Proximal tubule ATR regulates DNA repair to prevent maladaptive renal injury responses

Seiji Kishi, …, Ryuji Morizane, Joseph V. Bonventre


Graphical abstract

Find the latest version:

http://jci.me/122313/pdf
Proximal tubule ATR regulates DNA repair to prevent maladaptive renal injury responses

Seiji Kishi, Craig R. Brooks, Kensei Taguchi, Takaharu Ichimura, Yutaro Mori, Akinwande Akinfolarin, Navin Gupta, Pierre Galichon, Bertha C. Elias, Tomohisa Suzuki, Qian Wang, Leslie Gewin, Ryuji Morizane, and Joseph V. Bonventre

1Renal Division, Brigham and Women’s Hospital, Boston, Massachusetts, USA. 2Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA. 3Department of Nephrology, Graduate School of Biomedical Sciences, Tokushima University, Tokushima, Japan. 4Department of General Medicine, Kawasaki Medical School, Kurashiki, Japan. 5Division of Nephrology and Hypertension, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA. 6Harvard Stem Cell Institute, Cambridge, Massachusetts, USA. 7Sorbonne Université, INSERM UMR S1155, AP-HP, Hôpital Tenon, Paris, France.

Maladaptive proximal tubule (PT) repair has been implicated in kidney fibrosis through induction of cell-cycle arrest at G2/M. We explored the relative importance of the PT DNA damage response (DDR) in kidney fibrosis by genetically inactivating ataxia telangiectasia and Rad3-related (ATR), which is a sensor and upstream initiator of the DDR. In human chronic kidney disease, ATR expression inversely correlates with DNA damage. ATR was upregulated in approximately 70% of Lotus tetragonolobus lectin–positive (LTL+) PT cells in cisplatin-exposed human kidney organoids. Inhibition of ATR resulted in greater PT cell injury in organoids and cultured PT cells. PT-specific Atr–knockout (ATRRPTC–/–) mice exhibited greater kidney function impairment, DNA damage, and fibrosis than did WT mice in response to kidney injury induced by either cisplatin, bilateral ischemia-reperfusion, or unilateral ureteral obstruction. ATR+/– mice had more cells in the G2/M phase after injury than did WT mice after similar treatments. In conclusion, PT ATR activation is a key component of the DDR, which confers a protective effect mitigating the maladaptive repair and consequent fibrosis that follow kidney injury.

Introduction

Acute kidney injury (AKI) occurs in approximately 13% of hospitalized patients and is associated with high mortality, especially in critically ill patients (1). AKI also predisposes patients to chronic kidney disease (CKD), which affects approximately 10% of the world’s population (2–4). Renal proximal tubule epithelial cells (RPTECs) comprise the bulk of the renal parenchyma and are the primary targets of a large variety of ischemic and toxic insults (2, 5, 6). Studies from our laboratory that were also confirmed by others have implicated the DNA damage response (DDR) in the maladaptive repair process after injury (7, 8). However, to our knowledge, there have been no studies in which modification of a DDR component in a specific compartment of the kidney has been evaluated for its effect on the long-term response of the kidney to injury.

DNA damage is a feature of many forms of kidney injury (9–12). In response to DNA damage, the cell activates a unique DDR signaling pathway that varies depending on the type of DNA injury. The DDR is activated when 1 or more of the sensor kinases, including ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK), detect a DNA strand break. The sensor kinases then activate effectors that upregulate the checkpoint kinases, including checkpoint kinases 1 and 2 (Chk1 and Chk2), which regulate cell-cycle progression through the G1/S or G2/M checkpoint (13–18). In vitro studies suggest that the DDR is cytoprotective against ischemia and ATP depletion (9). Activation of the DDR generally results in DNA repair, but when DNA damage is severe, the ability of the DDR to repair DNA may be overcome. In this case, cells with prolonged DNA damage may enter a state of cell-cycle arrest or undergo apoptosis. We have shown that RPTEC G2/M cell-cycle arrest in response to injury results in the generation of proinflammatory and profibrotic cytokines, which promote the development of kidney fibrosis (7, 8).

ATR is a phosphoinositide 3-kinase–like kinase that is activated by recognition of single-strand breaks generated after replication fork stalling or as intermediates in nucleotide excision repair and homologous recombination repair. ATR largely acts through the phosphorylation of Chk1 to stop the cell cycle and activate the DDR (14, 15). Mutations of the ATR gene in humans have been linked to the development of Seckel syndrome (17), which is characterized by microcephaly, growth retardation, and congenital anomalies of the skeletal system. ATR is also involved in the regulation of cell-cycle arrest in response to DNA damage, which is essential for maintaining genomic stability and preventing tumor development.

Related Commentary: p. 4574

Authorship note: SK and CRB contributed equally to this work.
Conflict of interest: JVB and TI are co-inventors on KIM-1 patents (Molecules and methods for inhibiting shedding of KIM-1, patent no. 7696321; Kidney injury–related molecules, patent no. 6664385), which have been assigned to Partners Healthcare. JVB and RM are co-inventors on patents (PCT/US16/52350) on organoid technologies that are assigned to Partners Healthcare. JVB is a consultant to Aldeya, Anglon, Goldilocks, and Medimmune. He is also a consultant to and holds equity in MediBeacon, Sentien Biotech, Thrasos Therapeutics, and Goldfinch Bio and has received grant support from Boehringer Ingelheim.
Copyright: © 2019, American Society for Clinical Investigation.
Submitted: June 6, 2018; Accepted: July 23, 2019; Published: October 7, 2019.
by abnormally slow growth during fetal development and post-natally. Studies in mice showed that constitutive deletion of ATR in adulthood leads to increased DNA damage, defects in tissue homeostasis, and the rapid appearance of age-related phenotypes, such as hair-graying, alopecia, kyphosis, osteoporosis, thymic involution, and other abnormalities, similar to what is seen in progeroid (accelerated aging) syndromes in humans (19–21). ATR is activated in RPTECs in vitro and in vivo in response to cisplatin administration (10). Our goal was to better understand the role of ATR in the DDR to injury of RPTECs and its contribution to the maladaptive initiation and progression of interstitial fibrosis (7, 8, 22–25).

We report that humans with CKD have RPTEC activation of ATR and extensive DNA damage, marked by phosphorylation of the histone H2A variant H2AX (γH2AX), with ATR expression inversely related to the degree of tissue fibrosis. γH2AX is important for the recruitment and localization of DNA repair proteins (26). ATR and γH2AX are also markedly upregulated in the RPTECs of kidney organoids derived from human pluripotent stem cells after tubular injury is induced with cisplatin. We hypothesized that reduced RPTEC expression of ATR would result in more cells with unrepaired DNA damage and lead to increased maladaptive repair of the kidney following injury. To test this hypothesis, we specifically deleted the Atr gene from RPTECs in adult mice and then subjected these mice to tubular injury with either bilateral ischemia-reperfusion, cisplatin, or unilateral ureteral obstruction (UOO). We found that the animals with RPTEC deletion of the Atr gene had more cumulative DNA damage, apoptosis, acute impairment of kidney function, and worse kidney fibrosis following ischemia and UOO when compared with littermate controls with intact RPTEC Atr expression. These results were corroborated by in vitro studies of RPTECs and kidney organoids derived from human stem cells. Cumulatively, our findings suggest that after tubular injury, ATR plays an important protective role in RPTECs that leads to less maladaptive repair and kidney fibrosis.
PT Atr gene deletion exacerbates cisplatin-induced AKI. To evaluate whether ATR plays a role in cisplatin-induced RPTEC injury in vivo, we depleted the Atr gene specifically from mouse RPTECs. Atr conditional–knockout mice were generated by crossing Atrfl/fl mice with Atr heterozygous Slc34a1-GFP CreERT2 (Atr+/−/Slc34a1-GFP/+ (CE/−)) mice (19, 39). The resulting Atrfl/fl Slc34a1-GFP/+ mice were bred together to generate Atrfl/fl Slc34a1-GFP/+ or Atrfl/fl Slc34a1-GOE/+ mice, in which Atr was deleted in a tamoxifen-dependent manner (ATRPGC−−). These mice were compared with littermate controls, which carried the ATR-floxed allele but were not treated with tamoxifen (ATRCtrl). Treatment of ATRfl/fl Slc34a1-GFP/+ mice with 4-OH-tamoxifen resulted in efficient deletion of the Atr gene in a population of renal proximal tubular cells, as evidenced by increased numbers of KSP+ cells trended toward an increase (Figure 3A). The outer medulla of γH2AX+ and γH2AX+ foci were quantified and compared with the inner medulla in both regions of the kidney, with a greater increase in the outer medulla (Figure 3C). Ninety-six hours after cisplatin treatment, we observed no difference in the number of Ki67+ tubular or interstitial cells between ATRPGC−− and ATRPGC−+ mice (Figure 3D). As shown in Figure 3E, the majority of the Ki67+ cells did not stain for F4/80 or α-smooth muscle actin (α-SMA), indicating that the majority of cells were tubular cells and not macrophages or myofibroblasts.

Nine-six hours after cisplatin injection, we evaluated the p53/p21 signaling pathway, an effector pathway of the DDR that is downstream of ATR and known to initiate G2/M cell-cycle arrest. We detected higher p53 protein expression in ATRPGC−− mice when compared with ATRPGC−+ mice (Figure 3B). Caspase 3 activation was higher in the outer medulla than in the cortex in ATRPGC−− mice, whereas ATRPGC−+ mice showed increased caspase 3 activation in both regions of the kidney, with a greater increase in the outer medulla (Figure 2A). Kaplan-Meier survival analysis demonstrated that cisplatin administration (20 mg/kg i.p.) to ATRPGC−− mice led to significantly increased mortality by day 7 when compared with ATRPGC−+ mice (Figure 2B). In addition, although there were no significant differences in body weight between ATRPGC−− and ATRPGC−+ mice after saline vehicle treatment, following cisplatin treatment, ATRPGC−− mice lost significantly more weight when compared with their ATRPGC−− littermates (Figure 2C). Serum creatinine and blood urea nitrogen (BUN) levels in response to cisplatin-induced tubular injury were significantly higher in ATRPGC−− mice compared with levels in ATRPGC−+ mice (Figure 2, D and E). Consistent with greater kidney dysfunction, ATRPGC−− mice had higher tubular injury scores (composite of necrotic tubules, cast formation, and dilated tubules) when compared with scores for ATRPGC−+ mice.
Figure 3. ATRRPTC–/– mice have enhanced DNA damage and apoptosis after cisplatin injection. (A) Representative images of γH2AX-stained kidney sections from ATRCtrl and ATRRPTC–/– mice 96 hours after saline or cisplatin injection. Scale bar: 50 μm. Dot plots show quantification of γH2AX+ cells, percentage of cells with nucleus-wide γH2AX+ staining or γH2AX+ foci, and γH2AX+ cells in cortex versus outer medulla. Saline: ATRCtrl (n = 3), ATRRPTC–/– (n = 4); cisplatin: ATRCtrl (n = 9), ATRRPTC–/– (n = 11). (B) Representative images of cleaved caspase 3–stained kidney sections from ATRCtrl and ATRRPTC–/– mice 96 hours after saline or cisplatin injection. Scale bar: 50 μm. Dot plot shows corresponding quantification of cleaved caspase 3+ cells. Saline: ATRCtrl (n = 3), ATRRPTC–/– (n = 4); cisplatin: ATRCtrl (n = 9), ATRRPTC–/– (n = 11). (C) Immunostaining for cleaved caspase 3 and DAPI in kidneys from ATRCtrl and ATRRPTC–/– mice 96 hours after saline or cisplatin injection. Upper panels: Stitched images represent approximately 25% of the kidney cross-sectional area (original magnification, ×200). Lower panels: Higher-magnification images from the boxed regions in the stitched (upper) images (scale bar: 500 μm). Dot plot shows quantification of cleaved caspase 3 staining from the whole kidney section images in C relative to the LTL area of uninjured kidney tissue. Saline: ATRCtrl (n = 4), ATRRPTC–/– (n = 4); cisplatin: ATRCtrl (n = 6), ATRRPTC–/– (n = 8). (D) Representative images of Ki67-stained kidney sections from ATRCtrl and ATRRPTC–/– mice 96 hours after saline or cisplatin injection. Scale bar: 50 μm. Dot plot shows corresponding quantification of Ki67+ cells. Saline: ATRCtrl (n = 3), ATRRPTC–/– (n = 4); cisplatin: ATRCtrl (n = 9), ATRRPTC–/– (n = 9). (E) Representative images of F4/80- and Ki67-stained and α-SMA– and Ki67-stained sections of injured kidneys from ATRRPTC–/– mice 96 hours after cisplatin injection. Scale bar: 50 μm. (F) Representative Western blot. Each lane represents 1 sample from an individual mouse. Dot plots show quantification of Western blot band intensity for p53 and p21, 96 hours after cisplatin injection. ATRCtrl (n = 6), ATRRPTC–/– (n = 6). Also shown is a dot plot of quantitative RT-PCR analysis of p21 mRNA levels in ATRCtrl and ATRRPTC–/– kidneys. Saline: ATRCtrl (n = 3), ATRRPTC–/– (n = 4); cisplatin: ATRCtrl (n = 6), ATRRPTC–/– (n = 7). Data are presented as the mean ± SEM. Statistical significance was determined by 2-tailed, unpaired t test (A, B, D, and F). For cisplatin ATRCtrl vs. cisplatin ATRRPTC–/– and 1-way ANOVA followed by Tukey’s post-hoc test (C). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. See the complete unedited blots in the supplemental material.

We complemented the in vivo experiments with studies in cultured mouse RPTECs, pig kidney LLC-PK1 cells, and human kidney organoids. RPTECs and LLC-PK1 cells treated with cisplatin plus the ATR inhibitor VE-821 had significantly higher caspase 3 activation compared with cisplatin treatment alone (Figure 4, C and D). Next, we tested the effect of ATR inhibition on cisplatin-induced injury in human kidney organoids. Organoids treated with cisplatin plus VE-821 had a significant reduction in the number of LTL+ tubules and an increased percentage of LTL+ cells double-positive for pH3 and Ki67 (Figure 4, E–G). These data demonstrate that deletion of ATR in RPTECs, LLC-PK1 cells, and human kidney organoids significantly worsens cisplatin-induced kidney injury and increases the number of cells in the G2/M phase of the cell cycle.

ATR gene deletion promotes G2/M arrest in cisplatin-injured RPTECs. The results in Figure 4A suggest that there was an accumulation of cell-cycle–arrested RPTECs following injury in the ATRRPTC–/– mice. To determine whether RPTECs were arrested in G2/S or G2/M, the cell cycle was analyzed using the fluorescence ubiquitination–based cell-cycle indicator (FUCCI) (47). In primary RPTECs isolated from FUCCI2α mice, cisplatin treatment resulted in a marked increase in G2 and a reduction in S/G2/M-phase cells, suggesting activation of the G2/M checkpoint. When the ATR inhibitor VE-821 was added with cisplatin, we observed a marked increase in S/G2/M-phase cells (Figure 5A). Live microscopy cell-cycle analysis of HK-2 cells expressing Fucci revealed that inhibition of p53 by pifithrin-α resulted in an increase in mKO–Cd1+ G2-phase cells, with a corresponding decrease in mAG–geminin S/G2-phase cells. By comparison, incubation with rigosertib, a G2/M arrest inducer, increased the number of cells in the G2/M phase of the cell cycle (Figure 5, B and C). After establishment in primary culture, under basal conditions, we noted increased numbers of primary RPTECs from ATRRPTC–/– mice in G2/M relative to cells from WT mice, indicating an increased sensitivity of ATRRPTC–/– cells to the stresses of isolation and culture, activating the G2/M checkpoint (Figure 5, D–F). These findings indicated that ATR inhibition leads to increased G2/M cell-cycle arrest, which our laboratory and others have linked to maladaptive repair and fibrosis, and that p53 inhibition reduces G2/M arrest.

ATR gene depletion in RPTECs results in increased profibrotic changes after ischemia-reperfusion injury. ATR is upregulated following bilateral ischemia-reperfusion injury (IRI) in mice (Figure 6A). To investigate the role of Atr deletion in the transition from AKI to CKD after IRI, ATRRPTC–/– and control mice were subjected to 28 minutes of bilateral IRI. Decreased total kidney Atr mRNA was confirmed by qPCR in ATRRPTC–/– mice on day 28 after IRI (Figure 6B). We observed no differences in serum creatinine or BUN at 24 hours, indicating similar degrees of initial injury in the ATRRPTC–/– and ATRCtrl animals. However, 3 and 7 days after IRI, we observed delayed recovery among the ATR RPTC–/– mice (Figure 6, C and D), as reflected by persistent elevations in serum creatinine levels in ATRRPTC–/– mice compared with those in ATRCtrl mice. The differences in creatinine levels persisted for at least 7 days, and differences in BUN persisted up to day 21 after IRI in ATRRPTC–/– mice when compared with ATRCtrl mice (Figure 6D). Kidney histologic examination revealed substantially greater tissue damage (tubular dilation and loss of brush border) in ATRRPTC–/– mice on day 7 after ischemic injury (Figure 6E). Twenty-eight days after IRI, when compared with ATRCtrl mice, the ATRRPTC–/– mice had significantly more extracellular matrix collagen deposition, as quantified by Masson’s trichrome (MT) staining (Figure 6F). Immunofluorescence (IF) staining of kidney cortices for α-SMA expression was also greater in ATRRPTC–/– mice, reflecting an increase in myofibroblast activation when compared with ATRCtrl mice that had similar amounts of tubular injury at onset (Figure 6G). To quantify tubular cell loss, we measured the extent of KSP expression. ATRRPTC–/– mice had a greater reduction in KSP+ tubular cell areas when compared with ATRCtrl animals, which was consistent with increased tubular atrophy (Figure 6G). In addition, real-time PCR analysis of kidney samples 28 days after IRI showed increased, or a tendency toward increased, mRNA levels of genes related to interstitial fibrosis (TGFB-β, α-SMA, collagen type 1α1 [Coll1A1]) and the DDR (p21) in ATRRPTC–/– mice compared with mRNA levels in ATRCtrl mice (Figure 6, H–K). Twenty-eight days after IRI, more RPTECs were colabeled for both Ki67 (a marker of cell proliferation) and the phospho-histone H2AX (γH2AX) (Figure 6G). The Journal of Clinical Investigation
Figure 4. Atr gene deletion in RPTCs leads to increased cleaved caspase 3 and G2/M-phase cells after cisplatin injection. (A) Representative images of pH3-stained kidney sections from ATRCtrl and ATRRPTC–/– mice 96 hours after saline or cisplatin injection and the corresponding quantification of pH3+ nuclei. Scale bar: 50 μm. Saline: ATRCtrl (n = 3), ATRRPTC–/– (n = 4); cisplatin: ATRCtrl (n = 6), ATRRPTC–/– (n = 6). (B) Representative images of F4/80-stained kidney sections from ATRCtrl and ATRRPTC–/– mice 96 hours after saline or cisplatin injection. Scale bar: 50 μm. Representative Western blots of cleaved caspase 3 expression in mouse RPTCs (C) and LLC-PK1 cells (D) treated with cisplatin, VE-821, or a combination of both. n = 3 independent experiments. (E) Representative images of H9 cell–derived day-64 organoids treated with either cisplatin (5 μM) or vehicle (DMSO) for 24 hours, with or without 10 μM VE-821 pretreatment. Sections of the organoids were stained for LTL, pH3, Ki67, and DAPI. Scale bar: 50 μm; inset shows a high-power magnification of a triple-positive tubule. (F) Quantitation of LTL+ tubules and (G) percentage of pH3+, Ki67+, and LTL+ cells to LTL+ cells (n = 2 × 10 high-power fields [HPF] in each treatment group). Data are presented as the mean ± SEM. Statistical significance was determined by 2-tailed, unpaired t test (A and B, cisplatin ATRCtrl vs. cisplatin ATRRPTC–/–) and 1-way ANOVA followed by Tukey’s post-hoc test (C, D, F, and G) *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Figure 5. Atr deletion promotes G2/M arrest in cisplatin injured RPTECs. (A) Schematic diagram showing the timing of the FUCCI2a fluorescence reporters. Red indicates mCherry; green indicates mVenus. Dot plots show the cell-cycle profile and proportion of FUCCI RPTECs in G1, early S, and S/G2/M phases, as analyzed by flow cytometry. Primary RPTECs from FUCCI2a-gGT-Cre mice were synchronized in 1% FBS PTC media and then treated with cisplatin, with or without VE-821, or were left untreated for 48 hours. Cells were then fixed and analyzed by flow cytometry. Red and green colors in the graph indicate G1 and S/G2/M phases, respectively, and double-positive cells were considered to be in the early S phase. RFP, red fluorescent protein; GFP, green fluorescent protein. (B and C) Cell-cycle assessment by live microscopy of HK2 cells expressing the FUCCI system in control conditions (Ctrl, n = 4), or incubated with 50 nM pifithrin-α (PF50, n = 2) or 10 nM rigosertib (RG10, n = 2). (B) Representative images of the cells at 1 hour and 24 hours after incubation are shown. Images on the left show overlays of bright-field, monomeric Kusabira-Orange (mKO), and monomeric Azami-Green (mAG) channels. Images on the right show mKO and mAG channels (original magnification, ×10). (C) Graph shows the quantification over time of the cells in G1 (mKO-Cdt1, red), G2 (mAG-geminin, green), and S (mKO-Cdt1⁺ mAG-geminin, orange) phases of the cell cycle. (D) Cell-cycle analysis by propidium iodide staining and flow cytometry of mouse primary cells at baseline (top 2 graphs) and after treatment with cisplatin at 0.2 μg/mL for 48 hours (bottom 2 graphs). (E and F) Results of the cell-cycle analysis for 3 independent experiments. Data are presented as the mean ± SEM. Statistical significance was determined by 1-way ANOVA followed by Tukey’s post hoc test. *P < 0.05 and **P < 0.01.
Figure 6. Atr gene depletion in RPTECs results in increased profibrotic changes after IRI. (A) Representative images of ATR-stained sections of kidneys 48 hours after IRI or sham operation. Scale bar: 50 μm. (B) Real-time PCR analysis of Atr mRNA levels in ATRCtrl and ATRRPTC−/− kidneys on day 28 after IRI. ATRCtrl (n = 6), ATRRPTC−/− (n = 6). (C and D) Changes in serum creatinine and BUN following IRI. Sham operation: ATRCtrl (n = 3), ATRRPTC−/− (n = 3); IRI: ATRCtrl (n = 8), ATRRPTC−/− (n = 8). (E) Representative images of PAS-stained kidneys from ATRCtrl and ATRRPTC−/− mice on day 7 after IRI. Scale bars: 300 μm (top) and 50 μm (bottom). (F) Representative images of MT-stained kidneys from ATRCtrl and ATRRPTC−/− mice on day 28 after IRI or sham operation. Dot plot shows the quantification of the MT+ area. Scale bar: 100 μm. (G) Representative images of KSP- and α-SMA-stained kidney sections from ATRCtrl and ATRRPTC−/− mice 28 days after IRI or sham operation. Dot plots show the quantification of KSP+ and α-SMA+ areas. Scale bar: 50 μm. (H–K) RT-PCR analysis of TGF-β, α-SMA, Col1a1, and p21 mRNA levels in ATRCtrl and ATRRPTC−/− kidneys on day 28 following IRI. Sham operation: ATRCtrl (n = 3), ATRRPTC−/− (n = 3); IRI: ATRCtrl (n = 8), ATRRPTC−/− (n = 8). Data are presented as the mean ± SEM. Statistical significance was determined by 2-tailed, unpaired t test (IRI ATRCtrl vs. IRI ATRRPTC−/−). *P < 0.05 and **P < 0.01.
activity in ATRCtrl animals (Figure 7B). Taken together, these data suggest that a lack of ATR in RPTECs predisposes kidneys to maladaptive repair, with increased numbers of RPTECs in the G2/M cell-cycle phase, increased senescence, and increased fibrosis following tubular injury.

Deletion of the RPTEC Atr gene results in more severe kidney injury, increased DNA damage, apoptosis, G2/M-phase cells, and fibrosis after UUO. As the third model of kidney injury, we investigated the role of RPTEC ATR in the development of kidney fibrosis after UUO. DNA damage and DNA repair activity are related to the progression of kidney fibrosis after UUO (49, 50). ATRRPTC–/– mice had a higher histological tubular injury score 7 days after UUO compared with ATRCtrl mice (Figure 8A). In addition, KIM-1 was more highly expressed at the protein level 7 days after UUO in ATRRPTC–/– mice (Figure 8, B and C). Coincident with enhanced kidney injury, we found that the number of γH2AX+ cells on day 7 after UUO was significantly increased in ATRRPTC–/– mice when compared with ATRCtrl animals (Figure 8D). Furthermore, apoptosis, as measured by cleaved caspase 3 staining, was also significantly greater in ATRRPTC–/– mice (Figure 8E). These results indicated that the lack of ATR in RPTECs leads to an increase in the amount of DNA damage and apoptosis after UUO.

MT staining revealed greater collagen content in ATRRPTC–/– mice compared with ATRCtrl mice (Figure 9A). Moreover, ATRRPTC–/– mice had a significant increase in kidney α-SMA area after UUO and a decrease in KSP+ tubular cells compared with ATRCtrl mice (Figure 9B). The contralateral kidney (CLK) was used as a control for the obstructed kidney. We noted no significant differences between the CLKs of ATRCtrl and ATRRPTC–/– mice on day 28 following IRI and the corresponding quantification of Ki67 and pH3 nuclei. ATRCtrl (n = 7), ATRRPTC–/– (n = 5). Scale bar: 50 μm. (B) Representative images of SA-β-gal–stained kidney sections from ATRCtrl and ATRRPTC–/– mice 28 days after IRI or sham operation. Scale bar: 50 μm. Dot plots show the quantification of SA-β-gal–stained area. IRI: ATRCtrl and ATRRPTC–/– mice (n = 5 mice and 10 kidneys). Data are presented as the mean ± SEM. Statistical significance was determined by 2-tailed, unpaired t test (IRI ATRCtrl vs. IRI ATRRPTC–/–). *P < 0.05 and **P < 0.01.
**A**

ATR<sup>WT</sup> vs. ATR<sup>RPTC-/-</sup> for CLK and UUO.

**B**

ATR<sup>WT</sup> vs. ATR<sup>RPTC-/-</sup> for CLK and UUO.

**C**

Western blot for KIM-1 and β-Actin. UUO.

**D**

ATR<sup>WT</sup> vs. ATR<sup>RPTC-/-</sup> for CLK and UUO.

**E**

ATR<sup>WT</sup> vs. ATR<sup>RPTC-/-</sup> for CLK and UUO.

**αH2AX (DAPI)**

**Nucleus-wide**

**Foci**

**Cleaved caspase 3 (DAPI)**

**UUO**

**CLK**
Figure 8. Deletion of RPTEC Atr results in more severe kidney injury and increased DNA damage and cleaved caspase 3 after UUO. (A) Representative kidney histological images of PAS-stained kidney sections 7 days after UUO. Scale bar: 50 μm. Dot plot shows quantified tubular injury score. ATR Ctrl (n = 4), ATR RPTC–/– (n = 5). (B) Representative images of KIM-1-stained kidneys from ATR Ctrl and ATR RPTC–/– mice 7 days after UUO. Scale bar: 50 μm. (C) Western blotting was performed on UUO-injured kidney lysates to determine KIM-1 expression. Each lane represents 1 sample from an individual mouse. Dot plot shows the corresponding quantification of band intensity. ATR Ctrl (n = 3), ATR RPTC–/– (n = 5). (D) Representative images of H2AX-stained sections of CLKs and injured kidneys from ATR Ctrl and ATR RPTC–/– mice 7 days after UUO. Scale bars: 50 μm. Dot plot shows the corresponding quantification of H2AX+ cells and of nucleus-wide γH2AX+ foci. ATR Ctrl (n = 4), ATR RPTC–/– (n = 5). Scale bars: 50 μm. (E) Representative images of cleaved caspase 3–stained sections of CLKs and injured kidneys from ATR Ctrl and ATR RPTC–/– mice 7 days after UUO. Scale bar: 50 μm. Dot plot shows the corresponding quantification of cleaved caspase 3+ cells. ATR Ctrl (n = 4), ATR RPTC–/– (n = 5). Data are presented as the mean ± SEM. Statistical significance was determined by 2-tailed, unpaired t test (UUO ATR Ctrl vs. ATR RPTC–/–). *P < 0.05 and **P < 0.01. The complete unedited blots are provided in the supplemental material.

We identified TASSC formation in humans and animal models with CKD and linked it to the progression of kidney fibrosis in CKD (25). TASSC formation is critical for the AKI-to-CKD transition, since inhibition of TASSCs after the injury phase of AKI prevents CKD (25). To evaluate whether ATR inhibition increased TASSC formation, we treated kidney organoids with cisplatin. TASSC formation was significantly elevated 24 hours after cisplatin exposure and remained elevated over the long term (120 hours) (Figure 10A). We also examined TASSC formation in ATR RPTC–/– mice. Following UUO, we found that TASSC formation was significantly increased in ATR RPTC–/– mice compared with littermate controls (Figure 10B and Supplemental Video 1), as evaluated by super-resolution structured illumination microscopy (SIM). These data suggest that ATR inhibition or deletion promotes fibrosis in part by inducing the formation of TASSCs.

Discussion

This study reveals, for the first time to our knowledge, the importance of a DDR protein in post-injury repair in RPTECs, which comprise approximately 80% to 90% of the kidney cortex. Our previous study, as well as subsequent studies by other investigators, demonstrated that maladaptive DNA repair, with RPTECs arrested in the G2/M phase of the cell cycle, is an important mechanism underlying the progression from AKI to CKD (7, 52). We hypothesized that RPTEC-specific deletion of a DDR mediator would cause a reduction in DDR signaling, decrease adaptive repair after injury, and hasten chronic fibrotic changes to the kidney due to dysfunctional DNA repair. We found that ATR, a sensor of DNA damage and an initiator of the DDR signaling cascade (53, 54), was activated in patients with CKD and in mice with different models of CKD. We studied the role of RPTEC ATR in 3 different mouse models of tubular injury: cisplatin nephrotoxicity, ischemia-reperfusion, and UUO, as well as in RPTECs in culture and human kidney organoids. Each type of tubular insult has been shown to cause DNA damage in RPTECs through alkylation, oxidative stress, or oxygen free radical release (7, 9, 25, 55). Atr constitutive gene loss results in embryonic lethality (56), making it essential to use a conditional inducible system in mouse models of disease. At baseline, we found that similar levels of acute tubular injury were generated in either ATR RPTC–/– or ATR Ctrl mice after ischemia. Despite the similar degree of injury during the acute phase of injury, we observed a difference in the repair and chronic phases of injury in the ATR RPTC–/– mice, which developed more maladaptively injured cells in the G2/M phase and more fibrosis compared with ATR Ctrl mice. In the UUO model, which is characterized by greatly accelerated fibrosis, we observed increased tubular injury and fibrosis in ATR RPTC–/– mice. With cisplatin injury, the maladaptive repair in ATR RPTC–/– mice was associated with increased apoptosis, markers of kidney injury, and cell death when compared with ATR Ctrl mice. Experiments in both human kidney organoids and RPTEC lines confirmed that ATR inhibition promoted caspase activation and resulted in more cells in the G2/M phase of the cell cycle in response to cisplatin injury.

Previous studies by our group and others have shown that surviving TECs are stimulated to proliferate in order to replace lost RPTECs after AKI, and the severity of the injury determines the long-term outcome (3, 21–24). Maladaptive repair associated with G2/M arrest can lead to senescence and fibrosis. In the DDR pathway, ATM, ATR, and p53 play critical roles in regulating the balance between cell survival and death (24, 57). When DNA damage is relatively mild, the DDR can repair the DNA and prevent cell death. On the other hand, when the damage is severe, the DDR can induce tubular cells to go into cell-cycle arrest or apoptosis. In this study, we showed that, compared with WT mice, inhibition of the DDR in kidney RPTECs by genetic inactivation of Atr expression led to increased expression of markers of DNA damage and kidney injury as well as greater functional impairment following kidney injury. In effect, we reduced the number of cells that could contribute to recovery. In addition, deletion or inhibition of ATR also resulted in an increase in maladaptive repair, cell-cycle arrest at the G2/M checkpoint, and subsequent fibrosis and CKD. Thus, the maladaptive response includes inadequate salvaging of sublethally injured cells to replace lost cells as well as cell-cycle arrest, i.e., G2/M arrest, in surviving cells, which contributes to longer-term tissue injury and CKD (7, 57).

Following injury and induction of DNA damage, ATR-deficient cells have been shown to bypass the G2/M checkpoint first and undergo mitosis, but with daughter cells that have persistently unrepaired DNA damage that undergo more G2/M arrest (20, 48). Cumulative unresolved DNA damage can cause cells to undergo permanent cell-cycle arrest and senescence or, alternatively, may cause cell death through apoptosis (15, 56). We confirmed that G2/M arrest occurred in our models by performing flow cytometric analysis of primary RPTECs expressing FUCCI2a, using recently published methods that rule out other sources of >2n DNA such as polyploidy (58), which recent data suggest is not as prominent as had been previously reported (58). Thus, pathways such as the DDR that control the fate of cells can determine the outcome of kidney injury and provide important clues to therapeutic targets.

The DDR is a cellular mechanism that has evolved over time for the detection and repair of DNA strand breaks resulting from a myriad of endogenous and exogenous factors. Although the DDR generally protects against disease, hypo- or hyperactivation can result in a less efficient DDR that contributes to human patholo-
RESEARCH ARTICLE

A

ATR<sup>CH</sup> ATR<sup>PTC−/−</sup>

CLK

UUO

MT area (%)

B

ATR<sup>CH</sup> ATR<sup>PTC−/−</sup>

CLK

UUO

KSP area (%)

C

TGF-β mRNA (fold increase)

D

CTGF mRNA (fold increase)

E

α-SMA mRNA (fold increase)

F

Coll II mRNA (fold increase)

G

Fibronectin mRNA (fold increase)

H

PDGF-B mRNA (fold increase)

I

ATR<sup>CH</sup> ATR<sup>PTC−/−</sup>

CLK

UUO

K<sup>67</sup>/DAPI

J

F4/80/K<sup>67</sup>/DAPI α-SMA/K<sup>67</sup>/DAPI

K

ATR<sup>CH</sup> ATR<sup>PTC−/−</sup>

CLK

UUO

I<sup>67</sup>/DAPI

L

p53 mRNA (fold increase)

M

p21 mRNA (fold increase)
Figure 9. The increase in fibrosis and G$_{j}$/M phase cell cycle is greater in ATR$^{RPTC/-}$ mouse kidneys after UUO. (A) Representative kidney histological images of MT-stained sections 7 days after UUO and corresponding quantification of MT-areas. ATR$^{Ctrl}$ (n = 4), ATR$^{RPTC/-}$ (n = 5). Scale bar: 50 μm. (B) Representative images of KSP- and α-SMA-stained sections of CLKs and injured kidneys from ATR$^{Ctrl}$ and ATR$^{RPTC/-}$ mice on day 7. Scale bar: 50 μm. (C-H) Quantitative RT-PCR analysis of TGF-β, CTGF, α-SMA, Colla1, fibronectin, PDGF-β, p53, and p21 mRNA levels in kidneys from ATR$^{Ctrl}$ and ATR$^{RPTC/-}$ mice on day 7. ATR$^{Ctrl}$ (n = 3), ATR$^{RPTC/-}$ (n = 5). (I) Representative images of Ki67-stained sections of CKLs and injured kidneys from ATR$^{Ctrl}$ and ATR$^{RPTC/-}$ mice on day 7. Scale bar: 50 μm. (J) Representative images of pH3-stained sections of CLKs and injured kidneys from ATR$^{Ctrl}$ and ATR$^{RPTC/-}$ mice on day 7. Scale bars: 50 μm. (K) Representative images of α-actin-stained sections of CLKs and injured kidneys from ATR$^{Ctrl}$ and ATR$^{RPTC/-}$ mice on day 7. Scale bar: 50 μm. Data are presented as the mean ± SEM. Statistical significance was determined by 2-tailed, unpaired t test (UUO ATR$^{Ctrl}$ vs. UUO ATR$^{RPTC/-}$).

In conclusion, we have demonstrated that RPTEC ATR, a sensor of DNA damage, is crucial for adaptive repair and that, in its absence, there is enhanced maladaptive kidney repair with an increased number of senescent cells in the G$_{j}$/M phase of the cell cycle and an increased profibrotic secretory phenotype with consequent development of kidney tissue fibrosis.
Kidney organoids were generated from the H9 hESC line (WiCell; originally from James Thomson, University of Wisconsin, Madison, Wisconsin, USA) as previously described (37, 38, 77, 78). Kidney organoids were maintained in ultralow adhesion, 96-well plates (Corning) in 200 μL Advanced RPMI (Life Technologies, Thermo Fisher Scientific) supplemented with 1× GlutaMAX (Life Technologies, Thermo Fisher Scientific) and sustained with half exchanges (100 μL) of fresh media 3 times per week at regular intervals. Nephron organoids were treated with cisplatin (MilliporeSigma) (5 μM in Advanced RPMI/1× GlutaMAX) or vehicle (Advanced RPMI/1× GlutaMAX alone) for 24 hours, beginning on day 49 after induction of directed differentiation to the kidney cell lineages.

Kidney organoid tissue preparation. Nephron organoid samples were fixed with 4% paraformaldehyde for 1 hour at room temperature. Fixed samples (3 organoids per condition) were subsequently immersed in 30% (w/v) sucrose and then embedded in O.C.T. (Sakura FineTek), and 10-μm frozen sections were cut.
Kidney organoid IF analysis. Cryosections of 10-μm thickness were mounted onto Denville white-frosted, positively charged (Denville) microscope slides, air dried, and treated for IF analysis as described previously (79). Primary antibodies against the following proteins were used: ATR (goat polyclonal, 1:100, catalog sc1887, Santa Cruz Biotechnology); pATR (Ser428) (rabbit polyclonal, 1:100, catalog 2853, Cell Signaling Technology); pH3 (rabbit, 1:100, catalog 06-570, MilliporeSigma); H2AX (Ser139) (rabbit, 1:250, catalog 05-636, MilliporeSigma); Ki67 (Abcam ab15580); mTOR γH2AX) (mouse monoclonal, 1:250, MilliporeSigma); H2AX (Ser139) (mouse monoclonal, 1:250, MilliporeSigma; Ki67 (Abcam ab15580); mTOR γH2AX) (mouse monoclonal, 1:250, MilliporeSigma); H2AX (Ser139) (mouse monoclonal, 1:250, MilliporeSigma; Ki67 (Abcam ab15580); mTOR γH2AX) (mouse monoclonal, 1:250, MilliporeSigma). Sections were then blocked with BSA and normal donkey serum, followed by staining with anti-mTOR (Cell Signaling Technology, 7C10) and/or anti-GFP (Molecular Probes, 7C10) antibodies and DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, SP-2002). FITC-, Cy3-, or Cy5-conjugated secondary antibodies (Life Technologies, Thermo Fisher Scientific) were used according to the manufacturer’s instructions. Nuclei were counterstained using VECTASHIELD with DAPI (catalog H-1200, Vector Laboratories). Images were obtained by confocal microscopy (Nikon C1 Eclipse, Nikon).

Kidney organoid experiment with cisplatin and VE-821. Kidney organoids on day 64 were treated with 10 μM VE-821 or control DMSO in advanced RPMI for 6 hours (pretreatment). A 5-μM final concentration of cisplatin or control saline was added to the media. After 24 hours of exposure to cisplatin, media were replaced with new advanced RPMI. Twenty-four hours after changing media or 120 hours later (long term), the organoids were fixed and prepared as described above.

Genetically modified mice. To inactivate Atr in a tissue-specific fashion, mice carrying the PT-specific promoter Slc34a1 driving the eGFPCreERT2 fusion protein (Slc34a1GCE) (The Jackson Laboratory; originally from Benjamin Humphreys, Washington University, St. Louis, Missouri, USA) (39) were crossed with mice carrying only 1 normal Atr allele (Atr+/−) mice. The use of Atr−/−/-null alleles has previously been shown to enhance the phenotype of Atr tissue-specific knockouts (19). The Atr+/−Slc34a1GCE/+ progeny were mated to generate Atr+/−Slc34a1GCE+/− mice. Atr−/−Slc34a1GCE−/− mice were crossed to produce Atr−/−Slc34a1GCE−/− and Atr+/−Slc34a1GCE+/− mice, which were used for experiments (ATR−/−). Mice carrying the floxed allele but lacking Cre, Atr−/−Slc34a1GCE−/−, or Atr+/−Slc34a1GCE+/− were used as controls (ATR+/+). The Atr−/− and Atr+/− mice were provided by Eric J. Brown (Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA).

Animal experiments. To upregulate Slc34a1GCE expression, mice were fed a low-phosphorus (0.06%) diet (TestDiet) (39). For Atr deletion in RPTECs, adult mice (8-10 weeks of age) were i.p. administered every other day 3 doses (3-mg) of tamoxifen (MilliporeSigma) dissolved in 3% (vol/vol) ethanol-containing corn oil (MilliporeSigma), and cisplatin was injected 1 week after the last dose. For IRI and UUO, the mice were treated with 3 doses of tamoxifen by oral gavage (10 mg every other day), and surgery was performed 1 week after the last dose. In all cases, littermate control phenotypes were compared.

IRI. Ischemia for 28 minutes at 37°C was induced in both kidneys using a bilateral flank approach as previously reported (80). Warm saline (1 mL at 37°C) was injected i.p. after surgery for volume supplementation. The technical success of ischemia-reperfusion was monitored by checking the kidney color after clamping and after removing the clamps. Sham operations were performed with exposure of both kidneys, but without induction of ischemia.

Cisplatin administration to mice. Mice were administered cisplatin (MilliporeSigma; 1 mg/mL solution in sterile normal saline) or vehicle (normal saline) at 20 mg/kg in a single i.p. injection. Mice were sacrificed 96 hours after administration of cisplatin, and tissue and blood were collected for further analysis.

UUO. Mice were anesthetized and the left kidney exposed by flank incision. The ureter was ligated with 6-0 silk at 2 points, close to the renal pelvis (81).

Renal function. Serum creatinine was measured with a Beckman Creatinine Analyzer 2 using the Jaffé rate method. Serum BUN was measured using the Infinity Urea Kit (Thermo Fisher Scientific).

SIM. Paraﬃn-embedded tissue was sectioned at 4- to 6-μm thickness and mounted onto (3-aminopropyl) trimethoxysilane-treated coverslips (mounting the tissue to the coverslip is necessary, as super-resolution microscopes have limited working distances). Antigen retrieval was performed by incubating the sections under high temperature (120°C) and high pressure in citrate buffer using a pressure cooker. Sections were then blocked with BSA and normal donkey serum, followed by staining with anti-mTOR (Cell Signaling Technology, 7C10) and/or anti-LC3 (Nanotools, 5F10) antibodies and the corresponding secondary antibodies (Jackson Immunoresearch). Tissue sections were imaged on a Nikon structured illumination microscope (N-SIM) as a Z-stack at 0.12-μm thickness using a ×100 TIRF objective. The resulting Z-stack was rendered in 3D in NIS-Elements (Nikon) for image analysis. The movie was generated with Imaris software (Bitplane). SIM imaging was performed at the Nikon Center of Excellence at Vanderbilt University and the Vanderbilt Cell Imaging Shared Resource.

Primary RPTECs from FUCCI+ mice. FUCCI is a technology that visualizes the cell cycle by labeling distinct fluorescence — Kusabira-Orange2 in the G1 phase and Azami-Green1 during the S/G2/M phase — which allows for determination of G1, early S, and S/G2/M phases of the cell cycle. To generate RPTEC-specific FUCCI+ mice, we crossed B6;129-Gt (ROSA)26Sor<tm1(Fucci2aR)Jkn> mice (provided by Ian Jackson, University of Edinburgh, Edinburgh, United Kingdom) with γT-Cre mice (obtained from The Jackson Laboratory, stock Tg(Ggt1-cre)M3Egn/J). RPTECs were harvested from the mice as described previously (82). Briefly, kidney cortex was minced into small pieces, incubated with collagenase (1 mg/mL) and trypsin inhibitor (1 mg/mL) in oxygenated media at 37°C with vigorous shaking, and then centrifuged in 32% Percoll and diluted in DMEM/F-12 to isolate RPTECs from other fractions. These isolated tubules were plated on collagen-coated dishes and then maintained in RPTEC media (DMEM/F-12 medium supplemented with 5 μg/mL transferrin, 5 μg/mL insulin, 0.05 μM hydrocortisone, and 50 μM vitamin C). After expression of fluorescence was confirmed, isolated RPTECs were synchronized by incubation in RPTEC media containing 1% FBS. Cells were pretreated or not with VE-821 (10 μM) for 2 hours and then incubated with PBS or cisplatin (0.1 μg/mL) in the presence or absence of VE-821 (10 μM). After a 48-hour exposure to cisplatin, FUCCI RPTECs were harvested with 0.25% trypsin and 2.21 mM EDTA (Corning, 25-053-CI), fixed with BD Cytofix/Cytoperm (BD, catalog 554714), and stained with DAPI at a concentration of 1 μg/mL. The cell cycle was examined by flow cytometry using a 4-laser LSRFortessa (BD) and analyzed with FlowJo software (BD).

In vitro FUCCI cell analysis. HK2 (American Type Culture Collection [ATCC]) cells expressing FUCCI (47) were obtained by suc-
cessive transduction with lentiviral vectors expressing mKO-Cdt1 and mAG-geminin, respectively. Cells were imaged using a Nikon TE2000-S live microscope at 37°C in an ambient atmosphere in phe- nol red-free L15 Leibovitz’s medium supplemented with 10% FBS. Pifithrin-α (Selleckchem, S2929) was used at 50 nM for p53 inhibition and the PLK1 inhibitor rigosertib (Selleckchem, S1362) at 10 nM for induction of G2/M arrest. Triple acquisition for bright-field, mKO (excitation 550/20, emission 585/20), and mAG (excitation 495/10, emission 540/40) was achieved every 30 minutes. Background subtraction and correction of the fluorescence spill from mKO in the mAG channel was performed using Fiji (83), and the fluorescence signals were then quantified for each individual cell in every field for every time point using the TrackMate plug-in (84). M-phase cells (defined by cytidieresis) were excluded from analysis.

Statistics. Statistical analyses were performed using GraphPad Prism 6.07 (GraphPad Software). All results are reported as the mean ± SEM. Statistical significance was determined using a 2-tailed, unpaired t test or 1-way ANOVA followed by Tukey’s post hoc test. Survival curves were derived using the Kaplan-Meier method and compared using a log-rank test. Correlations were determined by Pearson’s correlation analysis. A P value of less than 0.05 was interpreted as statistically significant.

Study approval. The studies in animals were reviewed and approved by the IACUC and the Harvard Medical Area Standing Committee on Animals (protocol 03602). Human studies were approved by the IRB of the Ethics Committee of Tokushima University Hospital in Tokushima, Japan (protocol 680). All patients provided written informed consent.

Author contributions
The order of the co-first authors, SK and CRB, was determined on the basis of when they began working on the study (SK started the study and CRB joined the project later). JVB formulated and conceived of this study. SK, CRB, LG, TI, RM, and JVB designed experiments. SK performed experiments. CRB, KT, PG, YM, BCE, TI, RM, NG, TS, and QW performed experiments. SK, TI, QW, and TS maintained animals. RM maintained hPSCs and generated kidney organoids. YM and NG performed organoid experiments.

SK, CRB, TI, AA, RM, NG, and JVB analyzed the data. SK, CRB, RM, NG, and JVB wrote the manuscript. All authors helped to interpret results and approved the final version of the manuscript.

Acknowledgments
We thank Hiroshi Itoh and Toshiaki Monkawa (Keio University School of Medicine, Tokyo, Japan) for the mouse anti-KSP antibody and Lorraine Racusen (Johns Hopkins Hospital, Baltimore, Maryland, USA) for the HKC-8 cells. This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), NIH (R37 DK39773, RO1 DK072381, and UG TR002155, to JVB); a Grant-in-Aid for Scientific Research (C, 16K09620); a Research Fellowship from the Sumitomo Life Welfare and Culture Foundation (to SK); a NIH T32 Fellowship Training Grant (DK007527, to NG); a Harvard Stem Cell Institute (HSCI) Cross-Disciplinary Fellowship Grant (to NG); a Brigham and Women’s Hospital Research Excellence Award (to NG and RM); the Novartis Foundation for Gerontological Research (to SK); the Uehara Memorial Foundation (to RM); a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS) Post-doctoral Fellowship for Research Abroad (to RM); a Brigham and Women’s Hospital Faculty Career Development Award (to RM); a Harvard Stem Cell Institute Seed Grant (to RM); Ajinomoto Co. Inc. (to RM); the DiaComp Pilot and Feasibility Program (to RM); and the NIDDK, NIH (R01 DK121101, K01DK099473, and P30 DK114809, to CRB). PG is supported by a grant from the ATIP Avenir program (INSERM-CNRS). SIM imaging was performed at the Nikon Center of Excellence at Vanderbilt University and the Vanderbilt Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, DK59637, and EY08126).

Address correspondence to: Craig R. Brooks, MCN Room S-3223B, 1161 21st Avenue South, Nashville, Tennessee 37232, USA. Phone: 615.343.3868; Email: craig.brooks@vanderbilt.edu. Or to: Joseph V. Bonventre, Harvard Institutes of Medicine Room 570, 4 Blackfan Circle, Boston, Massachusetts 02115, USA. Phone: 617.525.5960; Email: joseph_bonventre@hms.harvard.edu.

The Journal of Clinical Investigation

RESEARCH ARTICLE


