DNA methyltransferase and is involved in the *INK4* locus via methylation of CpG islands [48]. Moreover, PcG is recruited to the *INK4* locus, thereby suppressing transcription via histone H3K27 methylation [49].

It has been suggested that antisense RNA transcribed near the *p15* gene controls transcription of *p15* [50]. Pasmant et al. identified an lncRNA, *ANRIL*, as an anti-sense transcript of the *p15* gene in the *INK4* locus [51]. Both our study and other research have revealed that *ANRIL* is involved in epigenetic repression of the transcription of the *INK4* locus [31, 32] (Table 1). We found that depletion of *ANRIL* by short hairpin RNA (shRNA) decreased the recruitment of SUZ12 to the *INK4* locus and promoted the expression of *p15* gene dramatically and *p16* gene moderately, but had no effect on *ARF* [31]. SUZ12 is a component of the PRC2 complex. In contrast, Yap et al. [32] demonstrated that *ANRIL* binds to CBX7, a component of the PRC1 complex, in the chromatin fraction, and recruits PRC1 to the *INK4* locus to mediate transcriptional suppression. Therefore, *ANRIL* binds to the PRC2 complex to recruit it to the *INK4* locus, and then histone H3K27 methylation is mediated by EZH2 in the PRC2 complex. Next, PRC2 with *ANRIL* is recognized by CBX7, and the PRC1 complex is recruited to the region. Further, histone H2AK119 monoubiquitination is induced to repress transcription of the *INK4* locus. Moreover, we demonstrated that depletion of *ANRIL* promotes growth arrest and induces senescence-associated beta-galactosidase in WI38 human fibroblasts [31]. Yap et al. [32] also suggested that CBX7-mediated suppression of the *INK4* locus is involved in regulating cellular senescence. These reports strongly suggest that *ANRIL* participates not only in cell proliferation but also in suppressing premature senescence via the recruitment of PRC1 and PRC2 to the *INK4* locus (Fig. 3a).

It is important to understand how *ANRIL* expression is regulated. We found that excess RAS signaling promoted by the introduction of activated H-RasG12V into WI38 fibroblasts suppressed *ANRIL* expression and induced p15 and p16, thereby arresting the cell cycle and inducing senescence-associated beta-galactosidase [31] (Fig. 3b). Recently, Wan et al. [52] reported that *ANRIL* is induced by DNA-damaging agents via the ATM-E2F1 pathway, but p53 is not induced. Moreover, they suggested that depletion of *ANRIL* decreases homologous recombination after DNA double-strand breaks, although it is unclear whether *ANRIL* promotes DNA repair via the recruitment of PRC1 and PRC2 to the *INK4* locus. Further studies are required on *ANRIL* function in response to cellular stresses. Moreover, Yang et al. found that *lncRNA-HEIH* is highly expressed in HBV-related hepatocellular carcinoma. It negatively regulates the expression of CDK inhibitors, such as p15, p16, p21, and p57, via interacting with EZH2, and then plays an important role in G0/G1 arrest [53] (Table 1).

$p18^{ink4c}$ (hereafter p18) is another INK4 family CDK inhibitor that also inhibits both CDK4 and 6 [3, 54]. Recently, *ink4c−/−* mice have been shown to develop spontaneous pituitary adenomas [55], the frequency of which is enhanced by deletion of other CDK inhibitor genes [56]. The combined deletion of the *p18* gene (*CDKN2C*) with the *p16* gene is also found in human cancers [57]. Moreover, the expression levels of p16 and p18 are often inversely correlated during the progression of senescence [58]. It has been reported that transcription of the *p18* gene is regulated by Menin-RET-signaling and the PI3K-AKT pathway [59]. Du et al. [60] reported that the

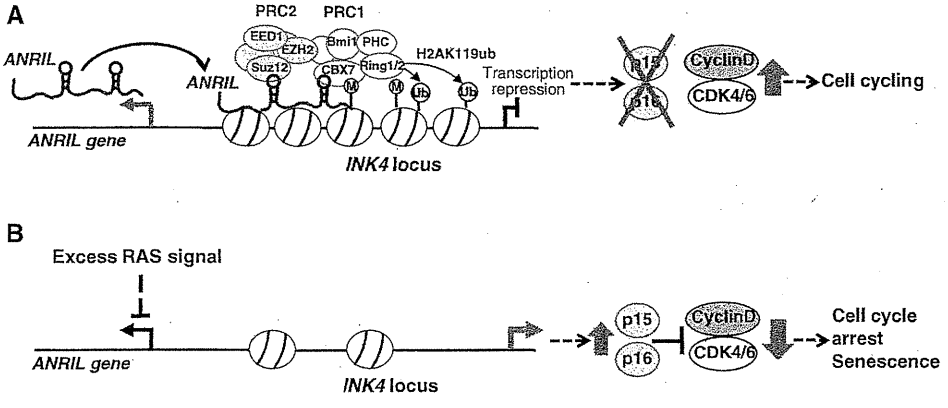

Fig. 3 Model showing the proposed mechanisms of *ANRIL*-mediated regulation of the *INK4* locus. **a** Model of *ANRIL*-mediated repression of the *INK4* locus. *ANRIL* binds to the PRC2 complex to recruit it to the *INK4* locus. Then, histone H3K27 methylation (M) is mediated by EZH2 in the PRC2 complex with *ANRIL*, which is recognized by CBX7 to recruit the PRC1 complex to the region. Histone H2AK119 monoubiquitination (Ub) is thereby induced to repress the transcription of *INK4*. **b** Excess RAS signaling suppresses the expression of *ANRIL*. Overexpression of activated H-RasG12V in WI38 fibroblasts promotes excess RAS signaling and suppresses *ANRIL* expression. Then, p15 and p16 are induced and the cell cycle undergoes arrest, inducing a premature senescence-like phenotype

lncRNA, *HULC*, negatively regulates the expression of *p18* gene, which is located near the region containing *HULC* (Table 1). *HULC* was identified as an lncRNA upregulated in human hepatocellular carcinoma (HCC) [61] that is transcribed in a CREB-dependent manner [62]. Moreover, the expression of p18 is induced and suppressed by depletion and overexpression of *HULC*, respectively. The expression of p18 is inversely correlated with the expression of *HULC* in human HCC tissue specimens. Furthermore, the hepatitis B virus oncogene product, HBx, activates the *HULC* promoter via CREB to suppress the transcription of the *p18* gene by upregulated HULC [60]. Downregulation of the *p18* gene by HBx via *HULC* induction may contribute to the development of HCC, although it is unknown how *HULC* suppresses the transcription of the *p18* gene.

Cip/Kip family inhibitors

The transcription of $p57^{Kip2}$ gene (*CDKN1C*), which is located at the *KCNQ1* domain, is epigenetically suppressed as an imprinted gene on the paternal chromosome [63]. *KCNQ1OT1* is paternally expressed as an antisense RNA of the *KCNQ1* domain containing *KCNQ1* and the $p57^{Kip2}$ gene [64]. *KCNQ1OT1* functions as a recruiter that associates with the chromatin modifiers, PRC2 and G9a, and recruits them to the *KCNQ1* domain to suppress the transcription of $p57^{Kip2}$ gene (Table 1). As described above, *lncRNA-HEIH* downregulates the expression of not only the INK4 family inhibitors, p15 and p16, but also the Cip/Kip family inhibitors, p21 and p57 [53].

LncRNAs regulating the pRB pathway

As described above, the tumor suppressor pRB is a critical regulator of G1/S progression [7, 65]. It is well known that the expression of the *RB* gene is epigenetically silenced by methylation of the promoter in some cancers, including retinoblastoma [66]. Hypermethylation of the CTCF binding site in the *RB* promoter is mediated by the CTCF protein [67]. CTCF also regulates the expression balance between the *IGF2/H19* locus together with DNA methylation of their promoters as an insulator of gene expression [68]. Interestingly, the *H19* gene encodes a 2.9-kb lncRNA, and the *H19* lncRNA is a precursor of miR-675 [69]. The expression of *H19* lncRNA is mediated by E2F1 and promotes cell proliferation [70], but the mechanism is unknown. Tsang et al. [71] reported that the *H19* lncRNA-derived miR-675 associates with the 3′ untranslated region of *RB* mRNA to negatively regulate pRB expression (Fig. 4; Table 1). In human colorectal cancer, *H19* lncRNA/miR-675 expression is inversely correlated with pRB expression [71]. Therefore, *H19* lncRNA/miR-675 may be a critical negative regulator of the RB tumor suppressor pathway (Fig. 3). Moreover,

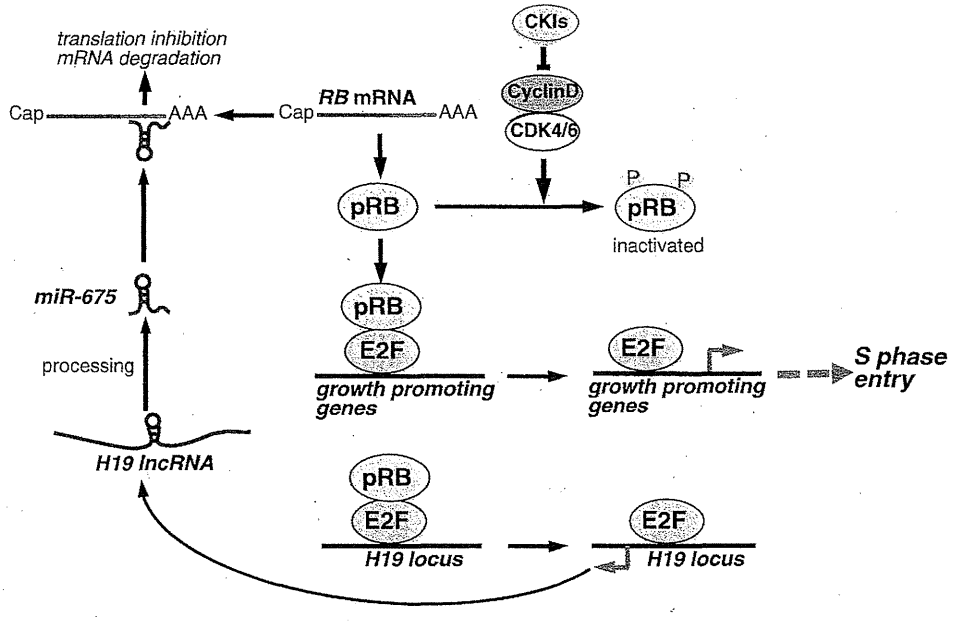

Fig. 4 Model showing the proposed mechanisms of lncRNA-mediated regulation of the RB pathway. pRB binds target transcription factors such as E2F and inhibits their activity in the G1 phase. Cyclin Ds-CDK4/6 phosphorylate pRB and activate E2F-mediated transcription in late G1, which regulates the expression of several growth-promoting genes and S phase entry. The transcription of *H19* lncRNA from the *H19* locus is mediated by E2F1. *H19* lncRNA is processed to generate miR-675, which binds to *RB* mRNA and inhibits its translation

pRB suppresses E2F-dependent transcription of *H19* transcription via repression of the *H19* promoter. Therefore, the *H19-RB* axis is self-regulated.

LncRNAs regulating the p53 pathway

Another important tumor suppressor, p53, functions as the gatekeeper of the genome to control cell cycle arrest and apoptosis in response to DNA damage [65, 72]. Although p53 is unstable, it is stabilized and activated via phosphorylation mediated by the ATM/ATR pathway in response to DNA damage. Moreover, p53 is also regulated via phosphorylation at various sites by specific kinases [73]. Zang et al. [74] reported that *lncRNA-RoR* negatively regulates p53 expression, thereby suppressing doxorubicin-induced G2/M arrest and apoptosis (Table 1). Depletion of *lncRNA-RoR* leads to p53 accumulation, and overexpression of *lncRNA-RoR* suppresses p53 expression. *LncRNA-RoR* binds to phosphorylated heterogeneous nuclear ribonucleoprotein I (p-hnRNP-I) in cytoplasm and thereby suppresses p53 translation. The 28-base RoR sequence is sufficient for its function. Additionally, wild-type p53 binds to the *RoR* promoter to promote transcription of *lncRNA-RoR*, but mutant p53 does not bind to this promoter. This is a novel autoregulatory feedback loop that controls p53 levels (Fig. 5).

Recently, Melo et al. [75] reported that enhancer RNAs (*eRNAs*) are required for coordinated promotion between p53 target genes and p53-bound enhancer regions distant from the target gene, and participate in p53-dependent cell cycle arrest (Table 1). LncRNA *loc285194* was suggested to have a tumor suppressor function, but its mechanism was unknown. Liu et al. found that *loc285194* is induced by binding of p53 to its binding site in the promoter (Table 1). Moreover, they indicated that *loc285194* binds to and inhibits miR-211, thereby downregulating miR-211-mediated cell proliferation [76]. *Loc285194* is downregulated in human colon cancer specimens, and thus may contribute to the tumor suppressive function of p53 to inhibit miR-211 [76].

Huarte et al. [77] identified *lncRNA-p21*, which is transcribed near the $p21^{Cip1}$ gene (*CDKN1A*) as a p53-target gene. p53 directly binds to its binding element in the *lncRNA-p21* promoter. Depletion of *lncRNA-p21* alters

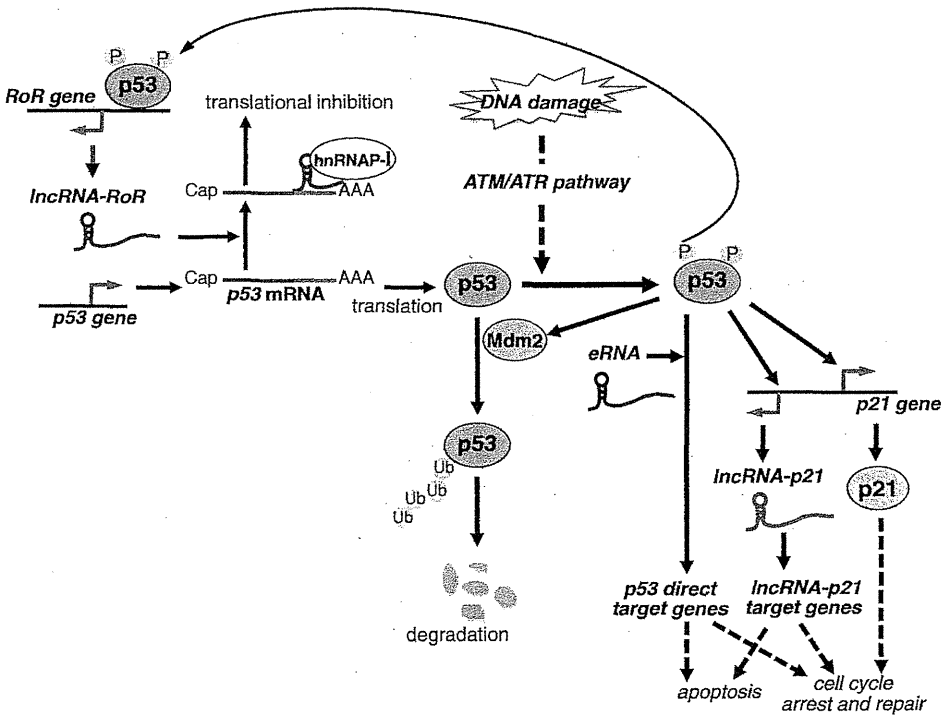

Fig. 5 Model showing the proposed mechanisms of lncRNA-mediated regulation of the p53 pathway. p53 controls cell cycle arrest, repair, and apoptosis in response to DNA damage. *lncRNA-RoR* binds to hnRNP-I and collaboratively suppresses p53 mRNA translation. This is an autoregulatory feedback loop that controls p53 levels. In response to DNA damage, p53 is stabilized and activated via phosphorylation mediated by the ATM/ATR pathway. p53 directly binds the target genes and regulates their expression to control cell cycle arrest, repair, and apoptosis. *eRNAs* are involved in promotion of p53-target genes in p53-dependent cell cycle arrest. p21 and *lncRNA-p21*, which is transcribed near the $p21^{Cip1}$ gene, are p53-target genes. *lncRNA-p21* controls the expression of some p53-target genes. p53 function is partially mediated by gene regulation via *lncRNA-p21*

the expression of some p53-target genes except for *p21* gene and inhibits apoptosis (Fig. 5; Table 1). *lncRNA-p21* binds to hnRNP-K and recruits it to the target genes, but the mechanism of target gene regulation is unknown. p53 function is partially mediated by gene regulation via *lncRNA-p21*-hnRNP-K. Moreover, Yoon et al. proposed that *lncRNA-p21* functions as a modulator of translation. *lncRNA-p21* associates with target mRNAs such as β-catenin and JunB in collaboration with Rck/p54 RNA helicase, and thus the translation of the target mRNAs is repressed [78]. Therefore, *lncRNA-p21* regulates both transcription in the nucleus and translation in the cytoplasm.

PANDA (p21-associated ncRNA DNA damage-activated) was identified as a *p21* promoter-derived transcript using ultra-high density tiling array of 56 cell-cycle genes. It is induced by DNA damage in a p53-dependent manner [79] (Table 1). *PANDA* binds to and inhibits NF-YA transcription factor, which limits the expression of proapoptotic genes such as *FAS* and *BIK* and results in the repression of apoptosis. *PANDA* is selectively induced in metastatic ductal carcinomas but not in normal breast tissue [79]. The results suggest that abnormal overexpression of *PANDA* may suppress apoptosis induced by DNA damage, which will accumulate and push the genome toward carcinogenesis.

Perspectives

Although the mechanisms of cell cycle regulation via cyclin–CDK, the p53/RB pathway, and the checkpoint pathway have been described in detail, recent studies on lncRNAs strongly suggest that lncRNAs control the expression of cell cycle regulators. Therefore, lncRNAs are critically involved in cell cycle regulation. However, it is unclear why lncRNAs might be deployed to regulate the cell cycle. As described in the "Introduction", lncRNAs involved in cell cycle regulation are classified into four groups. As shown in Table 1, *ANRIL*, *lncRNA-HEIH*, and *KCNQ1OT1* are involved in epigenetic regulation of target gene transcription by collaborating with chromatin modifiers, which are classified as epigenetic regulators. *ncRNA$_{CCND1}$*, *SRA*, *PANDA*, and *lncRNA-p21* directly interact with the transcriptional machinery on the target genes and collaboratively regulate transcription as transcription factor regulators. Post-transcription regulators including *gadd7*, *MALAT1*, *lncRNA-RoR*, and *loc285194* bind to their specific target mRNA to suppress translation and/or to modulate mRNA stability. *SRA* and *MALAT1* also promote protein–protein interactions and are classified as protein scaffolds. Because the general cell cycle is closely associated with various cellular events as well as biological processes, it should be accurately regulated. Post-transcription regulators such as *gadd7*, *MALAT1*, *H19 lncRNA*, and *loc285194* can rapidly and transiently suppress translation of their target genes. Transcription factor regulators such as *ncRNA$_{CCND1}$*, *SRA*, *PANDA*, and *lncRNA-p21* that directly interact with the transcription machinery on the target genes may also participate in transient regulation. Alternatively, epigenetic regulators such as *ANRIL*, *lncRNA-HEIH*, and *KCNQ1OT1* may have long-term effects on cellular senescence and imprinting because they mediate epigenetic regulation of cell cycle regulatory genes via chromatin modifiers. From this viewpoint, the cell cycle-regulated lncRNAs mainly control cellular levels of cell cycle regulators via various mechanisms, and may provide diversity and reliability to the general cell cycle.

It is interesting that many lncRNAs are associated with the DNA damage response. As shown in Table 1, 4 of 14 lncRNAs, *lncRNA$_{CCND1}$*, *gadd7*, *ANRIL* and *PANDA*, are induced by DNA damage. Another 4 lncRNAs, *lncRNA-RoR*, *lncRNAp21*, *p53-induced eRNA*, and *loc285194*, are induced in a p53-dependent manner, suggesting that they are induced by DNA damage. Therefore, these reported lncRNAs may participate in cell cycle arrest or induction of apoptosis as non-canonical DNA damage responses, whereas the ATM/ATR pathway is involved in a canonical DNA damage response to inactivate CDK activity as a DNA damage checkpoint. LncRNAs-mediated non-canonical pathways may ensure the response to DNA damage is diverse and reliable depending on the cellular context.

Considering the recent progress in lncRNA research, many lncRNAs that have a functional role in cell cycle regulation remain to be identified because the functions of only a small percentage of the total lncRNA population are understood. To clarify the roles of lncRNAs in cell cycle regulation, it should be determined how they regulate the target cell cycle regulators and which signaling pathways induce these lncRNAs. Since abrogation of the cell cycle is closely associated with cancer development and growth, cell cycle regulatory lncRNAs such as *ANRIL* and *PANDA* may have oncogenic properties. The importance of lncRNAs in cell cycle regulation will be clarified by further pathological studies. Moreover, these cell cycle regulatory lncRNAs may be novel candidate molecular targets for cancer therapy or diagnosis.

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Cell. Mol. Life Sci. (2013) 70:3277–3287
DOI 10.1007/s00018-012-1232-x

Cellular and Molecular Life Sciences

REVIEW

Roles of the Skp2/p27 axis in the progression of chronic nephropathy

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Received: 23 September 2012 / Revised: 14 November 2012 / Accepted: 3 December 2012 / Published online: 20 December 2012

Abstract S-phase kinase-associated protein 2 (Skp2) is an F-box protein component of the Skp/Cullin/F-box-type E3 ubiquitin ligase that targets several cell cycle regulatory proteins for degradation through the ubiquitin-dependent pathway. Skp2-mediated degradation of p27, a cyclin-dependent kinase inhibitor, is involved in cell cycle regulation. Tubular epithelial cell proliferation is a characteristic feature of renal damage that is apparent in the early stages of nephropathy. The p27 level is associated with the progression of renal injury, and increased Skp2 expression in progressive nephropathy is implicated in decreases of p27 expression. In $Skp2^{-/-}$ mice, renal damage caused by unilateral ureteral obstruction (UUO) was ameliorated by p27 accumulation, mainly in tubular epithelial cells. However, the amelioration of UUO-induced renal injury in $Skp2^{-/-}$ mice was prevented by p27 deficiency in $Skp2^{-/-}/p27^{-/-}$ mice. These results suggest that the Skp2-mediated reduction in p27 is a pathogenic activity that occurs during the progression of nephropathy. Here, we discuss the roles of the Skp2/p27 axis and/or related signaling pathways/components in the progression of chronic nephropathy.

Keywords Ubiquitin-proteasome · Ubiquitin ligase · Chronic nephropathy · p27 · Skp2

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Introduction

Cell proliferation is a fundamental biological mechanism that involves transit through the cell cycle. It is regulated by a network of proteins including cyclins, cyclin-dependent kinases (CDKs) [1], and CDK inhibitors (CKIs) [2]. The CKI $p27^{Kip1}$ (p27) is a negative regulator that halts progression from the G1 phase to the S phase in the cell cycle. p27 is abundantly expressed in most normal quiescent cells, whereas its level declines when cells are stimulated to proliferate in response to mitotic stimuli, allowing progression to the S phase [3, 4]. The ubiquitin-proteasome pathway for protein degradation plays an important role in regulating the abundance of cell cycle regulatory proteins [5, 6]. Protein degradation via the ubiquitin-proteasome pathway is rapid and substrate-specific, which is consistent with its role in controlling fluctuations in the intracellular concentrations of cyclins and CKIs. S-phase kinase-associated protein 2 (Skp2) is an F-box protein component of the Skp/Cullin/F-box (SCF)-type E3 ubiquitin ligase that plays important roles in regulating the progression to the S phase. p27 is phosphorylated at threonine residue 187 (Thr187) by CDK2/cyclin E. The SCF/Skp2 complex interacts with phosphorylated p27 to promote p27 degradation through the ubiquitin-proteasome pathway [7, 8]. The cdc kinase subunit 1 (Cks1) is an essential cofactor for SCF/Skp2 ubiquitin ligase to ubiquitylate p27. Cks1 recognizes and binds to Thr187-phosphorylated p27 and induces rigid binding between Skp2 and p27 [9, 10]. p27 is stabilized in Skp2-deficient mice [11]. Therefore, proteasomal ubiquitin-dependent degradation of p27 is specifically controlled by the SCF/Skp2/Cks1 complex.

In the kidney, cell proliferation is thought to represent a central response to renal injury culminating in end-stage renal disease caused by the progression of tubulointerstitial

fibrosis [12]. Disruption of the balance between cell proliferation and apoptosis leads to unchecked apoptosis of damaged tubular epithelial cells resulting in progressive tubular cell loss, renal tubular atrophy, and advanced interstitial fibrosis [13].

Unilateral ureteral obstruction (UUO) and anti-thymocyte serum (ATS) are models of chronic nephropathy

UUO is a widely used model of kidney disease associated with progressive tubulointerstitial damage. This method has been used to identify many of the cellular and molecular events that occur during the progression of renal fibrosis, including events associated with cell proliferation and apoptosis [14–16]. UUO kidneys show elevated expression levels of monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), which promote monocyte infiltration and kidney inflammation [17, 18]. It is generally believed that renal tubule dilation occurs as a result of increased hydrostatic pressure following obstruction. However, it was also reported that decreases in renal blood flow and the glomerular filtration rate both promote macrophage invasion into the renal interstitium. The infiltrated macrophages release various cytokines, including TNF-α [19]. The cytokine signals and hydrostatic pressure may act collaboratively to stimulate epithelial cell proliferation, which results in an increased number of tubular epithelial cells. We previously reported that tubule dilation is correlated with the increase in number of epithelial cells and enhanced tubular epithelial cell proliferation in the obstructed kidney [20]. Taken together, these results suggest that hydrostatic pressure and tubular epithelial cell proliferation are involved

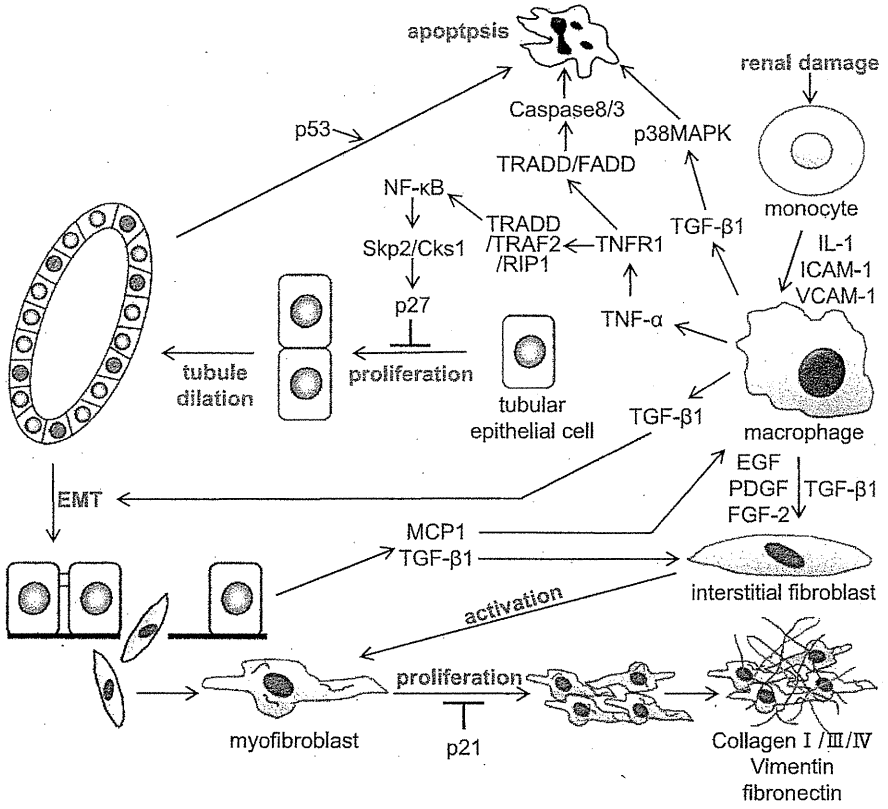

Fig. 1 The signal transduction pathways involved in the progression of chronic nephropathy. Following renal damage, infiltrated macrophages in the tubulointerstitium release cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1). TNF-α binds to TNF receptor 1 (TNFR1) and forms a complex with TNFR-associated death domain (TRADD), TNF associated factor 2 (TRAF2), and receptor interaction protein 1 (RIP1). This complex activates nuclear factor (NF)-κB, which induces Skp2 and Cks1. Upregulation of Skp2/Cks1 promotes p27 degradation in tubular epithelial cells, allowing proliferation of tubular epithelial cells and tubule dilation following the increase of tubular epithelial cell number. The tubular epithelial cells undergo epithelial-mesenchymal transition (EMT) by stimulation of TGF-β1, and the resulting fibroblasts migrate to the tubulointerstitium. Cytokines including TGF-β1 activate fibroblasts; activated myofibroblasts produce extracellular matrix components, such as collagen, vimentin, and fibronectin. Meanwhile, TNF-α and TGF-β1 induce tubular epithelial cell apoptosis. *IL-1* interleukin-1, *ICAM-1* intercellular adhesion molecule-1, *VCAM-1* vascular cell adhesion molecule-1, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor, *FGF-2* fibroblast growth factor-2, *FADD* Fas-associated death domain protein, *MAPK* mitogen-activated protein kinase

in tubule dilation. Renal tubular epithelial cell proliferation increases significantly and renal tubules start to dilate at 3 days after UUO [20, 21]. The extent of tubule dilation is related to the progressive increase in tubular epithelial cell number caused by proliferation. This process ultimately results in the fracture of the tubular basement membrane of the dilated renal tubules. In damaged kidneys, tubular epithelial cells trans-differentiate into mesenchymal cells that express α-smooth muscle actin (α-SMA) in response to kidney inflammation. These cells enter the tubular interstitium through the broken tubular basement membrane [22, 23]. The trans-differentiated tubular epithelial cells further differentiate into myofibroblasts (i.e., fibroblasts expressed α-SMA) in the interstitium. Concurrently, macrophages in the renal interstitium release several cytokines, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF-2), which activate fibroblasts. The interstitial myofibroblasts undergo hyperproliferation because of their high cell responsiveness, resulting in irreversible progression of renal interstitial fibrosis (Fig. 1). There are many reports of establishing UUO in knockout mice and the roles of many cell cycle-related molecules in renal damage have been investigated in UUO kidneys [24].

Another experimental model of chronic progressive glomerulonephritis can be induced in rats by repeated injections of ATS. In this model, irreversible glomerulosclerosis and tubulointerstitial fibrosis are induced after the second ATS injection and are associated with a gradual decline of renal function [25–27]. Alternatively, chronic renal failure can also be studied in the 5/6 nephrectomy model [28, 29] and in diabetic nephropathy [30, 31].

Signal transduction pathways involved in renal damage

Transforming growth factor-β1 (TGF-β1)/Smad pathway

TGF-β1 is a multifunctional signaling protein that regulates cell cycle, apoptosis, differentiation, and extracellular matrix accumulation [32]. TGF-β1 also has a significant role in the progression of renal fibrosis in clinical and experimental kidney diseases [25, 33]. Following the onset of nephropathy, TGF-β1 is released from macrophages in the damaged renal interstitium and influences the tubular epithelial cells. The damaged tubular epithelial cells also release TGF-β1, which exacerbates renal damage. TGF-β1 was also reported to stimulate the epithelial-mesenchymal transition (EMT) [34–36]. Finally, tubular epithelial cells that acquire a fibroblastic phenotype via EMT migrate into the interstitium, probably through the ruptured tubular basement membrane. TGF-β1 also promotes the differentiation of interstitial fibroblasts to myofibroblasts and their production of extracellular matrix [37, 38]. The accumulation of extracellular matrix in the tubulointerstitium and in the glomerulus is also stimulated by TGF-β1. Conversely, TGF-β1 promotes apoptosis of tubular epithelial cells via a p38 mitogen-activated protein kinase-dependent mechanism [39, 40]. Overall, upregulation of TGF-β1 contributes to EMT during renal fibrosis and apoptosis, and it induces the progression of nephropathy.

In terms of the TGF-β1 signaling pathway, Smad proteins play important roles as signal transducers downstream of TGF-β1 receptors [41, 42]. TGF-β1 binds to the TGF-β type II receptor, which recruits and phosphorylates the TGF-β type I receptor, ALK5. In turn, ALK5 phosphorylates Smad2 and Smad3, which then bind to Smad4 [43, 44]. The resulting complexes can then enter the nucleus [45–47]. Another Smad, Smad7, has an inhibitory role in the TGF-β1 signaling pathway [48]. It was also reported that chronic progressive renal injury can be suppressed by inhibiting the TGF-β/Smad axis using an anti-TGF-β antibody [27]. Although TGF-β1 signaling is also mediated by ALK1, another TGF-β type I receptor that phosphorylates Smad1/5 [49], little is known about the roles of the ALK1/Smad1/5 pathway in renal injury. It was also suggested that TGF-β promotes translocation of Skp2 into the nucleus, where it is degraded by the anaphase-promoting complex/cyclosome (APC/C)-Cdh1 E3 ligase. In addition, TGF-β decreases Cks1 mRNA expression, which allows p27 to accumulate following G1 arrest [50–52]. Taken together, these findings indicate that TGF-β is an important upstream signal that regulates the Skp2/p27 axis.

Tumor necrosis factor-α (TNF-α)/nuclear factor (NF)-κB pathway

TNF-α is a multifunctional cytokine that induces a wide range of cellular responses, including proliferation, differentiation, and activation of apoptosis [53]. TNF-α is produced by activated macrophages, and it stimulates the proliferation and apoptosis of renal tubular epithelial cells and interstitial cells in renal injury [54–56]. TNF-α binds to two different TNF receptors (TNFR), type 1 and type 2 receptors [57, 58]. On binding of TNF-α to TNFR1, TNFR1 recruits TNFR-associated death domain (TRADD) as an adaptor protein thorough death domain within 2 min. In turn, TRADD serves as an assembly platform protein to arborize TNFR1 signaling between apoptosis and anti-apoptosis/proliferation. TRADD recruits Fas-associated death domain protein (FADD) to its death domain and activates the Caspase-8/-3 cascade to induce apoptosis [59, 60]. TRADD also recruits TNF-associated factor 2 (TRAF2) and receptor interaction protein (RIP), leading to the activation of NF-κB, which has anti-apoptotic effects [57, 61]. It has been reported that the TNFR1/TRADD/TRAF2/RIP complex is produced more

quickly than the TRADD/FADD complex because of the antagonistic effects of the TNFR1/TRADD/FADD on apoptosis signaling pathways.

TNF-α can also bind to TNFR2, which recruits TRAF2 and activated NF-κB [62]. However, binding of TNF-α to TNFR2 promotes TRAF2 degradation through the ubiquitin-dependent proteasome pathway, resulting in the suppression of NF-κB activation by inhibition of TRADD/TRAF2/RIP complex formation. In addition, TNF-α decreases TRADD protein levels by enhancing its ubiquitin-dependent degradation in obstructive renal damage [20]. In the kidneys, it was reported that renal damage caused by cisplatin was less severe in TNFR2-deficient mice than in TNFR1-deficient mice [57]. However, renal damage in UUO mice was less severe in TNFR1-deficient mice than in TNFR2-deficient mice [63]. It was also reported that a reduction of TRADD inhibits TNFR1 signaling and that TNFR1-mediated TNF-α signaling may transfer to TNFR2 signaling in UUO mice [21]. Another report revealed that the two TNFRs may act collaboratively to regulate signal transduction [64, 65]. However, it has been unclear how TNFR2 regulates the TNF-α signaling pathway until now.

The transcription factor NF-κB, a downstream factor of TNF-α, is activated in renal damage and controls the activation of many genes related to inflammation [66, 67]. NF-κB is an inductive homo- or heterodimeric transcription factor composed of the Rel family members of DNA-binding proteins, including p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelA (p65), RelB, and c-Rel [68]. Activated NF-κB behaves as an important regulator of inflammation and immune responses by mediating the expression of pro-inflammatory genes, including cytokines, chemokines, growth factors, and adhesion molecules, which are implicated in the progression of renal inflammatory disease [69, 70]. The downstream targets of NF-κB are also important regulators of cell proliferation. For example, the IκB-inducing kinase (IKK)-regulated signaling pathway accelerates cell proliferation. Furthermore, IKK-α, an essential component of the NF-κB pathway, affects many physiologic activities in both healthy and disease states [71], including mammary epithelial cell proliferation [72]. In renal injury, NF-κB stimulates tubular epithelial cells and fibroblasts, and induces their proliferation and differentiation, which ultimately promote the progression of renal fibrosis [73]. It was reported that the NF-κB pathway regulates Skp2 expression [74, 75]. As described below, we have suggested that TNF-α stimulates Skp2 and Cks1 mRNA expression via the NF-κB pathway in chronic nephropathy [76]. Therefore, TNF-α is likely to participate in Skp2/Cks1-dependent degradation of p27 as a precipitating factor of chronic nephropathy.

Role of the Skp2/p27 axis in the progression of renal damage

Skp2

The SCF/Skp2 ubiquitin ligase complex targets several important regulator proteins that control the cell cycle, including p27, p21, p57, cyclin E, cyclin A, and cyclin D1 [77], by promoting their degradation via the ubiquitin proteasome-dependent pathway. In this way, Skp2 ubiquitin ligase promotes cell cycle progression to the S-phase by stimulating the degradation of negative cell cycle regulators, such as the CKI p27 [7, 8, 78] (Fig. 2). Moreover, it has been reported that Kip1 ubiquitination-promoting complex (KPC) [79] and Pirh2 [80] act as E3 ligases for p27, whereas it has not been clarified whether p27 is accumulated in their

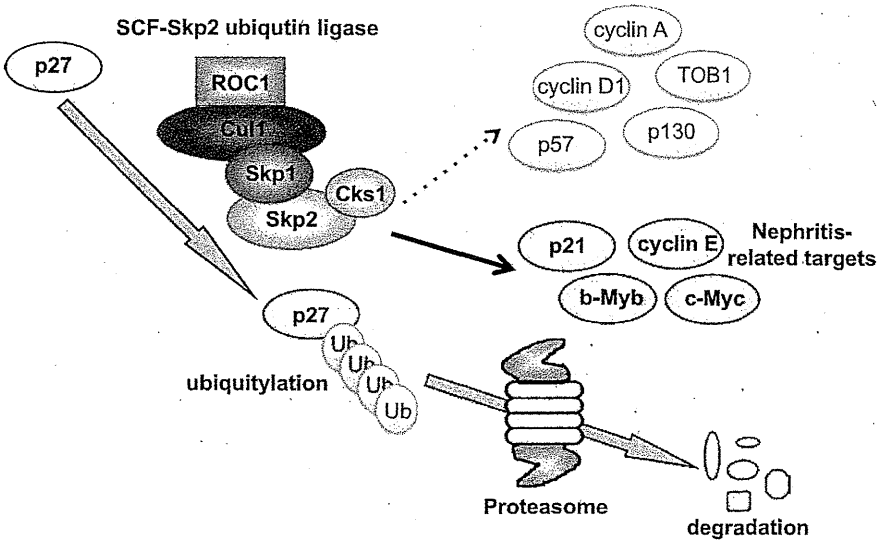

Fig. 2 The mechanism of p27 degradation by Skp2 E3 ubiquitin ligase. SCF (Skp1/Cul1/Skp2 as F-box) ubiquitin ligase induces p27 degradation by a proteasome-dependent pathway. Cks1 is an essential cofactor for p27 degradation by SCF/Skp2 that induces rigid binding between Skp2 and p27. Conversely, Skp2 has multiple targets and may also regulate p21, cyclin E, b-Myb, and c-Myc protein levels in unilateral ureteral obstruction (UUO). However, Skp2 did not affect the regulation of cyclin A, cyclin D1, TOB1, p57, or p130 in UUO kidneys

knockout mice. In human cancers, it was demonstrated that Skp2 overexpression stimulates the degradation of p27, indicating that Skp2 overexpression facilitates accelerated tumor growth and malignant potential [77]. However, the proteins that are targeted by Skp2 for degradation in specific biological processes or diseases have not been fully characterized.

We previously reported that Skp2 mRNA expression was increased in UUO kidneys in the early stages of renal damage and that the progression of tubulointerstitial fibrotic damage in UUO kidneys is attenuated in Skp2-deficient mice [20]. Furthermore, as described above, the mRNA and protein levels of Skp2 were increased in the ATS model of chronic nephropathy in rats [76]. It was reported that the NF-κB signaling pathway regulates the Skp2 promoter in cultured cells [74, 75]. TNF-α was reported to enhance mRNA expression of Skp2 in a normal rat epithelial kidney cell line (NRK) but not in control cells, which suggests that TNF-α facilitates the induction of Skp2 in nephropathy. In damaged kidneys, exposure to TNF-α significantly increased in cytoplasm of tubular epithelial cells. RelB and p52 proteins are known as NF-κB, and they are mainly seen in the nuclei of tubular epithelial cells. Skp2 is also expressed in the nuclei of tubular epithelial cells, similar to RelB and p52. Skp2 and RelB are colocalized in renal damage [76]. These data suggest that Skp2 is induced by the TNF-α/RelB/p52 signaling pathway in the early stages of renal injury and facilitates ubiquitin-dependent degradation of p27 in tubular epithelial cell proliferation and in the progression of chronic nephropathy (Fig. 3).

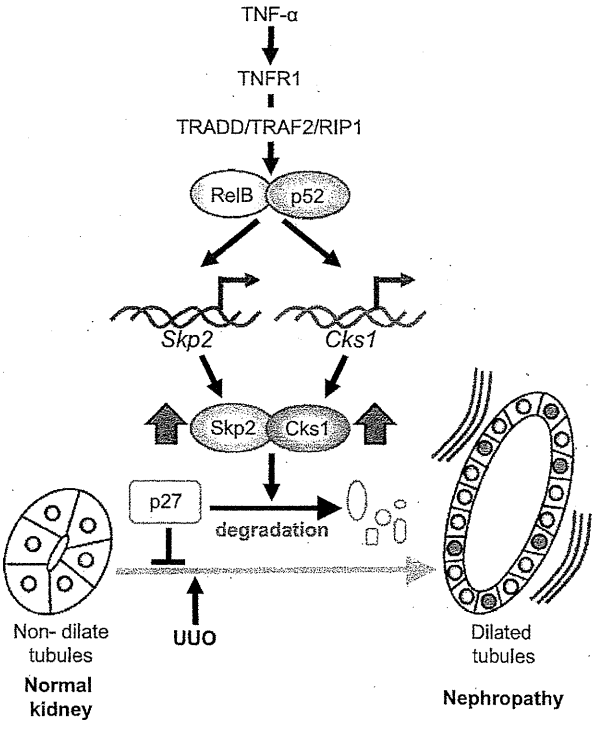

Fig. 3 Skp2/Cks1 is induced by the TNF-α/NF-κB signaling pathway in nephropathy. In normal kidneys, tubular epithelial cells highly express p27 in the quiescent phase of the cell cycle. Following renal damage, TNF-α activates RelB/p52, known as NF-κB, via TNFR1. The activated RelB/p52 complex induces the expression of both Skp2 and Cks1 in the nucleus. The induced Skp2/Cks1 degrades p27 in tubular epithelial cells in UUO kidneys, allowing tubular epithelial cell proliferation to increase [76]. Tubular dilation occurs as a result of the increase in the tubular epithelial cell number and ultimately leads to progressive nephropathy [20]

Cks1

Cks1 is an essential cofactor for ligation of ubiquitin to p27. It recognizes Thr187-phosphorylated p27 and is essential for the rigid binding between p27 and Skp2 that results in Skp2-mediated degradation of p27 [9, 10]. We previously reported that the mRNA and protein levels of Cks1 are increased in the early stages of renal damage [76]. Cks1 protein is mainly localized in the nuclei and to a lesser extent in the cytoplasm of tubular epithelial cells. Similar to Skp2, Cks1 colocalizes with RelB in the nuclei of tubular epithelial cells. These results suggest that Skp2 and Cks1 collaboratively promote p27 degradation via the ubiquitin proteasome pathway and induce tubular epithelial cell proliferation in the early stages of renal damage, resulting in tubular dilation in chronic nephropathy. The mRNA level of Cks1 is also significantly upregulated in TNF-α-stimulated NRK cells. We also reported that a sequence (GGGACTTCC) in the rodent Cks1 promoter is similar to the putative NF-κB element (GGGACTTTCC) at nine of the ten nucleotides. Therefore, it is seems likely that the TNF-α/NF-κB signaling pathway promotes the transcription of both Skp2 and Cks1 in renal injury [76].

p27

The CKI p27 is an important regulator of cell proliferation that negatively regulates the behavior of CDKs in the cell cycle [81, 82]. p27 is abundantly expressed in most normal quiescent cells, but its level decreases during progression to the S phase in response to a proliferative/mitotic stimulus [3, 4]. In vitro studies have shown that an experimental decrease of p27 protein enhances the proliferative response to mitogens [83, 84], while forced overexpression of p27 protein inhibits cell proliferation [4]. Additionally, p27 is destabilized in many types of human cancer, which is implicated in the aggressiveness and poor prognosis of tumors [77, 85–87]. The protein level of p27 is controlled transcriptionally and by proteolytic degradation of p27 protein via the ubiquitin-proteasome pathway. p27 is phosphorylated on Thr187 by CDK [9, 10], and Thr187-phosphorylated p27 is a specific target for the SCF/Skp2/Cks1 complex to induce its ubiquitin-dependent degradation [7, 11]. This is consistent with observations that Skp2-deficient mice and/or

Cks1-deficient mice exhibit cellular accumulation of p27 and a small body size compared with wild-type mice [9, 10].

In normal kidneys, p27 is expressed in most tubular epithelial cells to maintain their quiescent status. The level of p27 protein decreases rapidly in UUO kidneys, allowing proliferation of tubular epithelial cells and tubule dilation in the early stages of nephropathy. The mRNA and protein levels of p27 are subsequently upregulated in UUO mice [88, 89]. It was reported that renal tubular epithelial cell proliferation and apoptosis are markedly increased in the obstructed kidney of $p27^{-/-}$ mice [90]. Additionally, the magnitude of p27 protein upregulation in obstructed kidneys is greater in $Skp2^{-/-}$ mice than in $Skp2^{+/+}$ mice. In the UUO kidneys of $Skp2^{-/-}$ mice, tubular epithelial cell proliferation is inhibited by the accumulation of p27, preventing an increase in tubular epithelial cell number. Furthermore, apoptosis and tubulointerstitial fibrosis are markedly attenuated in the obstructed kidneys of $Skp2^{-/-}$ mice [20]. It is well known that renal fibroblast activation and proliferation are involved in the progression of chronic kidney disease [19]. We also reported that UUO stimulates renal interstitial cell proliferation and significantly increased the number of interstitial cells in the UUO kidney [20]. The enhanced interstitial cell proliferation and the increase in number of α-SMA-positive myofibroblasts were partially inhibited by Skp2-deficiency. p21 is the critical negative regulator of interstitial fibroblast proliferation [91]. We have shown that p21 accumulation in UUO kidneys is moderately enhanced by Skp2 deficiency [20]. In addition, the accumulation of p21 and p27 as a result of proteasome inhibition is associated with inhibition of interstitial fibroblast proliferation [92]. Therefore, p21 and p27 are negative regulators of interstitial cell proliferation while upregulated Skp2 in the UUO kidney enhances their degradation to promote interstitial fibroblast proliferation and myofibroblast formation as critical stages in the EMT. Taken together, these results suggest that Skp2 has important roles in the control of p27 and p21 in the kidney. In addition, Skp2, as induced by renal damage, promotes the proliferation of tubular epithelial cells and interstitial fibroblasts by enhancing the degradation of p27 and p21. Although further investigation is required to determine whether renal function was recovered by Skp2 deficiency, the histopathological features of $Skp2^{-/-}$ UUO kidney were apparently improved compared with the WT UUO kidney. Many other studies have demonstrated increased p27 expression in other models of renal disease, including diabetic nephropathy [30, 31] and cisplatin-induced acute renal failure [93]. In kidney cells, mesangial cells (MC) play a key role in glomerular hypertrophy in early diabetic nephropathy [94] by secreting extracellular matrix proteins that contribute to the development of glomerulosclerosis. Increased p27 expression in the glomerulus causes proliferation arrest and hypertrophy of MCs during early diabetic nephropathy. p27 is also highly expressed in the normal quiescent rat glomeruli, but its expression decreases in proliferating MCs in the ATS model of nephropathy [95]. The p27 expression level returns to the basal level after the resolution of MC proliferation [96]. Podocyte proliferation is also markedly increased in association with glomerulonephritis in $p27^{-/-}$ mice [90]. These data indicate that p27 regulates the proliferation of various types of renal cells, and its upregulation stops excessive renal cell proliferation to protect cells and tissues from inflammatory injury.

Renal damages in $Skp2^{-/-}p27^{-/-}$ mice

Unlike the marked amelioration of renal injury associates with renal accumulation of p27 in tubular epithelial cells in $Skp2^{-/-}$ mice, $Skp2^{-/-}/p27^{-/-}$ double knockout mice show marked progression of tubular dilatation as a result of the enhanced tubular epithelial cell proliferation that occurs through the loss of p27 [97]. Notably, the tubular epithelial cell number in UUO kidneys is much greater in $Skp2^{-/-}p27^{-/-}$ mice than in wild-type mice. Furthermore, interstitial cell proliferation in UUO kidneys is also greater in $Skp2^{-/-}p27^{-/-}$ mice than in $Skp2^{-/-}$ mice. The expression levels of vimentin, α-SMA, type I collagen, and fibronectin, components of the extracellular matrix, are significantly decreased in the UUO kidneys of $Skp2^{-/-}$ mice. While extracellular matrix production and macrophage infiltration are more pronounced in these mice, tubulointerstitial fibrosis progresses more in $Skp2^{-/-}p27^{-/-}$ mice compared with $Skp2^{-/-}$ mice [20, 91]. These results suggest that Skp2 may regulate extracellular matrix synthesis by modulating p27 expression/activity in renal diseases. Taken together, these results indicate that the ameliorative effects of Skp2 deficiency following UUO are canceled by p27 deficiency in $Skp2^{-/-}p27^{-/-}$ mice. As described above, it has been reported that proliferation is inhibited, and that the expression of p21 and p27 is increased by proteasome inhibitors in two nasal fibroblast cell lines. In these cell lines, treatment with a proteasome inhibitor suppressed fibrosis together with reduced MCP-1 production and TGF-β- and TNF-α-induced collagen mRNA expression. Moreover, the inflammatory response in fibroblasts is inhibited by suppression of IL-1β-/TNF-α-induced NF-κB activation and IL-1β-induced IL-6/8 production [92]. These results suggest that the accumulated p21 and p27 in fibroblasts can inhibit tissue inflammation and progressive fibrosis. In the UUO kidneys of $Skp2^{-/-}$ mice, extracellular matrix production, inflammation, and renal fibrosis may be ameliorated by p27 accumulation.

In addition to p27, Skp2 targets several other proteins that control the cell cycle, including p21, p57, cyclin E, cyclin A, and cyclin D1, for degradation via the ubiquitin-dependent proteasome pathway. Interestingly, the protein

levels of other Skp2 targets, including p57, p130, TOB1, cyclin A, and cyclin D1, in UUO kidneys were not significantly increased in $Skp2^{-/-}$ mice compared with wild-type mice. Although the levels of p21, c-Myc, b-Myb, and cyclin E, in the UUO kidneys were slightly increased in $Skp2^{-/-}$ mice, the magnitudes of the increments did not reflect the accumulation of p27 [97]. These findings suggest that p27 is the main target of Skp2 and that the reduction in p27 levels has a pathogenic role in the progression of nephropathy.

Other cell cycle regulators involved in nephropathy

p21

The CKI protein p21 has important roles in controlling cell proliferation, terminal differentiation, cellular senescence, and apoptosis [81]. p21 inhibits the cell cycle progression by binding to cyclin/CDK complexes. p21 also directly binds to proliferating cell nuclear antigen (PCNA), which inhibits the involvement of PCNA in DNA replication [98, 99]. The protein level of p21 increases in $Skp2^{-/-}$ mouse embryo fibroblasts during the S-phase, and its degradation is low in $Skp2^{-/-}$ cells, which suggests that p21 is a target of Skp2 degradation in the S-phase [100]. The p21 protein level is mainly controlled by transcription, but it is also subject to ubiquitin-independent and -dependent degradation [101]. In the kidneys, p21 is upregulated in the early stages of renal injury in UUO mice [102] and ATS nephropathy [95], as well as in ischemia [103] and cisplatin-treated mice [104]. p21 levels increase dramatically following growth arrest induced by the tumor suppressor protein p53 and in the early stage of differentiation [81, 105]. p21 is also induced in p53-mediated apoptosis, as the p53-dependent pathways are involved in transactivation of the p21 gene [106]. However, p21 mRNA expression was enhanced in p53-deficient mice with nephropathy, which suggests that p21 transcriptional activation occurs via a p53-independent pathway in renal damage [104]. Huge et al. also reported that the proliferation of interstitial cells, particularly myofibroblasts, was promoted in the UUO kidneys from $p21^{-/-}$ mice compared with wild-type mice resulting in progression of renal failure, although there was no difference in the rate of interstitial cell apoptosis between these two strains. Tubular epithelial cell proliferation and apoptosis were also unchanged in the obstructed kidney from $p21^{-/-}$ mice [91]. p21 plays a limited role in the proliferation of myofibroblasts in renal damage, and is not essential for the regulation of tubular epithelial cell proliferation or apoptosis following UUO. However, it was reported that p21 expression is increased in experimental diabetic nephropathy and inhibits mesangial cell proliferation [107]. Moreover, glomerular cell proliferation is significantly increased in glomerulonephritis in $p21^{-/-}$ mice [108]. Taken together, these results indicate that p21 regulates the proliferation of myofibroblasts and glomerular cells in nephropathy.

p57

The CKI protein p57 inhibits cell cycle progression into the S-phase. Overexpression of p57 induces G1-phase arrest [109, 110] and is implicated in cell cycle exit accompanying terminal differentiation [111, 112]. p57 is constitutively expressed in terminally differentiated normal mature podocytes [113, 114]. In glomerular diseases, p57 expression is decreased in podocytes, allowing mature podocytes to proliferate and acquire an immature phenotype in response to renal injury. In the ATS model, which is associated with podocyte injury, p57 expression is markedly decreased in proliferating podocytes [115]. However, the p57 protein level remains unchanged during differentiation in cultured podocytes. These properties suggest that p57 controls the proliferation of mature podocytes in nephropathy.

p53

p53 is associated with cell proliferation, DNA repair, maintenance of DNA integrity, and apoptosis [116]. p53 regulates the induction of p21 and growth-arrested DNA damage protein 45 (GADD45) to control cell replication [117, 118]. p53 mRNA expression increases rapidly after UUO. p53 induces apoptosis of severely damaged tubular cells to limit renal damage [102]. However, tubular apoptosis after UUO is also mediated by p53-independent pathways [119].

Perspectives

The number of patients with end-stage renal disease requiring renal replacement therapy is steadily increasing worldwide. However, the most effective therapies for this devastating disease are dialysis or kidney transplantation. Therefore, it is important to develop novel molecular targets for chronic kidney disease and avoid its progression to end-stage renal disease. Considering the results of that reports described above, it seems likely that proteasome inhibitors have some effects on Skp2-dependent protein degradation and may offer a new therapeutic drug for nephropathy, such as kidney obstruction. It has been reported that renal fibrosis is ameliorated by proteasome inhibitors in rat obstructive nephropathy [120]. Therefore, Neubert et al. [121] suggested that proteasome inhibitors are effective for treatment of nephropathy, and Pujols et al. [92] reported that a proteasome inhibitor could reduce proliferation, collagen production, and inflammatory responses in nasal fibroblasts.

However, proteasome inhibitors reportedly show severe side effects because they accumulate many proteins by inhibition of proteasome-mediated degradation [122]. A described above, renal damage in UUO kidneys, including interstitial fibrosis, is markedly attenuated in $Skp2^{-/-}$ mice compared with wild-type mice. The decreased tubular epithelial cell proliferation and reduced tubule dilation may effect the inhibition of EMT [22, 34–36] in the UUO kidneys of $Skp2^{-/-}$ mice. We suggest that the progression of renal damage is stopped at an early stage by Skp2 deletion, reducing the extent of renal fibrosis in UUO kidneys of $Skp2^{-/-}$ mice. Cks1 also increases p27 degradation in the early stage of renal damage, and Skp2 and Cks1 promote p27 degradation selectively in a collaborative manner. Therefore, we think an inhibitor for SCF-Skp2/Cks1 E3 ligase will offer a specific therapeutic target for renal injury and is likely to inhibit the progression of nephropathy.

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Clinical Study

Oncology

Oncology 2014;86:53–62
DOI: 10.1159/000356643

Received: July 1, 2013
Accepted after revision: September 26, 2013
Published online: January 8, 2014

Reduced Organic Anion Transporter Expression Is a Risk Factor for Hepatocellular Carcinoma in Chronic Hepatitis C Patients: A Propensity Score Matching Study

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Key Words

Hepatocellular carcinoma · SLC22A7 · Organic anion transporter 2 · Chronic hepatitis C · Hepatocarcinogenesis

Abstract

Objectives: Recent reports indicated that reduced SLC22A7 (a gene-encoding organic anion transporter 2) expression in noncancerous liver tissue predicts hepatocellular carcinoma (HCC) recurrence after curative resection. Our study aimed to elucidate the association between SLC22A7 expression and HCC development in chronic hepatitis C patients. ***Methods:*** HCC recurrence after local ablation therapy and SLC22A7 expression in noncancerous liver tissue were analyzed in 20 patients. Subsequently, the association between de novo HCC development and SLC22A7 expression was examined at baseline in 38 hepatitis C patients without HCC who subsequently developed HCC as well as in 76 hepatitis C patients who did not develop HCC and were matched for age, gender and stage of fibrosis. ***Results:*** In the patients whose HCC had been cured, reduced SLC22A7 expression in noncancerous liver tissue was significantly associated with a high incidence of multifocal HCC recurrence. In patients without HCC at baseline, cumulative incidence of de novo HCC development was significantly higher with a reduced SLC22A7 expression than with a normal expression ($p = 0.01$). This difference remained significant among patients without known risk factors for HCC like age and advanced fibrosis. ***Conclusion:*** Reduced SLC22A7 expression in the liver indicates a significant risk for HCC development in chronic hepatitis C, independently of other risk factors.

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0030–2414/14/0861–0053$39.50/0

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Introduction

Hepatocellular carcinoma (HCC) is the third most common cancer worldwide [1] and the most frequent primary liver cancer [2]. Chronic hepatitis C virus (HCV) infection is a major risk factor for developing HCC [3], increasing the risk by 17-fold when compared with healthy individuals [4, 5]. Among HCV-positive patients, several risk factors for HCC have been well documented, including age, obesity, sex, serum platelet count and stage of liver fibrosis [6–10]. Advanced fibrosis, in particular, is the most significant risk factor for HCC in chronic HCV patients. The response to interferon therapy is also related to HCC risk [11, 12], mainly because the treatment attenuates hepatitis in responsive individuals. However, despite the absence of known risk factors, younger patients and those with nonadvanced fibrosis also develop HCC. Thus, surveillance is insufficient and additional risk analyses are required for those chronic HCV patients without known risk factors for HCC.

As for curatively treated HCC patients, tumor differentiation or progenitor-cell feature markers of cancerous tissue have been identified as predictors of recurrence [13, 14]. In contrast, only several reports have mentioned the importance of background noncancerous liver tissue and the microenvironment; these are predictive of HCC recurrences [15, 16]. Moreover, no specific features of noncancerous liver tissue have been clarified to be associated with de novo HCC development.

A recent prospective study showed that reduced SLC22A7 (organic anion transporter 2, OAT2) activity in noncancerous liver tissue is associated with multifocal recurrence after curative resection, independently of age and stage of fibrosis [17]. Furthermore, this study revealed that reduced SLC22A7 expression indicates a high risk for poor prognosis [18]. This observation indicates that the function of the transporter in noncancerous liver tissue is related to hepatic carcinogenesis, which may explain HCC development in patients who have no other known risk factors.

In this study, the use of SLC22A7 as a biomarker for HCC recurrence after curative local ablation therapy was assessed in order to validate and extend previously reported observations. Subsequently, the propensity score matching method was used to match patients with and without HCC development as well as to elucidate the association between SLC22A7 expression in hepatitis tissue and the risk of HCC development in chronic HCV patients.

Patients and Methods

Distant Recurrence after Radio Frequency Ablation Therapy for HCC

Patients

To reveal the relationship between multifocal HCC recurrence and SLC22A7 expression in noncancerous liver tissue, we conducted a retrospective study enrolling patients who received curative local ablation therapy. Twenty of the patients who enrolled in this cohort fulfilled the following criteria: (1) their HCC was treated curatively by radio frequency ablation (RFA); (2) they were infected with HCV and (3) they underwent liver biopsy at least 6 months after curative RFA. Written informed consent was obtained from all patients. The study was approved by the Ethical Committee of the Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Data Collection and Histological Evaluation

Patient characteristics, treatment details and biochemical, hematological, virological and histological data were collected at enrollment.

Liver biopsy specimens were obtained using 13-gauge needles under laparoscopy or 15-gauge needles using an ultrasound guide. Liver biopsy specimens were scored by board-certified pathologists for stage of fibrosis and grade of inflammatory activity according to the classification by Desmet et al. [19].

Immunohistochemical Staining of SLC22A7

All liver biopsy specimens were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with anti-OAT2 (SLC22A) antibody (kindly provided by Dr. Anzai) at a 1:20 dilution. Immunohistochemical (IHC) staining was performed using an automated immunostainer (Ventana XT System; Ventana Medical Systems Inc., Tucson, Ariz., USA), with the same procedure as the previous study [17]. Cell staining was evaluated along the entire length of the biopsy core (>30 high-power fields). Staining was graded according to the following score: ≤25% = reduced staining of cells and >25% = normal staining of cells (fig. 1). Scoring of SLC22A7 staining was performed independently by two hepatologists (K.M. and A.K.) who were blinded to the clinical outcome, and average scores were used for analysis.

Surveillance for HCC

Patients were examined for HCC every 3–6 months by abdominal ultrasonography, dynamic computed tomography or magnetic resonance imaging. Serum alpha-fetoprotein levels were measured every 3 months. HCC diagnosis was confirmed from needle biopsies, surgical resection specimens or according to the typical radiological hallmarks of early enhancement and delayed washout. The start date of follow-up was the date of liver biopsy and the end date was HCC development or the latest medical attendance.

Relationship between SLC22A7 and de novo HCC Development in Chronic HCV without HCC at Baseline

Patients

To elucidate the relationship between SLC22A7 and de novo hepatic carcinogenesis, we conducted a study in an independent cohort. A consort diagram of this study is shown in figure 2. Since 1992, 1,512 chronic HCV patients provided liver biopsies prior to interferon therapy at Musashino Red Cross Hospital. A total of 1,003 of these patients did not achieve a sustained virological re-

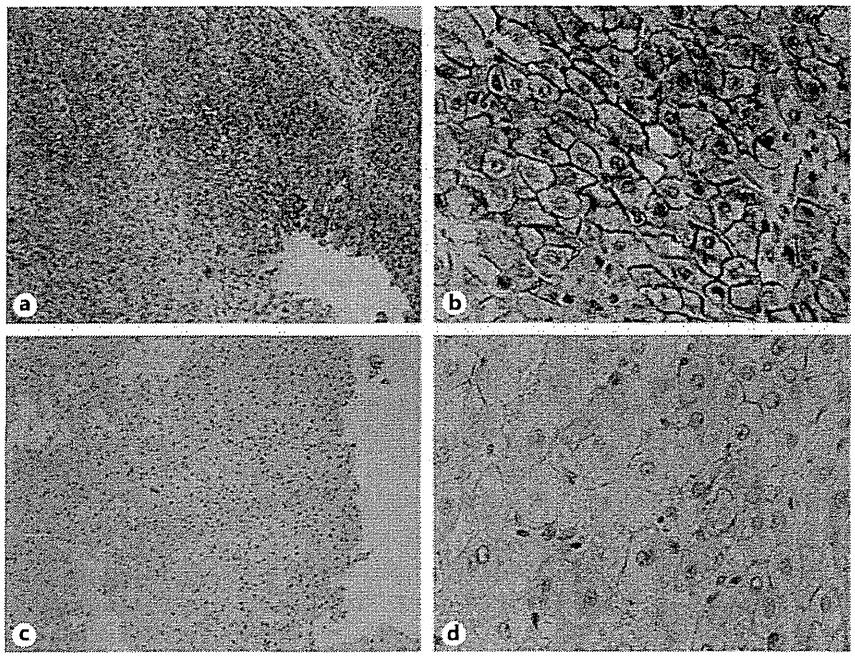

Fig. 1. IHC analysis of SLC22A7 in biopsy specimens. **a**, **b** Normal SLC22A7 expression (≥25% positive cells) **a** ×100. **b** ×400. **c**, **d** Reduced SLC22A7 expression (<25% positive cells). **c** ×100. **d** ×400.

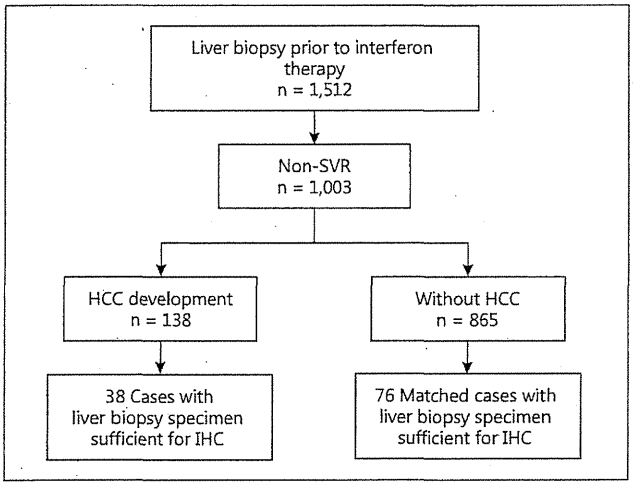

Fig. 2. Consort diagram of stratified analyses.

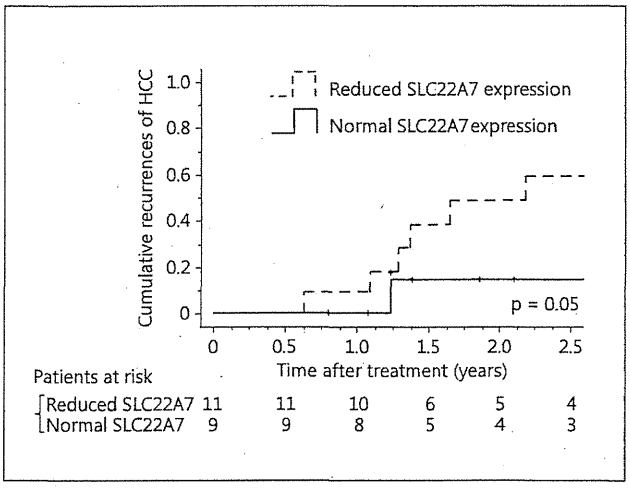

Fig. 3. Cumulative incidence of HCC recurrence after curative RFA was compared between patients with normal and reduced SLC22A7 expression.

sponse (SVR) to therapy and among these, 132 developed HCC. We enrolled 38 non-SVR patients who developed HCC and 76 matched non-SVR patients who did not develop HCC. Ninety-four patients who developed HCC were excluded because their liver biopsy specimens were of insufficient quality for IHC analyses. Matching was performed using a propensity score matching method. Histological evaluation, IHC staining and surveillance for HCC were performed as above. The average duration of follow-up was 6.6 years for all patients and 7.9 years for patients who did not develop HCC. As above, written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Propensity Score Matching

In multivariate analyses of 1,003 non-SVR patients, age, gender and stage of fibrosis were independent risk factors for HCC development. Using this multivariate logistic regression analysis, pro-

Table 1. Baseline characteristics of patients who underwent RFA

	Normal SLC22A7 expression (n = 9)	Reduced SLC22A7 expression (n = 11)	p value
Age, years	66.5±5.0	62.9±4.1	0.09
Gender (M/F)	4/5	3/8	0.64
Fibrosis (F0–2/F3–4)	5/4	4/7	0.65
Mean tumor size, mm	20.4±11.3	18.8±6.0	0.91
Albumin, g/dl	4.0±0.3	3.9±0.3	0.71
Bilirubin, mg/dl	0.7±0.2	0.9±0.4	0.09
AST, IU/l	82.0±47.1	74.2±30.6	0.84
ALT, IU/l	80.7±50.2	75.1±33.0	0.85
Glucose, mg/dl	100.3±11.6	123.5±38.7	0.25
Cholesterol, mg/dl	164.0±21.5	166.6±33.8	0.93
Alpha fetoprotein, ng/ml[a]	6.8 (3.7–106)	19.3 (5.9–87.3)	0.46
DCP, mAU/ml[a]	32 (14–129)	15 (14–26)	0.15

ALT = Alanine aminotransferase; DCP = des-gamma-carboxy prothrombin.
[a] Values are shown with median and range.

Table 2. Baseline characteristics of patients enrolled in study 2

	HCC cases (n = 38)	Non-HCC matching cases (n = 76)	p value
Age, years	64.6±7.1	64.6±6.4	0.98
Gender (M/F)	19/19	39/37	0.99
Fibrosis (F0–2/F3–4)	15/23	31/45	0.84
BMI	23.8±3.1	23.5±3.2	0.60
Albumin, g/dl	3.9±0.3	4.1±0.3	0.007
Bilirubin, mg/dl	0.7±0.3	0.7±0.3	0.42
AST, IU/l	83.5±39.2	66.2±37.7	0.07
ALT, IU/l	92.4±45.9	76.8±56.6	0.29
GGT, IU/l	74.6±59.0	63.2±54.0	0.42
Platelets, 10^4/μl	13.2±4.9	14.6±4.3	0.12
Glucose, mg/dl	116.8±20.9	112.4±24.1	0.16
Cholesterol, mg/dl	163.6±32.6	171.1±28.0	0.14

ALT = Alanine aminotransferase; BMI = body mass index; GGT = gamma-glutamyl transpeptidase.

pensity scores were calculated for each patient. These scores were used to match patients who developed HCC (HCC cases) with those who did not (non-HCC cases). Each HCC case was matched with 2 non-HCC cases whose propensity scores were similar to that of the HCC case (nearest-neighbor matching). Data analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, Ill., USA).

Statistical Analysis

Continuous variables are reported as the mean and standard deviation (SD) or median and categorical variables are shown as counts and proportions. Statistical significance was assessed using the Student t test (mean), the Mann-Whitney U test (median) or the Fisher exact test. In all tests, 2-sided p values were calculated and differences were considered statistically significant when $p < 0.05$. Statistically significant differences identified in univariate analyses were further assessed in multivariate logistic regression analysis. The stepwise and multivariate Cox proportional hazard models were used to explore independent factors that could be used to predict HCC development. Statistical analyses were performed using the SPSS software version 11.0.

Results

SLC22A7 Expression and Distant Recurrence after Curative RFA

Baseline characteristics of patients who received RFA are shown in table 1. No significant differences were observed between patients with normal SLC22A7 expression and those with reduced SLC22A7 expression. Figure 3 shows the cumulative rates of distant recurrences