A FANCD2/FANCI-Associated Nuclease 1-Knockout Model Develops Karyomegalic Interstitial Nephritis

Rannar Airik,* Markus Schueler,* Merlin Airik,* Jang Cho,* Jonathan D. Porath,* Elina Mukherjee,† Sunder Sims-Lucas,‡ and Friedhelm Hildebrandt*‡

*Department of Medicine, Boston Children’s Hospital, Boston, Massachusetts; †Department of Pediatrics, Children’s Hospital of Pittsburgh of the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; and ‡Howard Hughes Medical Institute, Chevy Chase, Maryland

ABSTRACT

Karyomegalic interstitial nephritis (KIN) is a chronic interstitial nephropathy characterized by tubulointerstitial nephritis and formation of enlarged nuclei in the kidneys and other tissues. We recently reported that recessive mutations in the gene encoding FANCD2/FANCI-associated nuclease 1 (FAN1) cause KIN in humans. FAN1 is a major component of the Fanconi anemia–related pathway of DNA damage response (DDR) signaling. To study the pathogenesis of KIN, we generated a Fan1 knockout mouse model, with abrogation of Fan1 expression confirmed by quantitative RT-PCR. Challenging Fan1−/− and wild-type mice with 20 mg/kg cisplatin caused AKI in both genotypes. In contrast, chronic injection of cisplatin at 2 mg/kg induced KIN that led to renal failure within 5 weeks in Fan1−/− mice but not in wild-type mice. Cell culture studies showed decreased survival and reduced colony formation of Fan1−/− mouse embryonic fibroblasts and bone marrow mesenchymal stem cells compared with wild-type counterparts in response to treatment with genotoxic agents, suggesting that Fan1 mutations cause chemosensitivity and bone marrow failure. Our data show that Fan1 is involved in the physiologic response of kidney tubular cells to DNA damage, which contributes to the pathogenesis of CKD. Moreover, Fan1−/− mice provide a new model with which to study the pathomechanisms of CKD.

Received October 9, 2015. Accepted February 17, 2016.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Rannar Airik, Children’s Hospital of Pittsburgh of the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania.

Copyright © 2016 by the American Society of Nephrology
Our previous work suggested that individuals with mutations in Fan1 might be more susceptible to environmental genotoxins, which contribute to the renal pathology of KIN. Indeed, in vitro studies with Fan1−/− deficient cells have shown cellular hypersensitivity to DNA interstrand crosslinking agents cisplatin and MMC.1,3–6 We, therefore, hypothesized that treatment of Fan1−/− mice with cisplatin would induce DNA ICL lesions and that repair in Fan1−/− mice would be deficient, leading to KIN. Cisplatin is one of the most widely used cancer therapeutics, but its use is limited because of severe nephrotoxicity, with about 25%–35% of patients displaying a transient decline in renal function after a single dose of cisplatin.11 Its toxicity arises from production of reactive oxygen species and generation of ICLs.12,13 We first asked whether Fan1−/− mice were more susceptible to AKI than wild-type animals by injecting them with cisplatin at a dose of 20 mg/kg (n=8 per experimental group). Fan1−/− mice showed 100% mortality in response to cisplatin treatment by postinjection day 7, with some mice dying already on day 2 (Figure 1A). Fan1−/− mice that survived until day 7 appeared severely dehydrated and cachectic, having lost 30% of their body weight (Figure 1B). In contrast, wild-type mice treated with cisplatin (20 mg/kg) showed 10% weight loss with no mortality (Figure 1B).

To examine the renal histology of cisplatin-injected mice, we performed histologic analysis of kidney sections using hematoxylin and eosin staining on postinjection days 3 and 7. Both wild-type and Fan1−/− mice had characteristic signs of AKI on the third day in response to cisplatin administration compared with uninjected control mice (Figure 1, C–F), and the affected kidneys contained dilated tubules filled with massive protein casts (Figure 1, D and F). In addition, Fan1−/− kidneys showed overt sloughing of the brush borders, vacuolization, and blebbing of the proximal tubules (Figure 1F). At day 7, cisplatin–treated wild-type mice showed signs of repair and only a few abnormalities, such as occasional tubular casts (Supplemental Figure 3B), whereas no recovery was observed in cisplatin–treated Fan1−/− kidneys (Supplemental Figure 3D). Together, our data show that Fan1−/− mice are more susceptible to cisplatin toxicity and that they fail to recover from cisplatin-induced AKI in contrast to wild-type littermates.

We next hypothesized that administering a reduced dose of cisplatin would avoid the acute toxicity of cisplatin and allow us to investigate a more subtle and chronic effect of drug toxicity on the kidney function in Fan1−/− animals. We next administered a dose of 2 mg/kg weekly. No mortality was observed in Fan1−/− mice at this dose initially. We monitored the mice for signs of general toxicity by weighing them weekly. By the third week, Fan1−/− mice in the cisplatin treatment group had reduced body weight compared with treated wild-type mice (Figure 2A); 5 weeks after initiating cisplatin treatment, Fan1−/− mice had lost 24% of their initial body weight and appeared moribund. In contrast, wild-type mice in the cisplatin treatment group showed normal age-related increases in body weight (106%) and appeared healthy, indicating that the administered dose was not sufficient to cause cachexia in wild-type animals (Figure 2A). Likewise, untreated Fan1−/− mice showed no weight loss (Figure 2A).

Individuals with mutations in the Fan1 gene have progressive renal failure and increased levels of BUN.14 We measured the BUN levels in Fan1−/− mice after they had been treated for 5 weeks with cisplatin. There was a steep increase in the BUN levels in Fan1−/− mice (ninefold over wild-type treated mice), although wild-type mice receiving cisplatin injections and untreated Fan1−/− mice had BUN levels indistinguishable from those of the wild-type mice (Figure 2B). The range of BUN increases was indicative of severe renal failure in Fan1−/− mice. To examine the renal histologic changes in Fan1−/− mice, we performed periodic acid–Schiff staining. Kidneys from wild-type mice treated with cisplatin had indistinguishable histology from wild-type and untreated Fan1−/− control mice (Figure 2, C–E). In contrast, Fan1−/− mice treated with cisplatin showed the characteristic features of KIN—formation of karyomegalic nuclei in the proximal tubule, presence of interstitial infiltrate, loss of brush borders, and tubular dilatation (Figure 2F). In addition, they presented with segmental thickening of the tubular basement membrane and frequent thickening of the Bowman’s capsule (Figure 2F, Supplemental Figure 4), a characteristic feature of NPHP-RC.

To further characterize the extent of renal fibrosis in Fan1−/− kidneys, we performed Masson trichrome staining. Although we observed a strong blue staining of collagen fibers in Fan1−/− kidneys (Figure 3D), no staining was detected in the kidneys from cisplatin–injected wild-type or Fan1−/− control mice (Figure 3, A–C). Thus, our data on the renal histology of Fan1−/− mice show that the mouse model of Fan1−/− recapitulates the human Fan1 deficiency phenotypes of KIN and interstitial fibrosis in the kidney.

It has been suggested that karyomegaly is caused by impaired progression of the cell cycle, which is characterized by reduced levels of the cell proliferation marker Ki67 and an increase in S-phase marker proliferating cell nuclear antigen in karyomegalic nuclei.14 However, these observations have not been confirmed by other groups.15 We investigated the levels of Ki67 by immunofluorescence in Fan1−/− kidneys. Neither wild-type (Figure 3E) nor Fan1−/− control kidneys (Figure 3G) contained Ki67-positive nuclei in the kidneys, indicative of very low levels of cell proliferation in adult kidneys. In contrast, kidneys from wild-type (Figure 3F) and Fan1−/− mice (Figure 3H) that were treated with cisplatin contained Ki67-positive nuclei, suggesting that cisplatin induces cell cycle activity in the renal tubular epithelium, probably as a repair response.16 The number of Ki67-positive nuclei was notably higher in Fan1−/− kidneys compared with wild-type kidneys (Figure 3, F and H). Moreover,
measuring the area of Ki67-positive nuclei revealed that they were significantly enlarged in Fan1−/− kidneys compared with in wild-type kidneys (Figure 3I). This result is consistent with induction of karyomegaly caused by impaired cell cycle regulation in Fan1−/− kidneys.3

To examine the effect of various genotoxins on Fan1−/− cell survival, we isolated mouse embryonic fibroblasts (MEFs) from wild-type and Fan1−/− embryos and treated them with MMC or diepoxybutane (DEB). Indeed, we observed reduced colony formation ability and survival of Fan1−/− cells compared with in wild-type control cells in response to these genotoxins (Figure 4, A and B).

Bone marrow failure in patients with FA includes abnormalities in the bone marrow mesenchymal stem cell (BM-MSC) niche.17,18 Thus, we investigated whether cisplatin administration to Fan1−/− mice induces bone marrow abnormalities in the mesenchymal stem cell compartment. We isolated BM-MSCs from wild-type and Fan1−/− mice and examined their in vitro clonogenic potential.19 The average number of colonies formed per mouse (cells were isolated from two femurs per mouse) in control and cisplatin-injected animals indicates that the BM-MSCs from cisplatin–treated Fan1−/− mice had severely depressed clonogenic ability compared with those from wild–type cisplatin–injected mice (Figure 4C) (P<0.001). Bone marrow cells from the Fan1−/− control group had the same colony formation capability as cells from wild–type control mice (Figure 4C). Together, our findings suggest that Fan1 function is critical for bone marrow maintenance by eliminating ICLs.

Phosphorylation of H2AX (γH2AX) is a signature of ATM/ATR–regulated DDR pathway activation. We next examined whether there is a difference between γH2AX levels in wild-type versus Fan1−/− kidneys on treatment with cisplatin. Indeed, we observed increased levels of γH2AX in cisplatin–treated Fan1−/− kidneys, whereas γH2AX levels in cisplatin–treated wild–type kidneys did not differ from the levels of untreated wild–type and Fan1−/− kidneys (Figure 4D). Together, it shows a prolonged and unquenched DDR activity in cisplatin–treated Fan1−/− kidneys, a likely cause for CKD.20,21

Our finding that Fan1−/− mice develop bone marrow abnormalities on treatment with cisplatin suggests that FAN1 mutations may underlie previously unrecognized susceptibility for bone marrow failure in humans. This warrants analysis of FAN1 mutations in patients with bone marrow failure. Thus far, FAN1 deficiency has been clinically
associated with microcephaly together with other developmental defects, KIN, and colorectal carcinoma.\textsuperscript{1,9,22} Our work also sheds light on the possible involvement of environmental genotoxins in determining the disease onset in individuals with FAN1 deficiency who are predisposed to genetic injury. On the basis of our studies in Fan1\textsuperscript{2/2} mice, we propose that exposure to subclinical doses of environmental genotoxins, such as food-derived genotoxins, genotoxic drugs, chemotherapeutics, etc., may insidiously lead to accumulation of irreparable DNA damage and KIN in humans. This is not without a precedent. For example, Fanca\textsuperscript{−/−} and Fancc\textsuperscript{−/−} mice do not recapitulate the spontaneous bone marrow disease of patients with FA.\textsuperscript{23,24} However, when challenged with genotoxic agents or inflammatory signals, these mice precipitated bone marrow failure.\textsuperscript{23,24} Together, these studies show that environmental factors are critical contributors to the onset of these diseases.

In conclusion, we show that Fan1\textsuperscript{2/2} mice provide a new model to study the pathomechanisms of drug-induced CKD and NPHP-RC. We show that Fan1\textsuperscript{2/2} mice are highly sensitive to genotoxic insults, which lead to KIN and bone marrow abnormalities.

**CONCISE METHODS**

**Mouse Breeding and Maintenance**

The experimental protocol was reviewed and approved by the Animal Care Committee of Boston Children’s Hospital. Targeted Fan1tm1a(KOMP)Wtsi ES cells were obtained from KOMP and injected into blastocysts to generate Fan1-transgenic mice. Chimeric mice were prepared by blastocyst microinjection and bred with C57BL/6J mice to obtain germline transmission. Founders were bred into a 129SvImJ congenic line for at least five generations before experiments were performed. Wild-type littermate mice were used as controls for Fan1 mutant mice.

**Cisplatin Injection**

Eight-week-old or 6-month-old Fan1\textsuperscript{−/−} mice were injected intraperitoneally with 2 or 20 mg/kg cisplatin, respectively (Teva Parenteral Medicines, Inc.), diluted in normal saline solution. Control mice received normal saline injections of equivalent volumes. Mouse body weight at the time of injection ranged from 25 to 40 g.

**Biochemical Analyses**

On the day of euthanasia, blood was immediately collected, and serum levels of BUN

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Chronic administration of low doses of cisplatin causes KIN in Fan1\textsuperscript{−/−} mice; 6- to 8-week-old mice were weekly given low doses of cisplatin (2 mg/kg). Renal histology and function were analyzed 5 weeks later. (A) Chronic cisplatin administration resulted in dramatic weight loss in treated Fan1\textsuperscript{−/−} mice but not in wild-type or untreated Fan1\textsuperscript{−/−} mice. Weight loss became apparent already 3 weeks after cisplatin was administered. (B) BUN values as a measure of renal function in control and cisplatin-treated mice after 5 weeks of treatment. Chronic administration of cisplatin did not affect kidney function in wild-type or untreated Fan1\textsuperscript{−/−} mice compared with control mice. In contrast, Fan1\textsuperscript{−/−} mice showed a ninefold increase in BUN values in response to cisplatin (wild type, 40.4±1.4 mg/dl; Fan1\textsuperscript{−/−}, 47.4±7.9 mg/dl; wild-type cisplatin, 35.2±6.5 mg/dl; Fan1\textsuperscript{−/−} cisplatin, 324.7±17.1 mg/dl). **P<0.01. (C–F) Histology of kidneys from wild-type and Fan1\textsuperscript{−/−} animals after 5 weeks of cisplatin treatment. Although cisplatin-treated wild-type and untreated Fan1\textsuperscript{−/−} kidneys appear histologically normal, Fan1\textsuperscript{−/−} kidneys treated with cisplatin (2 mg/kg) display the characteristic features of KIN, presence of interstitial infiltrate, and karyomegalic nuclei (green arrows). Notice the thickening of the tubular basement membranes (red arrows). CTRL, Control; wt, wild type.}
\end{figure}
Figure 3. KIN is characterized by renal tubular fibrosis and Ki67 positivity in Fan1^−/− mice. (A–D) Masson trichrome staining was used to examine kidney fibrosis. No fibrosis was observed in (A) wild-type, (B) Fan1^−/−, or (C) cisplatin-treated wild-type control mice. (D) In contrast, cisplatin-treated Fan1^−/− animals developed renal fibrosis (blue staining indicates collagen deposits). (E–H) Ki67 immunostaining (red) shows cell cycle activation in the renal epithelium of (F) cisplatin–treated wild-type and (H) Fan1^−/− kidneys but not in
(E) nontreated wild–type and (G) Fan1+/− kidneys. (H) However, the number of proliferating cells (Ki67 positive) is higher in Fan1+/− kidneys. Furthermore, a majority of the Ki67-positive nuclei in Fan1+/− kidneys are enlarged, representing karyomegalic nuclei. Notice a partial loss of LTA–positive brush borders (green arrows) from Fan1+/− kidneys. (I) Area of Ki67-positive nuclei in wild-type versus Fan1+/− kidneys treated with cisplatin. The mean size of Ki67-positive nuclei in Fan1+/− kidneys is significantly larger compared with that in wild-type kidneys (6086±341.9 versus 3924±180.0 pixels). ctrl, Control; LTA, Lotus tetragonolobus agglutinin; px, pixels; wt, wild type. ****P<0.001.

Western Blotting
Kidney tissues were lysed in RIPA Lysis Buffer (Pierce, Rockford, IL) and homogenized with a douncer. Cleared tissue lysates were used by centrifugation of the resulting samples at 16,000×g for 30 minutes at 4°C. Gel electrophoresis of tissue lysates was performed using the NuPAGE System (Invitrogen). Samples were run on 4%–12% Bis-Tris gels in 3-(N-morpholino)propanesulfonic acid buffer and transferred to a nitrocellulose membrane that was then probed for the protein of interest using antibodies diluted in Tris-buffered saline containing 5% milk and 0.1% Tween-20 (Sigma-Aldrich).

Statistical Methods
The t test was used to compare data between two groups. Significance was determined at P<0.05, and P<0.01, and P<0.001 are indicated. Where appropriate, data were presented as means±SEMs.
Colony Formation Assay
BM-MSC were cultured as described. Briefly, total numbers of isolated bone marrow cells from two femurs per mouse were seeded in 6-cm dishes and cultured for 7 days. Colonies were stained with crystal violet solution and counted.

DNA Damage Sensitivity Assay
MEFs were plated in a six-well plate in triplicate at a density of \(5 \times 10^6\) cells per well. Immediately after plating, MMC or DEB was added at a final concentration of 0 or 0.5 \(\mu\)M for MMC or 0–1 \(\mu\)g/ml for DEB. After 8 days of culture, surviving colonies (MM and DEB) and cell numbers (DEB) were counted. Colony number after DEB treatment was normalized to cell number in the untreated sample to give the percentage of survival.

ACKNOWLEDGMENTS
We thank Thomas L. Saunders, Elizabeth Hughes, Keith Childs, Galina Gavrilina, and Debra Vanheyningen for preparation of the embryonic stem cell mouse chimeras from Fan1\(^{–/–}\) (KOMP2/Whs) embryonic stem cell clone EPD0736_4_C08 and the Transgenic Animal Model Core of the University of Michigan’s Biomedical Core Facilities.

Core support was also provided by the Diabetes Research and Training Center and the O’Brien Renal Core Center, which are funded by National Institutes of Health (NIH) grants DK20572 and P30-DK08194, respectively. This support was provided by NIH grants DK099434 (to R.A.) and NIH grants DK20572 and P30-DK08194, respectively. This research was supported by NIH grant DK099434 (to R.A.) and NIH grants DK20572 and P30-DK08194, respectively. This research was supported by the National Institutes of Health (NIH) grants DK20572 and P30-DK08194, respectively. This research was supported by the OligoNucleotide Core of the University of Michigan Biomedical Core Facilities.

REFERENCES

DISCLOSURES
None.


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2015101108/-/DCSupplemental.