Up-Regulated Expression of Pro-Apoptotic Long Noncoding RNA lincRNA-p21 in Lupus Nephritis Patients and an Experimental Mouse Model

Yi-Cheng Chen
National Cheng Kung University College of Medicine
Pin-Yu Kuo
National Cheng Kung University College of Medicine
Chao-Liang Wu
National Cheng Kung University College of Medicine
Yu-Chi Chou
Academia Sinica
Hao-Earn Chong
National Cheng Kung University College of Medicine
Mei-Lin Yang
National Cheng Kung University College of Medicine
Ai-Li Shiau
National Cheng Kung University College of Medicine
Chrong-Reen Wang (z9109019@email.ncku.edu.tw)
National Cheng Kung University Hospial and Medical College https://orcid.org/0000-0001-9881-7024

Research article

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Abstract

Background: Accelerated cell apoptosis is a crucial pathogenic mechanism in lupus nephritis (LN) with dysregulated expression levels of long noncoding RNAs (lncRNAs). The expression of pro-apoptotic lincRNA-p21 and its competing endogenous RNA target miR-181a were studied in LN patients, human kidney cell and T-lymphocyte lines with CRISPR interference-conducted repression and lentiviral vector-mediated overexpression of lincRNA-p21, and a mouse LN model.

Methods: Clinical samples were collected from LN patients with higher disease activity and control subjects including lupus patients without renal involvement and age/sex-matched healthy controls (HCs). The expression of lincRNA-p21, H19 (anti-apoptotic lncRNA) and miR-181a were examined in peripheral blood mononuclear cells (PBMNCs) and urine cells, and analyzed for clinical correlation. Cell lines were treated with doxorubicin (Dox) to induce apoptosis and evaluate for the expression of lincRNA-p21, caspase 3 and p21. LincRNA-p21-silened HEK 293T and Jurkat transfectants were examined for apoptosis and miR-181a expression. LincRNA-p21-overexpressed HK-2 cells were examined for apoptosis and p53-related down-stream molecules levels. Female Balb/C mice were injected with pristane to induce LN, and examined for the expression of anti-DNA, proteinuria, lincRNA-p21, caspase 3 and p21 as well as *in situ* apoptosis.

Results: Up-regulated expression of lincRNA-p21 rather than H19 were identified in PBMNCs from LN patients, positively correlated with disease activity and proteinuria amount. Higher lincRNA-p21 levels were identified in LN CD4+T cells than other subpopulations. LN urine cells had greater lincRNA-p21 levels than HCs. There were lower miR-181a levels in PBMNCs from LN patients, negatively correlated with disease activity. Dox-induced apoptotic cell lines had up-regulated levels of lincRNA-p21, caspase 3 and p21, whereas down-regulated miR-181a expression with decreased TCRζchain and IL-2 levels was identified in Jurkat cells. LincRNA-p21-silenced transfectants displayed reduced apoptosis with up-regulated miR-181a expression. LincRNA-p21-overexpressed HK-2 cells revealed enhanced apoptosis with up-regulated expression of downstream PUMA and Bax molecules. LN mice had *in situ* apoptosis and progressively increased anti-dsDNA, proteinuria and renal lincRNA-p21 levels with up-regulated expression of caspase 3 and p21.

Conclusions: By using clinical samples, human cell lines and a mouse model, we demonstrate up-regulated expression of lincRNA-p21 in LN, implicating a potential activity biomarker and therapeutic target.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a loss of immune tolerance and formation of immune complexes (IC) containing nuclear autoantigens, resulting in inflammation at various organs and tissues, of which damage to the kidney as a consequence of lupus nephritis (LN) is the most common cause of morbidity and mortality [1, 2]. Current understanding of the

crucial pathogenic mechanisms includes an imbalance between production of apoptotic cells and disposal of apoptotic materials [2, 3]. Notably, apoptotic cell death with inefficient clearance results in the accumulation of self-double strand DNA (dsDNA), followed by a break of tolerance to induce production of dsDNA antibody with IC deposition in glomerular and tubular basement membranes, contributing to the development of LN [3, 4]. In particular, increased apoptotic circulating T-lymphocytes in SLE patients has been shown to be correlated with disease activity [5]. A therapeutic strategy to inhibit the apoptotic process, can not only reduce the production of nuclear autoantigens and avoid the IC formation, but also suppress the apoptotic death of phagocytes to restore the clearance of apoptosis-derived remnants [3].

Long noncoding RNAs (lncRNAs) can modulate a variety of cellular processes and activities through chromatin remodeling, epigenetic modification and gene transcription by their interaction with other intra-nuclear molecules [6]. Extensive functional potentials of lncRNAs are involved in autoimmune disorders [7]. Up- or down-regulated expression of certain lncRNAs have been reported to be correlated with disease activity in LN patients, and such molecules participate in targeting SLE-related signaling transduction such as the p53 gene pathway [8]. Lupus patients had higher p53 expression levels in circulating lymphocytes with a positive correlation with disease activity, suggesting a pathogenic role of p53 in inducing apoptotic death of lymphocytes [9]. LincRNA-p21, locating at upstream of a cell-cycle gene p21, is involved in the regulation of cell apoptosis [10]. It is directly induced by p53 through binding to the promoter with transcriptional activation, and has an action mechanism through p21. Upon DNA damage, decreased apoptosis has been identified in lincRNA-p21-depleted cells, whereas lincRNA-p21-overexpressed cells had increased apoptotic death [11]. Interestingly, reduced lincRNA-p21 levels in rheumatoid arthritis contribute to disease activity due to physically sequestrating NF-κB p65 mRNA by this lncRNA [12]. Notably, lncRNAs with microRNAs (miRNAs) response elements (MREs), can act as competing endogenous RNAs (ceRNAs) to degrade miRNAs and compete for the mRNA binding [13]. LincRNA-p21 with MREs for the miR-181 members, has been demonstrated to serve as their ceRNA [10]. Although miRNAs expression pattern is highly responsive to the cytokine environment [14], miRNA-181a levels have been demonstrated to be down-regulated in peripheral blood mononuclear cells (PBMNCs) from SLE [15], a disease with increased expression of abundant cytokines [16]. In sum, these findings implicate a pathogenic role of pro-apoptotic lncRNA-p21 in active lupus patients with accelerated cell apoptosis.

In this study, we investigated the expression of lncRNA-p21 and H19, a lncRNA counteracting apoptosis through the down-regulation of p53 expression [17], and miR-181a in SLE patients with LN, human kidney cell and T-lymphocyte lines with CRISPR interference (CRISPRi)-conducted repression and lentiviral vector (LV)-mediated overexpression of lincRNA-p21, and a pristane-induced murine model of LN.

Methods

SLE patients and healthy controls (HCs)

Thirty-four patients fulfilling the American College of Rheumatology revised Criteria for SLE [18], 30 females and 4 males aged from 28 to 65 years (44.4 ± 1.6), and age/sex-matched HCs were enrolled into this study. Their venous blood samples were collected for further examination. Medical records were reviewed for demographic, clinical and laboratory data, and the disease activity at the time of sample collection were assessed by SLEDAI-2K [19]. The diagnosis of LN was based on renal biopsy findings and/or a long-term follow-up of blood and urine examinations [20]. Seventeen patients in this study had LN, 15 females and 2 males aged from 28 to 60 years (44.0 ± 2.3), including 8 with class IV, 4 with class III, 3 with class V histopathological findings, and 2 without renal biopsy. Notably, LN patients had significantly higher SLEDAI-2K scores than SLE without renal involvement (8.4 ± 1.2 versus 3.5 ± 0.7, $P < 0.001$). Fresh urine specimens were collected from all LN patients and age/sex-matched HCs. This study was approved by the Institutional Review Board of our hospital with the informed consent from each participant.

Pristane-induced LN mouse model

Eight-week old female BALB/c mice were purchased from the Laboratory Animal Center of our medical college, and housed under specific pathogen-free conditions. Animal experiments were approved by the Institutional Animal Care and Use Committee of our university. Mice were intraperitoneally (i,p,) injected with 0.5 ml pristane (Sigma-Aldrich) to induce LN, whereas the control group was i.p. injected with 0.5 ml of phosphate-buffered saline (PBS) [21]. Blood and urine samples were periodically collected for examining anti-dsDNA levels and proteinuria concentrations, respectively. Mice were sacrificed at different time periods after induction, and their kidneys and spleens were removed for further studies.

Purification of human and mouse cells

Human MNCs were isolated from blood samples by Ficoll-Paque PLUS (GE Healthcare), and incubated with CD14 microbeads. CD14+ cells were eluted from the positive selection column of Magnetic Cell Sorter (Miltenyi Biotec). CD14- cells were incubated with CD4 microbeads, and CD4+ cells were eluted from the column. Mouse spleens were homogenized by using syringe plunger and mesh strainer. Mouse MNCs, were further incubated with PE-Cy5 anti-CD4 (BD Pharmingen) or FITC anti-CD19 (BD Pharmingen), and sorted by Moflo XDP Cell Sorter (Beckman Coulter) to obtain CD4+ or CD19+ cells. Purity of cell subpopulation was confirmed to be up to 95 % by flow cytometric analyses. Human urine cells were isolated from urine specimens by centrifugation and washing procedures to obtain cell pellets [22]. After removing capsules, mouse kidneys were minced into tiny pieces to obtain cortex tissues, followed by incubation with digestion buffer with collagenase (Sigma-Aldrich), and centrifuged to collect cell pellets [23].

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNAs from human or mouse cells were extracted by TRIzol reagent (Invitrogen), and complementary DNAs were obtained by using reverse transcriptase (Applied Biosystems). qRT-PCR was performed to quantify the target RNAs levels by using the SYBR qPCR Mix Kit (TOOLS) [24]. The

condition of PCR was: 95 ^{0}C for 5 min, 95 ^{0}C for 15 sec, primer-melting temperature (Tm) for 1 min with 40 cycles, and elongation at 72 ^{0}C for 20 sec. Primer sequences were as follows.

Human lincRNA-p21 (Tm 59 ^{0}C), forward 5'-GTGCAGAGCGTTTTGTTTGTCCAT-3'/reverse 5'-CCACAGCCTCTGGGAAG AAAATG-3'.

Human H19 (Tm 57^{0}C), forward 5'-GAAATGCTACCCAGCTCAAGC-3'/reverse 5'-CTGCTGTTCCGATGGTGTCTTTGA-3'.

Human Bax (Tm 58^{0}C), forward 5'-ATGCGTCCACCAAGAAGCTGAG-3'/reverse 5'-CCCCAGTTGAAGTTGCCATCAG -3'.

Human PUMA (Tm 58^{0}C), forward 5'-ACGACCTCAACGCACAGTACGA-3'/ reverse 5' CCTAATTGGGCTCCATCTCGGG -3'.

Human P53 (Tm 52^{0}C), forward 5'-CCCTTCCCAGAAAACCTACC-3'/reverse 5' CTCCGTCATGTGCTGTGACT-3'.

Human IL-2 (Tm 56^0 C), forward 5'-CATGCCCAAGAAGGCCACAG-3'/reverse 5'-T TGCTGATTAAGTCCCTGGGTC-3'.

Human TCR-□ chain (Tm 55^{0}C), forward 5'-CAGCCAGGGGATTTCCACCACTC-3' /reverse 5'-CCCTAGTACATTGACGGGTTTTTC-3’.

Human GADPH (Tm 54^{0}C), forward 5'-ACTTCAACAGCACACCCACT-3'/reverse 5' -GCCAAATTCGTTGTCATACCAG-3'.

Mouse lincRNA-p21 (Tm 57 ^{0}C), forward 5-'ccgacaggagtctcatgctcag-3'/ revers 5'-CTGACCCAGACCAGTCTGG GC -3'.

Mouse GADPH (Tm 56^{0}C), forward 5'-GTTGTCTCCTGCGACTTCAACA-3'/ reverse 5'-TTGCTGTAGCCGTATTCATTGTC-3'.

The relative abundance of a measured gene expression was normalized by GAPDH gene from each sample. The average levels of human HCs or PBS-injected control mice, and expression levels of cell lines without stimulation, CRISPRi-GFP-silenced transfectants, and LV-SFFV-Blast-overexpressed cells were determined as 100%.

For analyzing the expression levels of human and mouse miR-181a, total RNAs were reverse transcription (RT) by using the reverse transcriptase kit (Applied Biosystems) with 10 ng purified RNA, dNTP, MultliScribe reverse transcriptase, RT buffer, RNase inhibitor, random primers and gene-specific stem-loop RT primer with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in Smart Cycler (Cepheid) [24]. The reagents were incubated with 16 ^{0}C for 30 min, 42 ^{0}C for 30 min and 85 ^{0}C for 5 min.

The condition of PCR was: 95 ^{0}C for 10 min, 95 ^{0}C for 15 sec and 60 ^{0}C for 1 min with 40 cycles. Quantitative expression levels of miR-181a were analyzed with RNU6B small RNA (Applied Biosystems) as an endogenous control. The average levels of human HCs or PBS-injected control mice, and expression levels of cell lines without stimulation and CRISPRi-GFP-silenced transfectants were determined as 100%.

Construction of LV-based CRISPRi targeting and overexpression of lincRNA-p21 CRISPRi guide RNA spacer sequences targeting human lincRNA-p21 were designed as 5'-GCAAGGCCGCATGATGATGC-3', 71 bp from transcription start site (TTS) to 5' end of gRNAs in template (T) strand (71i) and 5'-GCTTGCTTTGCATGATTGTT-3', 184 bp from TTS in non-template (NT) strand (184i) [25]. The LV pALL-dCas9-KRAB.pPuro containing the catalytically dead Cas9/KRAB domain was obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan), and a 1.9 kb stuffer was removed from this vector for cloning of guide RNA spacer sequences targeting lincRNA-p21. LincRNA-p21 was generated from HEK 293T cells cDNA by PCR amplification and further cloned into LV-SFFV-Blast. To obtain CRISPRi-lincRNAp21 and LV-lincRNA-p21, the created guide RNA and lincRNA-p21 expressing vectors were transfected into sub-confluent HEK 293T cells, along with the packaging psPAX2 and envelope pMD2.G plasmids by using calcium phosphate precipitation to acquire recombinant LV [24,26]. After transfection for 48 to 72 hr, cell supernatants were harvested and stored at -80^{0}C until use. CRISPRi-GFP and LV-SFFV-Blast vectors were used as the control vector in this study.

Production of stable transfectants

Jurkat T-lymphocyte and HEK 293T kidney cells (American Type Culture Collection) with 5 × 10^5 cells/mL in 6-well plate, were transfected with LV-CRISPRi-lincRNA-p21 or LV-CRISPRi-GFP for 48 hr in the presence of polybrene (8 μg/ml, Sigma-Aldrich), and were incubated with 5 μg/ml and 0.5 μg/ml of puromycin, respectively [26]. The puromycin selection process was up to one month in order to select successfully transduced stable transfectants confirmed by qRT-PCR analyses.

Doxorubicin (Dox)-induced cell apoptosis

HEK 293T, HK-2, Jurkat cells or transfectants were seeded with 1 10^6 cells/mL in 6-well plate in the presence of Dox (TTY Biopharm) for 24 hr under 37 ℃, 5 % CO_2 incubation [11]. These cells were further stained with Annexin V and 7-AAD (BD Pharmingen) to detect apoptotic and dead cells, respectively, by flow cytometric analyses. Apoptotic cells were defined as Annexin V+ and 7-AAD- in this study.

Immunoblotting assessment

Cell lysates from human cell lines, transfectants or mouse cells were separated by electrophoresis on 10-15 % SDS-PAGE, transferred on PVDF membranes (Merck Millipore), blocked in 5 % of non-fat dry milk and incubated with primary antibodies anti-caspase 3 (Cell signaling), anti-procaspase 3 (Cell signaling), anti-p21 (Santa Cruz), or anti-□-actin antibodies (Sigma-Aldrich) at 4℃ for 16-18 hr [24,26]. After washing, the membranes were incubated with secondary antibodies (Jackson Immunoresearch) at room

temperature for 2 hr. Signal expression of protein-antibody complexes was detected by ECL system (Amersham) and visualized with Biospectrum imaging system (UVP). The relative protein expressions were measured by Image J (NIH).

Enzyme-linked immunosorbent assay (ELISA)

After pristane induction, serum samples from BALB/c mice were periodically examined for the presence of anti-dsDNA levels with an ELISA kit (Alpha Diagnosis).

Proteinuria detection

Urine samples from BALB/c mice were collected at different time periods, and proteinuria was detected by urine testing strips (Arkray). The results were determined by the semi-automated urine chemistry analyzer (Arkray RT-4010), and urine protein concentration (UPC) quantification data was transferred into 5 ranking including 0, 0.5, 1, 2 and 3.

Histopathological and immunofluorescence analyses

Paraffin-embedded sections were de-paraffinized in xylene, dehydrated in ethanol and rehydrated in distilled water. To determine glomerulonephritis (GN), mouse kidney sections were analyzed by Periodic acid-Schiff (PAS) staining. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect *in situ* apoptosis, antigens in the kidney sections were reactivated by proteinase K for 10 min, re-fixed by 4 % formaldehyde for 25 min, incubated with equilibrate buffer for 7 min, and finally labelled by the TUNEL detection cocktail (Promega) [26].

Statistical analyses

Data are expressed as the mean $\pm$ standard error of the mean (SEM). The expression levels of mRNA between patients and HCs or different groups of patients were analyzed by Mann-Whitney U test. Correlation analysis was analyzed by Spearman correlation coefficient test with linear regression analysis. The significant differences in other *in vitro* analyses were determined by Student's *t* test. The differences at different time points in i*n vivo* study were analyzed by repeated-measures analysis of variance. *P* values less than 0.05 is considered to be significant in this study with the symbols presenting as * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.

Results

Up-regulated expression of lincRNA-p21 in LN patients

Firstly, we examined PBMMCs from SLE patients and HCs for the expression of lincRNA-p21 and H19. Significantly higher lincRNA-p21 rather than H19 levels were found in SLE patients in comparison with HCs (Fig. 1a, $P = 0.002$). LN patients or those with class IV histopathology, had higher levels of lincRNA-p21 than those without renal involvement (Fig. 1b, LN versus Nil, $P = 0.013$, LN-IV versus Nil, $P = 0.016$).

There were no differences in H19 levels between SLE patients without renal involvement and those with LN, either class IV histopathology or others (Fig. 1c). Moreover, there was a significant positive correlation between lincRNA-p21 levels and SLEDAI-2K scores (Fig. 1d, r = 0.423, *P* = 0.013) or daily proteinuria amounts (Fig. 1e, r = 0.395, *P* = 0.021). No correlation was identified between H19 levels and SLEDAI-2k scores or proteinuria amounts (Fig, 1f and 1g).

In Fig. 2a, there were higher lincRNA-p21 levels in urine cells from LN patients than HCs (LN versus HC, 150.4 ± 78.0 versus 100.0 ± 27.1 %), and patients with class IV histopathology had significantly higher lincRNA-p21 levels than HCs (*P* = 0.028). No differences were found in H19 levels between HCs and LN patients or those with class IV histopathology (Fig. 2b). Next, we analyzed the expression of miR-181a in PBMNCs from SLE patients. There were decreased miR-181a levels in SLE patients (Fig. 2c, SLE versus HC, 56.8 ± 16.0 versus 100.0 ± 29.6 %), and LN patients had significantly lower levels than SLE without renal involvement (Fig. 2d, *P* = 0.011). A significant negative correlation existed between miR-181a levels and SLEDAI-2K scores (Fig. 2e, r = -0.383, *P* = 0.026).

MNC subpopulations from LN patients and HCs were examined for the expression of lncRNAs. CD4+T cells from LN patients had higher levels of lincRNA-p21 and H19 in comparison with HCs (Fig. 2f and 2g). Interestingly, overexpressing miR-181a, an intrinsic modulator of T cell receptor (TCR) signaling, in primed T-lymphocytes can up-regulate the expression of IL-2 [27]. Lower expression of TCR-□ chain has been identified in T cells from SLE patients with poor IL-2 production, and refilling this molecule can normalize IL2 levels *in vitro* [28]. Further analyses of CD4+T cells from LN patients revealed lower levels of miR-181a, IL-2 and TCR-□□□chain as compared with those from HCs (Fig. 2h). Collectively, these *ex vivo* findings from clinical samples in Fig. 1 and 2 demonstrated up-regulated expression of lincRNA-p21 in LN patients.

Up-regulated expression of lincRNA-p21 in apoptotic human T-lymphocyte and kidney cell lines

Under Dox-induced DNA damage to trigger p53-dependent cell apoptosis [11], we investigated lincRNA-p21 expression in a T-lymphocyte and two kidney cell lines. All of the results in Fig. 3 and Fig. 4 were representative of at least 2 independent experiments with similar findings.

By culturing Jurkat cells in the presence of Dox, there were dose-dependent up-regulated lincRNA-p21 levels and apoptotic cell ratios, and reciprocal down-regulation of miR-181a expression with reduced TCR-□□chain and IL-2 levels as well as an increase in expression of caspase 3 and p21 (Fig. 3a to 3f). Moreover, in two CRISPRi-lincRNA-p21 transfectants, the one (71i) with a higher silenced efficacy (70% knockdown in comparison with CRISPRi-GFP transfectants) had decreased Dox-induced apoptotic cell ratios and increased miR-181a levels (Fig. 3g).

Notably, TNF-□□has been identified to possess the strongest correlation with SLE activity among different tested plasma cytokines [29], and regulate the expression of abundant lncRNAs [30]. LncRNA-p21 levels were up-regulated in a dose-dependent manner by adding TNF-□ into Jurkat cells culture (Fig. 3h). In the presence of Dox, despite a simultaneous up-regulated expression of lincRNA-p21 and caspase 3, the

addition of a caspase 3 inhibitor (Z-DEVD-FMK) could reduce lincRNA-p21 levels (Fig. 3i), suggesting that caspase 3 activation can provide a feedback to enhance lincRNA-p21 expression in the apoptotic cell process.

Furthermore, we induced apoptosis in HEK 293T kidney cells with Dox treatment. There were dose-dependent up-regulated lincRNA-p21 levels, apoptotic cell ratios and caspase 3 expression (Fig. 4a to 4c). CRISPRi-lincRNA-p21 transduced HEK 293T transfectants (71i) with a better silencing effect (86% knockdown as compared with control cells) demonstrated reduced Dox-induced apoptotic cell ratios and enhanced miR-181a expression levels (Fig. 4d).

Another renal tubular HK-2 cell line was treated with Dox to induce apoptosis, resulting in dose-dependent increases in lincRNA-p21 levels, apoptotic cell ratios, and expression of caspase 3 and p21 (Fig. 4e to 4g). Since lincRNA-p21 can provide a feedback to enhance the p53 transcriptional activity [10], we examined the expression of p53 downstream molecules in Dox-treated HK-2 cells. There were up-regulated expression of p53, lincRNA-p21, PUMA and Bax (Fig. 4h). Moreover, lincRNA-p21-overexpressed HK-2 cells had increased expression of p53 downstream molecules PUMA and Bax (Fig. 4i) with enhanced Dox-induced apoptotic cell ratios (data not shown). Instead of using transiently transfected cells, further studies can apply blasticidin selection process to create stable lincRNA-p21 tranfectants and examine whether there are higher expression levels of these downstream molecules.

Altogether, these *in vitro* results indicated that up-regulated expression of lincRNA-p21 could enhance apoptosis in human T-lymphocyte and kidney cell lines.

Up-regulated expression of lincRNA-p21 in a LN mouse model

Pristane-injected Balb/c mice were periodically examined for dsDNA antibody and urinary protein. Significantly elevated proteinuria amounts and higher anti-dsDNA titers were noted at 5 and 6 months after induction, respectively (Fig. 5a). Their kidneys were removed upon sacrifice for histopathological and *in situ* apoptosis analyses. In Fig. 5b, kidneys from LN mice had GN with glomerular hyper-cellularity/mesangial expansion and the presence of *in situ* apoptotic cells at 6 months after pristane injection, an induced model with the presence of Fas-independent TUNEL-positive tissue cells [31]. Significantly up-regulated lincRNA-p21 and down-regulated miR-181a levels were shown in kidney cells from LN mice at 6 months after induction (Fig. 5c and 5d). Furthermore, there were increasingly up-regulated lincRNA-p21 levels in CD4+T cells from LN mice after pristane induction with significant higher levels at 6 months, as well as increased expression levels of caspase 3 and p21 (Fig. 5e). Taken together, these *ex vivo* data from LN mice indicated progressively up-regulated lincRNA-p21 expression in kidney and CD4+T cells.

Discussion

LN has multiple pathogenic pathways including aberrant apoptosis, autoantibody production and IC deposition with complement activation [1, 2]. Apoptotic cell death with roles in tissue damage and

immune dysregulation, is involved in the generation of autoantigens and the externalization of modified nuclear antigens [3]. In the development of LN, there are accelerated cell apoptosis in circulating lymphocytes [32], kidney cells (renal tubular and glomerular parenchymal cells) [33], and phagocytes for clearance of apoptotic cells [34]. LncRNAs are emerging as key players in controlling the cellular apoptotic process [6], and these molecules participate in the LN pathogenesis with aberrant expression levels [8]. In this study, up-regulated expression of pro-apoptotic lincRNA-p21, rather than anti-apoptotic H19, was identified in PBMNCs, especially in CD4 + T cells, from LN patients as well as a human T-lymphocyte line receiving Dox treatment to induce apoptosis. Moreover, higher lincRNA-p21 levels were detected in urine cells from LN patients and human kidney cell lines under the DNA damage response. Notably, transcriptome-wide studies have demonstrated that the expression of different lncRNAs is specific for cell types to exert their distinct regulatory functions [30]. Pro-inflammatory cytokines, TNF-α in particular, have been demonstrated to regulate the expression of lncRNAs, some of which have up-regulated levels in a NF-κB dependent manner [35], as demonstrated in this study with dose-dependent increases in lincRNA-p21 expression levels in Jurkat cells upon *in vitro* TNF-α stimulation (Fig. 3h). Indeed, in SLE patients, elevated cytokines levels can influence the expression of lncRNAs at different tissues and organs, leading to heterogeneous clinical involvement [16, 29, 30]. Furthermore, we observed increases in apoptotic cell ratios and expression levels of caspase 3 and p21 in Dox-treated T-lymphocyte and kidney cell lines. Up-regulated expression of lincRNA-p21 contributes to apoptotic cell death in circulating lymphocytes and renal cells, followed by production of autoantibodies, resulting in *in situ* IC accumulation and the formation of GN in SLE.

Owing to complex disease presentations and inherent limitations in clinical research, there are difficulties in performing direct studies in lupus patients. SLE mouse models have been developed to dissect pathogenic mechanisms and identify therapeutic targets [36]. Induced mouse models, particularly the pristane-induced mice with renal IC deposition causing GN, are useful tools to investigate the molecular pathogenesis with dysregulated signaling pathways and to screen therapeutic modalities in LN [21, 36]. In this study with Balb/c female mice, LN developed with increased anti-dsDNA levels, elevated proteinuria amounts, and the formation of GN after pristane induction. Moreover, there were *in situ* apoptosis with up-regulated expression of lincRNA-p21 in CD4 + lymphocytes and kidney cells as well as elevated caspase 3 and p21 levels. By using the lupus mouse model through a proof-of-concept approach, we demonstrated a progressive increase in renal expression of lincRNA-p21 during the development of LN. *In vitro* experiments by using CRISPRi-lincRNA-p21 transfected cell lines, revealed lower apoptotic cell ratios in the presence of DNA damage response, implicating a therapeutic strategy to treat LN by knocking down the expression of lincRNA-p21 to reduce cell apoptosis. Nevertheless, further efforts are needed to elucidate the potential of lincRNA-p21 as a therapeutic candidate by silencing its renal expression to examine whether there is improvement of GN in the LN mouse model.

Transcript RNAs with specific MREs can communicate with others via the miRNA messenger, and may serve as ceRNAs to de-repress the activity of other RNAs with similar MREs by competing for the same miRNAs [37]. LncRNAs harboring the MREs can serve as ceRNAs with the function to sponge or sequestrate miRNAs, and growing evidence has demonstrated that, an interaction between lncRNAs and

miRNAs can regulate miscellaneous cellular processes to affect human disease states [13, 37]. MiR-181a levels in PBMNCs have been shown to be down-regulated in SLE patients with higher disease activity [15], and also demonstrated in LN patients with a negative SLEDAI-2K correlation from this study. Furthermore, in cell lines under the DNA damage response, there were increases in lincRNA-p21 levels with a reciprocal decrease in miR-181a expression, and CRISPRi-mediated repression of lincRNA-p21 could restore the down-regulated expression of miR-181a. In addition, decreased mir-181a levels were observed in kidney cells form LN mice in this study. By transducing primed T cells with retroviral vector carrying miR-181a, IL-2 production can be up-regulated through targeting multiple negative regulators to augment T cell activation [27]. Impaired IL-2 production has been shown in T cells from SLE patients, and IL-2-treated LN mice have decreased autoantibody levels and reduced GN severity [39]. Notably, low-dose IL-2 treatment has recently been demonstrated to have the therapeutic efficacy in LN patients [40]. Accordingly, these findings suggest that the disease progression in LN can be reduced by inhibiting the expression of lincRNA-p21 to raise miR-181a levels for IL-2 restoration.

Conclusions

A better understanding of the LN pathogenesis can help to develop disease biomarkers in diagnosis and prognosis, and to identify novel therapeutics other than conventional immunosuppressive agents with significant failures and adverse effects. In this study, by using clinical samples, human cell lines and a mouse model, we demonstrate up-regulated expression of lincRNA-p21 in LN, implicating this pro-apoptotic lncRNA as a potential activity biomarker and therapeutic target.

Abbreviations

ceRNAs
competing endogenous RNAs; CRISPRi:CRISPR interference; dsDNA:double strand DNA; Dox:Doxorubicin; GN:Glomerulonephritis; HC:Healthy control; IC:Immune complexes; i.p.:intraperitoneally; LV:Lentiviral vector; lncRNA:Long noncoding RNAs; LN:Lupus nephritis; miRNA:MicroRNAs; PBMNCs:Peripheral blood mononuclear cells; PBS:Phosphate-buffered saline; qRT-PCR:quantitative real-time polymerase chain reaction; RT:Reverse transcription; SEM:standard error of the mean; SLE:Systemic lupus erythematosus; SLEDAI:SLE disease activity index; TCR:T cell receptor; TUNEL:Terminal deoxynucleotidyl transferase dUTP nick end labeling; UPC:Urine protein concentration

Declarations

Acknowledgments

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Availability of data and materials

All data supporting this study are within this manuscript.

Authors' contributions

YCC and PYK performed the experiments, participated in the study design and contributed to the results analyses. CLW participated in the study design and contributed to the results analyses. YCC participated in the CRISPRi design. HEC performed the experiments. MLY participated in the study design. ALS and CRW conceived and designed the study, analyzed the results and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate and publish

The study was approved by the Institutional Review Board of National Cheng Kung University Hospital, and the written informed consents were obtained from all participants to donate their clinical samples and publish their clinical data.

Competing interests

The authors declare that they have no competing interests.

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Figures

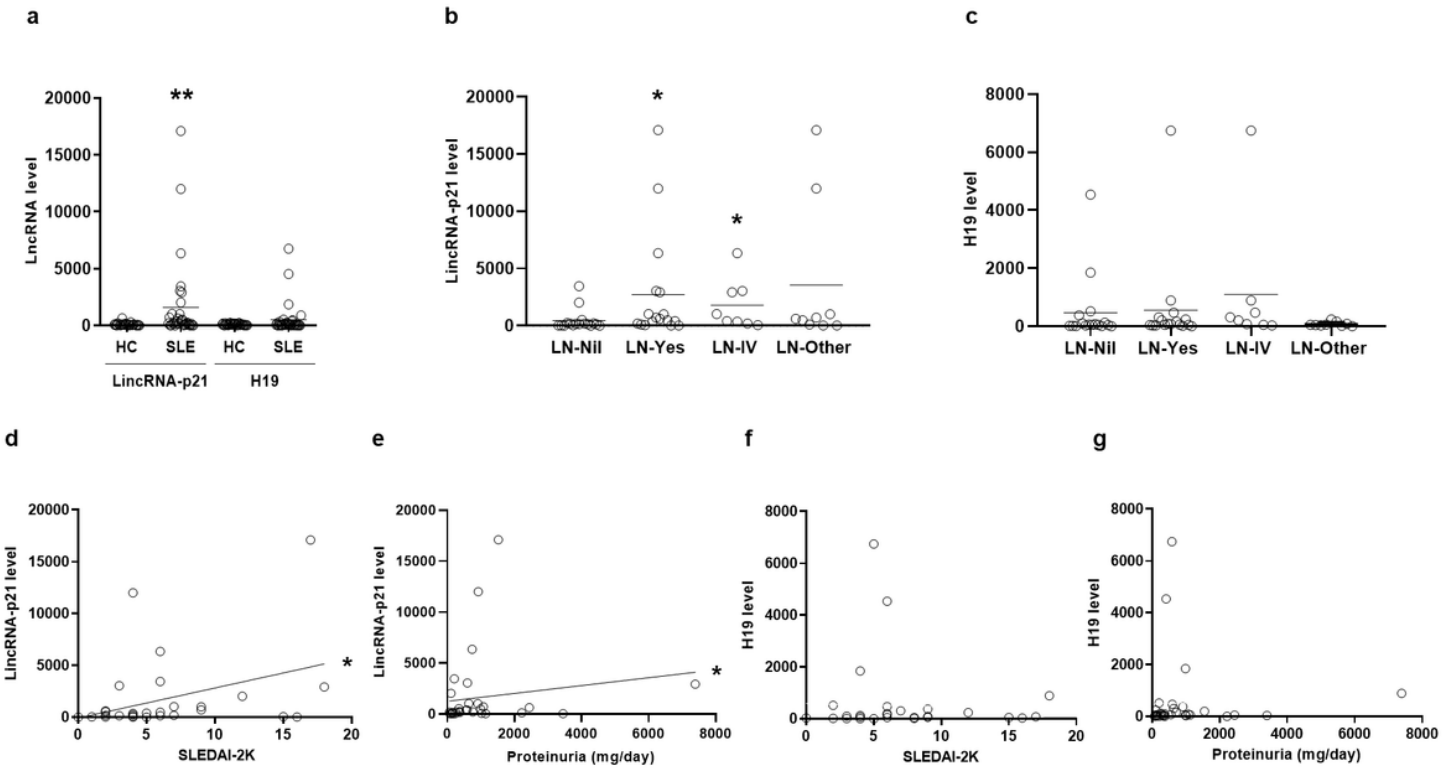

Figure 1

Up-regulated expression of lincRNA-p21 in PBMNCs from SLE and LN patients. a LincRNA-p21 and H19 levels in HCs and SLE patients. b LincRNA-p21 levels in SLE patients without renal involvement and LN patients, including IV histopathology and others. c H19 levels in SLE without renal involvement and LN patients including IV histopathology and others. d and e A significant positive correlation between lincRNA-p21 levels and SLEDAI-2K scores or daily proteinuria amounts in SLE patients. f and g No

correlation between H19 levels and SLEDAI-2K scores or daily proteinuria amounts in SLE patients. Horizontal lines in a to d are mean values from HCs and patients. n = 30 for HCs, n = 34 for SLE patients, n = 17 for LN patients, n = 17 for SLE without renal involvement, n = 8 for LN patients with IV histopathology and n = 9 for other LN patients. * P < 0.05, ** P < 0.01.

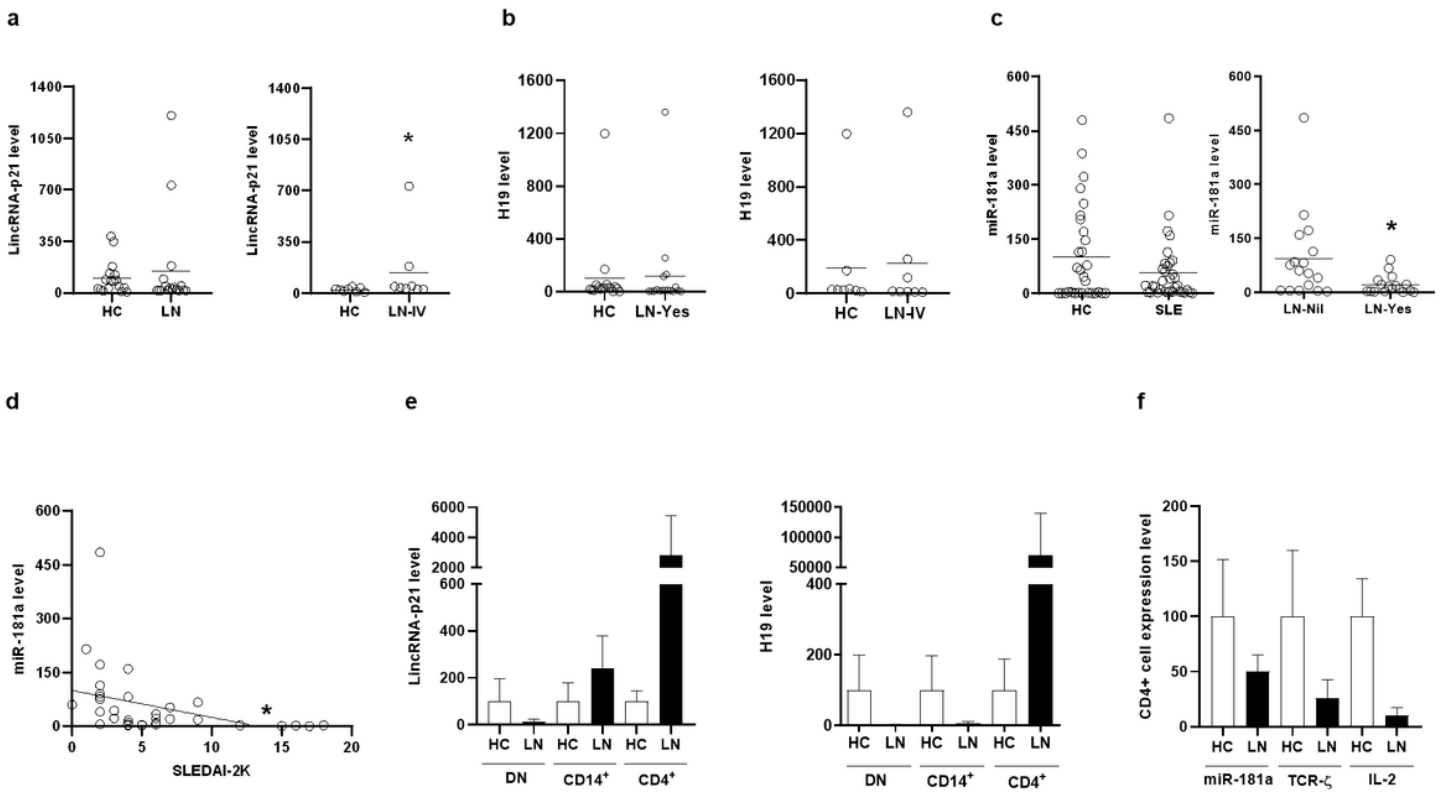

Figure 2

Up-regulated expression of lincRNA-p21 in urine cells and down-regulated expression of miR-181a in PBMNCs from LN patients. a LincRNA-p21 levels in urine cells from HCs and LN patients or IV histopathology. b H19 levels in urine cells from HCs and LN patients or IV histopathology. c miR-181a expression in PBMNCs from HCs and SLE patients, and from SLE patients without renal involvement and LN patients. d A significant negative correlation between miR-181a levels and SLEDAI-2K scores. e LincRNA-p21 and H19 levels in PBMNCs subpopulations including DN, CD14+ and CD4+ cells from HCs and LN patients. f MiR-181a, TCR-□ chain and IL-2 levels in CD4+ cells from HCs and LN patients. Horizontal lines in a to c are mean values from HCs and patients. Data are expressed in mean with SEM in e and f. n = 30 for HCs and n = 34 for SLE patients (in PBMNCs), n = 17 for HCs and LN patients (in urine cells), n = 8 for HCs and IV histopathology (in urine cells), n = 3 for HCs and LN patients (in PBMNCs subpopulations), and n =3 for HCs and LN patients (in CD4+ cells). DN: double negative. * P < 0.05.

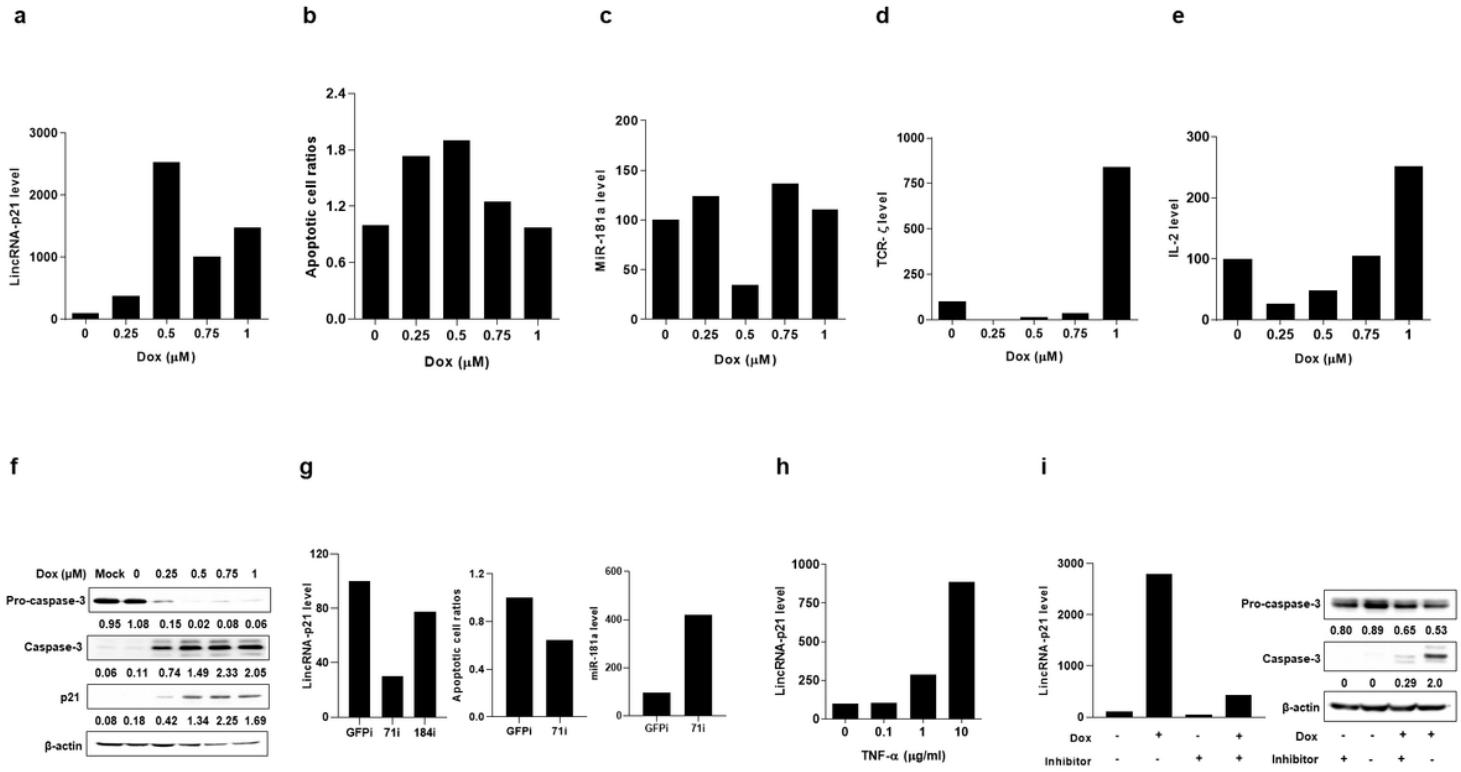

Figure 3

Up-regulated lincRNA-p21 expression in apoptotic T-lymphocyte cell line. a to f Dose-dependent up-regulated lincRNA-p21 levels, apoptotic cell ratios and caspase 3/p21 levels with reciprocal down-regulated miR-181a levels and reduced expression of TCR-□ chain and IL-2 in Jurkat cells culture under the Dox treatment. g Two CRISPRi-lincRNA-p21 transfectants and decreased Dox-induced apoptotic cell ratios with increased miR-181a levels shown in 71i with higher silenced efficacy. h Dose-dependent increases in lincRNA-p21 levels in cell culture in the presence of TNF-□□□i Down-regulated expression of lincRNA-p21 in the presence of caspase 3 inhibitor in the cell culture under the Dox treatment. All of the results in Fig. 3 were representative of at least two independent experiments with similar findings.

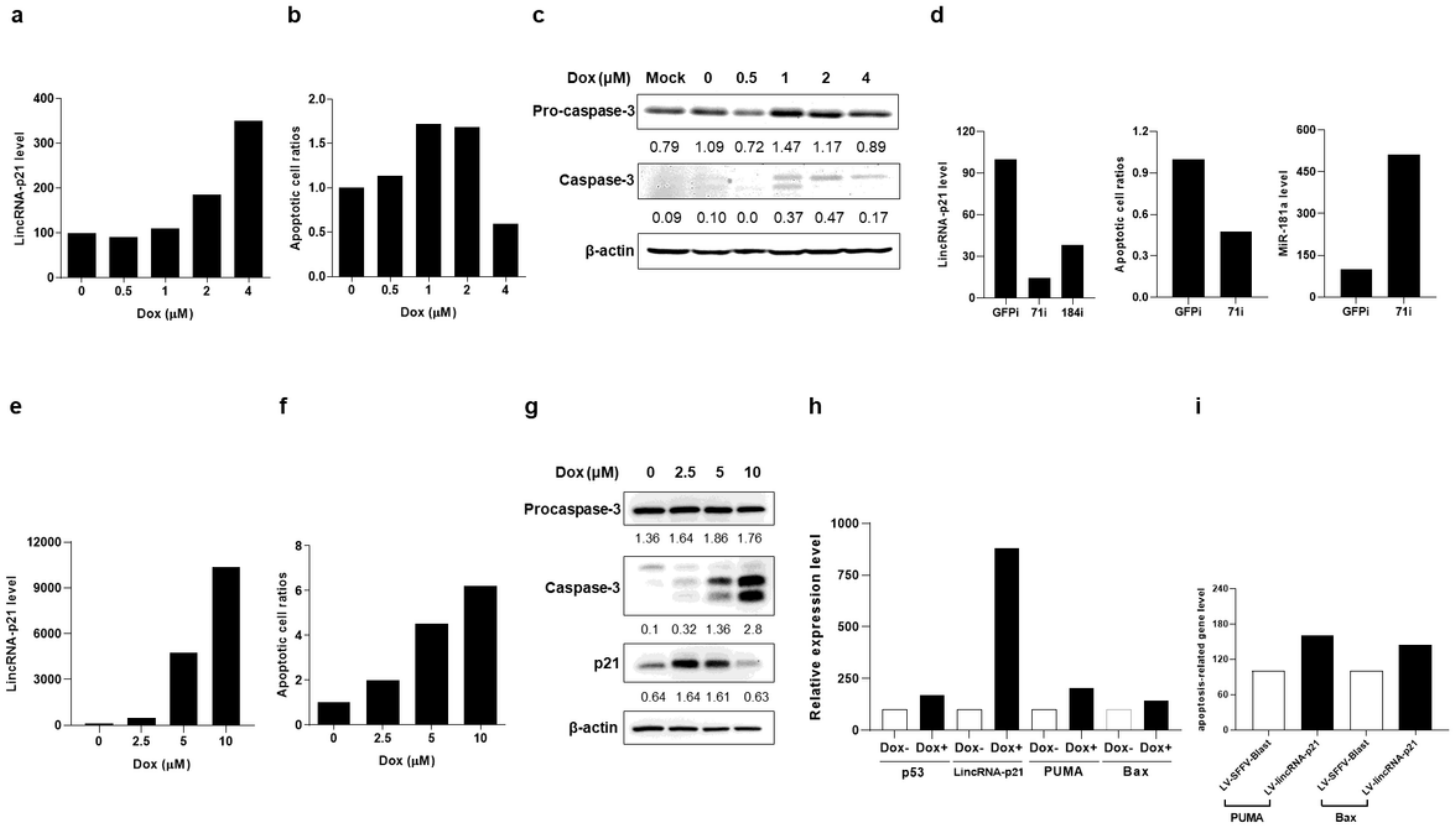

Figure 4

Up-regulated expression of lincRNA-p21 in apoptotic kidney cell lines. a to c Dose-dependent up-regulated lincRNA-p21 levels and apoptotic cell ratios, and increases in caspase 3 levels in HEK 293T cells culture under the Dox treatment. d Two CRISPRi-lincRNA-p21 transfectants and decreased Dox-induced apoptotic cell ratios with increased miR-181a levels shown in 71i with higher silenced efficacy. e to g Dose-dependent up-regulated lincRNA-p21 levels and apoptotic cell ratios, and increases in caspase 3/p21 levels in HK-2 cells under the Dox treatment. h Up-regulated expression of p53 down-stream molecules PUMA and Bax in HK-2 cells culture under the Dox 5 □M treatment. i Enhanced expression of p53 down-stream molecules PUMA and Bax in lincRNA-p21-overexpressed HK-2 cells. All of the results in Fig. 4 were representative of at least two independent experiments with similar findings.

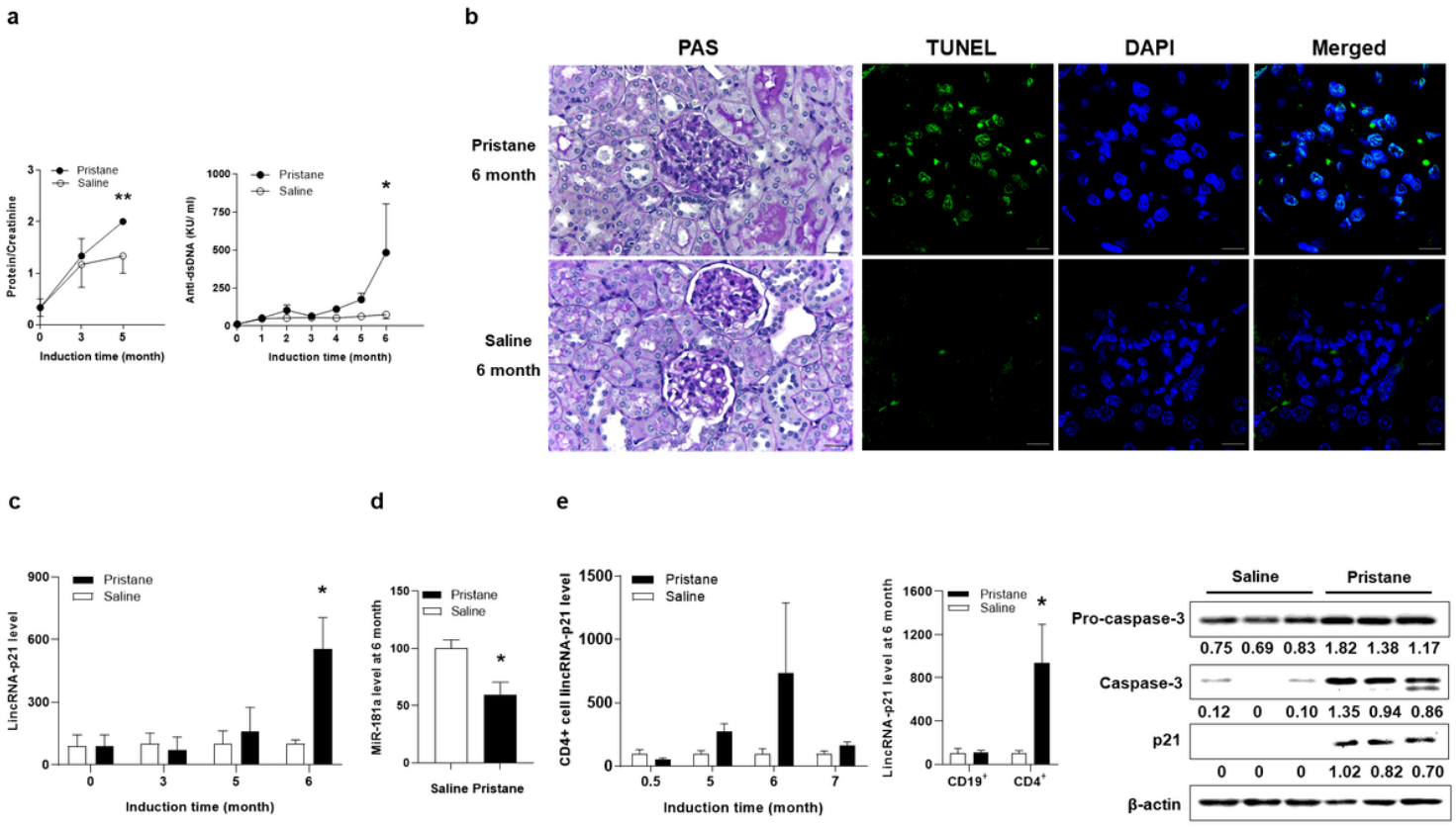

Figure 5

Up-regulated expression of lincRNA-p21 with in situ apoptosis in pristane-induced female Balb/C mice with LN. a Kinetic measurement of proteinuria amounts and anti-dsDNA titers after pristane induction. Values are the mean + SEM with 3 mice per group. b PAS staining of GN with glomerular hyper-cellularity/mesangial expansion (×400) and TUNNEL staining with in situ apoptotic glomerular parenchymal cells (×200) at 6 months after induction. Saline-injected sections as the control (lower panel). Bars shown on photomicrographs at ×200 and ×400 magnification correspond to 20□□m and 10 □m, respectively. Representative microphotographs from 3 mice per group. c Kinetic measurement of lincRNA-p21 levels from kidney cells after induction. Values are the mean + SEM with 5 mice per group. d Mir-181a levels from kidney cells at 6 months after induction. Values are the mean + SEM of 5 mice per group. e Kinetic measurement of lincRNA-p21 levels from CD4+ cells (left panel). LincRNA-p21 levels from CD19+ and CD4+ cells at 6 months after induction (middle panel), and expression of caspase 3 and p21 levels (right panel). Values are the mean + SEM of 3 mice per group for measurement of lincRNA-p21 levels, and 3 mice per group for caspase 3/p21 immunoblot assay. All of the results in Fig. 5 were representative of two independent experiments with similar findings.