RNA-binding protein Musashi Homologue 1 Regulates Kidney Fibrosis by Translational Inhibition of p21 and Numb mRNA

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RNA-binding proteins (RBPs) are recognized as key posttranscriptional regulators that not only modulate the spatiotemporal expression of genes during organism development but also regulate disease pathogenesis. Very limited information exists on the potential role of RBPs in modulating kidney fibrosis, which is a major hallmark of chronic kidney disease. Here, we report a novel mechanism in kidney fibrosis involving a RBP, Musashi homologue 1 (Msi1), which is expressed in tubular epithelial cells. Using two mechanistically distinct mouse models of kidney fibrosis, we show that Msi1 protein levels are significantly down-regulated in the kidneys following fibrosis. We found that Msi1 functions by negatively regulating the translation of its target mRNAs, p21 and Numb, whose protein levels are markedly increased in kidney fibrosis. Also, Msi1 overexpression and knock-down in kidney epithelial cells cause p21- and Numb-mediated cell cycle arrest. Furthermore, we observed that Numb looses its characteristic membrane localization in fibrotic kidneys and therefore is likely unable to inhibit Notch resulting in tubular cell death. Oleic acid is a known inhibitor of Msi1 and injecting oleic acid followed by unilateral ureteral obstruction surgery in mice resulted in enhanced fibrosis compared with the control group, indicating that inhibiting Msi1 activity renders the mice more susceptible to fibrosis. Given that deregulated fatty acid metabolism plays a key role in kidney fibrosis, these results demonstrate a novel connection between fatty acid and Msi1, an RNA-binding protein, in kidney fibrosis.

RNA-binding proteins are known to play a major role in development and most of our current knowledge on the functions of RNA-binding proteins (RBPs) originates from studies in Drosophila and Caenorhabditis elegans (1, 2). Based on genetic and biochemical studies several roles have been assigned to RBPs including biogenesis, surveillance, transport, localization, and degradation of RNA (3). RBPs function primarily by binding to either a specific sequence or structural elements within the coding, untranslated, or non-protein coding regions of the RNA in functional complexes called ribonucleoprotein complexes (3). A given RNA-binding protein can regulate the translation of multiple RNA targets (4), whereas a given RNA can in turn be regulated by multiple RNA-binding proteins (5). This highly dynamic posttranscriptional regulation by RBPs is therefore critical for normal functioning of various cellular processes.

Several RBPs have been identified to be associated with diseases: fragile X syndrome, spinal muscular atrophy, retinitis pigmentosa, opsoconus-myoconus ataxia, and cancers (6–11) and the Online Mendelian Inheritance in Man (OMIM) database lists ~150 RBPs as being linked to human diseases. Although the critical role of RBPs in neurological disorders or cancers has been investigated, very little is known about the regulatory role of RBPs in kidney disease.

In our quest to understand the molecular pathogenesis of kidney fibrosis, a major underlying process leading to chronic kidney disease, we identified Msi1 to be significantly up-regulated (18-fold, p = 0.003) in mouse kidneys subjected to folic acid-induced chronic progressive fibrosis (12). The objective of this study was to investigate the role of Msi1 in the progression of kidney fibrosis. Specifically, the aims were (i) to characterize the expression pattern of Msi1 in kidney fibrosis; (ii) to investigate whether Msi1 regulates kidney tubular cell apoptosis via translational regulation of its mRNA targets, p21 and Numb, and (iii) to identify the effect of the fatty acid metabolite (oleic acid), which modulates Msi1 expression, on kidney fibrosis.

**Experimental Procedures**

**Animals**—Male 8–10-week-old BALB/c mice (body weight: 25–29 g) were used for the unilateral ureteral obstruction (UUO) and for experiments involving folic acid injection. Animals were maintained in the central animal facility in transparent plastic cages free of any known chemical contaminants under conditions of 21 ± 1 °C and 50–80% relative humidity at all times in an alternating 12:12-h light/dark cycle. Commercial rodent chow and water were available to animals ad libitum.
animals were acclimated to this environment for at least 1 week before use in experiments. Animals were housed at 4–5 mice/cage, and each mouse was considered separately for data analysis. All animal maintenance and treatment protocols were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Harvard Medical School Animal Care and Use Committees (Institutional Animal Care and Use Committee).

**Animal Procedures**—UUO in mice was performed under general anesthesia (50 mg/kg i.p. of pentobarbital sodium) by ligation of the left ureter with two separate silk ties as previously described (12, 13). Mice were euthanized 3, 7, and 14 days after UUO. Mice were injected with 1 ml of normal saline subcutaneously (37 °C) immediately after surgery to replace the fluid loss. Pain medication was administered for 48 h post-surgery (buprenorphine (0.05 mg/kg subcutaneously) every 12 h; the first dose was administered with the saline injection immediately after surgery and subsequent doses were given in 50 μl of normal saline). Nephropathy was also induced chemically with a single intraperitoneal injection of 250 mg/kg of folic acid in 0.3 M sodium bicarbonate and animals were sacrificed at 3, 7, and 14 days post-injection. Normal mice, with no surgical or pharmacological interventions, were also included in the experiments. Euthanasia was performed under isoflurane anesthesia. Blood and kidney samples were collected, and the thoracic cavity was opened to make sure that the animal was deceased.

**Oleic Acid Treatment**—Male 8-week-old BALB/c mice were injected with either oleic acid (2 mg/kg body weight) or vehicle (20 μl of 100% ethanol). Twenty-four hours later, mice were subjected to UUO surgery and sacrificed 3 days post-surgery.

**Western Blotting Analysis**—Immunoblotting was performed as previously described (14). Protein concentrations were determined by the Bradford method and an equal amount of protein (25 μg) was run on either a 10 or 12% polyacrylamide gel (PAGE). The following primary antibodies were used: rat monoclonal anti-Msi1 (1:250) (2756, Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-ubiquitin (1:250) (2756, Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-Msi1 (1:250) (14-9896, eBioscience Inc., San Diego, CA), mouse monoclonal anti-SMA (1:500) (A2547, Sigma), and mouse monoclonal anti-α-tubulin (1:500) (T6793, Sigma). Molecular masses marked in each Western blot are in kilodaltons.

**Immunoprecipitation**—Immunoprecipitation was performed as previously described (14). Five hundred μg of protein was used for immunoprecipitation, and 25 μg was set aside as input. The sample volume was adjusted to 1 ml with immunoprecipitation buffer and 5 μg of rat monoclonal Msi1 or isotope-matched rat IgG was added and incubated overnight at 4 °C. Western blotting analysis was performed with anti-Msi1 and anti-ubiquitin antibodies. Rat IgG chains served as control for equal antibody loading.

**RNA Extraction and Quantitative Real-time PCR**—Total RNA was isolated from flash-frozen tissue using TRIzol (Invitrogen) following the manufacturer’s protocol. The RNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). One μg of total RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit from Qiagen (Valencia, CA). Quantitative RT-PCR was performed using a QuantiFast SYBR Green PCR kit (Qiagen) on a CFX96 RT-PCR instrument (Bio-Rad Laboratories) with the following temperature profile: 3 min of enzyme activation at 95 °C followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s. *Gapdh* was used as a reference gene for normalization. Primers used for qPCR are listed in Table 1.

**Cell Culture, siRNA, and Plasmid Transfections**—Primary human proximal tubular epithelial cells (HPTECs) were purchased from Biopredic (Paris, France) and cultured as described previously (15). Human embryonic kidney (HEK-293) cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM (Cellgro, Manassas, VA) with 10% FBS and incubated for 24 h at 37 °C in 5% CO₂. For MSI1 knockdown experiments, HPTECs were transfected with 100 nM scramble or Msi1 siRNA (Dharmacon, Lafayette, CO) with siPORT NeoFX transfection reagent (Life Technologies) following the manufacturer’s protocol. Cells were incubated further for 24 h and either harvested for Western blotting or RNA isolation followed by RT-PCR. For MSI1 overexpression experiments, HEK-293 cells were transfected with either pCMV Myc (pCMV) or pCMV Myc-FLAG-MSI1 (pCMV MSI1) plasmids (Origene Technologies, Rockville, MD) with Lipofectamine 2000 (Life Technologies). Cells were incubated

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### TABLE 1

| Gene       | Forward primer | Reverse primer |
|------------|----------------|----------------|
| Gapdh      | GAATACGCGTTACGACACAGG | GGTCTCGGATGTTGAATGTTG |
| Fibronectin (Fn) | AAGTTGACGACCTCTGAGATTG | GCCACCTGTTTGTCGGAAAGG |
| Collagen1α1 (Col1α1) | TACGCTGCAAGGCGGAGGATG | GTTCGAACTCTGAGGTGGAT |
| α-SMA      | GCCCAAGTTCTAGGGGGTTTA | AGCACCTGCGTTGCTCAGG |
| Msi1       | CTTCGCTGCAAGGCGGAGGATG | GTTCGAACTCTGAGGTGGAT |
| Numb       | CACACTGCTCAGGCGGAGGATG | GTTCGAACTCTGAGGTGGAT |
| CDK1α4 (p21) | GCCGCGAGATCCTCCAGG | AACACCTGCGTTGCTCAGG |
| Hes1       | CGGATTGCGAGTTCTGTTGAT | AGCACCTGCGTTGCTCAGG |
| HeyL       | GTGCGCAAGGCGGAGGAGG | AACACCTGCGTTGCTCAGG |
| GAPDH      | ATGGGCCTGCAAGGCGGAGGATG | GTTCGAACTCTGAGGTGGAT |
| MSI1       | CACACTGCTCAGGCGGAGGATG | GTTCGAACTCTGAGGTGGAT |
| NUMB       | GCCGCGAGATCCTCCAGG | AACACCTGCGTTGCTCAGG |
| CDK1α4 (p21) | GCCGCGAGATCCTCCAGG | AACACCTGCGTTGCTCAGG |
further for 24 h and either harvested for Western blotting or RNA isolation and RT-PCR.

\[^{35}S\]Methionine Labeling—HEK-293 cells were plated in a 100-mm dish and transfected with 10 \(\mu\)g of either pCMV or pCMV MS1U plasmid using Lipofectamine 3000 as per the manufacturer’s instructions. Briefly, 15 \(\mu\)l of Lipofectamine 3000 and 10 \(\mu\)g of plasmids were mixed in 500 \(\mu\)l of Opti-MEM and incubated for 5 min. Plasmid and lipofectamine were mixed and incubated for 10 min at room temperature and this complex was added dropwise on the cells. Cells were incubated for 24 h and washed twice with DMEM and further incubated for 24 h. Cells were trypsinized and plated in 6-well plates for Western blotting and the remaining cells were replated in a 100-mm Petri dish for \[^{35}S\]methionine labeling. After 24 h cells were blotted and the remaining cells were replated in a 100-mm Petri dish for \[^{35}S\]methionine labeling. After 24 h cells were washed twice with pulse labeling medium (DMEM without methionine in complete medium containing 10% dialyzed FBS) and incubated in this medium for 15 min. Cells were then labeled with pulse labeling medium containing 0.1 mCi/ml of \[^{35}S\]methionine for 30 min. Chase medium (DMEM without methionine in complete medium containing 10% dialyzed FBS and 15 mg/ml methionine) was added and incubated for 30 min. Cells were trypsinized and resuspended in 500 \(\mu\)l of RIPA buffer with protease inhibitors. One hundred \(\mu\)l of lysate was mixed to 200 \(\mu\)l of BSA/Na\(_2\) and incubated on ice and 1 ml of 10% TCA solution was added and vortexed vigorously and incubated further on ice for 30 min. The suspension was filtered onto 2.5-cm glass microfiber disks under vacuum. The disks were washed twice with 5 ml of ice-cold 10% TCA and twice with ethanol followed by air-drying. One microliter of \[^{35}S\]methionine (7 mCi/ml) from the vial was spotted on a glass microfiber as standard, and the incorporation of \[^{35}S\] methionine during protein synthesis was calculated. Competition assay was performed by incubating 15 mg/ml of cold methionine during the pulse labeling reaction. These glass microfiber disks were dipped in scintillation liquid and read on a scintillation counter. The counts were normalized to protein concentration and plotted.

Proteasomal Inhibition in HPTECs—HPTECs were plated and incubated for 24 h in 60-mm culture dish. Cells were treated with either dimethyl sulfoxide or 20 \(\mu\)M MG132 (Calbiochem) and incubated for 24 h. Cells were harvested and Western blotting analysis of MS1 was performed as described above.

Immunofluorescence—Immediately after euthanizing, kidneys from mice were collected and fixed in 4% paraformaldehyde on ice for 1–2 h, and transferred to sucrose solution (30%) overnight for cryoprotection following which the kidneys were frozen in Tissue-Tek O.C.T. (VWR, Radnor, PA). Five-\(\mu\)m thick kidney sections were permeabilized in 1X PBS containing Triton X-100 (0.1%) for 10 min. The sections were then labeled with primary antibodies, including rat anti-MS1 (1:200) (eBioscience Inc), mouse anti-p21 (1:200) (EMD Millipore), rabbit anti-Numb (1:200) (Cell Signaling), FITC-conjugated anti-\(\alpha\)-SMA (1:500) (F3777, Sigma), rabbit anti-CD11b (1:200) (ab133357, Abcam) fluorescein-labeled Lotus Tetragonolobus Lectin (1:500) (FL-1321, Vector Labs), and fluorescein-labeled dolichos biflorus agglutinin (1:200) (Fl-1031, Vector Labs, Burlingame, CA). Slides were subsequently exposed to species-specific FITC- or Cy3-conjugated secondary antibodies (1:500) (Jackson ImmunoResearch Laboratories) and mounted with 4’,6-diamidino-2-phenylindole-containing ProLong Gold Antifade Mountant (Life Technologies). FITC and Cy3 images were collected with a Yokogawa CSU-X1 spinning disk confocal with Borealis modification, mounted on a Nikon Ti-inverted microscope equipped with \(\times 60\) Plan Apo NA 1.4 objective lens, a Prior Proscan II motorized stage, and the Nikon Perfect Focus System for continuous maintenance of focus. FITC fluorescence was excited with an AOTF-controlled 488-nm solid-state laser and collected with a 525/50 emission filter (Chroma). Cy3 fluorescence was excited with an AOTF-controlled 561-nm solid-state laser and collected with a 620/60 emission filter (Chroma). For both FITC and Cy3, a Quad 405/491/561/642 dichroic mirror was used (Semrock). Widefield images of DAPI were collected alongside the confocal images using a 395/25 nm excitation and 460/25 emission filters (Chroma) and a Lumencor SOLA for illumination. Images were acquired with a Hamamatsu ORCA-AG-cooled CCD camera controlled with MetaMorph 7 software. Gamma, brightness, and contrast were adjusted on displayed images (identically for compared image sets) using MetaMorph 7 software.

Statistical Analysis—Data are expressed as average \pm S.E. Statistical difference (\(p<0.05\)) was calculated by non-parametric Student’s \(t\) test. \(p<0.05\) was considered significant and represented by an asterisk as compared with controls. All graphs were generated by GraphPad Prism (GraphPad, Inc., La Jolla, CA) or Microsoft Excel (Microsoft Corp., Redmond, WA).

Results

The Tubular Epithelial Cells Express MS1 and Its Protein Levels Are Reduced in Fibrotic Kidneys—We characterized the expression pattern of MS1 in two mechanically distinct mouse models of kidney fibrosis, single dose folic acid administration (250 mg/kg i.p.) and UUO (12, 13). The extent of fibrosis in these two models was determined by Masson’s trichrome staining as shown in Fig. 1, A and B. The mRNA levels of MS1 were highly up-regulated post-folic acid injection at day 3 (29-fold, \(p<0.05\)), day 7 (22-fold, \(p<0.05\)), and day 14 (12-fold, \(p<0.05\)) as compared with normal mice (Fig. 1C). Also in the UUO model we observed significant up-regulation of MS1 transcripts post-UUO surgery; day 3 (7-fold, \(p<0.05\)), day 7 (14-fold, \(p<0.05\)), and day 14 (11-fold, \(p<0.05\)) as compared with normal mice (Fig. 1D). In contrast, however, MS1 protein expression was significantly reduced in folic acid-treated mice kidneys as compared with normal kidneys (Fig. 1E). Similarly, MS1 protein expression was also decreased specifically in the UUO (fibrotic) kidneys in comparison to the contralateral kidneys (CoK) (Fig. 1F). To determine the cell-type specific expression of MS1 in the kidneys, we performed co-immunostainings in normal and fibrotic mouse kidneys. MS1 was found to be expressed in the proximal (co-localized with DBA) and distal tubules (co-localized with LTL) in normal, CoK, and UUO kidneys (Fig. 1G). To further assess whether MS1 is expressed in cells other than tubular epithelial cells, we performed MS1 co-immunostainings with either myofibroblast marker \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) or macrophage marker CD11b. MS1 did not co-localize with either \(\alpha\)-SMA or CD11b thereby establishing...
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A Normal Day 7 FA

B Day 7 CoK Day 7 UUO

C Fold over normal

E Msi1

Gapdh

D

F Msi1

Gapdh

G Normal Day 7 CoK Day 7 UUO

H Day 7 CoK Day 7 UUO

Msi1/LTL

Msi1/DBA

Msi1/α-SMA

Msi1/CD11b
Msi1 Negatively Regulates Its Targets, p21 and Numb, in Kidney Epithelial Cells and Causes Cell Cycle Arrest—To establish a functional role of Msi1 in epithelial cells, we performed in vitro overexpression and knockdown studies. Because Msi1 is not endogenously expressed in HEK-293 cells, we overexpressed the pCMV Msi1 plasmid and determined p21 and Numb protein expression 48 h post-transfection. Concurring with previous findings, Msi1 was specifically expressed in kidney tubules and that there is an inverse relationship between its mRNA and protein expression levels in fibrosis.

Proteasome-mediated Degradation Results in Decreased Msi1 Protein in UUO Kidneys—We next hypothesized that the decreased protein expression of Msi1 in kidney fibrosis despite the increased RNA levels (Fig. 1, C and D) could be because of its ubiquitin-mediated proteasomal degradation. The fibrotic kidneys from the UUO mouse model showed significantly high levels of ubiquitination as compared with the CoK at all time points during kidney fibrosis progression (Fig. 2A). Co-immunoprecipitation with anti-Msi1 antibody from day 3 CoK and UUO kidney lysates was followed by immunoblotting for ubiquitin and Msi1. IgG heavy and light chain served as loading controls for the antibody. Equal input lysates for immunoprecipitation were confirmed using Tubulin expression. Co-immunoprecipitation from kidney lysates by either Msi1 antibody or IgG control. D, Western blots showing an increase in Msi1 protein levels following 24 h treatment of HPTECs with the proteasomal inhibitor MG132 (20 μM) as compared with dimethyl sulfoxide (DMSO) control.

To assess the effect of decreased Msi1 protein expression in fibrotic kidneys, we investigated the level of protein expression of two of its targets, p21 and Numb (16, 17). Corresponding to the decrease in Msi1 protein (Fig. 1, E and F), p21 and Numb protein expression was significantly increased in mice kidneys with folic acid-induced kidney fibrosis compared with normal kidneys (Fig. 3A). A similar increase in p21 and Numb protein expression was also observed in UUO kidneys in comparison to the CoK (Fig. 3B), which does indicate that there is sufficient mRNA available for translation. These observations suggest that p21 and Numb protein expression levels are reciprocal to that of Msi1 (Fig. 1, E and F). We determined the mRNA levels of p21 and Numb and observed an increase in the UUO kidneys (Fig. 3, C and D). We further determined the cell-type specific expression of p21 and Numb in relationship to Msi1 by performing co-immunostainings of Msi1 with p21 and Numb. p21 and Numb were co-expressed with Msi1 in the tubules in both CoK and UUO kidneys at day 7 post-UUO (Fig. 3E). The most striking observation was the altered localization of Numb in the CoK and UUO kidneys. In the CoK it is expressed on the apical membrane of tubular epithelial cells, whereas the characteristic membrane expression is lost in the UUO kidneys and it appears more punctate. Together, the increased mRNA levels of p21 and Numb, their co-expression with Msi1 along with depleted levels of Msi1 (the translational regulator), potentially results in increased protein levels of p21 and Numb, ultimately triggering pathways contributing to fibrosis.

Msi1 is subjected to proteasomal degradation causing its decreased protein levels in fibrotic kidneys.

FIGURE 2. Msi1 ubiquitination levels are increased in fibrosis. A, Western blotting analysis of ubiquitin in CoK and UUO kidneys at days 3, 7, and 14 (n = 3 in each group), Gapdh was used as a loading control. B, immunoprecipitation in day 3 CoK and UUO kidney tissue lysates by Msi1 antibody followed by immunoblotting for ubiquitin and Msi1. IgG heavy and light chain served as loading controls for the antibody. Equal input lysates for immunoprecipitation were confirmed using Tubulin expression. C, immunoprecipitation from kidney lysates by either Msi1 antibody or IgG control. D, Western blots showing an increase in Msi1 protein levels following 24 h treatment of HPTECs with the proteasomal inhibitor MG132 (20 μM) as compared with dimethyl sulfoxide (DMSO) control.

FIGURE 1. Msi1 expression in normal and fibrotic kidneys. A, the degree of interstitial fibrosis in folic acid (day 7 post-injection), and B, UUO (day 7 post-surgery) models, as indicated by Masson’s trichrome stain (MTS) Bar represents 50 μm. C, Msi1 mRNA expression levels increase in the folic acid model of kidney fibrosis as compared with normal mice at days 3, 7, and 14 as measured by qPCR (n = 6 per time point in each group). D, qPCR data were normalized to Gapdh, data are presented as mean ± S.E. of fold changes over normal kidneys. *p < 0.05. D, Msi1 mRNA expression levels increase in the UUO model of kidney fibrosis as compared with CoK at days 3, 7, and 14 as measured by qPCR (n = 6 per time point in each group). E, qPCR data were normalized to Gapdh and presented as mean ± S.E. of fold changes over CoK kidneys, *p < 0.05. F, Western blotting analysis of Msi1 in normal and folic acid-treated mice at days 3, 7, and 14 (n = 3 per time point in each group). G, Western blotting analysis of Msi1 in CoK and UUO kidneys at days 3, 7, and 14 (n = 3 per time point in each group).

Finally, treatment of HPTECs with the proteasomal inhibitor MG132 significantly increased the levels of Msi1, thus confirming that Msi1 is indeed subjected to proteasomal degradation (Fig. 2D). These results demonstrate that Msi1 is subjected to proteasomal degradation causing its decreased protein levels in fibrotic kidneys.
with previous reports (16, 17), NUMB and p21 protein expression was significantly decreased in MSI1 overexpressing cells as compared with empty vector-transfected cells (Fig. 4A) without any significant change in the mRNA levels of both p21 and NUMB (Fig. 4B). Because Msi1 is expressed in tubular epithelial cells (Fig. 1G), we performed siRNA-mediated knockdown of MSI1 in previously characterized HPTECs (15). As shown in Fig. 4C, we achieved more than 50% MSI1 knockdown in MSI1 siRNA-transfected cells compared with scramble transfected cells. As expected, p21 and NUMB protein expression levels were significantly up-regulated in cells where MSI1 expression was knocked down compared with controls (Fig. 4C). Also, there was no significant change in the mRNA levels of either p21 or NUMB (Fig. 4D) confirming the role of translational regulation of p21 and NUMB by MSI1. We next performed propidium iodide staining to assess the effects of MSI1 overexpression and knockdown on cell cycle distribution. MSI1 overexpression resulted in cell cycle progression, whereas knockdown resulted in cell cycle arrest (Fig. 4, E and F). To assess the impact of MSI1 overexpression on global translation rates we conducted [35S]methionine incorporation studies in HEK-293 cells. We did not observe any significant change in radioactive counts between MSI1-transfected cells and controls (Fig. 4, G and H), thereby suggesting that Msi1 specifically targets translation rates. Together, these results confirm previous findings of p21 and Num binding regulation by Msi1 (16, 17) and further establish a key role for Msi1 in regulating kidney tubular epithelial cell cycle and apoptosis via p21 and also notch signaling via Numb.

Inhibition of Msi1 Function by Oleic Acid Results in Enhanced Fibrosis—Defective fatty acid oxidation results in the accumulation of lipids in epithelial cells likely contributing to kidney fibrosis (18). Clingman et al. (19) has shown that the fatty acid metabolite, oleic acid inhibits Msi1 function by binding to its RNA binding domain. To determine the effect of Msi1 inhibition on kidney fibrosis, mice were injected with oleic acid (2 mg/kg, i.p.) and 20 h later, subjected to UUO surgery. Three days post-surgery the oleic acid-treated mice exhibited significantly more fibrosis at the oleic acid-injected group compared with vehicle controls. We also observed increased fibrosis (Fig. 5C) and apoptosis (Fig. 5D) in the oleic acid-injected group compared with vehicle controls. This is consistent with the oleic acid-induced inhibition of Msi1 function and reciprocal increase in p21 expression that is known to play a critical role in cell cycle regulation and apoptosis. Because Msi1 also inhibits Numb mRNA translation as shown previously and given that Numb is known to negatively regulate Notch, we assessed the effect of Msi1 inhibition on Notch signaling by determining the mRNA levels of Hey1 and HeyL (Fig. 5E). Surprisingly, the levels of Hey1 and HeyL mRNA were increased in the oleic acid group indicating enhanced...
Notch signaling. These findings provide evidence for Msi1 in protecting epithelial cells against apoptosis via inhibiting p21 translation.

Discussion

In the present study, we show the role of RNA-binding protein Msi1 in kidney fibrosis and its link with deregulated fatty acid metabolism. Although Msi1 is well studied for its function as a RNA-binding protein in neurons (20, 21), there are no known reports to date on Msi1 (or any other RNA-binding protein) in kidney fibrosis. We report that ubiquitin-mediated proteasomal degradation is responsible for a significant decrease in Msi1 protein levels in tubular epithelial cells following kidney fibrosis. Because Msi1 is a RNA-binding protein we chose to decipher its role in fibrosis by studying the regulation of two of its known mRNA targets, the cell-cycle regulator p21 and the notch inhibitor Numb. We show that p21 and Numb proteins have reciprocal expression levels to that of Msi1 in fibrosis, consistent with their negative translational regulation by Msi1. Using Msi1 overexpression and knockdown in kidney epithelial cells, we show the effect of Msi1 on cell-cycle regulation mediated by translational regulation of p21 and Numb mRNAs. Finally, inhibiting Msi1 function in vivo by the fatty acid metabolite, oleic acid, resulted in increased fibrosis in the UUO model. The results are consistent with a model where Msi1 maintains the protein levels of p21 and Numb by regulating mRNA translation in normal kidney tubular epithelial cells.

While following insult, Msi1 protein is degraded by the proteasomal machinery resulting in a reciprocal increase of p21 and Numb protein expression. Persistent decrease in Msi1 and a corresponding increase in p21 leads to cell cycle arrest and/or apoptosis of tubular epithelial cells, thereby triggering a cascade of pro-fibrotic events (22–26) such as release of pro-fibrotic cytokines, inflammation, pericyte differentiation, fibroblast to myofibroblast transformation, and extracellular matrix deposition resulting in progressive fibrosis (Fig. 6).

Several groups working on kidney fibrosis have consistently shown the involvement of myofibroblasts as the primary cell type mediating fibrosis and resulting in extracellular matrix deposition, scar tissue formation, and eventually organ failure (27). Understandably, current efforts to find therapeutic targets to resolve fibrosis are focused on stemming fibroblast proliferation and their differentiation into myofibroblasts apart from identifying the source of fibroblasts within the kidney tissue (26). An alternative line of therapeutic intervention is targeting perturbed proteins in tubular epithelial cells, as they are the primary sites of injury resulting in the subsequent triggering of injury response pathways including fibroblast recruitment. In line with this reasoning, there is evidence for the role of several pathways that play a role in epithelial cell-mediated kidney fibrosis, cell cycle arrest (28), defective fatty acid metabolism (18), notch signaling (29), etc. Our findings present a connecting link between defective fatty acid...
metabolism, cell cycle arrest, and notch signaling via the RNA-binding protein Msi1.

Msi1 is known to be up-regulated in several cancers and is currently being explored as a potential therapeutic target (30). We found Msi1 to be expressed in tubular epithelial cells in the cytoplasm. In the normal kidney, Msi1 levels are high (compared with fibrotic kidneys) where it likely regulates the translation of its target mRNAs to maintain normal tubular epithelial cell function. There are several mRNA targets of Msi1 including \( \text{p21} \) and \( \text{Numb} \) and via these targets it regulates cell-specific functions by targeting multiple pathways (31). Msi1 negatively regulates \( \text{Numb} \) mRNA translation in the brain to maintain neuronal progenitor cells (17), whereas it positively regulates \( \text{Numb} \) mRNA translation in regenerating gastric mucosa (32). Msi1 is also expressed in the cytoplasm and nuclei of proliferating and non-proliferating sertoli cells in rat testis (33).

Although p21 has been shown to play a crucial role in mediating fibrosis (34), its immediate upstream translational regulators remain poorly understood. We present evidence that demonstrates that Msi1 regulates p21 in kidney tubular epithelial cells resulting in cell cycle arrest and fibrosis progression. Our \textit{in vitro} data (Fig. 4) show that MSI1 does not have an effect on the

**Figure 5.** Msi1 inhibition results in enhanced fibrosis in UUO model. 

**A.** mRNA levels of fibrotic markers: Fibronectin (Fn), Collagen1\( \alpha \)1 (Col1\( \alpha \)1), and \( \alpha \)-SMA in CoK and UUO kidneys in vehicle (V) and oleic acid (OA)-treated mice at day 3 as measured by qPCR \((n = 5 \text { in each group})\). qPCR data were normalized to Gapdh and presented as mean ± S.E. of -fold changes over normal, *, \(p < 0.05\). 

**B.** Western blotting analysis of Fn, Col1\( \alpha \)1, \( \alpha \)-SMA, Msi1, and p21 in CoK and UUO kidneys from vehicle and oleic acid-treated mice \((n = 4 \text { in each group})\).

**C.** The effect of OA treatment on the degree of interstitial fibrosis in the UUO kidneys (examined 3 days after UUO), as indicated by Masson’s trichrome stain. 

**D.** TUNEL staining to visualize apoptotic nuclei in UUO mice treated with either vehicle (V) or oleic acid (OA) and its quantitation, *, \(p < 0.05\). 

**E.** mRNA levels of notch pathway genes, \( \text{Hes1} \) and \( \text{HeyL} \) in UUO mice treated with either vehicle (V) or oleic acid (OA) as measured by qPCR \((n = 5 \text { in each group})\). qPCR data were normalized to Gapdh and presented as mean ± S.E. of -fold changes over normal, *, \(p < 0.05\).
Whereas following insult, Msi1 protein is degraded by the proteasomal machinery resulting in a reciprocal increase of p21 and Numb protein expression. Persistent decrease in Msi1 and a corresponding increase in p21 leads to cell cycle arrest and/or apoptosis of tubular epithelial cells, thereby triggering a cascade of pro-fibrotic events such as release pro-fibrotic cytokines, inflammation, pericyte differentiation, fibroblast to myofibroblast transformation, and extracellular matrix deposition resulting in progressive fibrosis. Pro-fibrotic events mentioned in the box outlined with dotted line are derived from published literature.

**FIGURE 6. Proposed model.** In normal kidney tubular epithelial cells, Msi1 maintains the protein levels of p21 and Numb by regulating mRNA translation. Whereas following insult, Msi1 protein is degraded by the proteasomal machinery resulting in a reciprocal increase of p21 and Numb protein expression. Persistent decrease in Msi1 and a corresponding increase in p21 leads to cell cycle arrest and/or apoptosis of tubular epithelial cells, thereby triggering a cascade of pro-fibrotic events such as release pro-fibrotic cytokines, inflammation, pericyte differentiation, fibroblast to myofibroblast transformation, and extracellular matrix deposition resulting in progressive fibrosis. Pro-fibrotic events mentioned in the box outlined with dotted line are derived from published literature.

mRNA levels of p21, although it has been shown to co-localize to processing bodies (P bodies), which are sites of mRNA degradation (35). It will be interesting to investigate if modulating Msi1 levels offer a novel approach to release the cell cycle block and thereby result in reversal of fibrosis.

Notch signaling plays an essential role in kidney development and has been shown to be up-regulated in fibrosis (36, 37). Numb is an inhibitor of notch signaling and Msi1 is known to regulate notch signaling via Numb mRNA translation (21). Numb is also known to regulate epithelial cell polarity and cell-cell adhesion (38). Our finding on the expression pattern of Numb in tubular epithelial cells in normal and fibrotic kidneys further highlights its potential role in cell polarity. In normal mouse kidney, Numb is predominantly expressed on the apical side of tubular epithelial cells and this characteristic membrane localization is lost in the fibrotic kidneys. This might have an impact on epithelial cell polarity and act as a potential trigger for partial epithelial to mesenchymal transition. Recent data on kidney fibrosis does show a strong contribution of partial epithelial to mesenchymal transition and cell cycle arrest in tubular epithelial cell apoptosis (39, 40). Given that Numb plays a role in targeting Notch intracellular domain (NICD) for degradation via α-adaptin (41), it is surprising to see increased Notch in fibrosis despite increased Numb levels. It will be interesting to investigate whether its membrane localization in tubular epithelial cells is essential for its activity and to also study its functions independent of Notch regulation. The fact that Msi1 plays a critical role in Numb mRNA translation, it likely acts as a master regulator of cellular differentiation in tubular epithelial cells as it does in neuronal cells (21). This study also presents a potential dual role for Msi1 in development and fibrosis. Given its role in regulating several cellular pathways, it will be of great interest to study the role of Msi1 in kidney development (if any) and fibrosis by identifying its novel mRNA targets in tubular epithelial cells.

In conclusion, we have identified a novel mechanism in kidney fibrosis involving a RNA-binding protein, which regulates the translation of a diverse set of mRNAs thereby regulating several cellular processes. We have also established a connection between fatty acid and kidney fibrosis via oleic acid inhibition of Msi1 function. Because Msi1 is subjected to ubiquitin-based proteasomal degradation in fibrotic kidneys, it will be of therapeutic importance to identify the E3 ubiquitin ligase tagging Msi1 and developing molecules that target the ligase and restore Msi1 levels.

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