

Immunohistochemical evaluation of phosphorylated SMAD2/SMAD3 and the co-activator P300 in human glomerulonephritis: correlation with renal injury

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Abstract

Background: Smad2 and Smad3 are transcription factors that mediate transforming growth factor beta (TGF- β) signals. Upon their activation, phosphorylated Smad2/Smad3 (pSmad2/Smad3), translocate to the nucleus and associate with co-activators such as p300, regulating the transcription of genes that contribute to the fibrotic processes. Methods: We investigated the immunohistochemical expression of pSmad2/Smad3 and the co-activator p300 in 152 renal biopsy specimens from patients with various types of glomerulonephritides (GNs) and in 15 normal kidney specimens. Patients' clinical data (serum creatinine levels and proteinuria) had been collected. Results: There was a dramatic increase in the expression of pSmad2/3 and p300 in all glomerular cell types in all GNs. pSmad2/3 expression was increased in all tubular segments (except for the proximal tubules in nonproliferative GNs), while p300 expression was significantly increased only in the proximal tubular cells in all GNs. Glomerular and tubular pSmad2/Smad3 and p300 were significantly increased in proliferative GNs (compared to the nonproliferative), particularly in the secondary group. The expression profile of p300 correlated positively with the expression of pSmad2/Smad3 in the diseased glomeruli and proximal tubules. pSmad2/3 and p300 were very often detected in segmental hyperplastic lesions, cellular crescents, microadhesions and segmental or global sclerotic areas. Glomerular and proximal tubular pSmad2/Smad3 was positively correlated with serum creatinine levels, while distal and collecting tubular pSmad2/3 and p300 correlated positively with tubular atrophy. Glomerular and proximal tubular pSmad2/3 expression and glomerular p300 expression correlated positively with lupus nephritis activity. Conclusion: Our results suggest that pSmad2/3-p300 pathway may play a pivotal role in the pathogenesis and progression of human glomerulonephritis.

Keywords: glomerulonephritis • pSmad2/3 • p300 • renal fibrosis

Introduction

The Smad pathway is the best characterized signaling pathway employed by transforming growth fac-

tor- β (TGF- β). TGF- β is a multifunctional growth factor involved in the regulation of cell proliferation, differentiation, extracellular matrix (ECM) formation and immune response [1, 2]. TGF- β has been proposed to mediate glomerulosclerosis and interstitial fibrosis in various renal diseases including glomerulonephritis [3–5]. TGF- β is overexpressed in human glomerular diseases including

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immunoglobulin A (IgA) nephropathy, lupus nephritis, focal and segmental glomerulosclerosis [6].

TGF- β binds to three different types of serine/threonine kinase receptors [7]. Upon TGF- β binding, the constitutively active TGF- β type II receptor associates with and phosphorylates the type I receptor [8]. The type I receptor kinase then phosphorylates the Smad2 and Smad3 proteins [9, 10]. The phosphorylated Smad2/3 form heteromeric complexes with Smad4 and translocate into the nucleus, [9, 11–15]. Smad3 and Smad4 interactions with DNA are weak and insufficient to convey promoter selectivity [16]. On the other hand, despite being highly homologous with Smad3, Smad2 is unable to bind to DNA directly. Thus, pSmad2/3-Smad4 complexes require other transcription factors and co-activators or co-repressors to target them to specific sequences. p300 is a transcriptional co-activator that appears to be involved in the transcriptional activation of Smad complexes [17]. It possesses histone acetyl transferase activity, suggesting that transcriptional activation by Smad complexes may require altering nucleosome structure and remodeling of the chromatin [18, 19].

Increased staining for Smad3 [20] has been described in the glomeruli of *db/db* mice, a genetic model of type 2 diabetes. Moreover, increased expression of pSmad2/3 has been reported in the glomerular epithelial cells of the sclerotic segments in renal biopsies from patients with focal and segmental glomerulosclerosis [21]. Earlier work demonstrated that Smads 2 and 3 can recruit the p300 co-activator to activate transcription [15]. Similarly, interaction of Smad4 with p300 has been reported [16]. Previous studies have also demonstrated that Smad4 contains an intrinsic 48-amino-acid p300 dependent SAD (Smad activation domain) [17]. Moreover, p300 may be involved in TGF- β 1/Smad-pathway-mediated type I collagen gene transcription in mouse mesangial cells [22]. However, no data exist on the expression patterns of p300 and phosphorylated Smads in human glomerulonephritis (GN).

Recent studies have demonstrated that Smad signaling is also a critical pathway for renal fibrosis induced by other pro-fibrotic factors, such as angiotensin II and advanced glycation end products (AGE) [23]. These pro-fibrotic factors can activate Smads directly and independently of TGF- β . They can also cause renal fibrosis *via* the

ERK/p38 MAP kinase-Smad signaling cross-talk pathway. Results from these studies demonstrate that Smad signaling is probably a key and final common pathway of renal fibrosis [23].

In an effort to extend previous knowledge on the activation of Smad signalling pathway in diseased human kidneys we investigated the expression of pSmad2/3 and p300 using immunohistochemical methods, in serial sections of a wide range of glomerulonephritides. We localised the expression of these molecules to several pathologic lesions. Correlations were performed between the expression profile of these molecules with clinical parameters of renal function and histologic injury.

Material and methods

Patients

Percutaneous renal biopsies were obtained from 152 patients with various types of GN. Renal biopsies were performed for diagnostic purposes and informed consent for the use of renal biopsy tissue, in excess of that required for diagnostic purposes, was obtained from the patients. 86 patients had primary GN and 66 patients secondary GN. Apart from taking into account the primary or the secondary nature of the glomerular disorder, GNs were further divided into proliferative (n = 85) and nonproliferative (n = 67). The non-proliferative group of primary GNs included minimal change glomerular disease (MCD, n = 10), membranous GN (MGN, n = 23), focal segmental glomerulosclerosis (FSGS, n = 16), while the respective group in SLE GNs consisted of cases with mesangial changes (WHO class II, n = 10) and diffuse membranous GNs (WHO class V, n = 8). The proliferative group of primary GNs included acute diffuse proliferative GN [in detail, acute post-infectious GN (PI, n = 6), membranoproliferative GN (MPGN, n = 7)] and IgA nephropathy (n = 24), while the proliferative group of secondary GNs comprised those pauci-immune GN due to vasculitis (Pauci-Im GN, n = 23) as well as focal segmental SLE GN (WHO class III, n = 9) and diffuse proliferative SLE GN (WHO class IV, n = 16). The classification and clinical parameters of patients with GN are given in Table 1. As a control group, totally normal kidney sections from 15 kidneys resected for renal cell carcinoma were examined.

Table 1 Classification and clinical parameters of patients with glomerulonephritis

Disease		No. of patients	Age (Years)	Gender (% male)	Serum Creatinine (mg/dL)	24-Hour Urinary Protein (g/d)
Non-proliferative primary GNs	MCD	10	39.5±19.6	90	0.92±0.12	5.7±3.1
	FSGS	16	36.5±14.7	69	1.21±0.58	3.3±3.35
	MGN	23	59.2±16.3	61	1.28 ±0.86	6.16±6.1
Proliferative primary GNs	IgA	24	33.5±13.9	71	0.96±0.38	1.74±1.67
	MPGN	7	41.6±28.2	14	1.65±0.20	6.34±5.3
	PostIn GN	6	40.6±25.1	84	3.95±2.19	3.35±0.91
Non-proliferative secondary GNs	NPLN	18	38.7±11.1	17	1.33±0.82	1.68±2.15
Proliferative secondary GNs	PLN	25	34±9.4	12	1.37±0.71	3.27±2.6
	Pauci-Im GN	23	52.2±19.6	52	3.69±2.26	0.91±0.97

MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; MGN, membranous GN; IgA, IgA nephropathy; MPGN, membranoproliferative GN; PostIn, postinfectious GN; NPLN, nonproliferative lupus nephritis (SLE II/V); PLN, proliferative lupus nephritis (SLE III/IV); and Pauci-Im GN, pauci-immune GN. Data are presented as mean ± 1 standard deviation. NOTE: To convert serum creatinine in mg/dL to µmol/L multiply by 88.4.

Immunohistochemistry

Immunohistochemical staining was performed on 4 µm thick formalin-fixed paraffin embedded sections, using a biotin free, two-step, HRP-labelled detection system (DACO EnVision™). Sections were deparafinized in xylene and rehydrated through graded alcohols. After quenching of endogenous peroxidase activity using a methanol/hydrogen peroxide solution (0.3% in TBS for 30 min), we proceeded to microwave heat-mediated antigen retrieval in 10 mM, pH = 6 citrate buffer and blockage of nonspecific binding by incubation in 10% normal horse serum in TBS (Vector Lab, Burlingame, CA, USA) for 30 min at room temperature. Subsequently, sections were incubated for 40 minutes at 4°C with the primary antibodies. The primary antibodies used in our study were: (1) anti-pSmad2/3 rabbit polyclonal antibody (#3101) (Cell Signaling Technology Inc, USA) at a dilu-

tion 1:500 and (2) anti-p300 rabbit polyclonal antibody (sc-585) (Santa Cruz Biotechnology Inc, UK) at a dilution 1:500. Sections were then incubated with a biotin free, two-step, HRP-labelled detection system (DACO EnVision™) for 30 min at room temperature followed by the addition of 3,3'-diaminobenzidine tetrachlorohydrate (DAB), in order to achieve visualization of the requested antigens. The biotin-avidin/streptavidin system is often used as an amplification step to increase sensitivity. However, in some tissues such as kidney, “nonspecific” interactions may be a problem due to high levels of endogenous biotin-containing proteins [24]. Thus, a biotin free detection system was used in our experiments. Finally, sections were rinsed, counterstained with hematoxylin and mounted. In cases where the identification of proximal *versus* distal tubules was difficult, consecutive serial sections were stained so that any remnants of brush border became detectable. Previously pSmad2/3 or p300

positive breast cancer tissue sections served as positive controls in each staining procedure while negative controls included substitution of primary antibody with non-immune mouse IgG diluted at the same concentration for each antibody, or the omission of primary antibodies.

Quantification of pSmad2/3 and p300 immunostaining in human renal biopsies

Serial sections were immunostained for each molecule. Nonglobally sclerosed glomeruli (mean, 15 ± 4.3 ; range 10 to 25) were counted. The intensity of glomerular staining of pSmad2/3 and p300 was evaluated according to the following 0 to 3 scale: **0**, <10% cells with nuclear staining; **1**, weak nuclear staining intensity or 11–50% of cells with nuclear staining; **2**, moderate nuclear staining intensity and >50% of cells with nuclear staining and **3**, strong nuclear staining intensity and >50% of cells with nuclear staining. The staining status of glomerular lesions was also assessed, with immunopositivity being recorded when some degree of immunostaining was noticeable. When no staining was observed, the respective lesions were characterized as negative. The staining intensity of the different parts of the tubules was evaluated according to the following: **0**, <10% cells with nuclear staining; **1**, weak nuclear staining intensity or 11–50% of cells with nuclear staining; **2**, moderate nuclear staining intensity and >50% of cells with nuclear staining and **3**, strong nuclear staining intensity and >50% of cells with nuclear staining. Sections were scored independently by two investigators (T.K. and L.N.) blinded to the patients' clinical profile.

Quantification of histologic injury in human renal biopsies

The human renal biopsy specimens were reviewed and analyzed by an independent anatomic pathologist who was blinded to the quantification of pSmad2/3 and p300 immunostaining. The severity of glomerulosclerosis was expressed as the percentage of globally sclerosed glomeruli out of the total number of glomeruli in the renal biopsy. The nonglobally sclerosed glomeruli were analyzed for the presence of proliferative segmental lesions, crescents, microadhesions, and segmental (or global) sclerotic lesions. The degree of interstitial inflammation, interstitial fibrosis and tubular atrophy

was scored between 0 and 3 according to the following: **0**, 0 to 5%; **1**, 6 to 20%; **2**, 21 to 40%; and **3**, >40% of the biopsy area demonstrating inflammation, fibrosis or atrophy, respectively. A previously described morphometric analysis based on point counting was used [25].

Statistical analyses

Data are presented as mean \pm 1 standard deviation. Comparisons between the groups were made with the Kruskal-Wallis test. Mann-Whitney U test was used to compare the molecules' expression between normal controls and every form of GN. The Spearman correlation analysis was used to detect possible correlations between staining scores at various nephron sites, clinical data and indices of histologic injury. The association of pSmad2/3 with p300 expression in glomerular lesions of serial sections was assessed with χ^2 test. Statistical significance was set at 5%.

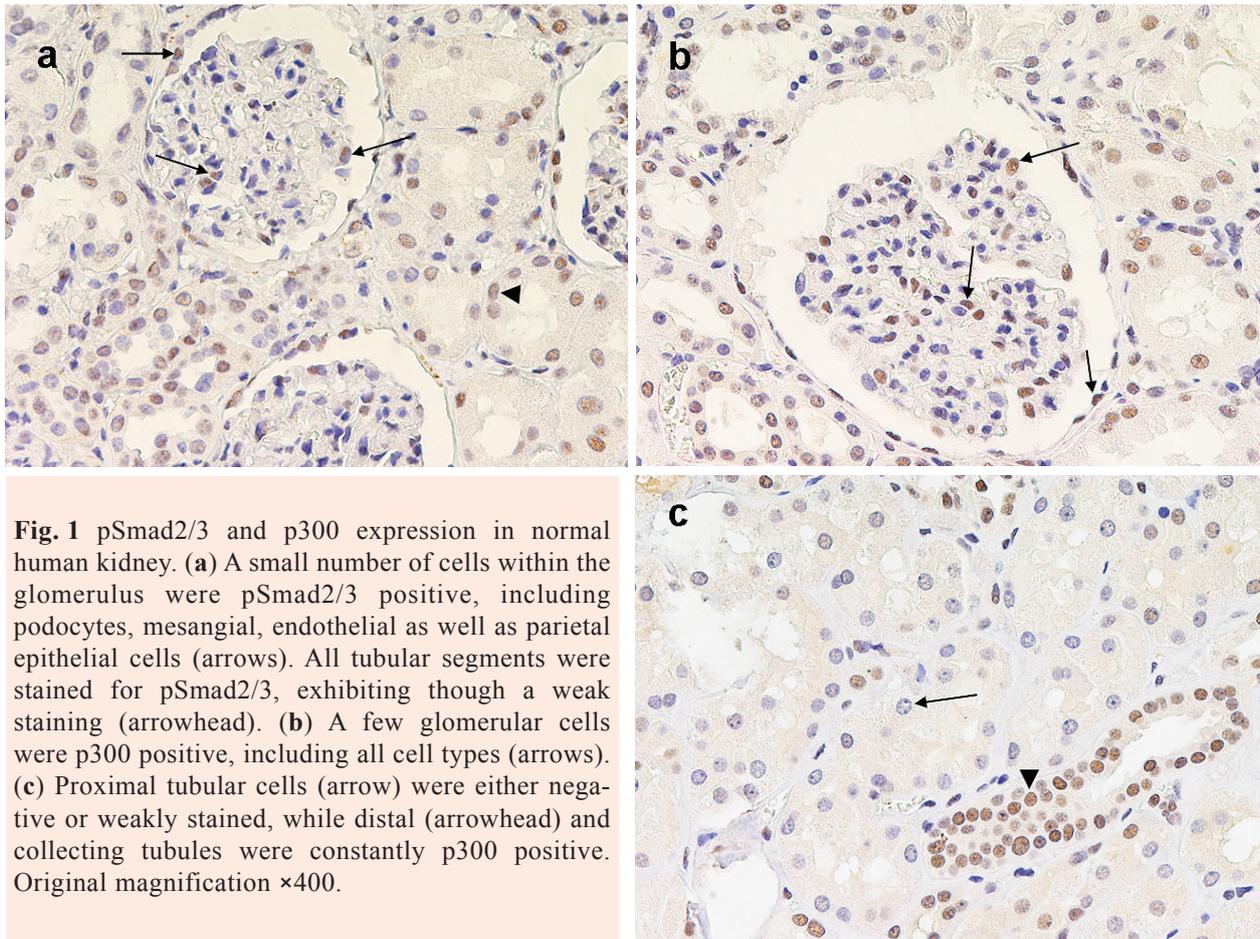
Results

Smad2/3 activation in normal human kidney

Within normal human kidney a small number of cells within the glomerulus (<10% of total glomerular cells) were pSmad2/3 positive, including podocytes, mesangial, endothelial as well as parietal epithelial cells. All tubular segments (proximal, distal and collecting) were stained for pSmad2/3, exhibiting though a weak staining (Fig. 1a). The staining pattern was nuclear as expected from *in vitro* studies demonstrating that phosphorylation of Smad2/3 leads to their translocation from the cytoplasm to the nucleus where they exert their effects [26].

p300 expression in normal human kidney

Less than 30% of glomerular cells were p300 positive, including all cell types. Proximal tubular cells were either negative or weakly stained. Distal tubular cells as well as collecting tubules were constantly p300 positive with a moderate to strong staining intensity (Fig. 1b and 1c). The staining pattern was nuclear as expected from previous immunohistochemical studies in other tissues [27].



pSmad2/3 and p300 expression in human glomerulonephritis

There was a dramatic increase in the expression of pSmad2/3 and p300 in all glomerular cell types in both proliferative and nonproliferative glomerulonephritides ($p < 0.0005$) (Fig. 2a). Proliferative glomerulopathies exhibited an increased pSmad2/3 expression in glomeruli ($p < 0.001$) and all tubular segments ($p = 0.003$, $p = 0.015$ and $p = 0.025$ for proximal, distal and collecting tubules respectively) and an increased p300 immunopositivity in glomeruli ($p = 0.005$) and proximal tubules ($p < 0.0005$) compared to nonproliferative GNs (Table 2). The expression of both molecules was more intense in the secondary group of proliferative GNs (Table 3). Similar immunostaining results were obtained comparing the nonproliferative group with the control group except for the fact that there was no difference in pSmad2/3 expression in proximal tubules ($p = 0.228$). In particular, only pauci-immune and

lupus proliferative nephritis exhibited an increased pSmad2/3 expression in proximal tubules ($p = 0.001$ and $p = 0.003$ respectively) (Fig. 2b). Taking into account all GNs, secondary glomerulopathies exhibited an increased pSmad2/3 and p300 expression in glomeruli ($p < 0.0005$ and $p = 0.015$ respectively) and proximal tubules ($p = 0.002$ and $p = 0.004$ respectively) compared to primary GNs. Compared to control no difference was detected in the expression of p300 in distal and collecting tubules of specimens with GN (Fig. 2c and 2d).

Comparisons between the different types of GN with the Kruskal-Wallis test showed a statistically significant difference in the expression of pSmad2/3 and p300 in glomeruli and proximal tubules ($p < 0.0005$, $p = 0.04$ and $p = 0.015$, $p = 0.001$ respectively). In detail, pSmad2/3 exhibited an increased glomerular expression in Pauci-Im GN compared with MCD, MGN, FSGS, and non-proliferative lupus nephritis (NPLN) and in proliferative lupus nephritis (PLN) compared with FSGS and an

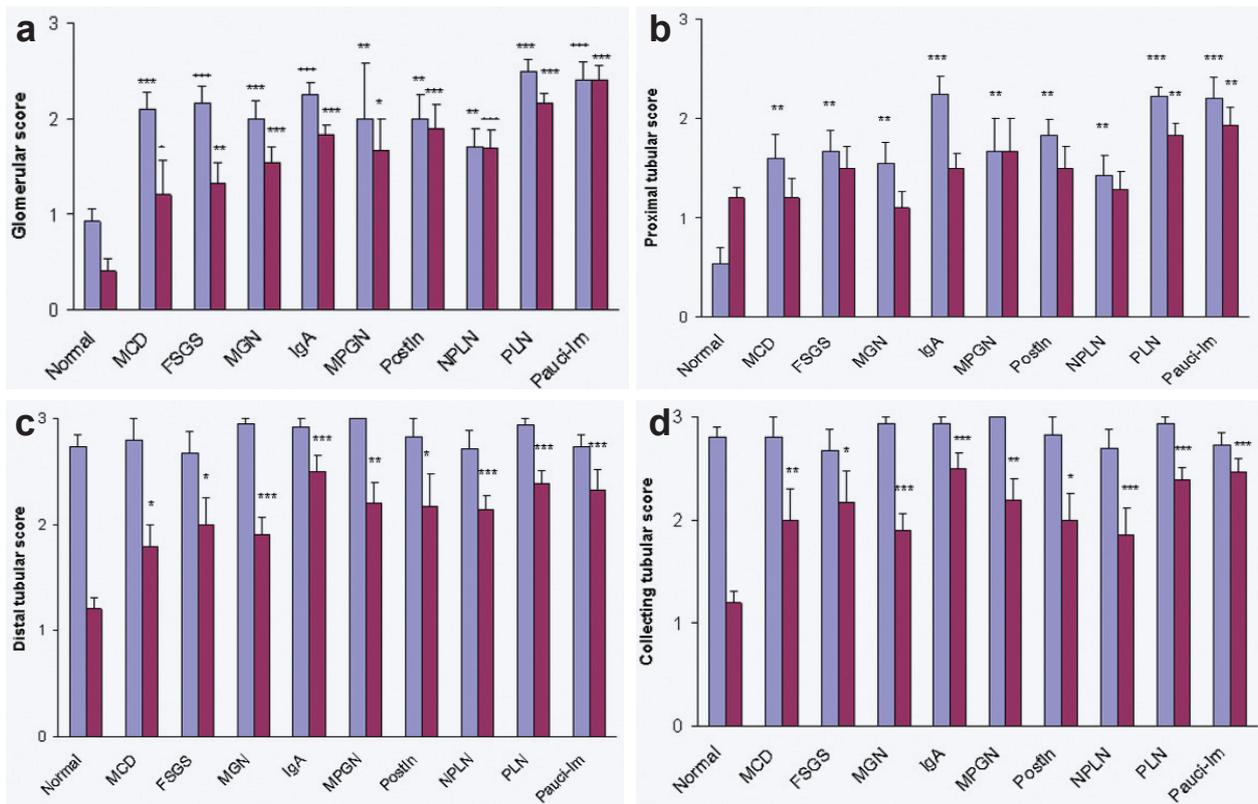


Fig. 2 Glomerular (a), proximal tubular (b), distal tubular (c) and collecting tubular (d) staining scores of p300 (■) and pSmad2/3 (■) in normal and diseased human kidneys. The error bars represent SE. *: P<0.05, **: P<0.01, ***: P<0.001. For abbreviations see legend to Table 1.

increased proximal tubular expression in Pauci-Im GN compared with MCD. Similarly, p300 glomerular expression was more intense in Pauci-Im GN and PLN compared with NPLN while its proximal tubular expression was increased in Pauci-Im GN compared with MGN, FSGS and NPLN.

With regard to pathologic lesions p300 and pSmad2/3 were almost constantly expressed in segmental hyperplastic lesions (100% and 97% respectively) (Fig. 3a and 3b), cellular and fibrocellular crescents (98% and 97% respectively) (Fig. 3c and 3d) and microadhesions (92% and 87% respectively) (Fig. 3e and 3f), while they were frequently detected in sclerotic glomeruli (80% and 79% for p300 and 63% and 73% for pSmad2/3 for segmentally or globally sclerosed glomeruli respectively) (Fig. 3g–j, Table 4). In cells overlying the sclerotic segments the expression of both molecules was prominent (Fig. 3g and 3h). There was a statistically significant association in the expression of both molecules in serial sections of glomeruli with

microadhesions and segmental sclerosis (Fisher's exact test, $p = 0.001$ and $p = 0.015$ respectively). Chi square test in respective cases with segmental hyperplasia or cellular crescents could not be performed due to impotence of the method (all serial sections with segmental hyperplasia and 90% of serial sections with cellular crescents were both positive for p300 and pSmad2/3).

Correlation of pSmad2/3 with p300 expression at correspondent nephron sites - Correlations between immunohistochemical, clinical, and histologic parameters

Taking into account all glomerulonephritides, there was a positive correlation between the expression profile of pSmad2/3 and p300 in diseased glomeruli and proximal tubular cells ($r = 0.357$, $p < 0.0005$ and $r = 0.259$, $p = 0.01$) (Table 5). Moreover, pSmad2/3 and p300 glomerular and

Table 2 Comparison of pSmad2/3 and p300 staining intensity in proliferative vs nonproliferative GNs

		Staining Score			
		pSmad2/3	P	P300	P
Glomerulus	Proliferative	2.03±0.61	<0.0005	2.28±0.65	0.005
	Nonproliferative	1.51±0.61		1.98±0.54	
Proximal tubules	Proliferative	1.76±0.67	0.003	2.12±0.64	<0.0005
	Nonproliferative	1.40±0.61		1.54±0.60	
Distal tubules	Proliferative	2.39±0.59	0.015	2.82±0.41	0.869
	Nonproliferative	2.13±0.56		2.82±0.38	
Collecting tubules	Proliferative	2.39±0.60	0.025	2.85±0.36	0.433
	Nonproliferative	2.14±0.63		2.79±0.41	

Staining scores are presented as mean ± 1 standard deviation.

Table 3 Comparison of pSmad2/3 and p300 staining intensity in secondary vs primary proliferative GNs

	Proliferative GN	Staining Score			
		pSmad2/3	P	P300	P
Glomerulus	Secondary	2.24±0.58	<0.0005	2.41±0.62	0.025
	Primary	1.72±0.53		2.07±0.65	
Proximal tubules	Secondary	1.93±0.63	0.009	2.22±0.64	0.096
	Primary	1.50±0.64		1.96±0.64	
Distal tubules	Secondary	2.42±0.59	0.623	2.83±0.39	0.780
	Primary	2.34±0.61		2.82±0.48	
Collecting tubules	Secondary	2.45±0.50	0.426	2.80±0.40	0.176
	Primary	2.29±0.71		2.92±0.27	

Staining scores are presented as mean ± 1 standard deviation.

proximal tubular immunostaining correlated positively with interstitial inflammation ($r = 0.243$, $p = 0.007$, $r = 0.304$, $p = 0.001$ for p300 and $r = 0.193$, $p = 0.043$, $r = 0.223$, $p = 0.02$ for pSmad2/3 respectively), while their expression in distal and collecting tubules exhibited a positive correlation with tubular atrophy ($r = 0.201$, $p = 0.022$, $r = 0.258$, $p = 0.005$ for p300 and $r = 0.19$, $p = 0.042$, $r = 0.203$, $p = 0.032$ for pSmad2/3 respectively). Finally, the expression of pSmad2/3 in the diseased glomeruli and proximal tubules correlated positively with serum creatinine values of the correspondent patients ($r = 0.334$, $p = 0.004$ and $r = 0.356$, $p = 0.002$ respectively) (Table 6).

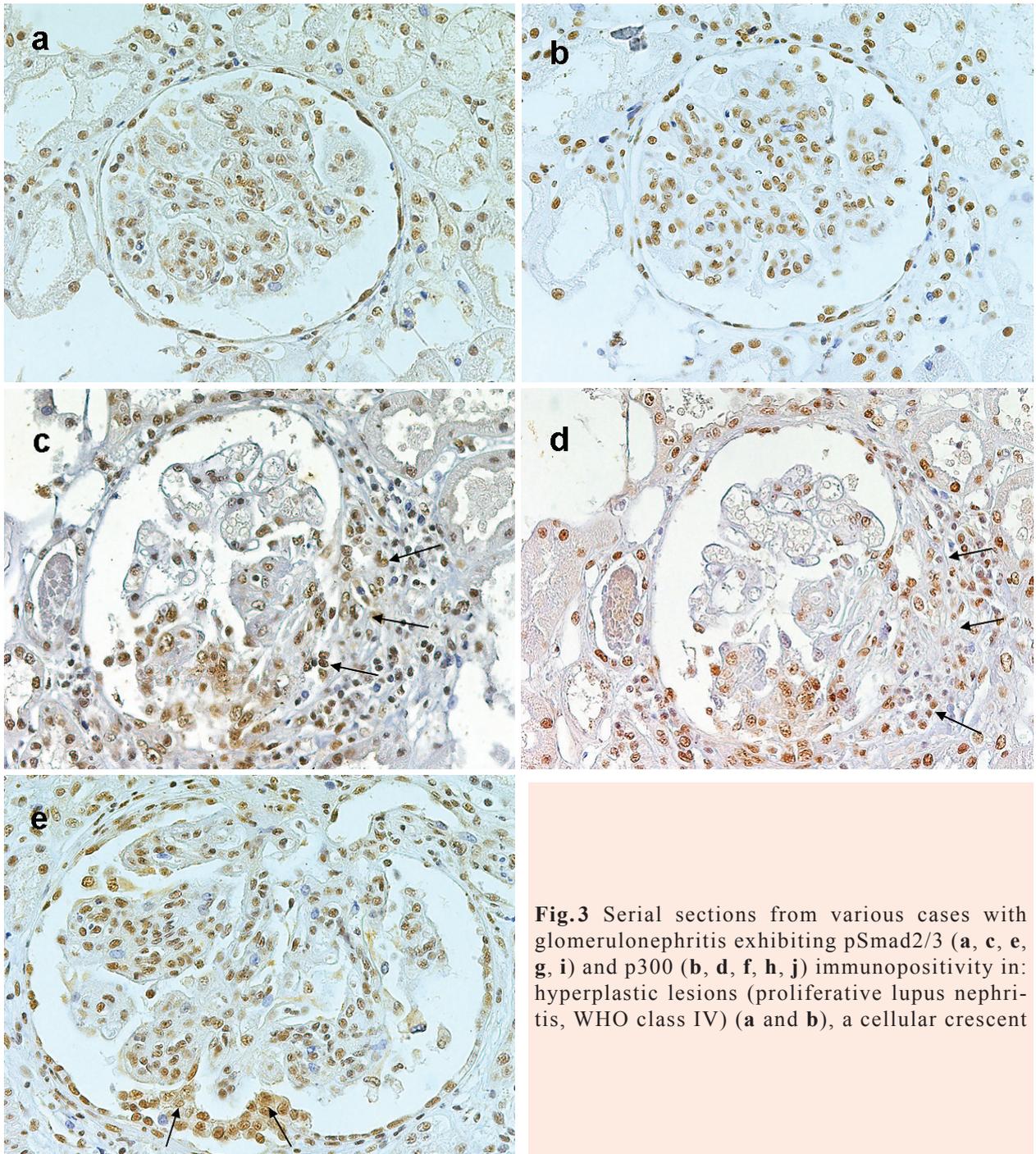
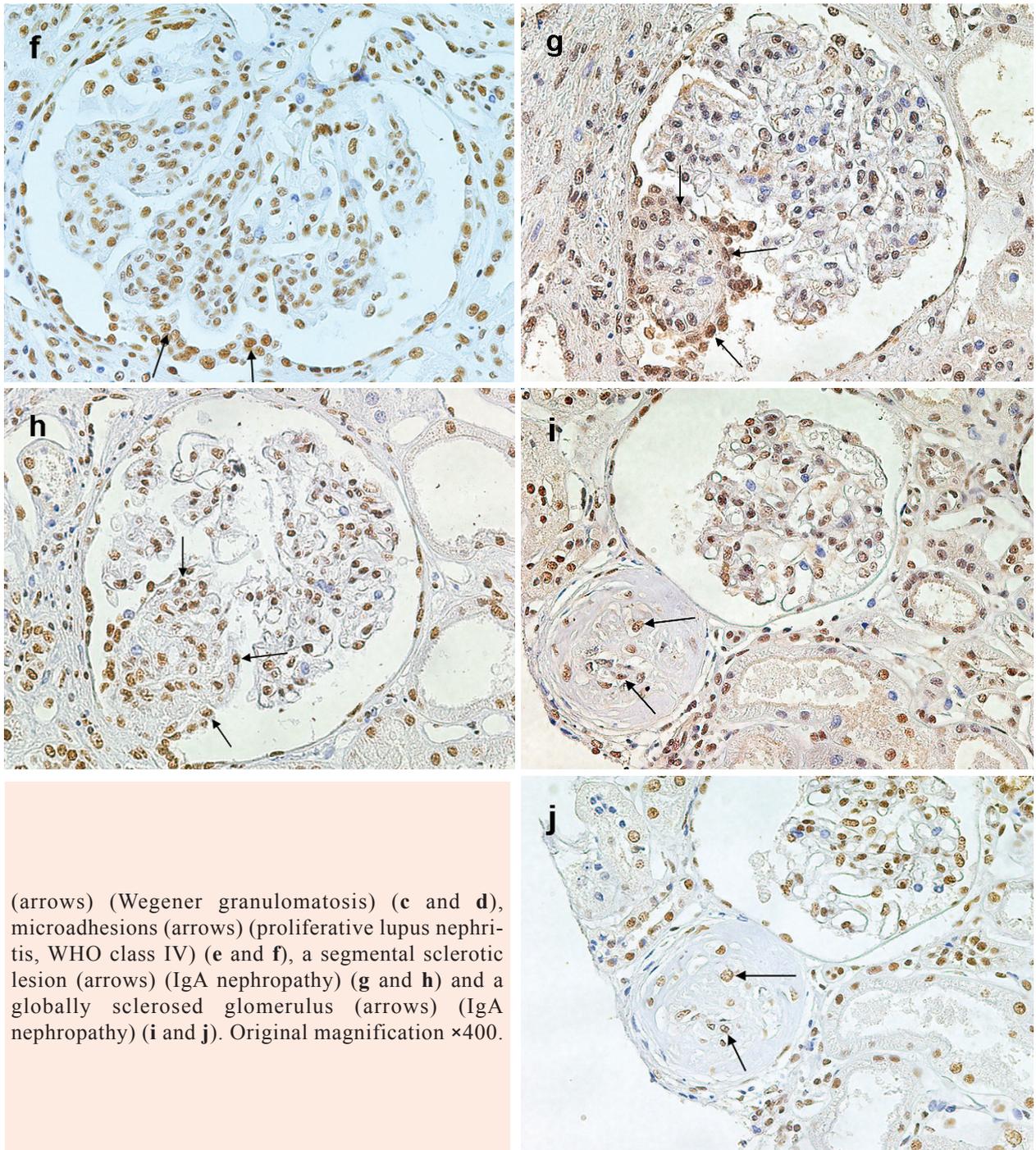


Fig.3 Serial sections from various cases with glomerulonephritis exhibiting pSmad2/3 (a, c, e, g, i) and p300 (b, d, f, h, j) immunopositivity in: hyperplastic lesions (proliferative lupus nephritis, WHO class IV) (a and b), a cellular crescent

However, subgroup analysis revealed that this correlation stood only for nonproliferative glomerulonephritides ($r = 0,406$, $p = 0.021$ and $r = 0.433$, $p = 0.013$). Moreover, the expression of p300 in distal and collecting tubules exhibited a positive correlation with tubular atrophy only in the proliferative group ($r = 0.304$, $p = 0.01$ and $r = 0.27$, $p = 0.01$). pSmad2/3

glomerular and proximal tubular expression as well as p300 glomerular expression correlated positively with lupus nephritis activity ($r = 0.45$, $p = 0.03$, $r = 0.47$, $p = 0.011$ and $r = 0.618$, $p = 0.042$ respectively). No correlation was detected between the expression of the two molecules with 24-hour urinary protein or interstitial fibrosis (Table 6).



Discussion

In this study, the activation of the transcription factors Smad2/3 and the expression of the co-activator p300 in normal human kidney and in specimens from patients with various forms of GN were examined.

A previous study on human renal tissue reported no immunostaining for pSmad2/3 in normal specimens [21]. These results contrast with the findings of the current study. This divergence is most likely due to differences in the immunostaining protocol or the usage of a different primary antibody. Specifically, Kim *et al.* baked sections in a microwave oven for 5 minutes, incubated the sec-

Table 4 The percentages of p300/pSmad2/3 immunopositivity incidence in the various lesions of the different groups of GN

	Glomeruli					
	Total number of cases	Segmental hyperplasia	Cellular crescents	Micro-adhesions	Segmental sclerosis	Global sclerosis
Non-proliferative primary GNs	49			70%/70%	71%/60%	82%/70%
Non-proliferative SLE GNs	18			100%/100%		
Proliferative primary GNs	37	100%/100%	100%/100%	91%/78%	90%/67%	71%/67%
Proliferative secondary GNs	48	100%/96%	97%/96%	97%/94%	78%/62%	80%/78%
All GNs	152	100%/97%	98%/97%	92%/87%	80%/63%	79%/73%

tions with the primary antibody at a dilution of 1 in 1000 and used an avidin-biotin-peroxidase method for antibody localization. In our study sections were baked for 15 minutes, the primary antibody was used at a dilution of 1 in 500 and a biotin free, two-step, HRP-labelled detection system was used for antibody localization. This system has been reported to have in many instances greater sensitivity than the avidin-biotin system [28, 29]. Regarding animal studies, it has been demonstrated that Smad2 and 3 are weakly expressed in glomeruli and tubules in the 15-day mouse embryo [30]. In another study, they were detected at all embryonic and postnatal stages examined, including the adult mouse kidney [31]. TGF- β 1 (whose signals are mostly mediated by Smad2/3) and its receptors are

expressed in all glomerular cell types as well as in tubular cells in the rat kidney [32]. Furthermore, previous reports have shown that TGF- β has an impact on tubular function [33, 34]. Thus, it is possible that Smad2 and Smad3 are important factors for structural and functional homeostasis in the nephron, although their physiological functions in the kidney are not well understood.

p300 expression in mouse mesangial cells has been documented in a previous study [22]. However, no data exist on its expression in normal or diseased human kidneys. p300 is a transcriptional “integrator” involved in a wide range of cellular activities, such as DNA repair, cell growth, differentiation, and apoptosis [35]. Thus, its expression in normal glomerular, distal and collecting tubular

Table 5 Correlation of pSmad2/3 with p300 expression at correspondent nephron sites

Glomerulus	Proximal tubules	Distal tubules	Collecting tubules
All/NP/P	All/NP/P	All/NP/P	All/NP/P
0.357***/0.228/0.254*	0.259**/0.275/0.083	0.11/0.042/0.123	0.183/0.163/0.142

The pSmad2/3 immunohistochemical staining score was correlated with p300 staining score at correspondent nephron sites using the Spearman single correlation coefficient. All: All glomerulonephritides, NP: nonproliferative GN, P: proliferative GN; *: P<0.05, **: P<0.01, ***: P<0.001.

Table 5 Correlations between immunohistochemical, clinical, and histologic parameters

	Scr	Prot	Glomscl	IF	TA	II	
	All/NP/P	All/NP/P	All/NP/P	All/NP/P	All/NP/P	All/NP/P	
p300	Glomerulus	0.108/0.095/0.051	0.01/0.02/0.098	-0.109/-0.065/-0.116	0.091/0.181/0.079	0.088/0.068/0.114	0.243**/0.169/0.192
	Proximal tubules	0.158/0.132/-0.001	-0.135/-0.181/0.083	0.01/0.126/-0.002	0.027/0.064/0.043	0.020/0.040/0.023	0.304**/0.231/0.208
	Distal tubules	-0.020/-0.044/-0.059	0.056/-0.083/0.212	0.037/0.067/0.023	0.09/0.047/0.131	0.201*/0.074/0.304*	0.031/-0.034/0.076
	Collecting tubules	-0.053/-0.092/-0.158	0.023/-0.067/0.176	0.088/0.105/0.1	0.174/0.166/0.183	0.258**/0.238/0.270**	0.069/-0.045/0.114
pSmad2/3	Glomerulus	0.334**/0.406*/0.246	-0.157/-0.002/-0.216	-0.075/-0.057/-0.089	-0.129/-0.045/-0.182	-0.051/-0.044/-0.070	0.193*/0.024/0.100
	Proximal tubules	0.356**/0.433*/0.232	-0.043/-0.123/0.06	-0.059/-0.043/-0.071	-0.083/-0.085/-0.113	-0.038/0.002/-0.106	0.223*/0.148/0.108
	Distal tubules	0.097/0.231/-0.083	-0.005/-0.055/0.097	-0.003/0.084/-0.057	0.79/0.038/0.078	0.190*/0.088/0.220	0.137/-0.02/0.111
	Collecting tubules	0.132/0.263/-0.041	0.019/0.037/0.085	0.060/0.086/0.031	0.123/0.130/0.095	0.203*/0.173/0.195	0.143/-0.078/0.184

The immunohistochemical staining score at various nephron sites was compared with clinical data (serum creatinine levels and 24-hour urinary protein) and indices of histologic injury, using the Spearman single correlation coefficient in all glomerulonephritides (All), nonproliferative GN (NP) and proliferative GN (P). Values are given as r; *, P<0.05, **, P<0.01, ***, P<0.001. Scr: Serum creatinine, Prot: 24-hour urinary protein, glomscl: glomerulosclerosis, IF: interstitial fibrosis, TA: tubular atrophy, II: interstitial inflammation. (Some Spearman r values in the All GNs column have superior P-values than corresponding higher r values in NP GNs column. This is due to the larger amount of All GNs).

cells in our study suggests a potential role for p300 in maintaining the normal function of these cells.

This study provides the first demonstration of Smad2/3 activation and p300 overexpression in a broad spectrum of human glomerulonephritides. Although the activation of Smad2/3 is supposed to exert antiproliferative effects [36], in this study pSmad2/3 exhibited a more intense expression in proliferative GNs, particularly in the secondary group. However, this finding is in context with an earlier study demonstrating that glomerular TGF-β1 expression was more severe in proliferative compared to nonproliferative GNs [6]. Moreover, although TGF-β in the initial phases of tumorigenesis acts as a tumor suppressor, in late stage tumors, it has been shown to act as a tumor promoter [37]. Smads, like TGF-β, depending on cellular context, may thus exert proliferative or antiproliferative

effects [38]. Similarly, although several lines of evidence suggests that p300 is a negative regulator of the cell cycle [39, 40], its ability to serve also as mediator of cell proliferation remains a paradox [41]. Moreover, the increased expression of pSmad2/3 and p300 in glomeruli as well as in tubules of proliferative GNs and particularly of the secondary group may be due to the increased inflammation that accompanies the disease process. This is supported by the positive correlation of pSmad2/3 and p300 glomerular and proximal tubular immunostaining with interstitial inflammation. Except for its pro-fibrotic effect TGF-β is also known to possess anti-inflammatory and immunoregulatory properties [23]. It is involved in the resolution of renal injury in the anti-Thy1 model of GN [42]. TGF-β also exerts its anti-inflammatory effect by counteracting inflammatory cytokine pro-

duction and by inhibiting IL-1 β and TNF- α -induced NF κ B activation [43]. Exactly how TGF- β regulates the immune response remains poorly understood, but Smad3 may play a critical role in immune-regulation by TGF- β [44–46]. On the other hand, p300 except for its recruitment by Smad complexes it is also implicated in the NF κ B signaling pathway by engaging to the activated p65 subunit [47]. NF κ B is a transcription factor that plays an important role in the transcriptional regulation of genes involved in inflammation. Previous studies have shown that NF κ B is activated in tubules and glomeruli in various experimental models of renal injury [48, 49]. Thus, there might be a more intense Smad2/3 activation and p300 expression in conditions associated with increased inflammation.

In segmental hyperplastic lesions as well as in cellular and fibrocellular crescents which are hyperplastic lesions composed of epithelial cells and macrophages, pSmad2/3 as well as p300 immunopositivity was prominent. Based on the considerations made above the increased expression of these molecules in these lesions may either imply a potential mitogenic influence of these factors in cells forming cellular crescents or it may be due to the amplified inflammatory status and the subsequent release of cytokines (such as TGF- β), that accompanies the crescent formation process.

The common expression of pSmad2/3 and p300 in microadhesions may imply a role of these two molecules in the early stages of glomerulosclerosis. The statistical significant association in the expression of both molecules in serial sections of glomeruli with microadhesions and segmental sclerotic lesions suggests a possible recruitment of p300 by pSmad2/3 in these lesions in order to activate the transcription of genes responsible for glomerulosclerosis. As far as the chronic phase of glomerular injury is concerned, sclerotic glomeruli were almost constantly p300 positive, whereas pSmad2/3 immunopositivity was frequently detected. This suggests a possible role for these two molecules in advanced fibrotic events. This is the first study to demonstrate the colocalization of p300 and pSmad2/3 in early and advanced sclerotic lesions.

The positive correlation of glomerular and proximal tubular pSmad2/3 with the severity of renal dysfunction along with the positive correlation of pSmad2/3 and p300 with tubular atrophy in

distal and collecting tubules may imply a role of these molecules in the progression of renal disease. This is also supported by the positive correlation of glomerular and proximal tubular pSmad2/3 and glomerular p300 with lupus nephritis activity and the increased expression of both molecules in glomeruli and proximal tubules of severe forms of GNs such as Pauci-IM GN and PLN. Moreover, the positive correlation of pSmad2/3 with p300 immunopositivity in the diseased glomeruli and proximal tubules along with their common expression at sites of microadhesions, cellular crescents, segmental hyperplastic and sclerotic lesions, argues that these molecules cooperate in inducing renal injury. Interestingly, it has been recently demonstrated that p300 is an essential component of the cellular TGF- β signal transduction pathways mediating stimulation of collagen synthesis in fibroblasts [50].

In summary, this study has identified the expression of pSmad2/3 and p300 in normal human kidney and a marked increase in their expression in various nephron sites in many forms of GN. The correlation of Smad2/3 activation and p300 with clinical and histologic parameters suggests that this pathway plays an important role in the pathogenesis and the progression of human GN. Thus, blockade of Smad2/3 activation or pSmad2/3-p300 interaction may provide a novel target in the treatment of human GN. The latter treatment would have the potential of inducing minimal side effects as this interaction seems to be one of the late steps in the Smad signaling pathway.

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