

## Recombinant fibromodulin has therapeutic effects on diabetic nephropathy by down-regulating transforming growth factor- $\beta$ 1 in streptozotocin-induced diabetic rat model

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### ABSTRACT

**Objective(s):** Diabetic nephropathy is an important long-term complication of diabetes mellitus which appears to be partially mediated by an increase in secretion of transforming growth factor- $\beta$  (TGF- $\beta$ ). Fibromodulin, the small leucine-rich proteoglycan, has been proposed to be the potent TGF $\beta$ 1 modulator. In this study, the therapeutic effects of recombinant adenoviral vectors expressing fibromodulin on TGF- $\beta$ 1 expression on diabetic nephropathy were assessed.

**Materials and Methods:** Forty-eight Sprague-Dawley rats were divided into 4 groups: STZ-induced diabetic rats (diabetic-control), fibromodulin adenovirus vector treated STZ rats (Ad- fibromodulin), and Ad-lacZ-treated STZ rats (Ad-lacZ), and vehicle control (PBS-control). At 10 weeks after STZ treatment, we measured urinary albumin excretion (UAE), urine creatinine was measured by Jaffe method. We also measured kidney TGF- $\beta$ 1 levels by reverse transcription polymerase chain reaction and Real-time PCR.

**Results:** Urine albumin to creatinine ratio or UAE level were listed in four groups. UAE difference between healthy and diabetic rats in all three groups were significant ( $P \leq 0.005$ ) and between the control group and treated groups were not significant. Our results indicated that TGF- $\beta$ 1 gene expression in diabetic rats were increased and difference between normal group and diabetic group were significant ( $P \leq 0.001$ ). Fibromodulin gene transfection mediated by a recombinant adenovirus decreased TGF- $\beta$ 1 level in STZ-induced diabetic rats and TGF- $\beta$ 1 mRNA in diabetic kidney were reduced 2 weeks after Ad-fibromodulin injection.

**Conclusion:** Intraperitoneal injection of adenoviral vectors expressing fibromodulin reduced TGF- $\beta$ 1 level in diabetic rat models. The molecular mechanisms involved in this process require further study.

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### Introduction

Diabetic nephropathy (DN) is the prominent cause of end-stage renal disease and common cause of death in diabetes (1). It is characterized by progressive proteinuria, decline in kidney function, arterial hypertension, and increased risk of cardiovascular disease (2). Although there have been significant advances in the last decade with regard to the cure and prevention of diabetic kidney disease, pathophysiological mechanisms of DN were not fully understood (3). Transforming growth factor-beta (TGF- $\beta$ ) is an important cytokine widely associated with the development of fibrosis and glomerulo-

sclerosis in DN (4). TGF- $\beta$  has multiple subtypes, but TGF- $\beta$ 1 is one of the most important (5). TGF- $\beta$ 1 is a potent mediator in fibrotic processes. In addition, it plays a key role in induction of both (6) albuminuria (7-9) and apoptosis in podocytes (6). Elevated renal TGF- $\beta$ 1 mRNA and protein levels have been reported in various animal models and the human form of DN (10). This molecule has the potential to be useful for therapeutic applications. Much effort has been put into finding inhibitors of TGF- $\beta$ 1 such as TGF- $\beta$ 1 antibodies and soluble TGF- $\beta$ 1 type-II receptors (11). Decorin is a natural inhibitor of TGF- $\beta$ 1 found in the interstitial extracellular matrix (12).

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Studies of decorin gene transfer in diabetic nephropathy have demonstrated significant inhibition of TGF- $\beta$ 1 (13). In the context of fibromodulin for treatment of diabetic nephropathy, few studies have been done (14-15), Fibromodulin is a more effective competitor for TGF- $\beta$ 1 binding than decorin in heart disease (12). There are different routes for introducing genes into the kidney: renal artery, renal vein and direct injection into the kidney parenchyma. However, all these routes require major surgical invasion. Previous work has shown that intraperitoneal injection of an adenoviral vector leads to transgene expression in multiple organs; expression levels were highest in the liver, followed by the kidney, spleen, prostate and lung. Fibromodulin (FMOD) is a 59 kd proteoglycan which is present in many types of connective tissues such as tendon, skin, cartilage, sclera, and cornea. FMOD is a small leucine-rich proteoglycan (16) which along with other small leucine-rich proteoglycans such as cytokines, ECM components, cell-surface receptors, and growth factors such as TGF- $\beta$  ligands to modulate their activity side (14).

## Materials and Methods

### Recombinant adenovirus construct

The construct of recombinant adenovirus expression (Rads) comprising fibromodulin or lacZ cDNA were developed by Paul Kingston of the Gene Therapy Unit at the University of Manchester. Rad vectors were E1/E3-deleted first-generation adenoviruses, in which a recombinant transgene and promoter were inserted in place of the E1 region. LacZ and FMOD expression were compelled by a murine CMV (mCMV) promoter. The Woodchuck hepatitis-virus post-transcriptional regulatory element (WPRE) bring about significantly higher lacZ expression levels (eight fold) in primary endothelial cells (18). The efficiency of these vectors was confirmed in a previous study (19).

### Cell culture

Human kidney 293 cells were obtained from the Pasteur Institute (IRAN), maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, and grown in 5% CO<sub>2</sub> at 37 °C (20). Confluent HEK cells, 2×10<sup>5</sup> cells/60 mm-dish, in conditioned 2% medium were treated with Rad-FMOD (MOI=1000) or Rad-lacZ. After 5 hr of incubation, the supernatant was discarded. Five ml of standard medium per 60-mm dish was added. Uninfected cells cultured in the same conditions served as negative control (13). The cells were collected at 72 hr to detect FMOD mRNA using reverse transcriptase polymerase chain reaction (RT-PCR).

### RT-PCR

Total RNA was extracted from renal tissue by means of RNeasy Mini Kit, according to company protocols (Qiagen Cat no: 74104). The concentration

of total RNA was measured by NanoDrop 2000 (Thermo Scientific). The sequences of the primers were: Fmod Forward 5'-TGA AGG CAG CAC CTG ACC GC-3' Fmod Reverse 5'-ACG CCT TGG CTT CTC CTG CC-3(196 bp)  $\beta$ actin Forward5'- CAC CCG CGA GTA CAA CCT TC-3'  $\beta$ actin Reverse5'- CCC ATA CCC ACC ATC ACA CC-3' (207bp). The PCR product was run on a 1% (w/v) agarose gel and then visualized with ethidium bromide staining under UV-light (21).

### Bioactivity of rads encoding fibromodulin in vitro

The medium was removed from 25 cm<sup>2</sup> flasks containing HEK 293 cells, and replaced with 3 ml of DMEM supplemented with 0.1% bovine-serum albumin and 2 mM of glutamine. The cells were then infected with RAds-encoding fibromodulin or  $\beta$ -galactosidase at an MOI of 1000. After 5 hr of incubation, the supernatant was discarded and 5 ml of standard medium per 25 cm<sup>2</sup> flask was added. The cells were incubated at 37 °C in 5% CO<sub>2</sub> for 72 hr. Then, the cells were collected to detect mRNA by reverse transcriptase PCR (22).

### Animal model

Sprague-Dawley rats (adult male) with a body weight ranging from 250 to 300 g were used for experiment. All animals were kept at constant temperature and humidity, under a 12/12 hr light-dark cycle, and had free access to food and water. The rats were made diabetic with a single intraperitoneal (IP) dose of streptozotocin (STZ; Sigma, 55 mg/kg) (23), while control rats received an equivalent volume of the vehicle (citrate buffer, pH 4.5). Blood samples were obtained from the tail vein of each rat 72 hr after STZ injection, and blood glucose levels were measured with Glucometer (Accu-Chek Active). Eight weeks after the STZ injection, 36 rats were randomly divided into three groups (n=12). One group received a 0.6 ml (1 × 10<sup>9</sup> PFU/ml) IP injection of Rad-fibromodulin. Another group received a 0.6 ml (1 × 10<sup>9</sup> PFU/ml) IP injection of recombinant adenovirus encoding lacZ (1 × 10<sup>9</sup> PFU/ml). The last group received a 0.6 ml IP injection of PBS. The 12 control rats that were given diabetes also received a 0.6 ml IP injection of PBS (3). At 10 weeks after STZ injection, individual rats were placed in metabolic cages for urine to be collected every 24 hr. The animals were then sacrificed and the blood and kidneys collected for examination. The urine samples were reserved at -20 °C until later measurements of creatinine (Jaffé assay) and albumin (competitive ELISA). The kidneys were quickly removed, thoroughly washed with saline to remove the blood, and kept at -80 °C for RT-PCR and real-time PCR analysis. The kidney hypertrophy index (KHI) was considered as the ratio of kidney weight (g) to body weight (kg). Urine albumin excretion (UAE) values were considered as

the ratio of urinary albumin/urinary creatinine (mg/mg).

**Real-time PCR**

After extraction of total RNA from tissue samples or cells, first-strand cDNA was produced for each sample with the QuantiTect Reverse Transcription Kit, according to company protocols (Qiagen Cat No: 205311). Quantitative real-time PCR experiment was performed using an ABI 7300 sequence detector (Applied Biosystems). The PCR reactions were performed in 25 µl containing 1x of Quanti Fast SYBR Green PCR Master Mix, 10 pM of each forward and reverse primers, and cDNA corresponding to 100 ng RNA. PCR reactions for TGF-β1 and β-actin were performed using the following cycle conditions: 95 °C/5 min, followed by 35 cycles at 95 °C/10 sec and 60 °C/30 sec. The sequences of the primers were: TGFβ1 Forward 5'- GGT AAC CGG CTG CTG ACC-3' TGFβ1 Reverse 5'- GCC CTG TAT TCC GTC TCC TTG-3' (101bp) β-actin Forward 5'-GCTACAGCTTCACCACCA CAG-3' β-actin Reverse 5'- GGT CTT TAC GGA TGT CAA CGTC-3' (288 bp)(24).

**Statistical analysis**

All the data were analysed by IBM SPSS statistics version 19.0. The Mann-Whitney U test was used to compare mean values between two groups. The ANOVA or Kruskal-Wallis test was used to compare mean values between more than two groups. The calculation of Spearman's rank correlation coefficient was applied to evaluate the relationship between quantitative parameters. Data are expressed as SEM means. All reported P values less than 0.05 were considered statistically significant.

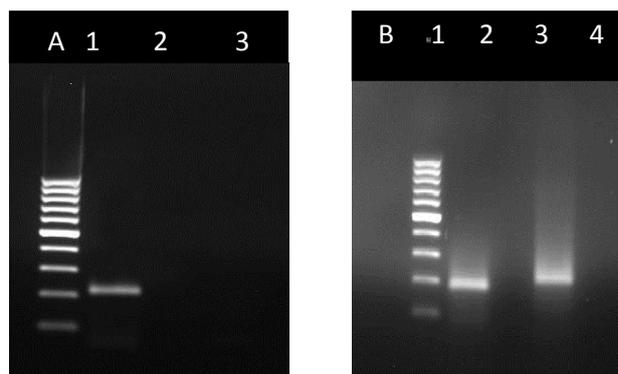
**Results**

**Fibromodulin expression by adenovirus vector in cultured cells**

RT-PCR was done by means of mRNA, which extracted at 72 hr after cell transfection to verify the expression of fibromodulin mRNA in HEK 293 cells. The mRNA signal for fibromodulin was detected to be expressed in Ad-fibromodulin-infected HEK 293 cells, but was not found in Rad-lacZ-transfected cells or uninfected cells (Figure 1).

**Effect of Rad-fibromodulin on rat's clinical data**

The STZ-induced diabetic rats showed typical



**Figure 1.** Identification of the expression of Rad-fibromodulin in HEK 293 cells. A: uninfected cells: (lane 1) marker 100 bp; (lane 2) β actin band, 207 bp; (lane 3) non-template control. B: RT-PCR of fibromodulin mRNA of HEK cells infected by Rad-fibromodulin. There is a band of 196 bp on the lane of Ad-fibromodulin in Agarose electrophoresis: (lane 1) marker, 100 bp; (lane 2) Rad-fibromodulin infected cells; (lane 3) non-template control; (lane 4) β actin band, 207 bp

diabetic symptoms of polyuria, polydipsia and weight loss. These symptoms are related to the presence of hyperglycemia. Blood glucose significantly was different between normal rats and diabetic rats, diabetic rats+Rad/LacZ, diabetic rats+Rad/FMOD. Urine volume was significant between normal groups and other diabetic and treated groups. There was significant differences between diabetic rat group and diabetic rats+Rad/FMOD group. Water consumption showed significant differences between diabetic rat group and diabetic rats+Rad/FMOD group. Body weight was not significant between normal group and diabetic rats and diabetic rats+Rad/lacZ and diabetic rats+Rad/FMOD groups (Table 1).

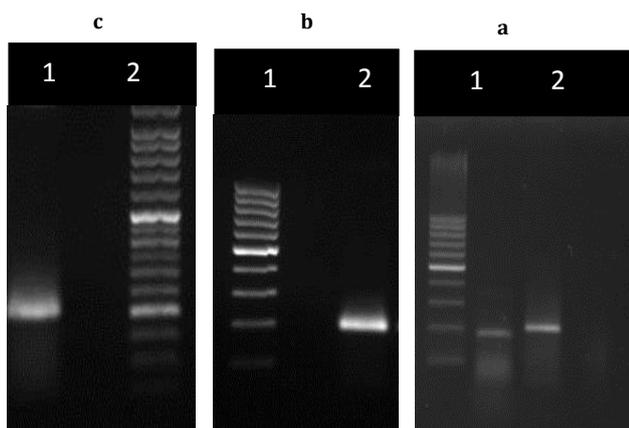
**Effect of Ad- fibromodulin on a kidney hypertrophy index (KHI) and UAE**

As expected, the KHI measures in all diabetic rat groups, including the Ad- Fibromodulin and Ad-lacZ groups, were higher than those in the PBS-control group. The KHI of the Ad-Fibromodulin group was slightly lower than that of the diabetic-control group. However, there were no significant differences of KHI measures among all diabetic rat groups (Ad- Fibromodulin, Ad-lacZ, diabetic-control). Albuminuria is an early hallmark of DN.UAE measures in the diabetic-control group were significantly larger

**Table 1.** Effect of Rad-FMOD and Rad-lacZ on rat's clinical data

|                   | Normal rats | Diabetic rats | Diabetic rats +rad/lacZ | Diabetic rats + rad/FMOD | P-value |
|-------------------|-------------|---------------|-------------------------|--------------------------|---------|
| Blood glucose     | 89.5±5.24   | 581.83±44.49  | 586.67±24.22            | 561.50±64.66             | 0.001*  |
| Urine volume      | 5.67±2.18   | 57.5±14.40    | 65±21.44                | 41.50±14.61              | ≤0.001* |
| Body weight       | 325.7±53.46 | 238.17±40.82  | 181.33±37.19            | 228±38.46                | 0.164   |
| Water consumption | 8.75±3,46   | 153.43±66.24  | 155.83±69.31            | 146.67±49.66             | ≤0.001* |

\*sign showed significant difference versus normal group. Significant data indicated with P≤ 0.001



**Figure 2.** Identifying the expression of Rad-fibromodulin in rat kidney. a: healthy rats: (lane1) Marker band 100bp; (lane 2) non-template control; (lane 3)  $\beta$  actin band, 207 bp. b: Rad-lacZ rats: (lane 1)  $\beta$  actin band, 207 bp; (lane 2) non-template control; (lane 3) marker 100 bp. c: Rad-fibromodulin rats (there is a band of 196 bp on the lane of Ad-fibromodulin in agarose electrophoresis): (lane 1) marker, 100 bp; (lane 2) Ad-fibromodulin; (lane 3)  $\beta$  actin band, 207 bp; (lane 4) non-template control

than in the PBS-control group. UAE measures in the Ad-fibromodulin group showed significantly smaller UAE values than the diabetic-control group. By the ten weeks, UAE of the Ad- Fibromodulin group decreased to 0.28 mg/mg ( $P < 0.05$ , when compared to that of the diabetic-control group), which was similar to the value of PBS-control group at this time point. There were no significant differences between the UAE measures of the Ad- fibromodulin group and the Ad-lacZ group (22).

**Fibromodulin expression by adenovirus vector in kidney samples**

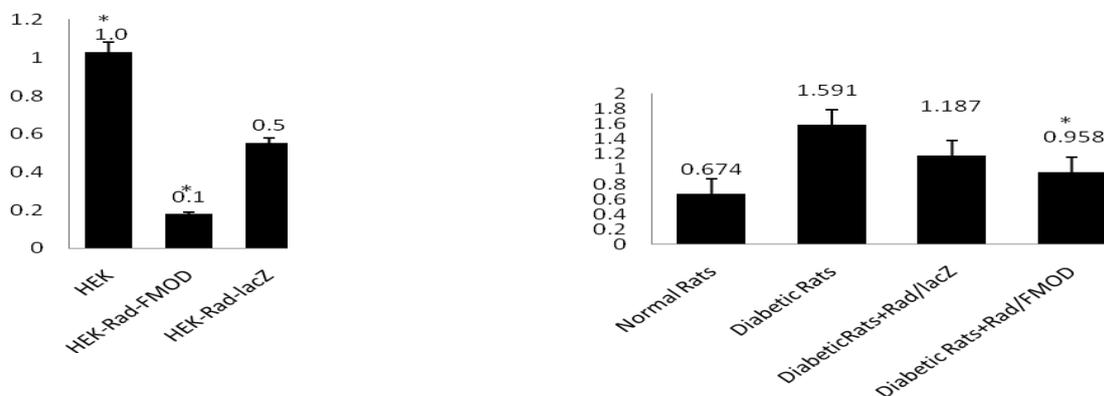
RT-PCR was performed at two weeks after viral infection to verify the expression of rat fibromodulin mRNA in the kidney. Signal for fibromodulin mRNA was detected in RAD-Fibromodulin-infected rats but was not found in the Rad-lacZ rats or the uninfected rats (Figure 2).

**Expression of TGF-  $\beta$ 1 in HEK 293 cells and diabetic kidneys by real-time PCR**

TGF- $\beta$ 1 mRNA expression was elevated in the kidney of the diabetic control group and HEK 293 cells. TGF- $\beta$ 1 mRNA decreased at the 10<sup>th</sup> week (two weeks after transfection) in the Ad- Fibromodulin group compared to the diabetic control group. To assess TGF- $\beta$ 1 activity, we measured the ratio of TGF- $\beta$ 1 to b actin in kidney and HEK 293 cells by real-time PCR. TGF- $\beta$ 1 measurements were taken in all three cell-lines groups, including the Ad- Fibromodulin and Ad-lacZ groups and the uninfected group. TGF- $\beta$ 1 levels in uninfected HEK cells were significantly higher than in other groups (PE0/05). By 10 weeks, the TGF- $\beta$ 1 of the Rad- FMOD group decreased to 0.17 compared to that of the control groups in HEK293 (Figure 3). TGF- $\beta$ 1 mRNA expression was also elevated in the kidneys of the diabetic control group. There were no significant differences in the TGF- $\beta$ 1 measurements of all diabetic rat groups (Ad-decorin, Ad-lacZ, diabetic control) but there were significant differences between diabetic rats and Rad- FMOD group (Figure 3).

**Discussion**

Diabetes mellitus (DM) is one of chronic metabolic disease that characterized by hyperglycemia mainly due to complete (Type 1 DM) or relative (Type 2 DM) absence of insulin. World Health Organization (WHO) estimates that more than 346 million people affected with DM in the worldwide. This amount is likely to more than double by 2030 without any intervention (25). Hyperglycemia, can affect different organs and cause retinopathy, neuropathy and nephropathy (26). Diabetic nephropathy (DN) is the leading cause of kidney transplant and death due to complications of diabetes is more common in nephropathy (27). DN is clinically characterized by proteinuria, albuminuria, elevated creatinine levels, and uncommon glomerular filtration rates. The important pathological features of DN include glomerular hypertrophy, podocyte, diffuse glomerular basement membrane thickening,



**Figure 3.** Expression of TGF-  $\beta$ 1 in HEK 293 cells by real-time PCR. Left: HEK 293 showed high level of TGF- $\beta$ 1; expression of infected cells (HEK-Ad-FMOD) decreased significantly ( $P \leq 0.01$ ). Right: expression was elevated in the kidney of the diabetic control group. There were no significant differences in TGF- $\beta$ 1 measurements among diabetic rat groups.

mesangial matrix expansion, nodular glomerulosclerosis, mesangiolysis and glomerular microaneurysms, interstitial fibrosis, tubular atrophy and foot process effacement. Inflammation and endothelial dysfunction have important roles in DN pathogenesis. Albuminuria and afterwards proteinuria associated to glomerular changes and interstitial fibrosis are marks of DN (28). These complex and progressive pathologic alterations are mostly induced by (I) hyperglycemia and higher formation of advanced glycation end products (AGE); (II) high activity of angiotensin II (Ang II) within the renin-angiotensin system; (III) excessive TGF $\beta$ -signaling; (IV) chronic inflammation associated to high recruitment of leukocytes and release of proinflammatory cytokines, chemokines and growth factors (29-30).

Oxidative stress is relating most of the molecular event underlining the pathological manner in DN, related to hyperglycemia and AGE, the renin-angiotensin system, TGF $\beta$  signaling, and chronic inflammation. Glomerular and tubular hypertrophy mostly due to extracellular matrix deposition, mesangial cells accumulation, apoptosis and podocyte dysfunction, altogether are redox-induced alterations leading to proteinuria, glomerulosclerosis, albuminuria, and tubulointerstitial fibrosis (28). On the basis of our research in hopes of finding a new approach to the prevention of diabetic nephropathy we used adenoviral vectors that express fibromodulin for down deregulating TGF- $\beta$  gene activity.

In 2012, Park and colleagues by IP injection of streptozotocin at a dose of 50 mg/kg of body weight, established nephropathy model. In this study 72 hr after STZ injection, blood glucose tested animals. If blood glucose levels were higher than 250 mg/dl, considered as the model of diabetes (31). In our study, animal model established with a single IP injection of STZ at a dose of 55 g per body weight. Rats which blood glucose levels were above 250 milligrams per deciliter, considered as a diabetic model. Our study have shown an increase of blood glucose in diabetes is the third day after STZ injection. Changes in body weight, urine output, water consumption and glucose levels among the all groups showed statistically significant differences. The results showed that the treatment had no effect on blood glucose levels of animals. In 2010, a study by Zhang *et al* was used to treat nephropathy from adenovirus expressing decorin also had no effect on blood glucose (3).

Other studies have shown that expression of TGF $\beta$ 1 in renal cortex of STZ-induced diabetic rats was markedly enhanced, and the level of TGF $\beta$ 1 mRNA might be related with the evolution of diabetic nephropathy (8). The present study also found that

TGF $\beta$ 1 mRNA production was notably enhanced in HEK 293 cell lines and diabetic kidneys.

Fibromodulin is a naturally occurring human proteoglycan that may be more appropriate for clinical therapeutic use than TGF- $\beta$ 1 antibodies. The low half-life of fibromodulin presents a challenge to the clinical goal of maintaining a high expression in the kidney. To resolve this problem, we examined the adenovirus as a potential vector delivery system for fibromodulin. As a delivery system, adenovirus vector has multiple advantages (32). The ease of genetic manipulation, high transduction efficiency into nondividing cells, ease of high titer production, copious gene expression *in vitro*, and storage stability. Our previous work established that Rad-FMOD has a potential therapeutic role in diabetic animal model. Subsequently, other researchers tracked adenovirus expression with green fluorescent protein and compared the intraperitoneal, intra-arterial and intramuscular injection routes for optimal kidney expression, and found that the intraperitoneal route was the most effective (3).

Our data in the diabetic rat model indicate that fibromodulin can effect on DN. Observations in the Rad-FMOD group suggest that fibromodulin gene transfer via IP injection markedly attenuates some pathological diabetic manifestation. Biglycans are capable of binding to TGF $\beta$  ligands with high affinity, resulting in the formation of inactive TGF $\beta$  complexes. Biglycan therefore acts in an analogous manner to TGF $\beta$  neutralizing antibodies (33). In addition, the lower UAE level in the Rad-fibromodulin group compared to the diabetic control indicates added amelioration of albuminuria with fibromodulin therapy. This protective effect of fibromodulin did not include any improvement in blood glucose, as the Ad-Fibromodulin group did not show any difference from diabetic controls. Furthermore, the therapeutic effect of Rad-Fibromodulin was independent of the adenoviral vector, as the Rad-lacZ group did not show any amelioration in albuminuria and KHI. The therapeutic mechanism of fibromodulin in DN remains undefined. Fibromodulin along with other small leucine-rich proteoglycans such as decorin can interact with a number of different cell surface receptors, ECM components, cytokines, and growth factors, such as TGF- $\beta$  ligands to modulate their activity (14).

Down-regulation of TGF- $\beta$ 1 by fibromodulin indicates that this protein exerts its effect on proteins stated above through TGF- $\beta$ 1 interaction with some of this proteins and can be a therapeutic target in DN. The possibility is that fibromodulin can inhibit the expression of TGF- $\beta$ 1 mRNA, which has been confirmed in other studies.

Our results are consistent with these studies. We found that TGF- $\beta$ 1 mRNA was decreased in the Rad-Fibromodulin group at the 10<sup>th</sup> week. However, this inhibition of TGF- $\beta$ 1 only appeared at the 10<sup>th</sup> week. Perhaps only high titers fibromodulin can decrease the TGF- $\beta$ 1 expression. Real-time PCR showed that TGF- $\beta$ 1 mRNA expression was elevated in the kidneys of the diabetic control group and HEK 293 cells. TGF- $\beta$ 1 mRNA decreased at the 10<sup>th</sup> week (two weeks after transfection) in the Rad-Fibromodulin group compared to those in the diabetic control group. To assess TGF- $\beta$ 1 activity, we measured the ratio of TGF- $\beta$ 1 to b actin in kidney and HEK293 cells. TGF- $\beta$ 1 is a potent mediator of fibrotic processes through stimulation of the production of extracellular matrix components. TGF- $\beta$ 1 also induces albuminuria (34). Elevated renal TGF- $\beta$ 1 mRNA and protein levels have been found in several animal models and the human form of diabetic nephropathy.

### Conclusion

Rad-fibromodulin ameliorate renal albuminuria by inhibition of TGF- $\beta$ 1 in rat. TGF- $\beta$ 1 has important role in mechanisms of nephropathy and it is essential to study the effects of fibromodulin on other signaling pathway. However, the molecular mechanisms involved in this route require further study.

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