Autosomal dominant polycystic kidney disease (ADPKD) is characterized by renal cyst formation, inflammation, and fibrosis. Macrophages infiltrate cystic kidneys, but the role of these and other inflammatory factors in disease progression are poorly understood. Here, we identified macrophage migration inhibitory factor (MIF) as an important regulator of cyst growth in ADPKD. MIF was upregulated in cyst-lining epithelial cells in polycystin-1–deficient murine kidneys and accumulated in cyst fluid of human ADPKD kidneys. MIF promoted cystic epithelial cell proliferation by activating ERK, mTOR, and Rb/E2F pathways and by increasing glucose uptake and ATP production, which inhibited AMP-activated protein kinase signaling. MIF also regulated cystic renal epithelial cell apoptosis through p53-dependent signaling. In polycystin-1–deficient mice, MIF was required for recruitment and retention of renal macrophages, which promoted cyst expansion, and Mif deletion or pharmacologic inhibition delayed cyst growth in multiple murine ADPKD models. MIF-dependent macrophage recruitment was associated with upregulation of monocyte chemotactic protein 1 (MCP-1) and inflammatory cytokine TNF-α. TNF-α induced MIF expression, and MIF subsequently exacerbated TNF-α expression in renal epithelial cells, suggesting a positive feedback loop between TNF-α and MIF during cyst development. Our study indicates MIF is a central and upstream regulator of ADPKD pathogenesis and provides a rationale for further exploration of MIF as a therapeutic target for ADPKD.
Macrophage migration inhibitory factor promotes cyst growth in polycystic kidney disease

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by renal cyst formation, inflammation, and fibrosis. Macrophages infiltrate cystic kidneys, but the role of these and other inflammatory factors in disease progression are poorly understood. Here, we identified macrophage migration inhibitory factor (MIF) as an important regulator of cyst growth in ADPKD. MIF was upregulated in cyst-lining epithelial cells in polycystin-1-deficient murine kidneys and accumulated in cyst fluid of human ADPKD kidneys. MIF promoted cystic epithelial cell proliferation by activating ERK, mTOR, and Rb/E2F pathways and by increasing glucose uptake and ATP production, which inhibited AMP-activated protein kinase signaling. MIF also regulated cystic renal epithelial cell apoptosis through p53-dependent signaling. In polycystin-1-deficient mice, MIF was required for recruitment and retention of renal macrophages, which promoted cyst expansion, and Mif deletion or pharmacologic inhibition delayed cyst growth in multiple murine ADPKD models. MIF-dependent macrophage recruitment was associated with upregulation of monocyte chemotactic protein 1 (MCP-1) and inflammatory cytokine TNF-α. TNF-α induced MIF expression, and MIF subsequently exacerbated TNF-α expression in renal epithelial cells, suggesting a positive feedback loop between TNF-α and MIF during cyst development. Our study indicates MIF is a central and upstream regulator of ADPKD pathogenesis and provides a rationale for further exploration of MIF as a therapeutic target for ADPKD.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited disease characterized by formation and progressive growth of innumerable cysts and is associated with renal interstitial inflammation and fibrosis. ADPKD is caused by mutations in PKD1 and PKD2, genes that encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively (1). Multiple signaling pathways have been identified in regulating cystic renal epithelial cell proliferation and apoptosis, and targeting these pathways delays cyst growth in animal models of PKD (2–4). Interstitial inflammation, which has been consistently reported in human and animal models of PKD, also regulates cyst growth and is associated with functional impairment. The selective depletion of macrophages in kidneys of Pkd1 conditional KO mice and congenital polycystic kidney (cpk) mice caused a significantly lower cystic index, reduced proliferation of cyst-lining cells, and improved renal function (5, 6). However, the mechanisms promoting recruitment of macrophages to pericystic and interstitial sites within cystic kidneys, and the specific roles macrophages and other infiltrating inflammatory cells play in cystogenesis, have not been defined.

Macrophage migration inhibitory factor (MIF) as a pleiotropic proinflammatory cytokine (7), possessing tautomerase activity, plays an important role in the recruitment of innate and adaptive immune cells to sites of inflammation (8). MIF was originally identified as a soluble factor in the culture medium of activated T lymphocytes that inhibited the random migration of macrophages. In addition to T lymphocytes, MIF is also expressed and secreted by other cell populations, including macrophages/monocytes (9, 10), endothelial cells (ECs) (11), epithelial cells (12), smooth muscle cells (13), synovial fibroblasts (14), and anterior pituitary cells (14). In adults, the predominant sites of MIF expression are the proliferating and differentiating epithelial linings of various organs (15). The broad expression of MIF suggests that it is involved in a wide array of physiological and pathophysiological processes.

MIF plays a critical pathogenic role in kidney diseases through mechanisms involving the innate and adaptive immune systems; the induction of cytokines, chemokines, and adhesion molecules; and interactions with glucocorticoids and the hypothalamic-pituitary–adrenal axis (16). High MIF production is found in human and experimental kidney disease and contributes to macrophage and T cell accumulation, as well as progressive renal injury (16). Upregulation of MIF was also reported in the kidney tissue of IgA nephropathy patients, compared with healthy controls and patients with anti-neutrophil cytoplasmic antibody–associated (ANCA-associated) glomerulonephritis (17). The functional importance of MIF in kidney disease is demonstrated by the findings that treatment with a neutralizing anti-MIF antibody prevents or reverses renal injury in crescentic anti-GBM glomerulonephritis (18). In addition, mice null for MIF are protected against immune-mediated lupus nephritis (19).
MIF expression was upregulated in Pkd1 mutant renal epithelial cells and tissues, as well as in ADPKD kidneys. To determine if MIF plays a role in PKD and regulates PKD-relevant signaling pathways, we investigated the expression of MIF in Pkd1 mutant renal epithelial cells and tissues, as well as in ADPKD kidneys. We found that MIF expression was increased in Pkd1 mutant mouse embryonic kidney (MEK) cells and postnatal Pkd1 homozygous PN24 cells compared with Pkd1 wildtype (WT) MEK cells and postnatal Pkd1 heterozygous PH2 cells, as analyzed with Western blot (Figure 1A) and quantitative reverse transcription PCR (qRT-PCR) (Figure 1B). The expression of MIF was also upregulated in kidneys or in cyst-lining epithelia in kidneys from Pkd1fl/fl Ksp-Cre mice or Pkd1fl/fl Pkhd1-Cre mice compared with age-matched controls, as analyzed with qRT-PCR (Figure 1C) and immunohistochemistry staining (Figure 1, D and E). Similar elevation of MIF was observed in human kidney cysts obtained from ADPKD patients (Figure 1F). We further found that MIF was detected in the fractions of plasma membrane and cytosol but not in the nuclear fraction of the cystic renal epithelial cells, as shown by cell fraction-
An inhibitor of MIF, isoxazolines (ISO-1), delays cyst growth in Pkd1 mutant kidneys. Given the robust evidence of upregulation of MIF in cystic renal epithelial cells and tissues, it is reasonable to investigate whether targeting MIF with its inhibitor delayed cyst growth in vivo. Current therapeutic strategies for targeting MIF focus on inhibiting its tautomerase activity by small molecules, including ISO-1, which acts by competing with the substrate for the catalytic site (36). We first tested whether inhibiting the activity of MIF with ISO-1 could reduce cyst growth in a Pkd1fl/fl Ksp-Cre mouse model, an aggressive cyst-progression animal model of ADPKD (37). We injected Pkd1fl/fl Ksp-Cre pups i.p. with ISO-1 (35 mg/kg) or DMSO daily from postnatal day 3 (PN3) to PN6, and kidneys were harvested and analyzed at PN7. We found that administration of ISO-1 (n = 6) delayed cyst growth characterized by partial preservation of renal parenchyma (Supplemental Figure 2A) and a significant decrease in cystic index (Supplemental Figure 2B) and the kidney weight/body weight ratios (KW/BW ratios) (Supplemental Figure 2C) in the Pkd1fl/fl Ksp-Cre mice compared with DMSO-injected Pkd1fl/fl Ksp-Cre mice, whereas ISO treatment had no effect on the body weight of these mice (Supplemental Figure 3A). Reduction in cyst growth correlated with significantly improved renal function, as indicated by lower blood urea nitrogen (BUN) levels in ISO-1–treated mice (Supplemental Figure 2D). In addition, the proliferation of the cystic-lining epithelial cells, as analyzed by Ki67 staining (Supplemental Figure 2E), was significantly decreased, but the apoptosis of the cyst-lining epithelia as analyzed by TUNEL assay, was significantly induced (Supplemental Figure 2F) in ISO-1–treated kidneys of Pkd1fl/fl Ksp-Cre mice compared with the DMSO-treated mice.

Next, we determined if ISO-1 reduced cyst growth in Pkd1fl/fl Pkhd1-Cre mice, a milder Pkd1 conditional KO mouse model. The cyst growth in Pkd1fl/fl Pkhd1-Cre mice begins around PN10, followed by rapid cyst development over the subsequent 2 weeks (6). We treated Pkd1fl/fl Pkhd1-Cre pups with daily i.p. injection of ISO-1 (35 mg/kg) or DMSO (control) from PN8–PN24. The kidneys

Figure 2. Treatment with ISO-1 delayed cyst growth in Pkd1fl/fl Pkhd1-Cre mice. (A) Histologic examination of PN25 kidneys from Pkd1fl/fl Pkhd1-Cre mice treated with vehicle (DMSO) (n = 10) or ISO-1 (n = 10). Scale bar: 2 mm. (B) Quantification of the percentage of cystic areas over total kidney-section areas of PN25 kidney sections from Pkd1fl/fl Pkhd1-Cre mice treated as in A. Shown is mean ± SEM of all sections quantified for each condition. P < 0.01. (C and D) KW/BW ratios (C) and BUN levels (D) were decreased in Pkd1fl/fl Pkhd1-Cre mice at PN25 treated with ISO-1 compared with mice treated with DMSO (control). P < 0.01. (E) ISO-1 treatment reduced cyst-lining epithelial cell proliferation in Pkd1fl/fl Pkhd1-Cre mouse kidneys at PN25 as detected by Ki67 staining. P < 0.001. Scale bars: 100 μm. Arrows indicate Ki67-positive cells. (F) ISO-1 treatment induced cyst-lining epithelial cell apoptosis in Pkd1fl/fl Pkhd1-Cre mouse kidneys at PN25 as detected by TUNEL assay. P < 0.001. Scale bars: 100 μm. Statistical analysis was performed using an unpaired 2-tailed Student’s t test.
were harvested and analyzed at PN25, and we found that ISO-1 treatment did not affect the body weight, compared with DMSO-treated mice (Supplemental Figure 3B). However, administration of ISO-1 (n = 10) delayed renal cyst growth (Figure 2A) characterized by significantly decreasing cyst index (Figure 2B), the KW/BW ratios (Figure 2C), and BUN levels (Figure 2D), as well as by inhibiting cystic epithelial cell proliferation, as analyzed with Ki67 staining (Figure 2E), and inducing cyst-lining epithelial cell apoptosis, as analyzed with TUNEL assay (Figure 2F), in kidneys from Pkd1fl/fl Pkhd1-Cre mice compared with the age-matched kidneys from DMSO-injected Pkd1fl/fl Pkhd1-Cre mice (n = 10).

Last, we determined if ISO-1 reduced cyst growth in Pkd1nl/nl mice, a milder Pkd1 hypomorphic mouse model (38). We treated Pkd1nl/nl pups with daily i.p. injection of ISO-1 (35 mg/kg) or DMSO (control) from PN5–PN27. The kidneys were harvested and analyzed at PN28, and we also found that ISO-1 treatment did not affect the body weight compared with DMSO-treated mice (Supplemental Figure 3C). However, administration of ISO-1 (n = 6) delayed renal cyst growth (Figure 3A), characterized by significantly decreasing cyst index (Figure 3B, P < 0.001), the KW/BW ratios (Figure 3C, P < 0.001), and BUN levels (Figure 3D, P < 0.001), as well as by inhibiting cystic epithelial cell proliferation, as analyzed with Ki67 staining (Figure 3E), and inducing cyst-lining epithelial cell apoptosis, as analyzed with TUNEL assay (Figure 3F) in kidneys from Pkd1nl/nl mice compared with the age-matched kidneys from DMSO-injected Pkd1nl/nl mice (n = 12). These results suggest that targeting MIF with pharmacological inhibitors may delay cyst growth in ADPKD patients.

MIF and Pkd1 double KO delays renal cyst growth. To further explore the in vivo function of MIF in a Pkd1 KO mouse model, we crossed the Pkd1fl/+ Ksp-Cre Mif–/– male mice to generate Pkd1fl/fl Ksp-Cre Mif–/– mice. We found that cyst formation was significantly delayed in the absence of MIF in Pkd1fl/fl Ksp-Cre Mif–/– mice (n = 6) at PN7 compared with age-matched Pkd1fl/fl Ksp-Cre Mif+/+ mice (n = 10) (Figure 4A and B). The KW/BW ratios from Pkd1fl/fl Ksp-Cre Mif–/– mice were dramatically reduced compared with Pkd1fl/fl Ksp-Cre Mif+/+ mice (Figure 4C). In addition, BUN levels were also significantly reduced in Pkd1fl/fl Ksp-Cre Mif–/– mice (Figure 4D). These results suggest that targeting MIF with pharmacological inhibitors may delay cyst growth in ADPKD patients.
addition to decreasing cell proliferation and inducing apoptosis in cyst-lining epithelia in vivo, treatment with ISO-1 also dramatically reduced the accumulation/recruitment of macrophages to pericystic sites and interstitium in kidneys from Pkd1fl/fl Ksp-Cre mice and Pkd1fl/fl Pkhd1-Cre mice compared with DMSO-treated Pkd1 fl/fl Ksp-Cre mice and Pkd1fl/fl Pkhd1-Cre mice (Supplemental Figure 5, B–D).

ISO-1 was reported to inhibit the tautomerase activity of MIF by competing with the substrate for the catalytic site (36). However, we found that ISO-1 treatment also decreased the levels of MIF in kidneys from Pkd1fl/fl Ksp-Cre mice and Pkd1 fl/fl Pkhd1-Cre mice, as determined by Western blot (Supplemental Figure 6, A and B) and immunohistochemistry staining (Supplemental Figure 6, C and D). These results suggest a role of MIF in regulating the recruitment of macrophages to pericystic sites and interstitium of ADPKD.

MIF can be secreted into cell-culture mediums of renal epithelial cells and is highly enriched in cyst fluids and urines derived from 2 distinct orthologous mouse models of ADPKD. The proliferative effect of macrophages on cyst growth does not require cell-cell contact but can be mediated via unknown soluble factors (5). Western blot analysis of conditioned media showed that renal epithelial cells can provide essential factors to promote cell proliferation, as indicated by the increased expression of PCNA in cyst-lining epithelial cells from Pkd1 conditional KO kidneys compared with WT kidneys (Supplemental Figure 5A). We also found that ISO-1 treatment reduces macrophages at interstitial and pericystic regions in Pkd1 conditional KO mouse kidneys (Figure 4E).

ISO-1 treatment reduces macrophages at interstitial and pericystic regions in Pkd1 conditional KO mouse kidneys. It has been reported that large numbers of renal macrophages are recruited to interstitial and pericystic regions in orthologous models of PKD1, PKD2, and cpk mice (5, 6), and that an abundance of M2-like macrophages promotes cystic renal cell proliferation and cyst growth (5). We found that double KO of Pkd1 and Mif decreased the accumulation/recruitment of macrophages to pericystic sites and interstitium in kidneys compared with kidneys from Pkd1 single KO Pkd1fl/fl Ksp-Cre mice (Supplemental Figure 5A). We also found that in mice compared with Pkd1fl/fl Ksp-Cre Mif+/+ mice, which indicated that renal function was normalized in Pkd1fl/fl Ksp-Cre Mif–/– mice (Figure 4D). At the same time, the expression of MIF could not be detected in kidneys from Pkd1 and Mif double KO mice, as analyzed by Western blot (Supplemental Figure 4). PCNA staining revealed that the proliferation of cyst-lining epithelial cells was significantly decreased in Pkd1fl/fl Ksp-Cre Mif–/– neonates (Figure 4E). On average, the percentage of PCNA-positive nuclei in cyst-lining epithelial cells was calculated from 1,000 nuclei per mouse-kidney section, and only strongly stained nuclei were considered as PCNA-positive. Shown is mean ± SEM. P < 0.001. Scale bars: 50 μm. Arrows indicate the PCNA-positive cells. (F) KO of Mif induced cyst-lining epithelial cell apoptosis in Pkd1fl/fl Ksp-Cre mouse kidneys, as detected by TUNEL assay. P < 0.05. Scale bars: 100 μm. Statistical analysis was performed using an unpaired 2-tailed Student’s t test.
MIF is a soluble factor secreted by ADPKD cells into ADPKD-conditioned medium and promotes macrophage migration and the expression of MCP-1 and TNF-α. To investigate whether MIF present in ADPKD conditioned medium (CM) may contribute to macrophage recruitment in our established system as described (5), we tested the effects of ISO-1 on the monocyte chemoattractant activity present in this CM. For these experiments, transwell migration assays of THP-1 cells, a human monocyte cell line, were carried out. We found that while ADPKD CM promoted robust migration of monocytes, this effect was significantly diminished by the presence of ISO-1 (Supplemental Figure 9). These results support that MIF, as a factor secreted by ADPKD cells, promotes the migration of macrophages.

Accumulation of macrophages was associated with the increased expression of MCP-1 in the kidneys of Han:SPRD polycystic rats, a nonorthologous model of ADPKD (39). We found, by Western blot analysis, that the expression of MCP-1 was elevated in Pkd1 mutant renal epithelial cells (Supplemental Figure 10A). We found similar results by immunohistochemistry staining in cyst-lining epithelia in kidneys from Pkd1 fl/fl Pkd1-Cre mice, as well as in kidneys from ADPKD patients (Supplemental Figure 10, B-D). Treatment with ISO-1 decreased the expression of MCP-1 in Pkd1 mutant kidneys from Pkd1 fl/fl Pkd1-Cre mice, but not in age-matched Pkd1 WT mice (Supplemental Figure 10C). The mRNA levels of these genes were normalized to mRNA levels in WT kidneys, which is set as 1.

Figure 5. MIF promotes glucose uptake and ATP generation in renal epithelial cells and tissues. (A) Knockdown of MIF with siRNA or inhibition of MIF with ISO-1 decreased the uptake of glucose in Pkd1 mutant MEK cells compared with untreated Pkd1 mutant MEK cells. n = 3, P < 0.001; ANOVA post hoc test. (B) Knockdown of MIF with siRNA or inhibition of MIF with ISO-1 decreased the levels of intracellular ATP in Pkd1 mutant MEK cells compared with untreated Pkd1 mutant MEK cells. n = 3, P < 0.001; ANOVA post hoc test. (C and D) Treatment with ISO-1 decreased the levels of intracellular ATP in kidneys from Pkd1 fl/fl Ksp-Cre mice at PN7 (C) and from Pkd1 fl/fl Pkd1-Cre mice at PN25 (D). n = 5, P < 0.001; ANOVA post hoc test. (E) qRT-PCR analysis of the expression of Hk1, Ldha, and Pkm2 mRNA in PN25 kidneys from Pkd1 fl/fl Pkd1-Cre mice treated with ISO-1 or DMSO. n = 3, P < 0.05; ANOVA post hoc test.

MIF is a soluble factor secreted by ADPKD cells into ADPKD-conditioned medium and promotes macrophage migration and the expression of MCP-1 and TNF-α. To investigate whether MIF secreted in ADPKD cells secreted more MIF than Pkd1 WT renal epithelial cells when MIF levels were normalized to cell number (Supplemental Figure 7A). Moreover, MIF was highly enriched in cyst fluid collected from Pkd1 fl/fl Ksp-Cre mice and Pkd1 fl/fl Pkd1-Cre mice (Supplemental Figure 7B). MIF was only detected in urine from Pkd1 fl/fl Ksp-Cre mice and Pkd1 fl/fl Pkd1-Cre mice, but not from age-matched Pkd1 WT mice (Supplemental Figure 7C). Furthermore, MIF was detected in cyst fluids from ADPKD patients (Supplemental Figure 8A) and in the urine of ADPKD patients (Supplemental Figure 8B). The MIF levels in the urines of normal volunteers were very low, as shown by weak bands in some samples (Supplemental Figure 8B). These results suggested that MIF in cyst fluids and urines might be secreted by renal epithelial cells and/or macrophages accumulated in pericystic sites and interstitium of kidneys in Pkd1 KO mice and ADPKD patients, and MIF might work as a soluble mediator to regulate intracellular signaling pathways through its receptors.
Cre mice (Supplemental Figure 10B) and Pkd1<sup>fl/fl</sup> Pkhd1-Cre mice (Supplemental Figure 10C) compared with age-matched control kidneys treated with DMSO. In addition, we found that treatment with ISO-1 decreased the levels of MCP-1 mRNA and protein in kidneys from Pkd1<sup>fl/fl</sup> Pkhd1-Cre mice compared with DMSO-treated control (Supplemental Figure 11). We also found that MIF induced the expression of MCP-1 and TNF-α in Pkd1 mutant renal epithelial cells (Supplemental Figure 12, A and B), whereas TNF-α also induced the expression of MIF in renal epithelial cells (Supplemental Figure 12C). It has been reported that MIF induces macrophage recruitment through chemokine CCL2 (MCP-1) (26). Our results suggested that MIF is an upstream regulator of MCP-1 and may regulate MCP-1-mediated recruitment/retention of macrophages to the kidneys of ADPKD patients.

**MIF promotes glucose uptake and ATP generation in cystic renal epithelial cells and tissues.** MIF has been reported to regulate glucose uptake in non-small cell lung carcinomas (23) and to stimulate glycolysis during sepsis (40). Deprivation of glucose abrogated the higher ATP content of Pkd1 mutant mouse embryonic fibroblasts (MEFs), and glucose addition increased the intracellular ATP in these cells (33). We found glucose uptake and ATP levels were elevated in Pkd1 mutant (Null) MEK cells compared with Pkd1 WT MEK cells (40).

**Figure 6. MIF regulates renal epithelial cell proliferation through activation of ERK, mTOR, and Rb/E2F signaling pathways.** (A and B) Western blot analysis of the expression of ERK and phospho-ERK, as well as AMPK and phospho-AMPK, from whole-cell lysates of Pkd1 null MEK (Null-MEK) cells (A) and Pkd1 mutant PN24 cells (B) induced with MIF (10 ng/ml) at indicated time points. (C) Western blot analysis of the expression of ERK and phospho-ERK, AMPK and phospho-AMPK, S6 and phospho-S6, and Rb and phospho-Rb from whole-cell lysates of Pkd1 mutant PN24 cells treated with ISO-1 (100 μM) at indicated time points. (D) Western blot analysis of the expression of ERK and phospho-ERK, AMPK and phospho-AMPK, S6 and phospho-S6, and Rb and phospho-Rb from whole-cell lysates of Pkd1 mutant PN24 cells transfected with MIF siRNA or control siRNA. (E and F) Western blot analysis of the expression of ERK and phospho-ERK, AMPK and phospho-AMPK, S6 and phospho-S6, and Rb and phospho-Rb in kidneys from 3 different Pkd1<sup>fl/fl</sup> Ksp-Cre mice (E) and Pkd1<sup>fl/fl</sup> Pkhd1-Cre mice (F) treated with DMSO or ISO-1.
Pkhd1-Cre mice, compared with DMSO-treated Pkd1 fl/fl Pkd1-Cre mice (Figure 5E). Our results suggested that MIF regulated glucose uptake and ATP levels in renal epithelial cells and tissues.

MIF regulates renal epithelial cell proliferation through activation of ERK, mTOR, and Rb/E2F signaling pathways. MIF, as a pleiotropic proinflammatory cytokine, regulates not only the recruitment of innate and adaptive immune cells to the place of inflammation, but also cell proliferation, cell differentiation, and apoptosis (41), which are the drivers of cyst growth (2, 4), thus suggesting that multiple MIF-mediated mechanisms and signaling pathways are likely to be involved in MIF-mediated cyst growth. MIF exerts its biological activities through signal transduction, initiated by direct binding to the CD74/CD44 receptor complex to activate the downstream signaling pathways (42). MIF binds to CD74, a type II transmembrane protein, inducing its phosphorylation and the recruitment of CD44, leading to phosphorylation of ERK1/2 and the further activation of downstream effector proteins involved in inflammatory processes and cell proliferation (20, 21). We found that MIF induced rapid

Figure 7. MIF regulates cystic epithelial survival and death via p53. (A and B) Inhibition of MIF with ISO-1 or knockdown of MIF with siRNA-induced apoptosis in Pkd1 null MEK cells (A, left panel) while knockdown of p53 with siRNA prevented ISO-1- or MIF siRNA-induced apoptosis in these cells as detected by TUNEL assay, which were transfected with p53 siRNA for 24 hours and then treated with 100 μM ISO-1 or MIF siRNA for 24 hours. P < 0.001; ANOVA post hoc test. However, inhibition of MIF with ISO-1 or knockdown of MIF with siRNA did not induce apoptosis in Pkd1 WT MEK cells (A, right panel). Scale bar: 100 μm. (C) Western blot analysis of the expression of p53 and active caspase-3, as well as the cleaved poly (ADP-ribose) polymerase (PARP), a substrate of caspase-3 in Pkd1 null MEK cells treated with ISO-1 (100 μM) at indicated time points. The expression of p53 and active caspase-3, as well as the cleaved PARP, were gradually increased in Pkd1 null MEK cells treated with ISO-1 up to 12 hours, compared with untreated cells (0 hour).
and transient activation of ERK — a canonical PKD1 effector (33, 35) — in Pkd1 mutant renal epithelial cells at 10 minutes (Figure 6, A and B), while inhibition of MIF with ISO-1 or knockdown MIF with siRNA decreased the phosphorylation and activation of ERK in these cells (Figure 6, C and D, and Supplemental Figure 14A). To support that MIF as a soluble factor that is able to induce the phosphorylation of ERK, we found that CM collected from MIF knockdown cystic renal epithelial cells did not stimulate the phosphorylation of ERK in Pkd1 null cells and PN24 cells, whereas the CM collected from control siRNA transfected cells did induce the phosphorylation of ERK in these cells (Supplemental Figure 14B). This observation was confirmed in vivo; targeting MIF with ISO-1 decreased the phosphorylation of ERK in kidneys from Pkd1<sup>fl/fl</sup> Ksp-Cre mice (Figure 6E) and Pkd1<sup>fl/fl</sup> Pkd1<sup>Cre</sup> mice (Figure 6F) as compared with kidneys from DMSO-injected Pkd1<sup>fl/fl</sup> Ksp-Cre mice and Pkd1<sup>fl/fl</sup> Pkd1<sup>Cre</sup> mice (Figure 6, E and F).

Next, we found that endogenous MIF is necessary for the phosphorylation of S6, a substrate of mTOR complex 1, in Pkd1 mutant renal epithelial cells. As shown in Figure 6, C and D, inhibition of MIF with ISO-1 or knockdown MIF with siRNA resulted in a pronounced reduction of phosphorylation of S6 in Pkd1 mutant renal epithelial cells, which indicated that MIF was an upstream regulator of mTOR signaling in those cells. It has been reported that mTOR activity can be inhibited by activated AMPK that is a regulator of mTOR signaling in those cells. It has been reported that mTOR activity can be inhibited by activated AMPK that is a regulator of mTOR signaling in those cells. Upregulated MIF in Pkd1 mutant renal epithelial cells activates ERK and mTOR signaling, which may result in activation of ERK-mTOR-mediated cell proliferation and glycolysis, as has been suggested. It also increases glucose uptake, which results in promoting glycolysis and the generation of ATP to inhibit AMPK; inhibits p53-dependent cystic epithelial cell death; is responsible for the recruitment of macrophages to pericystic and interstitial regions in Pkd1 mutant mouse kidneys, a process likely to be enhanced by MIF-mediated upregulation of MCP-1 and/or TNF-α; and is a target of ISO-1, which delays cyst growth in Pkd1 KO mouse kidneys. In addition, 2 double-negative feedback loops downstream of MIF can be observed: among ERK-mTOR-glycolysis-AMPK-ERK and among mTOR-glycolysis-AMPK-mTOR. Arrow indicates the positive effect; “T” indicates the negative effect. Dashed lines indicate putative signaling mechanisms.

Further, we found that treatment with ISO-1 decreased the activation and phosphorylation of S6 (Figure 6, E and F) but sustained activation of AMPK in Pkd1 mutant kidneys (Figure 6, E and F). These results suggest that MIF regulates the interplay between mTOR and AMPK signaling in regulating Pkd1 mutant renal epithelial cell proliferation, and this process involves MIF-mediated glucose uptake and ATP production.

MIF has been reported to interfere with the Rb/E2F pathway (28), which was recently shown to regulate cystic renal epithelial cell proliferation (2, 3). We found that inhibition of MIF with ISO-1 or knockdown of MIF with siRNA decreased the phosphorylation of Rb in Pkd1 mutant renal epithelial cells (Figure 6, C and D) and in Pkd1 KO kidneys upon ISO-1 treatment (Figure 6, E and F). These results suggested that MIF also affected Pkd1 mutant renal epithelial cell proliferation through Rb/E2F-dependent mechanisms.

**MIF inhibition induces p53-dependent cystic renal epithelial cell apoptosis.** Treatment with ISO-1 induced cystic renal epithelial cell death in Pkd1 KO renal tissues (Figure 2F, Figure 3F, and Supplemental Figure 1F). Previous studies demonstrated that MIF protects macrophages from p53-mediated apoptosis (24). Thus, we examined whether MIF regulated p53-dependent cystic renal epithelial cell survival and death. We found that inhibition of MIF with ISO-1 or knockdown of MIF with siRNA significantly induced Pkd1-null (Null) MEK cell apoptosis (Figure 7, A and B), whereas no apoptotic cells were observed in Pkd1 WT MEK cells with all these treatments, as analyzed by TUNEL assay (Figure 7A). However, knockdown of p53 with siRNA significantly decreased the apoptosis of Pkd1 mutant renal epithelial cells induced by inhibi-
tion of MIF with ISO-1 or MIF siRNA-mediated knockdown (Figure 7, A and B), while knockdown of p53 alone had no effect on cell survival and death. The knockdown efficiency of p53 siRNA and MIF siRNA was confirmed by Western blot (data not shown). Treatment with ISO-1 increased the expression of p53 and active caspase-3 in Pkd1 mutant cystic renal epithelial cells (Figure 7C). Caspase-3 activation was further confirmed by the appearance of cleaved poly (ADP-ribose) polymerase (PARP), a substrate of caspase-3 (Figure 7C).

Discussion
In this study, we provide evidence that MIF is a key regulator of cyst growth in ADPKD and upstream of ERK, AMPK, mTOR, Rb/E2F, and p53 signaling pathways; it is also a regulator of the processes of glucose uptake and macrophage recruitment, and the production of TNF-α and MCP-1. MIF upregulation in renal cyst epithelial cells activates ERK and mTOR signaling, which may result in activation of ERK-mTOR-mediated cell proliferation and glycolysis, as has been suggested (33); increases glucose uptake, which results in promoting glycolysis and production of ATP to increase the ratio of ATP/AMP, which inhibits AMPK; increases the phosphorylation of Rb, which releases E2F from Rb/E2F, thereby increasing S-phase entry; inhibits p53-dependent apoptosis of cyst epithelial cells; and contributes to the recruitment of macrophages to pericystic and interstitial regions in Pkd1 mutant mouse kidneys, a process possibly regulated by MIF-mediated upregulation of MCP-1 and/or TNF-α (Figure 8). KO of Mif or targeting MIF with the inhibitor, ISO-1, delayed cyst growth and improved renal function in Pkd1 conditional KO mouse models and Pkd1 hypomorphic mouse model. The treated kidneys were characterized by diminished cyst mural cell proliferation, induced cyst mural cell apoptosis, diminished renal ATP generation, and diminished recruitment of macrophages to the kidneys (Figure 8).

PKD is not primarily an inflammatory disorder; however, recent studies suggest that the chronic interstitial inflammation in PKD contributes to cyst expansion and renal impairment (5, 6). Rather than serving as a primary cause of cyst initiation and expansion, interstitial inflammation secondarily promotes disease progression in response to cyst-initiated macrophage recruitment. Renal cyst growth may involve 2 phases: initiation of cyst formation and progressive cyst enlargement. Initiation is induced by unscheduled proliferation of tubule cells in response to the malfunction of polycystins 1 and 2. As the tubule wall expands and bulges into the interstitium, gene array and proteomic studies have revealed that a large number of secondary processes are set in motion, some of which have deleterious effects. In this regard, the formation of a cyst can be viewed as a microscopic injury to the kidney, provoking an injury response resembling those observed when a wound is created in any organ. In PKD, the lesions never heal and relentlessly enlarge, obstructing the flow of blood, lymph, and urine in nearby vessels (42). Cyst formation induced by genetic mutation of Pkd1 may set in motion several primary effects, including MIF initiation and regulated activation of ERK, mTOR, and Rb/E2F signaling, leading to increased cyst epithelial cell proliferation; increased glucose uptake and glycolysis that inhibit AMPK signaling; the inhibition of p53 that prevents apoptosis of Pkd1 mutant renal epithelial cells (Figure 8); and, secondarily, MIF-mediated recruitment of macrophages in an injury response (43) that promotes cyst expansion and disease progression (Figure 8). By this account, MIF promotes signaling pathways that directly influence cyst initiation and growth of renal epithelial cells, as well as calling to action troublesome inflammatory mediators (Figure 8). The central and upstream position of MIF in the pathogenesis of ADPKD suggests that therapeutically targeting MIF could ameliorate the progression of ADPKD.

MIF regulation in ADPKD. We found that MIF was upregulated in the kidneys of Pkd1 mutant mice and ADPKD patients (Figure 1). Elevated MIF in ADPKD may be caused by increased transcription due to unknown transcriptional regulators and/or epigenetic regulation, including histone acetylation and methylation, or by protein stabilization due to unknown factors. Our results that TNF-α could induce the expression of MIF in renal epithelial cells (Supplemental Figure 12C) suggested that cyst fluid TNF-α might stimulate the expression of MIF in cyst-lining epithelia in vivo (Figure 8). Together with the result that MIF also stimulates the expression of TNF-α (Supplemental Figure 12A and 12B), a positive feedback loop may exist between MIF and TNF-α in vivo (Figure 8). In addition, HSP90 is upregulated in ADPKD kidneys, and HSP90 inhibition slows cyst growth in a mouse model of ADPKD (44). It has been reported that inhibiting HSP90 destabilizes MIF in breast cancer cells (45), suggesting that the elevated MIF levels in ADPKD may be mediated through HSP90 stabilization (Figure 8). In support of this idea, we found that HSP90 inhibition decreased MIF levels in Pkd1 mutant renal epithelial cells (Supplemental Figure 15).

MIF and the recruitment of macrophages, as well as the expression/secretion of cytokines. MIF stimulates the expression and secretion of proinflammatory cytokines from macrophages (41). Macrophages secrete TNF-α (46), and increased MCP-1 levels are present in a rat model of PKD (39). Urinary levels of MCP-1 correlate with disease severity, indicating that MCP-1 is a potential biomarker for disease progression (34). Although MIF may be expressed by interstitial T cells and macrophages in the setting of PKD, it is unlikely that macrophages are the only source of cytokines in PKD kidneys. The current results that MIF was secreted by renal epithelial cells into culture medium and cyst fluids (Supplemental Figure 7, A and B); that MCP-1 was elevated in Pkd1 mutant mouse cells and in mouse and human ADPKD kidneys; that TNF-α expression was upregulated in Pkd1 mutant cystic epithelial cells (4); and that MIF induced the expression of MCP-1 and TNF-α in Pkd1 mutant renal epithelial cells (Supplemental Figure 12) suggest that Pkd1 mutant renal epithelial cells might be another source for the origin of chemotactic cytokines, which are regulated by MIF in ADPKD kidneys. MIF induces macrophage recruitment through chemokine CCL2 (MCP-1) and through monocyte recruitment cytokines, including TNF-α, IL-1β, and IL-2, which were found in cyst fluids from human kidneys (26). KO of MIF or inhibition of MIF with ISO-1 diminished the levels of macrophages present within pericytic and interstitial regions of Pkd1 KO mouse kidneys (Supplemental Figure 5) and reduced migration of human THP-1 monocytes in vitro (Supplemental Figure 9). These results suggest that MIF contributes to the recruitment of macrophages in the cystic kidneys of Pkd1 KO mice (Figure 8). Whether MIF recruits renal macrophages directly in vivo or through its effects on MCP-1 and/or TNF-α signaling is unknown.
The metabolic effects of MIF. MIF induces cellular glucose uptake and stimulates glycolysis during sepsis (40). It has been suggested that Pkd1-deficient cells reprogram their glucose metabolism to use aerobic glycolysis (33), a phenomenon often called the Warburg effect, which has been recognized in cancer for more than 80 years (47). Warburg’s observation indicates that unlike most of the normal cells, which channel pyruvate into the tricarboxylic acid (TCA) cycle, cancer cells preferentially generate energy through “fermenting” glucose into lactate and reducing pyruvate into lactate, even in the presence of sufficient oxygen. Due to the lower efficiency of aerobic glycolysis in terms of ATP production per glucose molecule versus the TCA cycle, a definitive explanation for Warburg’s observation has remained elusive, at least in part because the energy requirements of cell proliferation appear at first glance to be better met by complete catabolism of glucose using mitochondrial oxidative phosphorylation to maximize ATP production. It has been proposed that the metabolism of cancer cells, and indeed all proliferating cells, is adapted to facilitate the uptake and incorporation of nutrients, particularly glucose, that meet or exceed the bioenergetic demands of cell growth and proliferation (48). The reported finding that the cystic kidneys from Pkd1<sup>−/−</sup> Ksp-Cre mice showed a higher uptake of glucose and more conversion to lactate compared with control kidneys (33), and our results that glucose uptake was increased in Pkd1 mutant renal epithelial cells (Figure 5A) not only suggested a switch to the Warburg effect but also supported this prediction. PKD1 mutation–mediated glycolysis may depend on ERK-mediated inhibition of AMPK and activation of the mTOR-glycolytic cascade (33). However, the upstream factor(s) that regulate the uptake of glucose have not been explored in ADPKD. Based on our observation that MIF not only regulated the uptake of glucose and the generation of ATP in renal epithelial cells (Figure 5), but also activated ERK and mTOR signaling and inhibited the AMPK pathway in Pkd1 mutant renal epithelial cells and tissues (Figure 6), we think that MIF is a key upstream activator of all of these processes and pathways and may act in a dual mechanism to inhibit AMPK in Pkd1 mutant renal epithelial cells. MIF may induce glucose uptake to directly increase glycolysis and the generation of ATP, which then acts to inhibit the activation of AMPK. MIF-mediated activation of ERK signaling is likely to activate the mTOR-glycolytic cascade, as has been proposed (33), which also inhibits the activation of AMPK. Inhibition of AMPK will relieve its inhibition on ERK or mTOR (23) to form double feedback loops to increase cystic epithelial cell proliferation (Figure 8). Activation of ERK signaling promotes cyst growth (35), while targeting mTOR with rapamycin or activating AMPK with metformin delays cyst growth in several different models of ADPKD (30, 31). Although the interaction among the mechanisms involved remains elusive, our results favor the view that MIF impacts defective glucose metabolism, ATP generation and cyst epithelial cell proliferation by mediating the interplay among ERK, mTOR, and AMPK in ADPKD.

MIF and its receptor CD74-mediated pathways. MIF promotes either the transient or the sustained activation of ERK1/2 mitogen–activated protein kinase (MAPK) pathway (20, 21), and this process is dependent on MIF interaction with its membrane receptor, CD74 (49). We found that the expression of CD74 was upregulated in Pkd1 mutant renal epithelial cells (Supplemental Figure 16, A and B) and was elevated in ADPKD kidneys (Supplemental Figure 16C). We also found that MIF induced rapid and transient activation of ERK in Pkd1 mutant renal epithelial cells in vitro (Figure 6, A and B) but sustained activation of ERK in Pkd1 KO mouse kidneys in vivo (Figure 6, E and F). We further found that treatment with CD74 antibody blocked the MIF-induced phosphorylation of ERK but did not affect the MIF-induced MCP-1 expression (Supplemental Figure 16D). It has been reported that activation of the ERK/MAPK signaling pathway by MIF in fibroblasts and macrophages is dependent on SRC kinase activity (21), which suggests that MIF is an upstream regulator of SRC-mediated ERK MAPK signaling. Inhibition of SRC kinase activity delayed renal cyst growth in PKD animal models (50). Whether activation of ERK by MIF is dependent on CD74-mediated activation of SRC or other mechanisms in cystic renal epithelial cells awaits investigation. In addition, CD74 is responsible for signaling to AMPK activation by MIF (22), since silencing CD74 with siRNA had the same effects of enhanced AMPK phosphorylation as MIF silencing with siRNA in non–small cell lung carcinomas cells (23). The elevation of CD74 in Pkd1 mutant renal epithelial cells and ADPKD kidneys (Supplemental Figure 16) implies that MIF may also signal through CD74 to inhibit AMPK phosphorylation and activity in those cells.

MIF and the p53 pathway. MIF has been described as an inhibitor of p53-dependent apoptosis in macrophages (51). It was hypothesized that upregulation of MIF at sites of chronic inflammation might impair p53-dependent cellular responses toward DNA damage and induce inappropriate proliferation and, thus, promote the accumulation of oncogenic mutations (52). We found that inhibition of MIF with ISO-1–induced p53-dependent Pkd1 mutant renal epithelial cell apoptosis, since knockdown of p53 abolished ISO-1–induced cell death in these cells (Figure 7); this suggested that, in addition to its effects on macrophages, MIF also regulated p53-dependent cystic renal epithelial cell death. However, the mechanisms for the upregulation of p53 and the activation of caspase-3 in Pkd1 mutant renal epithelial cells upon ISO-1 treatment remain to be determined.

Methods

Cell culture and reagents. Pkd1 WT and Pkd1 null MEK cells, which were generated from Jing Zhou’s laboratory at Harvard Medical School (Boston, Massachusetts, USA) and were used in our recent publications, were maintained as previously described (2, 53). Pkd1 heterozygous PH2 cells and homozygous PN24 cells, provided by Stefan Somlo through the George M. O’Brien Kidney Center at Yale University (New Haven, Connecticut, USA), were cultured as described (35, 54). ISO-1 was purchased from EMD Millipore. Recombinant mouse MIF was purchased from R&D Systems. Pkd1 null MEK cells and PN24 cells were treated with ISO-1 or recombinant MIF for 24 hours and then were harvested and analyzed by Western blot. The CM from Pkd1 null MEK cells transfected with control siRNA or MIF siRNA for 72 hours were collected by centrifuging at 3,000 g for 10 minutes and then used to stimulate the Pkd1 null MEK cells and PN24 cells. PN24 cells were pretreated with normal IgG or CD74 antibody for 2 hours and then treated with recombinant MIF (10 ng/ml), and the whole cell lysates were analyzed by Western blot. Pkd1 WT MEK cells treated with or without ISO-1, as well as the cells transfected with p53 siRNA or MIF siRNA for 24 hours, were analyzed by TUNEL.
 assay. The Pkd1 null MEK cells with p53 siRNA or control siRNA transfection for 24 hours were treated with ISO-1 or transfected with MIF siRNA for another 24 hours and analyzed by TUNEL assay. Primary cells obtained from the cavities of cysts on the surfaces of ADPKD kidneys (ADPKD cells) were supplied by the PKD Research Biomaterials and Cellular Models Core at the KUMC (Kansas City, Kansas, USA) and cultured up to 3 passages in DMEM/F-12 (Cellgro 15-090-CV; Corning) supplemented with 5% FBS, 15 mM HEPES, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (ITS; BD Biosciences) plus penicillin (100 U/mL), streptomycin (Pen/Strep; 130 μg/mL). CM was prepared from ADPKD cells incubated in DMEM (Sigma-Aldrich) containing 10% FBS, 2 mM additional glutamine, and Pen/Strep for 3 days. THP-1 monocytes were maintained in RPMI-1640 media (Sigma-Aldrich) containing 10% FBS, 2 mM L-glutamine, and Pen/Strep.

Subcellular fractionation. The Pkd1 null MEK cells were washed twice with PBS and harvested by scraping, followed by centrifugation. The cell pellets were resuspended in 10 volumes of Buffer A, containing 2.0 mM MgCl₂, 25 mM KCl, 10 mM HEPES (pH 7.5), and protease inhibitors. Cells were homogenized in a homogenizer after swelling for 10 minutes on ice. The homogenate was centrifuged at 1,000 g for 10 minutes to generate the first pellets and the first supernatant. The first pellets—which were resuspended in 3 ml of Buffer N, containing 0.25 M sucrose in buffer A, and were layered over 2 ml of medium containing 1.1 M sucrose in buffer A—were centrifuged at 1,000 g for 10 minutes to collect the nuclear pellet. The first supernatant was further fractionated by centrifugation at 35,000 g for 40 minutes to generate the second pellets and the second supernatant. The second supernatant represented soluble cytoplasmic proteins, whereas the second pellets contained plasma membrane proteins. The nuclei, cytoplasmic, and plasma membrane fractions were resuspended in SDS sample buffer for Western blot analysis.

Western blot analysis. We performed Western blotting on whole-cell lysates as previously described (3,5). Briefly, cells were lysed at 4°C with RIPA buffer consisting of 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1% glycerol, 0.5 mM dithiothreitol, and 1 mM sodium vanadate plus protease inhibitor (Roche Applied Science). The cell pellets were resuspended in 10 volumes of Buffer A, and were layered over 2 ml of medium containing 1.1 M sucrose in buffer A—and were centrifuged at 1,000 g for 10 minutes to collect the nuclear pellet. The first supernatant was further fractionated by centrifugation at 35,000 g for 40 minutes to generate the second pellets and the second supernatant. The second supernatant represented soluble cytoplasmic proteins, whereas the second pellets contained plasma membrane proteins. The nuclei, cytoplasmic, and plasma membrane fractions were resuspended in SDS sample buffer for Western blot analysis.
was analyzed using the Amplex Red glucose assay kit (Invitrogen), according to the manufacturer’s protocol. Glucose uptake was shown by subtracting the remaining glucose from total glucose in the fresh medium. For ISO-1 treatment, the cells treated by ISO-1 for 24 hours were replaced with fresh medium with ISO-1. After an additional 24 hours of incubation, the glucose was analyzed as described above.

**ATP assay.** The cells were transfected with MIF siRNA or ISO-1 for 48 hours and lysed by RIPA buffer. The intracellular ATP levels of cell lysates were assessed using the ATP Determination Kit (Invitrogen), according to the manufacturer’s protocol. The intracellular ATP levels were shown by normalizing to protein amount.

**TUNEL assay.** TUNEL assay for Pkd1 WT MEK cells, Pkd1 null MEK cells, and kidneys treated with ISO-1 or DMSO were performed according to the manufacturer’s protocols (In Situ Death Detection Kit; Roche Diagnostics). ProLong Gold Antifade reagent with DAPI (Invitrogen) was used. If images were obtained with a NIKON ECLIPSE 80i microscope.

**Mouse strain and treatment.** Pkd1fl/fl Ksp-Cre mice were used to test the effect of ISO-1 on cyst progression at PN7. Pkd1fl/fl and Ksp-Cre transgenic mice were generated as described previously (35, 55).

**Statistics.** Data are presented as mean ± SEM. Unpaired 2-tailed Student’s t test was used to determine significance of differences between 2 groups. One-way ANOVA post hoc test (IBM SPSS Version 22.0) was used to determine significance of differences in the variables among multiple groups. P values less than 0.05 were considered significant.

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