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Identification of Novel Long Noncoding RNAs Associated with TGF- β /Smad3-Mediated Renal Inflammation and Fibrosis by RNA Sequencing

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We have previously shown that transforming growth factor- β /Smad3-dependent miRNAs play a critical role in renal inflammation and fibrosis. However, off-target effects of miRNAs limit their therapeutic application. Recently, emerging roles of long noncoding RNAs (lncRNAs) in diseases have been recognized. In this study, we used high-throughput RNA sequencing to identify the Smad3-dependent lncRNAs related to renal inflammation and fibrosis in *Smad3* knockout mouse models of unilateral ureteral obstructive nephropathy and immunologically induced anti-glomerular basement membrane glomerulonephritis. Compared with wild-type mice, 151 lncRNAs in the unilateral ureteral obstructive nephropathy kidney and 413 lncRNAs in kidneys with anti-glomerular basement membrane glomerulonephritis were significantly altered in *Smad3* knockout mice. Among them, 21 common lncRNAs were up-regulated in wild-type, but down-regulated in *Smad3* knockout, kidneys in both disease models in which progressive renal inflammation and fibrosis were abolished when the *Smad3* gene was deleted or suppressed. Real-time PCR confirmed these findings and revealed the functional link between Smad3-dependent lncRNAs np_5318/np_17856 and progressive kidney injury. Results demonstrate that the identification and characterization of functional lncRNAs associated with kidney disease may represent a promising research direction into renal disorder and may lead to the development of new lncRNA therapies for kidney diseases. (*Am J Pathol* 2014; 184: 409–417; <http://dx.doi.org/10.1016/j.ajpath.2013.10.007>)

Increasing evidence shows that chronic kidney disease (CKD) is a major problem and health care burden worldwide. More than 10% of the entire population experiences different stages of CKD. Renal inflammation and fibrosis are the common manifestations of CKD.¹ Transforming growth factor- β 1 (TGF- β 1), a well-studied profibrogenic cytokine, exerts its biological effects by activating its downstream mediators, Smad2 and Smad3, which is counterregulated by an inhibitory Smad7.^{2,3} Although Smad3 is a key transcription factor in response to many fibrogenic mediators, targeting Smad3 may cause autoimmune disease by impairing immunity.^{2,4–6} Thus, alternative approaches to inhibit TGF- β actions should be developed in an attempt to suppress renal inflammation and fibrosis without impairing the immune system.

Emerging evidence shows that noncoding RNAs (ncRNAs) may play a critical role in the development of kidney diseases.

The identification of disease-related miRNAs in nephropathy was thought to be a specific therapy for treating the kidney disease.^{3,7–16} However, the off-target effects hinder the development of specific miRNA therapy, because one miRNA is able to regulate many target genes.^{7,10}

Since the discovery of Xist, a long noncoding RNA (lncRNA) required for mammalian X chromosome inactivation,¹⁷ many

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lncRNAs have been reported in mammals and other vertebrates.¹⁸ At present, many lncRNAs, defined as RNAs >200 nucleotides (nt) that do not encode any protein, are found to be able to control gene expression.¹⁸

The development of high-throughput RNA sequencing (RNA-Seq) accelerates the discovery of novel ncRNAs because RNA-Seq can achieve single-base resolution and capture all of the transcripts in the samples.^{19,20} In this study, we used this technique to search for *Smad3*-dependent lncRNAs during renal injury in mouse models of immunologically induced anti-glomerular basement membranous glomerulonephritis (anti-GBM GN) and nonimmune disease of unilateral ureteral obstructive nephropathy (UUO). The results of real-time PCR validation, sequence analyses, and chromatin immunoprecipitation (ChIP) assays revealed a functional link between *Smad3*-dependent lncRNAs and progressive kidney injury. Thus, outcomes from this study suggested a novel research direction into renal disorder and may promote new therapeutic strategies for kidney diseases by targeting lncRNAs.

Materials and Methods

Mouse Model of UUO and Anti-GBM GN

The animal models of UUO and anti-GBM GN were induced in both sexes of C57BL/6J wild-type (WT) and *Smad3* knockout (KO) mice at 8 to 10 weeks of age, as previously described.^{21,22} Briefly, for the UUO model, the left ureter from *Smad3* WT or KO mice ($n = 8$ per group, both sexes, 8 weeks of age, 22 to 25 g body weight) was ligated and the

UUO kidney was harvested at day 5 after surgery for further analysis. In addition, a group of eight *Smad3* WT or KO mice, sham operated on, were used as a control. For the anti-GBM GN model, groups of eight *Smad3* WT or KO mice were pre-immunized with sheep IgG in Freund's complete adjuvant (Sigma Chemical Co, St. Louis, MO), followed 5 days later by i.v. administration of sheep anti-mouse GBM immunoglobulin at a dose of 60 μ g/g of body weight (termed day 0). Diseased mice were euthanized at day 10 for further analysis. Groups of age- and weight-matched normal *Smad3* WT or KO mice were used as normal controls ($n = 8$). The experimental procedures for both models were performed following the approved protocol by the Animal Experimentation Ethics Committee at The Chinese University of Hong Kong (Hong Kong).

Ultrasound-Mediated Gene Transfer of Inducible *Smad7* Overexpression Plasmids into the Ligated Kidneys

The mixture of *Smad7* and tetracycline-on (tet-on) plasmids was transferred into the ligated kidneys of UUO, as described previously.²³ The experimental procedures were approved by the Chinese University of Hong Kong's Animal Experimental Ethics Committee.

Smad7 Knockout Mice

Mutant mice deficient in exon 1 of the *Smad7* gene from a CD1 background (provided by Prof. Rainer L. Heuchel,

Table 1 Real-Time PCR Primers for 21 lncRNAs and GAPDH

Gene	Forward primer	Reverse primer
np_18588	5'-CGGAGGATTGATAGCCAAAA-3'	5'-CCTGGTTGTGCTGTTTCCTT-3'
np_26992	5'-GACCAATGTTTCGTAGCAGCA-3'	5'-TCCTTCTCTTTGTGCGTGTG-3'
na_2414	5'-CTGCCTCTGTGCTGCATTTA-3'	5'-TCTTTGTACCCCAAGCAGTG-3'
np_29908	5'-TGGATTTGGCCTCAGTTTTC-3'	5'-CAGCTTCGAGTTTGTACCA-3'
np_16555	5'-CAGCCTAGCTCACCTTCTGG-3'	5'-TGTTTCATCTGTGGCTCTTGG-3'
np_5340	5'-TTCCTGGGCTTCACAAGTTT-3'	5'-GCGTGGCTTCAGGTATTTGT-3'
np_17234	5'-TGTTTTAACCCATTGTATCATTTCA-3'	5'-CAACCCCTTTGGAAGACTC-3'
np_18603	5'-CCTTTACAAGCAAAAGCCAAA-3'	5'-CCCTTGAGACACTGCCTGAC-3'
na_9884	5'-ACCACAGCAGATACCAAGC-3'	5'-CAGCAAGTCCTTTTTTCCAC-3'
np_5392	5'-GGGTGTGAACGGAGACTGTT-3'	5'-GGCGAGGAATGTGTTTGT-3'
np_18004	5'-CTCCATAAAGAGCCCCATGA-3'	5'-TTGGGCCTTCCAACAATAAC-3'
np_28730	5'-TCTGGGTCCAACCTCATTTT-3'	5'-CCTGTTTAAATTGGGGGAAA-3'
np_4334	5'-AGAGGGCAAGCAACATGTGTC-3'	5'-GCTGTGGGGAGAGCAACTAA-3'
np_18636	5'-CAACCTAAGGCTGCTCTCTCC-3'	5'-CCATCAAGAAACCTGGCAAT-3'
np_17856	5'-GAAGCCTGCAGGAAGATGAC-3'	5'-ACCAGTCACAAAAGCCAACC-3'
np_18537	5'-TTCCAGTAGTGCTGCACAGG-3'	5'-TCCAGGTTTCCAACCTTTTGC-3'
np_5318	5'-AACTCGCCACAGAAATCCAC-3'	5'-ACAACCCCAAAACAAGCTGTC-3'
np_19902	5'-TGTTGCTAGAATGGCAGGTG-3'	5'-TCCTGCCTGGGAATACTGTC-3'
np_28233	5'-GATGGGGAAGAAACCCATTT-3'	5'-CAGGGCCAGTTATGTCCAGT-3'
np_2108	5'-AGATCTCCCCCTCTTGGGAAA-3'	5'-CTCCCTCTCCACACTGCTTC-3'
np_28496	5'-AGCAGGCTAAGTCAGGTGA-3'	5'-GGGTTGAGCTGCCATGTATT-3'
GAPDH	5'-TGCTGAGTATGTCGTGGAGTCTA-3'	5'-AGTGGGAGTTGCTGTTGAAATC-3'

Karolinska Institutet, Stockholm, Sweden) have been described recently.²⁴ All mice were from a CD1 background. A mouse model of UUO was induced in groups of six to eight *Smad7* KO or WT mice (male, 8 weeks of age, 22 to 25 g body weight), as previously described.^{21,25} The experimental procedures were approved by the Chinese University of Hong Kong's Animal Experimental Ethics Committee.

RNA Preparation for Sequencing

Kidney tissues of *Smad3* KO/WT mice ($n = 2$ in each group) were collected from a normal, UUO model at day 5, and an anti-GBM GN model at day 10 for RNA sequencing. Total RNA from the kidney tissue was extracted by TRIzol reagent (Life Technologies, Carlsbad, CA), as per the manufacturer's instructions. Additional DNase I digestion and clear-up steps were performed per the protocol from the Beijing Genomics Institute (Shenzhen, China). RNA quality

and purity were determined by the ND-1000 Nanodrop (NanoDrop, Wilmington, DE). For each sample, RNAs were fractionated with a denaturing polyacrylamide gel. To maximize the ncRNA database for analysis, RNAs >150 nt were used for cDNA synthesis. After adenylating the 3' end of RNAs and ligating the adapter, the RNA samples were preceded to library construction following the protocol at Beijing Genomics Institute (Supplemental Figure S1). The sequencing was performed by the Genome Analyzer (serial No. Hiseq2000; Illumina, San Diego, CA).

Analysis of Sequencing Data and Assembling RNA Transcripts

Low-quality reads, >50% of the sequence reads (including adapters) with a quality score of ≤ 10 and $N > 5\%$, were discarded. Ribosome RNA sequences were filtered from the raw fragments. Remaining clean reads were mapped to a genome (University of California, Santa Cruz, CA, genome

Figure 1 Results of RNA-Seq in *Smad3* WT/KO kidneys in UUO and anti-GBM GN models. **A:** The number of transcripts aligned to the known ncRNA database and transcripts predicted to be ncRNAs. **B:** Comparisons of differentially expressed lncRNAs (length >200 nt) between normal and diseased kidney and between *Smad3* KO and WT mice in two kidney disease models. **C:** Venn diagrams demonstrate overlapped lncRNAs (common lncRNAs) versus nonoverlapped lncRNAs related to *Smad3* that were differentially expressed in both UUO and anti-GBM GN models. $P < 0.01$ (**B** and **C**). **D:** Real-time PCR validation of 21 common lncRNAs in anti-GBM and UUO models of *Smad3* WT (S3WT)/*Smad3* KO (S3KO) mice ($n = 8$ in each group). The relative levels of target lncRNAs were calculated as the ratio to GAPDH. * $P < 0.05$ versus S3WT mice. Nor, normal.

browser, mouse genome July 2007, mm9) by using Tophat [1.3.0.Linux_x86_64 (bowtie-0.12.7)] and then assembled with Cufflinks (1.3.0.Linux_x86_64; Berkeley, CA). To discriminate coding from noncoding transcripts, a support vector machine-based classifier (coding potential calculator) was used to predict novel ncRNAs.²⁶ The prediction of novel lncRNA was assembled based on the SwissProt database (Hinxton, Cambridge, UK). All of the ncRNAs were annotated by aligning the genomic sequence against RFAM, a collection of multiple sequence alignments and covariance models representing ncRNA families (<http://rfam.sanger.ac.uk>, last accessed August 31, 2013), as previously described.²⁷ The RPKM value was used to obtain a comparable measure of gene expression, and Pearson correlation was used to analyze RPKM value correlation. ncRNAs >200 nt were selected as lncRNAs for further analysis because approximate 200 nt is the common cutoff to define lncRNA in the literature.¹⁸ The sequencing data obtained were deposited in the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/bioproject/223210>; accession number PRJNA223210).

Chromosomal Location and Gene Ontology of lncRNAs

We used an evolutionary conserved region (ECR) browser²⁸ to detect any conserved Smad binding motif prediction in mouse regulatory regions. Chromosomal location and gene ontology of the 21 common lncRNAs and their linked genes were searched on the EMBL-EBI database (<http://www.ebi.ac.uk>,

last accessed August 31, 2013) and the ECR Browser (<http://ecrbrowser.dcode.org>, last accessed August 31, 2013).²⁸

ChIP Analysis

ChIP was performed with a transcription factor ChIP kit, as previously described.^{15,29} In brief, cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C, quenched with glycine, and then sonicated using a Bioruptor (Diagenode, Liège, Belgium) to generate 300- to 600-bp DNA fragments. Immunoprecipitation was performed with the antibody against Smad3 (Upstate/Millipore, Billerica, MA), and a normal IgG was used as a control. Precipitated DNAs were detected by PCR using specific primers: Smad binding site (SBS) for np_17856, 5'-GAAGAGATCTTATACAT-TTCCCCCTA-3' and 5'-TTCCTCTTCCCAATGTGGTC-3'; and SBS for np_5318, 5'-CTCTCTCAAACAGCCTGTGG-3' and 5'-GAAATTTGGAGGTGCAATCAA-3'.

RT-PCR and Real-Time PCR Analysis

lncRNA expression was quantified by real-time PCR with SYBR Green (Life Technologies, Carlsbad, CA) as the fluorescent reporter. Total RNA (1 µg) was used to synthesize the first strand of cDNA using M-MLV Reverse Transcriptase (Life Technologies). The real-time PCR was performed as previously described.²³ Primers for 21 common lncRNAs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. The relative levels of target

Table 2 Comparison of Expression Levels of 21 Common lncRNAs in S3WT/S3KO Kidneys with UUO and Anti-GBM GN

lncRNA	Length (nt)	Log ₂ fold change			
		S3WT (UUO vs Nor)	S3KO (UUO vs Nor)	S3WT (GBM vs Nor)	S3KO (GBM vs Nor)
np_18588	574	2.146*	-3.660**	2.834***	-4.600**
np_26992	1228	2.174**	-2.982**	2.419***	-4.922**
na_2414	528	2.196**	-3.245***	2.320**	-2.085***
np_29908	1413	2.196**	-4.075**	2.213**	-5.015***
np_16555	818	2.229**	-4.245***	3.132***	-5.185***
np_5340	2157	2.360***	-3.708***	3.104***	-3.384***
np_17234	269	2.381***	-3.397***	2.789***	-3.337***
np_18603	606	2.381*	-5.535***	2.497**	-2.474**
na_9884	436	2.474*	-2.175***	3.156***	-3.337***
np_5392	2917	2.544***	-2.075**	3.320***	-5.600***
np_18004	621	2.644***	-3.883**	2.294***	-4.822**
np_28730	436	2.703***	-4.776**	2.227***	-4.715**
np_4334	1393	2.722**	-2.277**	4.337***	-3.538***
np_18636	1495	2.866**	-2.535**	3.950***	-5.474***
np_17856	1002	2.966**	-3.163**	3.834***	-5.102***
np_18537	345	3.307**	-2.660***	3.879***	-3.600***
np_5318	2180	3.392***	-2.798***	3.642***	-6.059***
np_19902	747	3.917***	-4.776**	3.589***	-4.715**
np_28233	1508	4.428***	-4.660**	3.419	-4.600**
np_2108	737	5.381***	-3.535***	4.642**	-2.474**
np_28496	658	6.146***	-2.982**	5.004***	-4.922**

* $P < 0.01$, ** $P < 0.001$, and *** $P < 0.0001$ for the comparison between groups.

Nor, normal; S3KO, *Smad3* KO; S3WT, *Smad3* WT.

lncRNAs were calculated using the $\Delta\Delta C_T$ method, and the data were normalized with GAPDH.

Statistical Analysis

For lncRNA expression by RNA-Seq, significance levels were indicated as $P < 0.01$ and greater than twofold change. Data obtained from real-time PCR are expressed as the means $\pm$ SEM and analyzed using a t -test in Microsoft Excel (Redmond, WA).

Results

In this study, high-throughput RNA-Seq was used to search for Smad3-dependent lncRNAs during renal injury in mouse models of anti-GBM GN and UUO. Kidneys of Smad3 KO/WT mice with UUO and anti-GBM GN were collected for sequencing, as described in Supplemental Figure S1. Total RNA of all 12 samples of Smad3 KO/WT kidneys was sequenced by Illumina, each generating >50 million reads. Low-quality reads and rRNA sequences were filtered from the raw fragments. Total clean reads were then mapped to genome by using Tophat, an alignment program that maps RNA-Seq reads across the splice junction without relying on gene annotation.³⁰ As shown in Supplemental Table S1, the proportion of clean reads that mapped to the genome ranged from 71.55% to 85.82% in 12 samples. The mapped reads were

assembled into transcripts with Cufflinks, putative transcripts containing both coding and noncoding transcripts.³¹

The identification and characterization of lncRNAs are an experimental and computational challenge. In the present study, all assembled transcripts were aligned to a ncRNA database using blastn (1×10^{-10}). The filter was set as identity >0.9 and coverage >0.8 . Reads mapped to multi-noncoding RNA databases using the previously described criteria were identified as noncoding RNAs. Approximately 20.03% to 35.71% clean reads were mapped to the ncRNA database (obtained from fnadb, lncrnadb, NONCODE, RNAdb, and ensembl) (Supplemental Table S1). All ncRNAs were then annotated by aligning a genomic sequence against RFAM, a collection of multiple sequence alignments and covariance models representing ncRNA families. The resulting hits were then used as supporting evidence for ncRNAs. At last, 12,131 transcripts were aligned to the known ncRNA database, and 32,957 transcripts were predicted to be ncRNAs with a length of >150 nt (Figure 1A).

We next identified lncRNAs that differentially expressed, in both models of anti-GBM GN and UUO kidneys, the transcript abundances measured by reads per kb per million (RPKM; $\text{RPKM} = 10^9 \times C/NL$) where C is the reads number of the transcript, L is the length of the transcript, and N is the total reads number of the sample (Supplemental Table S2).³² Pearson correlation analysis showed that the RPKM values between these groups correlated moderately

Table 3 Chromosomal Location and Gene Ontology of 21 Common lncRNAs and Their Linked Genes

lncRNA	Status	Chromosomal location	Linked gene	Gene Ontology*
np_18588	Intronic	Chr1: 55, 627, 209-55, 627, 782	<i>Plcl1</i>	Signal transducer activity and lipid metabolic process
np_26992	Intronic	Chr10: 40, 123, 552-40, 124, 779	<i>Slc16a10</i>	Transmembrane transport
na_2414	Intronic	Chr4: 117, 190, 004-117, 190, 531	<i>Kif2C</i>	Cell division and chromosome segregation
np_29908	Intronic	Chr1: 55, 657, 642-55, 659, 054	<i>Plcl1</i>	Signal transducer activity and lipid metabolic process
np_16555	Intronic	Chr2: 135, 078, 612-135, 079, 429	<i>Plcb1</i>	Intracellular signal transduction and interleukin-mediated signaling pathway
np_5340	Intronic	ChrX: 162, 039, 814-162, 041, 970	<i>NHS</i>	Cell differentiation
np_17234	Intronic	Chr12: 8716, 669-8716, 937	<i>Pum2</i>	Translation regulation and RNA binding
np_18603	Intronic	Chr1: 61, 800, 641-61, 801, 246	<i>Pard3b</i>	Cell cycle, cell division, and tight junction
na_9884	Sense	Chr5: 52, 973, 057-52, 992, 294	<i>5033403H07Rik</i>	None
np_5392	Intronic	Chr3: 30, 449, 176-30, 452, 092	<i>Mecom</i>	Apoptosis, differentiation, and transcription
np_18004	Intronic	Chr16: 51, 132, 993-51, 133, 613	<i>Tbc1d5</i>	Positive regulation of RabGTPase activity
np_28730	Intronic	Chr16: 77, 503, 372-77, 503, 807	<i>2810055G20Rik</i>	None
np_4334	Intronic	Chr4: 76, 295, 267-76, 296, 659	<i>Ptprd</i>	Cell-cell adhesion and differentiation
np_18636	Intronic	Chr1: 68, 836, 861-68, 838, 355	<i>Erbb4</i>	Apoptosis, lactation, and transcription
np_17856	Intronic	Chr16: 77, 377, 192-77, 378, 193	<i>2810055G20Rik</i>	None
np_18537	Intronic	Chr1: 187, 858, 330-187, 858, 674	<i>Esrrg</i>	Multicellular organismal, development, and transcription
np_5318	Intronic	Chr1: 69, 073, 523-69, 075, 702	<i>Erbb4</i>	Apoptosis, lactation, and transcription
np_19902	Intergenic	Chr7: 37, 982, 813-37, 983, 559	<i>Tshz3</i>	Regulation of transcription and DNA dependent
np_28233	Intronic	Chr14: 22, 305, 506-22, 307, 013	<i>1700112E06Rik</i>	None
np_2108	Intergenic	Chr1: 133, 222, 856-133, 223, 592	<i>PPP1R15B</i>	Translational initiation, signal transduction, and hydrogen peroxide
np_28496	Sense	Chr15: 96, 351, 836-96, 352, 493	<i>ARID2</i>	Chromatin modification, nucleosome disassembly, and regulation of transcription

*Gene ontology was searched on the EMBL-EBI database (<http://www.ebi.ac.uk>, last accessed August 31, 2013).

Chr, chromosome.

(Supplemental Figure S2), which may be due to low expression levels of lncRNAs within the diseased kidney because higher expression levels of mRNAs (0.8 to 1.0) in these diseased kidney tissues were detected by the Pearson correlation analysis (data not shown). Further studies revealed that, compared with normal mice, many lncRNAs were differentially expressed in the diseased kidney of *Smad3* WT mice, but this was less abundant in *Smad3* KO mice (Figure 1B). Interestingly, deletion of *Smad3* largely altered the expression levels of lncRNAs in both disease models (Figure 1B). Of these, 151 lncRNAs (plus or minus twofold to eightfold) in UUO and 413 lncRNAs (plus or minus twofold to ninefold) in anti-GBM GN were differentially expressed in WT and *Smad3* KO kidneys (Figure 1C). Among them, 21 lncRNAs were altered in both UUO and anti-GBM GN models, being *Smad3*-dependent common lncRNAs (Figure 1C). Expression levels of these common lncRNAs were up-regulated in WT (plus twofold to approximately sixfold), but suppressed in *Smad3* KO mice with UUO (−twofold to approximately fivefold) and anti-GBM GN (−twofold to approximately sixfold) ($P < 0.01$) (Table 2). To confirm the expression levels of these 21 common lncRNAs *in vivo*, real-time PCR assays were used. The relative expression levels of 21 lncRNAs were significantly up-regulated in WT, but inhibited in *Smad3* KO kidneys in both anti-GBM GN and UUO models (Figure 1D).

According to the relationship between lncRNAs and their associated protein-coding genes, lncRNAs are classified as sense, antisense, intronic, divergent, and intergenic.^{33,34} In the present study, we focused on the 21 common lncRNAs because they shared all features of both anti-GBM GN and UUO models. The genomic status of 21 common *Smad3*-dependent lncRNAs included two sense, two intergenic, and 17 intronic lncRNAs (Table 3). Some of them were clustered closely within the same chromosome, such as np_18588 and np_29908, and np_18636 and np_5318.

Because 17 lncRNAs were intronic, we then determined whether the linked protein-coding genes of these lncRNAs were involved in inflammation and fibrosis. We blasted 21 common *Smad3*-dependent lncRNAs in the University of California, Santa Cruz, genome browser (December 2011), GRCm38/mm10. The gene ontology terms for their host genes included cell differentiation, interleukin-mediated signaling transduction, and tight junction, which are likely relevant to inflammation and fibrosis (Table 3), and suggest that these lncRNAs may co-express their linked genes during renal injury.

Although it is known that distant transcriptional activators or repressors (*trans*-acting) play a role in regulating gene expression, lncRNAs are typically co-expressed with their neighboring genes via *cis*-acting regulation.^{18,35–37} These reports supported the notion that these 21 common lncRNAs may co-express with the linked genes. We have previously reported that there are several *Smad3*-dependent miRNA-associated renal fibroses.^{22,23} We then examined whether these linked genes were regulated by *Smad3* by determining

any conserved *Smad* or *Smad3* binding sites in the promoter region of lncRNAs and linked genes. We used an ECR browser²⁸ to detect any conserved *Smad* binding motif prediction in mouse regulatory regions. Of 21 lncRNAs, 8 had at least one *Smad3* binding site and 13 had at least one SBS within the 2-kb upstream regions (Table 4). Despite the poorly conserved promoter regions of lncRNAs among species,^{18,35–37} np_17856 and np_5318 contained conserved *Smad3* binding sites in both human and mouse genomes (Figure 2, A–C). To confirm this finding, we used a ChIP assay to determine the interaction of *Smad3* with the promoter regions of np_17856 and np_5318 in mouse embryonic fibroblast (MEFs). As shown in Figure 2D, the antibody against *Smad3* could successfully immunoprecipitate the DNA fragments from MEFs containing the potential SBSs in the promoter regions of np_17856 and np_5318, supporting that *Smad3* could physically interact with their promoter regions.

To examine the potential relationship between expression of these *Smad3*-dependent lncRNAs and progressive renal injury, we validated their expression levels in the UUO kidney from mice with alternation of TGF- β /*Smad* signaling. Consistently, expression of np_5318 and np_17856 in the UUO kidney was both up-regulated in WT mice with severe renal inflammation and fibrosis and abolished in *Smad3* KO mice and in mice with overexpression of *Smad7* (Figure 2E), in which *Smad3* signaling is blocked and renal inflammation

Table 4 *Smad* Binding Sites in the 2 kb Upstream of 21 Common lncRNAs

lncRNA	ECR region (upstream 2 kb)*	No. of <i>Smad3</i> binding sites	No. of <i>Smad</i> binding sites
np_18588	Chr1: 55626524-55626634	6	5
np_26992	Chr10: 40124874-40124971	1	1
na_2414	None	0	0
np_29908	None	0	0
np_16555	Chr2: 135077190-135077499	1	1
np_5340	ChrX: 162042604-162042923	0	3
np_17234	None	0	0
np_18603	None	0	0
na_9884	Chr5: 52994103-52994294	0	3
np_5392	Chr3: 30452304-30454092	0	3
np_18004	Chr16: 51134121-51134534	1	0
np_28730	None	0	0
np_4334	Chr4: 76297322-76297735	2	0
np_18636	Chr1: 68839334-68840018	1	3
np_17856	Chr16: 77376543-77376645	5	5
np_18537	Chr1: 187857672-187857899	0	1
np_5318	Chr1: 69077396-69077702	1	2
np_19902	None	0	0
np_28233	Chr14: 22304667-22305288	0	4
np_2108	Chr1: 133222674-133222856	0	3
Np_28496	Chr15: 96350567-96350689	0	2

*The ECR region was searched on the ECR Browser (<http://ecrbrowser.dcode.org>, last accessed August 31, 2013).

Chr, chromosome.

and fibrosis are inhibited.^{16,23} In contrast, expression levels of np_5318 and np_17856 were further enhanced in the UUO kidney of Smad7 KO mice, in which activation of Smad3 signaling and renal inflammation and fibrosis are enhanced, as previously reported.³⁸ These results implicate that these Smad3-dependent lncRNAs may have a functional role in the development of renal inflammation and fibrosis, although regulatory mechanisms remain largely unexplored.

Discussion

TGF- β /Smad signaling plays a critical role in renal fibrosis and inflammation in CKD. Recently, Smad3 is shown to be a key mediator of downstream TGF- β /Smad signaling and mediates renal inflammation and fibrosis via several miRNAs^{13,15,16}; however, the involvement of TGF- β /Smad3 on lncRNA expression during renal injury remains unclear. By using the

high-throughput RNA sequencing and Smad3 KO mouse models with immune or nonimmune renal diseases, we identified that 151 lncRNAs in UUO and 413 lncRNAs in anti-GBM GN were differentially expressed in WT and Smad3 KO kidneys. Among them, 21 novel lncRNAs were co-expressed in both disease models. It is likely that lncRNAs differentially expressed in the UUO kidney may be fibrogenesis related because progressive renal fibrosis in the UUO kidney is Smad3 dependent^{16,18,30}; those differentially expressed in anti-GBM GN may be immune and inflammation associated because TGF- β /Smad signaling has been shown to play a crucial role in T-cell and macrophage-mediated anti-GBM GN.¹⁵ It is also possible that 21 common lncRNAs that co-expressed in both disease models may be associated with TGF- β /Smad3-mediated renal inflammation and/or fibrosis. However, the functional role of these lncRNAs that differentially expressed in anti-GBM GN and UUO nephropathy is largely unknown and requires further studies.

Increasing evidence shows that there are several relationships between lncRNAs and their associated protein-coding genes.^{33,34} In this study, we showed that the genomic status of 21 common Smad3-dependent lncRNAs included two sense, two intergenic, and 17 intronic lncRNAs (Table 2). Interestingly, some of them were clustered closely within the same chromosome. For example, np_18588 and np_29908 were located in the same intron of inactive phospholipase C-like protein 1, whereas np_18636 and np_5318 were located within the same intron of tyrosine kinase-type cell surface receptor 4 (Erbb4), implying that they may co-express and be regulated by the same transcriptional regulators.

Many ncRNAs, such as miRNAs and small-nucleolar RNAs, exhibit strong conservation across diverse species. Most of the miRNAs have 90% sequence identity between human and mouse snRNAs conserved at levels between 80% and 90%.³⁹ However, lncRNAs lack such strong conservation, including Air and Xist.^{39,40} This observation suggests that, unlike mRNAs and miRNAs, which have conserved nucleotides to ensure codon use or target identity, the lack of conservation of lncRNAs may work differently to exert their functions.^{39–41}

Because the RNA-Seq was performed with whole kidney samples, this information does not provide any information about where these lncRNAs are expressed. Further studies need to be performed to clarify the spatial and temporal expression patterns of these potential lncRNAs within both normal and diseased kidneys. Moreover, it seems that there were low expression levels of lncRNAs within the kidney because the Pearson correlation in our studies was moderate (values from 0.4 to 0.7). This observation was consistent with other studies in which the Pearson correlations of lncRNAs were <0.6.^{42–44}

In conclusion, our study identifies the Smad3-dependent lncRNAs related to renal inflammation and fibrosis. Results from this study demonstrate, for the first time to our knowledge, that lncRNAs may be involved in TGF- β /Smad3-mediated renal inflammation and fibrosis. Further studies to characterize these Smad3-associated lncRNAs should provide valuable information for a better understanding of the insightful mechanisms of TGF- β -mediated renal inflammation and fibrosis. Moreover, identification of the functional lncRNAs associated with renal inflammation and/or fibrosis may represent a promising research direction into renal disorder and may lead to the development of new lncRNA therapies for kidney diseases.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.10.007>.

References

- Eddy AA: Progression in chronic kidney disease. *Adv Chronic Kidney Dis* 2005; 12:353–365
- Roberts AB, Tian F, Byfield SD, Stuelten C, Ooshima A, Saika S, Flanders KC: Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine Growth Factor Rev* 2006; 17:19–27
- Lan HY, Chung AC: TGF-beta/Smad signaling in kidney disease. *Semin Nephrol* 2012; 32:236–243
- Yang F, Huang XR, Chung AC, Hou CC, Lai KN, Lan HY: Essential role for Smad3 in angiotensin II-induced tubular epithelial-mesenchymal transition. *J Pathol* 2010; 221:390–401
- Chung AC, Zhang H, Kong YZ, Tan JJ, Huang XR, Kopp JB, Lan HY: Advanced glycation end-products induce tubular CTGF via TGF-beta-independent Smad3 signaling. *J Am Soc Nephrol* 2010; 21:249–260
- Meng XM, Huang XR, Chung AC, Qin W, Shao X, Igarashi P, Ju W, Bottinger EP, Lan HY: Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis. *J Am Soc Nephrol* 2010; 21:1477–1487
- Lorenzen JM, Haller H, Thum T: MicroRNAs as mediators and therapeutic targets in chronic kidney disease. *Nat Rev Nephrol* 2011; 7: 286–294
- Kato M, Natarajan R: MicroRNA circuits in transforming growth factor-beta actions and diabetic nephropathy. *Semin Nephrol* 2012; 32: 253–260
- Wei Q, Mi QS, Dong Z: The regulation and function of microRNAs in kidney diseases. *IUBMB Life* 2013; 65:602–614
- Chung AC, Yu X, Lan HY: MicroRNA and nephropathy: emerging concepts. *Int J Nephrol Renovasc Dis* 2013; 6:169–179
- Lan HY, Chung AC: Transforming growth factor-beta and Smads. *Contrib Nephrol* 2011; 170:75–82
- Meng XM, Chung AC, Lan HY: Role of the TGF-beta/BMP-7/Smad pathways in renal diseases. *Clin Sci* 2013; 124:243–254
- Qin W, Chung AC, Huang XR, Meng XM, Hui DS, Yu CM, Sung JJ, Lan HY: TGF-beta/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. *J Am Soc Nephrol* 2011; 22:1462–1474
- Zhong X, Chung AC, Chen HY, Dong Y, Meng XM, Li R, Yang W, Hou FF, Lan HY: miR-21 is a key therapeutic target for renal injury in a mouse model of type 2 diabetes. *Diabetologia* 2013; 56:663–674
- Zhong X, Chung ACK, Chen H-Y, Meng X-M, Lan HY: Smad3-mediated upregulation of miR-21 promotes renal fibrosis. *J Am Soc Nephrol* 2011; 22:1668–1681
- Chung ACK, Huang XR, Meng X, Lan HY: miR-192 mediates TGF- β /Smad3-driven renal fibrosis. *J Am Soc Nephrol* 2010; 21: 1317–1325
- Brown SD: XIST and the mapping of the X chromosome inactivation centre. *Bioessays* 1991; 13:607–612
- Guttman M, Rinn JL: Modular regulatory principles of large non-coding RNAs. *Nature* 2012; 482:339–346
- Wang Z, Gerstein M, Snyder M: RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; 10:57–63
- Marguerat S, Bahler J: RNA-seq: from technology to biology. *Cell Mol Life Sci* 2010; 67:569–579
- Huang XR, Chung AC, Wang XJ, Lai KN, Lan HY: Mice over-expressing latent TGF-beta1 are protected against renal fibrosis in obstructive kidney disease. *Am J Physiol Renal Physiol* 2008; 295: F118–F127
- Huang XR, Chung AC, Zhou L, Wang XJ, Lan HY: Latent TGF-beta1 protects against crescentic glomerulonephritis. *J Am Soc Nephrol* 2008; 19:233–242
- Chung AC, Dong Y, Yang W, Zhong X, Li R, Lan HY: Smad7 suppresses renal fibrosis via altering expression of TGF-beta/Smad3-regulated microRNAs. *Mol Ther* 2013; 21:388–398
- Li R, Rosendahl A, Brodin G, Cheng AM, Ahgren A, Sundquist C, Kulkarni S, Pawson T, Heldin CH, Heuchel RL: Deletion of exon I of SMAD7 in mice results in altered B cell responses. *J Immunol* 2006; 176:6777–6784
- Chung AC, Huang XR, Zhou L, Heuchel R, Lai KN, Lan HY: Disruption of the Smad7 gene promotes renal fibrosis and inflammation in unilateral ureteral obstruction (UUO) in mice. *Nephrol Dial Transplant* 2009; 24:1443–1454

26. Kong L, Zhang Y, Ye ZQ, Liu XQ, Zhao SQ, Wei L, Gao G: CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. *Nucleic Acids Res* 2007, 35: W345–W349
27. Burge SW, Daub J, Eberhardt R, Tate J, Barquist L, Nawrocki EP, Eddy SR, Gardner PP, Bateman A: Rfam 11.0: 10 years of RNA families. *Nucleic Acids Res* 2013, 41:D226–D232
28. Ovcharenko I, Nobrega MA, Loots GG, Stubbs L: ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. *Nucleic Acids Res* 2004, 32:W280–W286
29. Li R, Chung AC, Dong Y, Yang W, Zhong X, Lan HY: The micro-RNA miR-433 promotes renal fibrosis by amplifying the TGF- β /Smad3-Azin1 pathway. *Kidney Int* 2013, 84:1129–1144
30. Trapnell C, Pachter L, Salzberg SL: TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 2009, 25:1105–1111
31. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 2010, 28: 511–515
32. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B: Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 2008, 5:621–628
33. Zhang Y, Liu XS, Liu QR, Wei L: Genome-wide in silico identification and analysis of cis natural antisense transcripts (cis-NATs) in ten species. *Nucleic Acids Res* 2006, 34:3465–3475
34. Rinn JL, Chang HY: Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 2012, 81:145–166
35. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Morales DR, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL: Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci U S A* 2009, 106:11667–11672
36. Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang QH, Guigo R, Shiekhattar R: Long noncoding RNAs with enhancer-like function in human cells. *Cell* 2010, 143:46–58
37. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL: Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Gene Dev* 2011, 25:1915–1927
38. Chung AC, Huang XR, Zhou L, Heuchel R, Lai KN, Lan HY: Disruption of the Smad7 gene promotes renal fibrosis and inflammation in unilateral ureteral obstruction (UUO) in mice. *Nephrol Dial Transplant* 2009, 24:1443–1454
39. Pang KC, Frith MC, Mattick JS: Rapid evolution of noncoding RNAs: lack of conservation does not mean lack of function. *Trends Genet* 2006, 22:1–5
40. Nesterova TB, Slobodyanyuk SY, Elisaphenko EA, Shevchenko AI, Johnston C, Pavlova ME, Rogozin IB, Kolesnikov NN, Brockdorff N, Zakian SM: Characterization of the genomic Xist locus in rodents reveals conservation of overall gene structure and tandem repeats but rapid evolution of unique sequence. *Genome Res* 2001, 11:833–849
41. Ponting CP, Oliver PL, Reik W: Evolution and functions of long noncoding RNAs. *Cell* 2009, 136:629–641
42. Gellert P, Ponomareva Y, Braun T, Uchida S: Noncoder: a web interface for exon array-based detection of long non-coding RNAs. *Nucleic Acids Res* 2013, 41:e20
43. Petazzi P, Sandoval J, Szczesna K, Jorge OC, Roa L, Sayols S, Gomez A, Huertas D, Esteller M: Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model. *RNA Biol* 2013, 10:1197–1203
44. Khaitan D, Dinger ME, Mazar J, Crawford J, Smith MA, Mattick JS, Perera RJ: The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. *Cancer Res* 2011, 71: 3852–3862