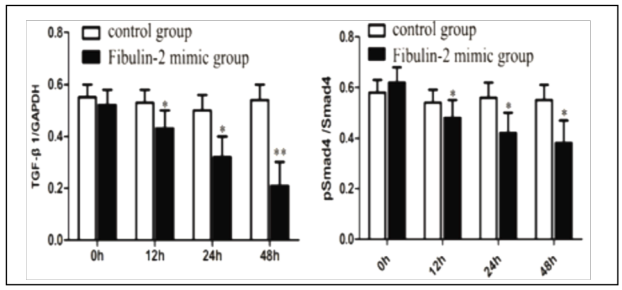

Figure 4. Western blot was used to detect the expression levels of TGF-β1 and pSmad4/Smad4 in the control group and fibulin-2 mimic groups at 0, 12, 24 and 48 h, respectively. *represents $P < 0.05$, with a significant difference; **represents $P < 0.01$, with an extremely significant difference.

Also, the expression of TGF-β1 mRNA was significantly higher in the fibulin-2 mimic group than in the control group at 12 h and 24 h. In addition, as time prolonged, the ratio of pSmad4/Smad4 was much lower in the fibulin-2 mimic group than in the control group. The findings indicated that the fibulin-2 mimic inhibited the TGF-β1/Smad4 signalling pathway.

Effect of fibulin-2 mimics on the proliferation and apoptosis of gastric cancer cells

In order to investigate the effect of fibulin-2 on

the proliferation of gastric cancer cells, the effect of fibulin-2 mimics on the proliferation of gastric cancer cell line SGC7901 was determined by MTT assay. We measured fibulin-2 mimics and the control groups at 0, 24, 48 and 72 h and found that the proliferation of gastric cancer cell line SGC7901 was significantly inhibited in the fibulin-2 mimic group, and the cell proliferation at 48 h in the fibulin-2 mimic group was close to two times more than that in the control group (Figure 5).

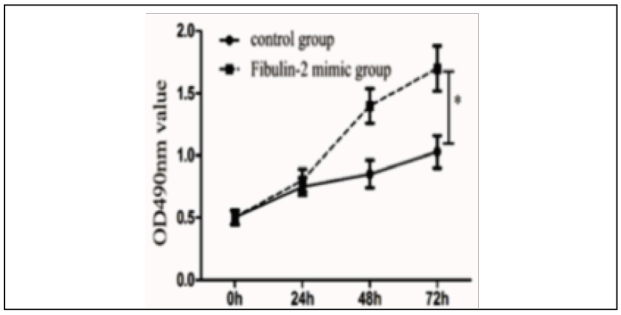

Figure 5. Cell proliferation was detected at 0, 24, 48 and 72 h with MTT.

Fibulin-2 promotes the apoptosis of gastric cancer cells through inhibiting the TGF-β1/ Smad4 signal pathway

In order to further clarify whether fibulin-2 promotes the apoptosis of gastric cancer cells by inhibiting the TGF-β1/Smad4 signalling pathway, we set up a control group, fibulin-2 mimic group, TGF-β1/Smad4 signalling pathway promoter group and fibulin-2 mimic + TGF-β1/Smad4 signalling pathway promoter group for detection and determined the apoptosis rate of SGC7901 cells at 0, 24 and 48 h by flow cytometry.

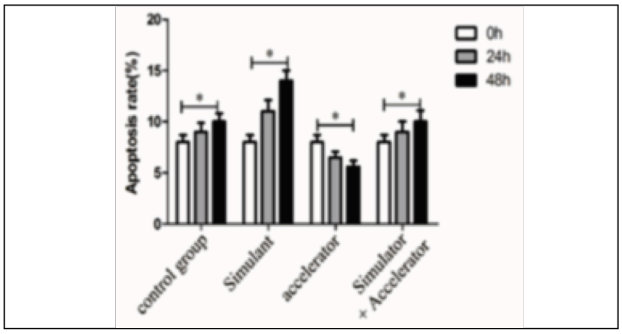

Figure 6. Apoptosis rate of gastric cancer cells detected by flow cytometry. *represents $P < 0.05$, with a significant difference.

The findings suggested that the fibulin-2 mimic group greatly advanced the apoptosis of SGC7901 cells, and as time prolonged, the apoptosis rate was largely decreased in the TGF-β1/Smad4 signalling pathway promoter group. There was no significant difference in the apoptosis rate in the fibulin-2 mimic + TGF-β1/Smad4 signalling pathway promoter group. These findings fully demonstrated that fibulin-2 promoted the apoptosis of gastric cancer cells by inhibiting the TGF-β1/Smad4 signal pathway (Figure 6).

Discussion

Fibulin-2, an ECM protein, interacts with a variety of other ECM proteins and can merge into various extracellular structures[15]. It plays an important role in embryonic development, particularly in the site of epithelial-mesenchymal transition and tissue remodelling of adults as well as in skin wound repair and the pathological induction of vascular lesions[16]. Furthermore, fibulin-2 is induced by TGF-β1 both in vitro and in vivo, but no constitutive expression of fibulin-2 is observed in any of the tested models. Therefore, it is concluded that fibulin-2 may play a causative role after TGF-β secretion, rather than during basic physiological adult neurogenesis.

Multicellular organisms have many mechanisms to prevent tumourigenesis. Apart from the strict control of the cell cycle, cells must also be monitored and maintain the integrity of their genetic information. Cancers are activated through suicidal or forced aging sensations and abnormal responses. Tight control of genes determines cell lifespan, and the proper functions and spatial relationship between it and other cells are assessed.

TGF-β is necessary for maintaining genomic stability, inducing replicative senescence and inhibiting telomerases[17]. In tissues with a higher level, TGF-β1 appears to be important for preventing early tumourigenesis in the colon by keeping the tissue structure normal[18]. Epithelial Smad4 is critical for preventing inappropriate angiogenesis[19]. Obviously, we must consider the simple changes in cell turnover now, and start to determine other molecular mechanisms impacted, so as to fully understand the role of TGF-β in restricting tumours.

In general, both alleles of a tumour-suppressing gene must be inactivated to form a tumour. The TGF-β pathway may not operate in this simple switching mode due to its dual tumour suppressors and carcinogenesis. For mice, only the loss of one allele in TGF-β1 is sufficient to alleviate growth inhibition and endanger tumour suppressor function, thus predicting changes in TGF-β1 levels may

impact human's susceptibility to cancer. A gradient descent in TGF-β1 production from ascending colon to rectum may lead to a higher incidence of colon tumours in the distal colon[20]. A possible threshold effector mechanism is elucidated in endothelial cells, and the elevated level of TGF-β may actually involve different type I receptors, resulting in the activation of various downstream mediators[21-23].

To sum up, we have confirmed that fibulin-2 mediates the proliferation and apoptosis of gastric cancer cells. It may advance the apoptosis of gastric cancer by inhibiting the TGF-β1/Smad4 signal pathway. However, the specific intrinsic mechanism is unclear, thus deserving further study.

References

1) Yasmin, Maskari RA, McEniery CM, Cleary SE, Li Y, et al. The matrix proteins aggrecan and fibulin-1 play a key role in determining aortic stiffness. Sci Rep 2018; 8: 8550.
2) Susek KH, Korpos E, Huppert J, Wu C, Savelyeva I, et al. Bone marrow laminins influence hematopoietic stem and progenitor cell cycling and homing to the bone marrow. Matrix Biol 2018; 67: 47-62.
3) Tan H, Zhang J, Fu D, Zhu Y. Loss of fibulin-2 expression is involved in the inhibition of breast cancer invasion and forms a new barrier in addition to the basement membrane. Oncol Lett 2017; 14: 2663-2668.
4) Mouton AJ, Rivera OG, Lindsey ML. Myocardial infarction remodeling that progresses to heart failure: a signaling misunderstanding. Am J Physiol Heart Circ Physiol 2018; 315: 71-79.
5) Kelly RJ, Giaccone G. Lung cancer vaccines. Cancer J 2011; 17: 302-308.
6) Ozkan K, Eralp L, Kocaoglu M, Ahishali B, Bilgic B, et al. The effect of transforming growth factor β1 (TGF-β1) on the regenerate bone in distraction osteogenesis. Growth Factors 2007; 25: 101-107.
7) Theron AJ, Anderson R, Rossouw TM, Steel HC. The role of transforming growth factor beta-1 in the progression of HIV/AIDS and development of non-AIDS-defining fibrotic disorders. Front Immunol 2017; 8: 1461.
8) Qiao YC, Chen YL, Pan YH, Ling W, Tian F, et al. Changes of transforming growth factor beta 1 in patients with type 2 diabetes and diabetic nephropathy: a PRISMA-compliant systematic review and meta-analysis. Medicine 2017; 96: 6583.
9) Gerlach CV, Das SR, Volz DC, Bisson WH, Kolluri SK, et al. Mono-substituted isopropylated triaryl phosphate, a major component of Firemaster 550, is an AHR agonist that exhibits AHR-independent cardiotoxicity in zebrafish. Aquat Toxicol 2014; 154: 71-79.
10) Bagchi RA, Roche P, Aroutiounova N, Espira L, Abrenica B, et al. The transcription factor scleraxis is a critical regulator of cardiac fibroblast phenotype. BMC Biology 2016; 14: 21.
11) Busato KC, Gomes RA, Ladeira MM, Duarte MS, Freita NC, et al. Expression of genes related to the regulation of muscle protein turnover in Angus and Nellore bulls. J Anim Sci 2016; 94: 1472-1481.
12) Wu J, Jackson-Weaver O, Xu J. The TGFβ superfamily in cardiac dysfunction. Acta Biochim Biophys Sin (Shanghai) 2018; 50: 323-335.
13) Chen L, Paquette N, Mamoor S, Rus F, Nandy A, et al. Innate immune signaling in Drosophila is regulated by TGFβ-activated kinase (Tak1)-triggered ubiquitin editing. Journal of Biological Chemistry 2017; 292: 8738-8749.
14) Yamada Y, Matsumoto T. House dust mites induce production of endothelin-1 and matrix metalloproteinase-9 in keratinocytes via proteinase-activated receptor-2 activation. Int Arch Allergy Immunol 2017; 173: 84-92.
15) Tan H, Zhang J, Fu D, Zhu Y. Loss of fibulin-2 expression is involved in the inhibition of breast cancer invasion and forms a new barrier in addition to the basement membrane. Oncol Lett 2017; 14: 2663-2668.
16) Markova DZ, Pan TC, Zhang RZ, Zhang G, Sasaki T, et al. Forelimb contractures and abnormal tendon collagen fibrillogenesis in fibulin-4 null mic. Cell Tissue Res 2016; 364: 637-646.
17) Matsumoto H, Fukui E, Yoshizawa M. Molecular and cellular events involved in the completion of blastocyst implantation. Reprod Med Biol 2016; 15: 53-58.
18) Xie L, Zhou D, Xiong J, You J, Zeng Y, et al. Paraquat induce pulmonary epithelial-mesenchymal transition through transforming growth factor-β1-dependent mechanism. Exp Toxicol Pathol 2016; 68: 69-76.
19) Carl C, Flindt A, Hartmann J, Dahlke M, Rades D, et al. Ionizing radiation induces a motile phenotype in human carcinoma cells in vitro through hyperactivation of the TGF-beta signaling pathway. Cell Mol Life Sci 2016; 73: 427-443.
20) Thomson JP, Ottaviano R, Unterberger EB, Lempiäinen H, Muller A, et al. Loss of Tet1-associated 5-hydroxymethylcytosine is concomitant with aberrant promoter hypermethylation in liver cancer. Cancer Res 2016; 76: 3097-3108.
21) Raven N, Schillberg S, Rasche S. Plant cell-based recombinant antibody manufacturing with a 200 L orbitally shaken disposable bioreactor. Methods Mol Biol 2016; 1385: 161-172.
22) Feng J, Fu Z, Guo J, Lu W, Wen K, et al. Overexpression of peroxiredoxin 2 inhibits TGF-β1-induced epithelial-mesenchymal transition and cell migration in colorectal cancer. Mol Med Rep 2014; 10: 867-873.
23) Khan SA, Dong H, Joyce J, Sasaki T, Chu ML, et al. Fibulin-2 is essential for angiotensin II-induced myocardial fibrosis mediated by transforming growth factor (TGF)-β. Lab Invest 2016; 96: 773-783.

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