ELSEVIER

Cell Biology International 31 (2007) 30–34

Cell Biology International

www.elsevier.com/locate/cellbi

# Connective tissue growth factor and fibronectin secretion in renal tubular epithelial cells induced by TGF-β1: Suppressive effects of troglitazone

Jia-li Wei[1], You-ming Peng*, Fuyou Liu[1]

*Department of Nephrology, The Second Xiangya Hospital of Central South University, Middle Ren-Min Road No. 86, Changsha, Hunan 410011, PR China*

Received 9 October 2005; revised 5 August 2006; accepted 5 September 2006

**Abstract**

Although some studies have suggested that troglitazone could retard the progression of glomerulosclerosis, its effects on renal tubulointerstitial fibrosis have not been completely clarified. The aim of this study was to investigate the effects of troglitazone on the secretion of connective tissue growth factor (CTGF) and fibronectin (FN) in human renal proximal tubular epithelial (HK-2) cells induced by transforming growth factor-β1 (TGF-β1). The mRNA of CTGF and FN were measured by semi-quantitative RT-PCR. CTGF and FN protein were detected by Western blot and ELISA, respectively. Our results revealed that troglitazone could inhibit CTGF and FN expression in a dose-dependent manner in human renal proximal tubular epithelial cells induced by TGF-β1, which may be one of the mechanisms of troglitazone contributing to retard the progression of renal tubulointerstitial fibrosis.
© 2006 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.

*Keywords:* Peroxisome proliferator activated receptor gamma; Transforming growth factor-β1; Connective tissue growth factor; Fibronectin; Renal proximal tubular epithelial cells; Renal tubulointerstitial fibrosis

## 1. Introduction

The three types of peroxisome proliferator activated receptor (PPAR) alpha, beta (or delta) and gamma, each with a specific tissue distribution, compose a subfamily of the nuclear hormone receptor gene family (Schoonjans et al., 1996). It is reported that PPARgamma ligands express in many cells such as endothelial cells, vascular smooth muscle cells and renal epithelial cells with beneficial effects in anti-inflammation, anti-proliferation, anti-neoplasia (Schoonjans et al., 1997; Elstner et al., 1998; Takahashi et al., 1999; Guan et al., 1999) and related to adipogenesis (Camp et al., 2002), glycometabolism (Rangwala and Lazar, 2004) and angiogenesis (Fauconnet et al., 2002).

Renal tubulointerstitial fibrosis is a major determinant in the progression of renal damage regardless of the origin (Eddy, 1996). It has been shown that tubulointerstitial fibrosis was a more consistent predictor of functional impairment than glomerular damage (Nath, 1992; Ong and Fine, 1994). TGF-β1, a multifunctional growth factor, has been regarded as a central mediator in the pathogenesis of fibrosis. TGF-β1 is up-regulated in renal tubulointerstitial fibrosis and plays a crucial role in the excretion of cellular factors and the expansion of extracellular matrix (ECM) (Tamaki et al., 1994; Fan et al., 1999). It was reported that PPARgamma ligands could retard or even retroconvert the progression of glomerulosclerosis (Imano et al., 1998; McCarthy et al., 2000). However, the anti-fibrosis role of PPARgamma in renal tubulointerstitial fibrosis has not been completely clarified. Our study was to investigate the effects of troglitazone, a synthetic PPARgamma ligand, on the growth factors and ECM secretion in human renal tubular epithelial cells induced by TGF-β1.

* Corresponding author. Tel.: +86 139 7318 9719.
*E-mail address:* pengym104@yahoo.com.cn (Y.-m. Peng).
[1] Jiali Wei and Fuyou Liu contributed equally to this work.

1065-6995/$ - see front matter © 2006 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2006.09.004

## 2. Materials and methods

### 2.1. Materials

Human renal proximal tubular epithelial cells (HK-2) were kindly provided by the Division of Nephropathy, Vanderbilt University, USA. Recombinant human TGF-β1 was obtained from R&D Systems, USA. Troglitazone (TGL) was kindly provided by Professor You-Fei Guan, Division of Nephropathy, Vanderbilt University, USA.

### 2.2. Cell culture

HK-2 cells were maintained at 37 °C in an atmosphere of 95% air and 5% $CO_2$ in DMEM/F12 containing 10% FBS (Gibco BRL, USA). After digestion with 0.25% trypsin (Amresco) and 0.02% EDTA, $2 \times 10^6$ cells were grown in 60 mm culture dishes and $1.0 \times 10^6$ cells were grown in 6 well plates. For experiments, subconfluent cells were starved for 24 h, then divided into five groups as follows: control group, TGF-β1 (5 ng/ml) group, TGF-β1 (5 ng/ml) plus (TGL 1 μmol/L, 5 μmol/L, 10 μmol/L) group. Cells were incubated with the indicated concentrations of TGL for 30 min before exposure to TGF-β1 for 24 h. After the incubation, cells were harvested for the determination described below.

### 2.3. MTT assay

HK-2 cells were plated at $5 \times 10^4$ cells/well in 96-well plates in DMEM/F12 containing 10% FBS. After 24 h, the culture medium was replaced by fresh medium containing TGL (1 μmol/L, 5 μmol/L, 10 μmol/L). Three duplicate wells were set up in each sample. After 24 h incubation, 20 μL MTT (5 g/L) was added to each well and incubated for 4 h. Supernatant was then removed, 100 μL DMSO was added, then shaken for 10 min until the crystal was dissolved. Cell growth state was completed using $A_{570\ nm}$ value (mean ± SD) as the ordinate.

### 2.4. Semi-quantitative RT-PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. RNA samples were dissolved in DEPC-treated water and the RNA concentration in each sample was determined spectrophotometrically. 1.0 μg total RNA was analyzed for CTGF, FN, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of the sense and antisense primers used for amplification were summarized in Table 1. Amplification consisted of 5 min at 95 °C, 30 s of denaturation at 95 °C, 30 s at 56 °C, 50 s at 72 °C, followed by 26, 21 cycles respectively. The final extension was set for 10 min at 72 °C. Those PCR products were electrophoresed on 1.5% (w/v) agarose gel. 5 independent cultures for the mRNA analysis were performed. All data were expressed as the relative differences between control and treated cells after normalization to the GAPDH expression.

### 2.5. Western blot analysis

24 h after incubation with TGF-β1, cells were washed with PBS, collected by scraping and centrifuged at 1000 × *g* for 5 min, then lysed in ice-cold RIPA buffer containing 150 mM Nacl, 10%Triton X-100, 0.5% Deoxycholate, 0.1% SDS and 50 mM Tris (pH 7.5–8.0) for 30 min and centrifuged at 12,000 × *g* for 30 min. The supernatant was used for protein determination by the Bradford procedure (Bio-Rad) and western blot. 50 μg total protein was suspended in 5 × reducing sample buffer, boiled for 10 min, electrophoresed on 12% SDS-PAGE gels, and then transferred to polyvinylidene difluoride membrane by electroblotting. Blocked membranes were incubated with monoclonal anti-CTGF antibody (1:1000, R&D, USA) for 1 h, washed, and then incubated with HRP-conjugated secondary antibody (1:10,000 dilution, KPL,USA) for 1 h. Antigens were detected with a chemiluminescent substrate system (enhanced chemiluminescence, ECL, Pharmacia, USA).

Table 1
Primers used in the RT-PCR analysis

| Primer | Sequence | Length |
|---|---|---|
| CTGF-F | 5′-CAT CTT CGG TGG TAC GGT GT-3′ | 374 bp |
| CTGF-R | 5′-AGG AGG CGT TGT CAT TGG TA-3′ | |
| FN-F | 5′-AGC CGC CAC GTG CCA GGA TTA C-3′ | 439 bp |
| FN-R | 5′-CTT ATG GGG GTG GCC GTT GTG G-3′ | |
| GAPDH-F | 5′-gAT TCC ACC CAT ggC AAAT-3′ | 260 bp |
| GAPDH-R | 5′-TTC ACA CCC ATg ACg AACAT-3′ | |

### 2.6. ELISA assay

Fibronectin (FN) in conditioned medium was quantitatively measured by ELISA according to the instructions provided with the fibronectin-ELISA Detection Kit (Jingmei Biotech, China).

### 2.7. Statistical analysis

All data were presented as mean ± SD. One-way analysis of variance followed by the Q test using SPSS 10.0 software was performed to determine statistical significance. A $P$ value <0.05 was considered statistically significant.

## 3. Results

### 3.1. Results of MTT assay

As shown by MTT assay, TGL could inhibit the growth of HK-2 cells in dose-dependent manner (Table 2).

### 3.2. Effects of TGL on TGF-β1-induced CTGF, FN mRNA expression in HK-2 cells

The levels of CTGF, FN mRNA were significantly increased in HK-2 cells induced by TGF-β1 compared with control group ($P < 0.001$). TGL could inhibit CTGF, FN mRNA expression in a dose-dependent manner compared with TGF-β1 group ($P < 0.01$) (Fig. 1). A lower level of CTGF, FN mRNA expression was seen between TGL (10 μmol/L) and control group, but there was no significance.

### 3.3. Effects of TGL on TGF-β1-induced CTGF protein expression in HK-2 cells detected by Western blot

The levels of CTGF protein were significantly increased in HK-2 cells induced by TGF-β1 compared with control group

Table 2
Results of MTT assay ($\overline{X} \pm SD$)

| Groups | OD index at 570 nm |
|---|---|
| DMEM/F12 | 0.5345 ± 0.00779 |
| DMEM/F12 + TGL 1 μmol/L | 0.5216 ± 0.00704* |
| DMEM/F12 + TGL 5 μmol/L | 0.5121 ± 0.01230# |
| DMEM/F12 + TGL 10 μmol/L | 0.5025 ± 0.01116& |

After 24 h incubation with various concentration of TGL, MTT assay was performed. Three duplicate wells were set up in each sample. Cell growth state was completed using $A_{570\ nm}$ value in ELISA reader. All data were shown as (mean ± SD) from 5 independent experiments. *: compared with DMEM/F12 group, $P < 0.01$; #: compared with TGL 1 μmol/L group, $P < 0.05$; &: compared with TGL 5 μmol/L group, $P < 0.05$.

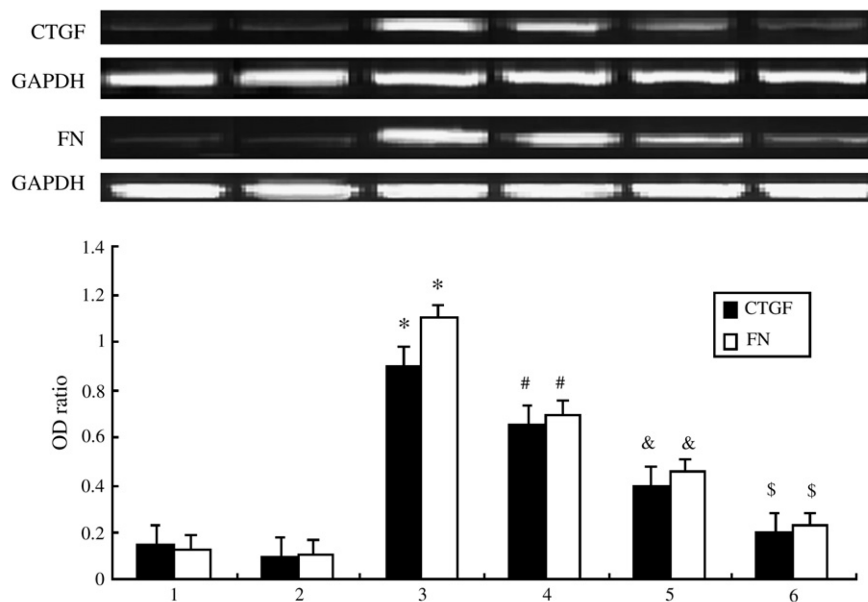


Fig. 1. Effects of TGL on TGF-beta1-induced CTGF, FN mRNA expression in HK-2 cells by semi-quantitative RT-PCR analysis. The levels of CTGF, FN mRNA were significantly increased in HK-2 cells induced by TGF-beta1 compared with control group. TGL could inhibit CTGF, FN mRNA expression in dose-dependent manner compared with TGF-beta1 group. GAPDH served as control. Top panels are representative of 5 separate experiments. Bottom panels are the summary of data (mean ± SD) from these 5 experiments. Lane 1: DMEM/F12 group; Lane 2: TGL (10 μmol/L) group; Lane 3: TGF-beta1 group (5 ng/ml); Lane 4: TGF-beta 1 (5 ng/ml) plus TGL (1 μmol/L) group; Lane 5: TGF-beta1 (5 ng/ml) plus TGL (5 μmol/L) group; Lane 6: TGF-beta1 (5 ng/ml) plus TGL (10 μmol/L) group. *: compared with DMEM/F12 group $P < 0.001$; #: compared with TGF-beta1 group, $P < 0.01$; &: compared with TGF-beta1 plus TGL 1 μmol/L group, $P < 0.01$; $: compared with TGF-beta1 plus TGL 5 μmol/L group, $P < 0.01$.

($P < 0.001$). TGL could inhibit CTGF protein expression in dose-dependent manner compared with TGF-β1 group ($P < 0.01$) (Fig. 2). The level of CTGF protein was a little decreased in the TGL (10 μmol/L) group when compared with control, but there was no significance.

### *3.4. Effects of TGL on TGF-β1-induced FN protein expression detected by ELISA*

The levels of FN protein were significantly increased in HK-2 cells induced by TGF-β1 compared with control group ($P < 0.01$). TGL could inhibit FN protein expression in dose-dependent manner compared with TGF-β1 group ($P < 0.05$). Although the level of FN protein was a little decreased in the TGL (10 μmol/L) group when compared with control, there was no significance (Table 3).

## 4. Discussion

In various kinds of chronic renal diseases, renal tubulointerstitial fibrosis is greatly associated with renal function. Therefore to retard the progression of renal tubulointerstitial fibrosis as early as possible may display great significance in the prevention of end stage renal disease (ESRD). It is reported that TGF-β1 is up-regulated in renal tubulointerstitial fibrosis, which can promote the excretion of ECM and profibrotic inflammation factors (Fan et al., 1999; Tamaki et al., 1994). Thus down-regulating the expression of ECM and profibrotic inflammation factors may provide a potential treatment for tubulointerstitial fibrosis.

PPARgamma is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors. TGL, a synthetic ligand of PPARgamma, plays its pharmacologic action by activating nucleus PPARgamma with high selectivity. Data indicated that PPARgamma agonist had anti-fibrosis effects, whose mechanism might be associated with down-regulation the expression of profibrotic factors such as TGF-β1, CTGF and PAI-1 (Broeders and Abramowicz, 2002). Our study revealed that TGL could inhibit the expression of CTGF in dose-dependent manner in renal tubular epithelial cells induced by TGF-β1. It has been reported that CTGF was a PPARgamma-regulated gene. The suppression of CTGF expression is mediated by PPARgamma and PPARgamma inhibited TGF-β-induced CTGF expression in human aortic smooth muscle cells by directly interfering with the Smad3 signaling pathway (Fu et al., 2001). Because CTGF is a key factor in the regulation of extracellular matrix production, this repression of CTGF expression by PPARgamma activation may be one of the mechanisms through which PPARgamma agonists inhibit extracellular matrix formation after renal tubuloepithelial cell lesion.

Previous studies have shown that pioglitazone inhibited TGF-β1-induced FN expression in human mesangial cells by inhibiting AP-1 activation dependent on PPARgamma (Guo et al., 2004; Maeda et al., 2005). Our results also confirmed that TGL inhibited the increased expression of FN in renal tubular epithelial cells induced by TGF-β1, which might contribute to retard the progression of renal tubulointerstitial fibrosis. Given the fact that CTGF secreted by tubular cells may act in a paracrine mode on myofibroblasts and fibroblasts

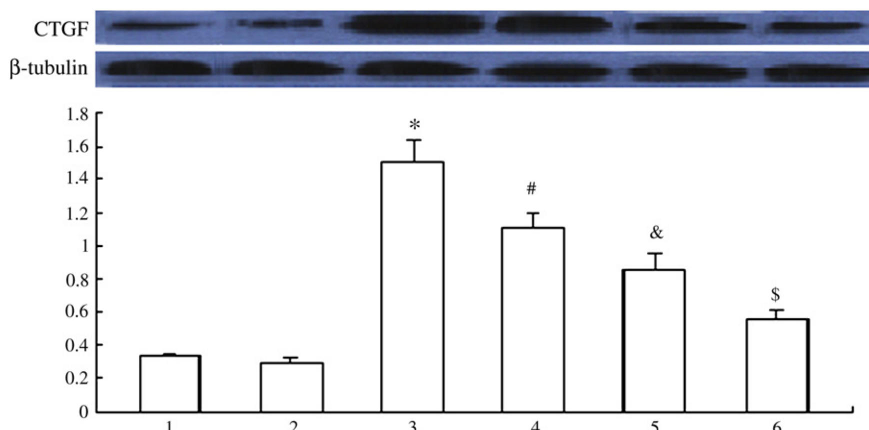


Fig. 2. Effect of TGL on TGF-beta1-induced CTGF protein expression in HK-2 cells detected by Western blot. The levels of CTGF protein were significantly increased in HK-2 cells induced by TGF-beta1 compared with control group. TGL could inhibit CTGF protein expression in dose-dependent manner compared with TGF-beta1 group. β-tubulin served as loading control. Top panels are representative of 5 separate experiments. Bottom panels are the summary of data (mean ± SD) from these 5 experiments. Lane 1: DMEM/F12 group; Lane 2: TGL (10 μmol/L) group; Lane 3: TGF-beta1 group (5 ng/ml); Lane 4: TGF-beta 1 (5 ng/ml) plus TGL (1 μmol/L) group; Lane 5: TGF-beta1 (5 ng/ml) plus TGL (5 μmol/L) group; Lane 6: TGF-beta1 (5 ng/ml) plus TGL (10 μmol/L) group. *: compared with DMEM/ F12 group, $P < 0.001$; #: compared with TGF-beta1 group, $P < 0.01$; &: compared with TGF-beta1 and TGL 1 μmol/L group, $P < 0.01$; $: compared with TGF-beta1 and TGL 5 μmol/L group, $P < 0.01$.

in the renal interstitium (Badid et al., 2001), blocking CTGF production may inhibit both tubular ECM synthesis directly and reduce the ECM produced by myofibroblasts and fibroblasts. All these effects would lead to the amelioration of renal tubulointerstitial fibrosis.

PPAR gamma is involved in the cellular growth. It is reported that PPAR gamma activation with its agonists inhibited the proliferation and differentiation of cultured rat mesangial cells in dose-dependent manner, resulting in growth retardation (Asano et al., 2000). PPARgamma beneficial effects in glomerulosclerosis are associated with regulation of glomerular cell proliferation, hypertrophy by decreases in glomerular p21 mRNA and p27 protein (Ma et al., 2001). In our study, although TGL could inhibit the growth of renal tubular epithelial cells in dose-dependent manner and the expression of CTGF and FN in TGL (10 μmol/L) was a little lower when compared with the control, there was no significance, indicating the suppression effects of TGL mainly due to a direct effect on CTGF and FN expression rather than the inhibition of cell growth.

Table 3
The levels of FN protein detected by ELISA

| Groups | FN (μg/L) |
|---|---|
| DMEM/F12 | 21.1230 ± 1.24013 |
| TGL 10 μmol/L | 17.5231 ± 1.43152 |
| TGF-beta1 | 66.4453 ± 6.27784 |
| TGF-beta1 + TGL 1 μmol/L | 58.9334 ± 5.8243* |
| 5:TGF-beta1 + TGL 5 μmol/L | 53.1945 ± 6.11451# |
| 6:TGF-beta1 + TGL 10 μmol/L | 44.5231 ± 5.5376& |

Effects of TGL on TGF-beta1-induced FN protein expression in the media detected by ELISA. Three duplicate wells were set up in each sample. All data were shown as (mean ± SD) from 5 independent experiments. $: compared with DMEM/F12 group, $P < 0.01$; *: compared with TGF-beta1 group, $P < 0.05$; #: compared with TGF-beta1 and TGL 1 μmol/L group, $P < 0.05$; &: compared with TGF-beta1 and TGL 5 μmol/L group, $P < 0.05$.

Taken together, CTGF and FN down-regulation by PPARgamma activation might be one of the mechanisms by which PPARgamma agonists inhibit renal tubular epithelial cell injury.

## Acknowledgements

We are grateful to Professor JIA-HUI Xia for the supply of equipment in the National Laboratory of Genetics, China.

## References

Asano T, Wakisaka M, Yoshinari M, Iino K, Sonoki K, Iwase M, et al. Peroxisome proliferator-activated receptor gamma1 (PPARgamma1) expresses in rat mesangial cells and PPARgamma agonists modulate its differentiation. Biochim Biophys Acta 2000;1497:148−54.

Badid C, Vincent M, Fouque D, Laville M, Desmouliere A. Myofibroblast: a prognostic marker and target cell in progressive renal disease. Renal Fail 2001;23:543−9.

Broeders N, Abramowicz D. Peroxisome proliferators-activated receptors (PPARs): novel therapeutic targets in renal disease. Kidney Int 2002;61: 354−5.

Camp HS, Ren D, Leff T. Adipogenesis and fat-cell function in obesity and diabetes. Trends Mol Med 2002;8:442−7.

Eddy AA. Molecular insights into renal interstitial fibrosis. J Am Soc Nephrol 1996;7:2495−508.

Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, et al. Ligands for peroxisome proliferator-activated receptor-gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. Proc Natl Acad Sci USA 1998;95: 8806−11.

Fan JM, Ng YY, Hill PA, Nikolic-Paterson DJ, Mu W, Atkins RC, et al. Transforming growth factor-β regulates tubular epithelial-myofibroblast transdifferentiation *in vitro*. Kidney Int 1999;56:1455−67.

Fauconnet S, Lascombe I, Chabannes E, Adessi GL, Desvergne B, Wahli W, et al. Differential regulation of vascular endothelial growth factor expression by peroxisome proliferator-activated receptors in bladder cancer cells. J Biol Chem 2002;277:23534−43.

Fu M, Zhang J, Zhu X, Myles DE, Willson TM, Liu X, et al. Peroxisome proliferator-activated receptor gamma inhibits transforming growth factor beta-induced connective tissue growth factor expression in human aortic smooth muscle cells by interfering with Smad3. J Biol Chem 2001;276: 45888−94.

Guan YF, Zhang YH, Breyer RM, Davis L, Breyer MD. Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in human transitional bladder cancer and its role in inducing cell death. Neoplasia 1999;1:330−9.

Guo B, Koya D, Isono M, Sugimoto T, Kashiwagi A, Haneda M. Peroxisome proliferator-activated receptor-gamma ligands inhibit TGF-β 1-induced fibronectin expression in glomerular mesangial cells. Diabetes 2004;53: 200−8.

Imano E, Kanda T, Nakatani Y, Nishida T, Arai K, Motomura M, et al. Effect of troglitazone on microalbuminuria in patients with incipient diabetic nephropathy. Diabetes Care 1998;21:2135−9.

Ma LJ, Marcantoni C, Linton MF, Fazio S, Fogo AB. Peroxisome proliferator-activated receptor-gamma agonist troglitazone protects against non-diabetic glomerulosclerosis in rats. Kidney Int 2001;59: 1899−910.

Maeda A, Horikoshi S, Gohda T, Tsuge T, Maeda K, Tomino Y. Pioglitazone attenuates TGF-β(1)-induction of fibronectin synthesis and its splicing variant in human mesangial cells via activation of peroxisome proliferator-activated receptor (PPAR)gamma. Cell Biol Int 2005;29: 422−8.

McCarthy KJ, Routh RE, Shaw W, Walsh K, Welbourne TC, Johnson JH. Troglitazone halts diabetic glomerulosclerosis by blockade of mesangial expansion. Kidney Int 2000;58:2341−50.

Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. Am J Kidney Dis 1992;20:1−17.

Ong AC, Fine LG. Loss of glomerular function and tubulointerstitial fibrosis: cause or effect. Kidney Int 1994;45:345−51.

Rangwala SM, Lazar MA. Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. Trends Pharmacol Sci 2004;25:331−6.

Schoonjans K, Staels B, Auwerx J. The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta 1996;26:93−109.

Schoonjans K, Martin G, Staels B, Auwerx J. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr Opin Lipidol 1997;8:159−66.

Tamaki K, Okuda S, Ando T, Iwamoto T, Nakayama M, Fujishima M. TGF-b in glomerulosclerosis and interstitial fibrosis of adriamycin nephropathy. Kidney Int 1994;45:525−36.

Takahashi N, Okumura T, Motomura W, Fujimoto Y, Kawabata I, Kohgo Y. Activation of PPARgamma inhibits cell growth and induces apoptosis in human gastric cancer cells. FEBS Lett 1999;455:135−9.