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Journal

Proceedings of the National Academy of Sciences of the United States of America, 110(27)

ISSN

0027-8424

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Publication Date

2013-07-02

DOI

10.1073/pnas.1309561110

Peer reviewed

Matrix metalloproteinase-9 deficiency phenocopies features of preeclampsia and intrauterine growth restriction

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Contributed by Zena Werb, May 27, 2013 (sent for review April 3, 2012)

The pregnancy complication preeclampsia (PE), which occurs in approximately 3% to 8% of human pregnancies, is characterized by placental pathologies that can lead to significant fetal and maternal morbidity and mortality. Currently, the only known cure is delivery of the placenta. As the etiology of PE remains unknown, it is vital to find models to study this common syndrome. Here we show that matrix metalloproteinase-9 (MMP9) deficiency causes physiological and placental abnormalities in mice, which mimic features of PE. As with the severe cases of this syndrome, which commence early in gestation, MMP9-null mouse embryos exhibit deficiencies in trophoblast differentiation and invasion shortly after implantation, along with intrauterine growth restriction or embryonic death. Reciprocal embryo transfer experiments demonstrated that embryonic MMP9 is a major contributor to normal implantation, but maternal MMP9 also plays a role in embryonic trophoblast development. Pregnant MMP9-null mice bearing null embryos exhibited clinical features of PE as VEGF dysregulation and proteinuria accompanied by preexisting elevated blood pressure and kidney pathology. Thus, our data show that fetal and maternal MMP9 play a role in the development of PE and establish the MMP9-null mice as a much-needed model to study the clinical course of this syndrome.

ectoplacental cone | fetus

Preeclampsia (PE) is one of the most common pregnancy complications worldwide, affecting ~3% to 8% of all pregnancies, and is a leading cause of perinatal and maternal morbidity and mortality (1). PE is characterized by placental hypoperfusion and shallow trophoblast invasion of uterine blood vessels (2) that is particularly evident in the severe cases that commence early in pregnancy (3). Adequate trophoblast invasion is vital to provide the embryo with access to oxygen and nutrients, and, in human and mouse, the placenta is thereby in direct contact with maternal blood. The clinical diagnostic criteria of this syndrome include widespread maternal endothelial dysfunction as evidenced by hypertension, proteinuria, and peripheral and/or cerebral edema (4). In addition to the maternal signs, PE is also frequently associated with intrauterine growth restriction (IUGR) and prematurity (5). The etiology of PE is unclear and the only known cure is delivery of the placenta. The upstream regulatory mechanisms remain elusive, as do the downstream consequences that lead to the maternal signs. Nevertheless, there is substantial evidence for contributing factors including abnormal placentation, particularly the invasive component. Restricted invasion is thought to be a reflection of defects in the cytotrophoblast (CTB) differentiation pathway that is required for uterine interstitial and endovascular invasion. Specifically, CTBs, which are epithelial cells of ectodermal origin, acquire vascular-like properties, and this transformation is dysregulated in PE (3, 6). The rudimentary endovascular invasion is thought to lead to the release of pathologic factors such as vasculogenic and angiogenic substances and their inhibitors into the maternal circulation (2). These factors can induce an inflammatory response as

well as generating reactive oxygen and nitrogen species within the vasculature, resulting in the maternal symptoms of PE.

Among the factors that may facilitate trophoblast invasion are matrix metalloproteinases (MMPs) (7). MMPs are well-established mediators of tissue remodeling and angiogenesis, several of which exhibit modified expression in placentas of patients with PE. MMPs target the extracellular matrix (ECM) and are involved in normal physiology and in various pathologies. MMPs are secreted as inactive proenzymes that are activated when cleaved by extracellular proteinases (8). A remarkably broad spectrum of MMPs and tissue inhibitors of MMPs are expressed at the human fetomaternal interface, specifically by uterine natural killer cells, decidual cells, and trophoblasts (9). Their significance in mediating placental development is bolstered in results of *in vitro* experiments that used a broad-spectrum pharmacological MMP inhibitor (10). MMP9 (92-kDa gelatinase B or type IV collagenase) is a key effector of ECM remodeling that degrades types IV, V, and IX collagens, denatured collagens (gelatin), and elastin (8). Active MMP9 is highly expressed at the embryo implantation site by human and mouse trophoblasts, and is implicated in their invasive behavior (10, 11). Multiple lines of evidence point to a role for MMP9 in PE, as CTBs in PE produce less MMP9 (12) and MMP9 inhibition or gene silencing blocks CTB invasion *in vitro* (13). MMP9 is also consistently deficient in the plasma of patients with PE (14), and, recently, an MMP9 variant was shown to be a useful biomarker of susceptibility to severe PE and its early onset (15).

Accordingly, we investigated the role of embryonic and maternal MMP9 in embryo implantation and placentation.

Results

MMP9-Null Placentas and Fetuses Exhibit Severe Abnormalities. We first confirmed the previously reported fertility disorders associated with MMP9 insufficiency (16) in several mouse backgrounds. For pure or mixed 129SV/J backgrounds, we observed as much as a 20% reduction in litter size in MMP9-null homozygous litters. For C57BL/6J, we observed as much as a 50% reduction in litter size in homozygous × heterozygous matings, similar to that observed for homozygous matings (16). Heterozygous matings resulted in the expected Mendelian frequency for all genotypes in all backgrounds

Author contributions: V.P., J.R., R.K., and Z.W. designed research; V.P., J.R., J.D., M.F., M.S., K.K., and W.N. performed research; V.P., J.R., J.D., M.F., M.S., K.K., and W.N. analyzed data; and V.P. and Z.W. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1309561110/-DCSupplemental.

tested, similar to that of C57BL/6 and pure 129SV/J (16). In keeping with the previous observations, we conclude that MMP9 should be eliminated from maternal and fetal sides to observe subfertility characterized by reduced litter size.

We examined MMP9-null placentas at embryonic day (E) 10.5, the first time point during pregnancy at which the mature placenta is fully formed with its two distinct layers: labyrinth and junctional zone, which are integrated with the decidua (2). To eliminate the contribution of maternal MMP9, we studied MMP9 null \times null matings. We observed runted, pale, and poorly developed MMP9-null embryos (Fig. 1 *A* and *B*). The implantation chambers were reduced in size, and the runted embryos exhibited significantly reduced weight characteristic of IUGR (Fig. 1*C*). We also observed many resorptions in MMP9-null litters but rarely in WT litters.

Placentas from the runted conceptuses were significantly smaller (Fig. 1 *D* and *F*; quantified in Fig. 1*H*). WT placentas had a single layer of trophoblast giant cells (TGCs; Fig. 1 *G* and *E*) that was significantly expanded to multiple layers in MMP9-null placentas

(Fig. 1*G*; quantified in Fig. 1*I*). In contrast, the spongiotrophoblasts (STs) and the labyrinthine layer were significantly reduced in the runted placentas (Fig. 1 *E* and *G*; quantified in Fig. 1*I*).

Before Placental Maturation, MMP9 Null Mice Exhibit Abnormalities in Embryonic Development and Formation of the Maternal-Fetal Interface. MMP9 production by TGCs and its subsequent activation is a prerequisite for trophoblast invasion in vitro. Our group showed previously that MMP9 is produced by the blastocyst and is first detected at the E5.5 implantation site (10). Its expression peaks in TGCs invading the decidua between E7.5 and E8.5 (17) and then decreases to low but detectable levels at E10.5 (18). To determine whether the delayed development evident in MMP9-null placentas at E10.5 stems from an earlier defect, we examined the initial stages of trophoblast invasion. Considering that severe cases of PE are characterized by shallow trophoblast invasion and IUGR, which commences as early as the second trimester in humans (19), we examined E7.5 implantation sites (Fig. 2). MMP9-null implantation sites and the embryos proper were significantly reduced in size. By using timed pregnancies from mice that were mated and plugs checked during a 2-h time window, followed by a morphometric analysis of histological sections of implantation sites, we observed that embryos in all null implantation sites (Fig. 2 *C*, *E*, and *G*) lagged 6 to 12 h behind their WT counterparts (Fig. 2*I*; quantified in Fig. 2*J*).

As the first migratory cell type in the mouse embryo, parietal endoderm (PED) cells synthesize and deposit components of the Reichert membrane, a basement membrane that lies between the PED and the trophoblast. As the embryo continues to grow (after PED ceases migration at E6.5), new PED cells are derived from the transdifferentiation of borderline visceral extraembryonic endoderm cells. PED is a source for MMP9 secretion, and MMP9 regulates PED differentiation and migration (20). We observed impaired PED migration and expansion along with that of the visceral endoderm in MMP9-null embryos (Fig. 2 *A* and *B* vs. *C-H*). Thus, impaired PED and visceral endoderm migration may hamper the migration of trophoblasts from the extraembryonic ectoderm to the base of the EPC and play a role in the developmental delays observed for E7.5 MMP9-null embryos.

TGCs have intense phagocytic activity, leading to the erosion and displacement of the uterine epithelial and decidual cells (21). In the absence of MMP9, the matrix surrounding the decidual cells that is normally engulfed by TGCs failed to be cleared, leading to excessive debris. We detected misoriented ectoplacental cones (EPCs) accompanied by blood pools around the EPC areas that were filled with debris between the tip of the EPC and the mesometrial decidua in MMP9-null embryos (Fig. 2*B* vs. *D*, *F*, and *H*), similar to fibrin accumulation typical of PE placentas (22). Moreover, among various characteristics indicative of the depth of trophoblast invasion, MMP9 nulls exhibited 34% smaller EPC areas than WT (Fig. 2*I*).

MMP9 Deficiency is Associated with Impaired Trophoblast Differentiation and Maternal Vascular Defects. Next, we examined MMP9-null EPCs to determine the cause of these early defects in trophoblast invasion. The EPC commences its growth from the extraembryonic ectoderm at E5.5. The base of the EPC comprises precursor cells that, as they migrate to the tip of the cone, differentiate first into intermediate cells, then into secondary giant cells and later into STs (23). TGCs make initial contacts with the maternal blood sinuses that form in the decidua at E7.5 (21). We did not observe significant differences in proliferation (Fig. 3*A* and Fig. S1*A*) or apoptosis (Fig. 3*B* and Fig. S1*B*) of MMP9-null EPCs compared with age-matched WT EPCs. As the cells migrated from the base of the cone and began to differentiate into invasive trophoblasts, they increased in size. The secondary giant cells that will form connections with the maternal blood sinuses not only enlarge, but cease to proliferate, form focal adhesions, and undergo endoreduplication of their DNA (24). We observed that differentiation of MMP9-null E7.5 EPCs was impaired, as the trophoblast vascular bed length was

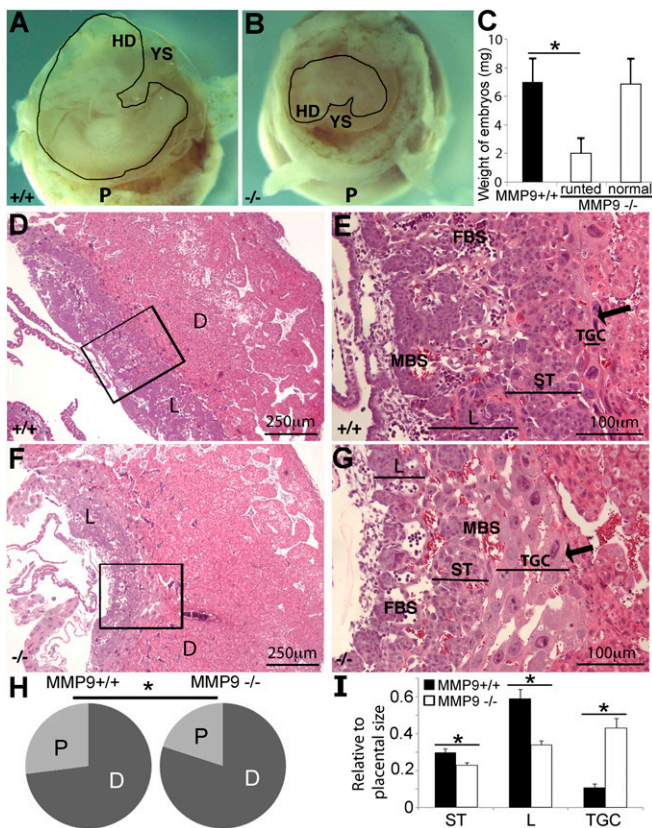
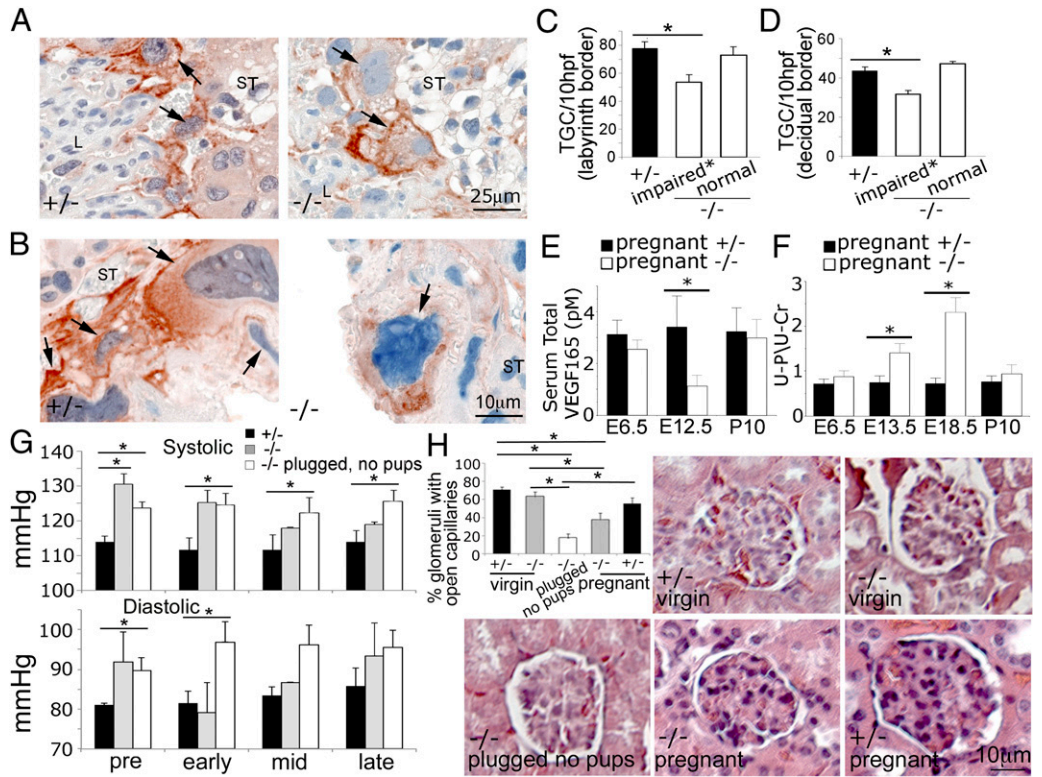


Fig. 1. Placental abnormalities in E10.5 MMP9-null placentas. Animals were mated for 2 h/d to synchronize embryonic development. Representative E10.5 embryos from MMP9 WT \times WT ($+/+$) (*A*) and from null \times null ($-/-$) (*B*) matings, inside the yolk sac and resting on top of the placenta. The embryos are depicted with a black line. (*C*) A total of 18.4% of the embryos from $-/-$ matings were runted compared with $+/+$ matings at E10.5 ($n = 6$ null and $n = 4$ WT pregnant mice). (*D* and *F*) Placental transverse mid-sections of WT and runted null E10.5 embryos, respectively. Box demarcates the enlarged area seen in *E* and *G*, respectively. D, decidua; FBS, fetal blood sinus; HD, head; L, labyrinth; MBS, maternal blood sinus; P, placenta; YS, yolk sac. Arrows indicate multiple layers of TGCs in runted $-/-$ vs. single normal layer in $+/+$ placentas. (*H*) Pie charts of placenta (labyrinth, STs, TGCs) vs. decidua lengths calculated from the total length of implantation chambers of each genotype. The $-/-$ placentas were 20% of the entire length of the implantation chamber vs. 27% in $+/+$ ($*P = 0.002$, $n = 6$ placentas per genotype). (*I*) Length of labyrinth and ST layers relative to total placental length of each genotype was significantly decreased in $-/-$ placentas; the opposite was for TGC ($*P < 0.02$).

Fig. 5. Pregnant MMP9-null mice exhibit clinical diagnostic features of PE. Cytokeratin staining of TGCs (arrows) from E20.5 placentas in control (+/-) and MMP9-null (-/-) mice on (A) the inner border of the ST layer (labyrinth border) or (B) the outer border of the ST layer (decidual border). Number of TGCs found in the placentas of the impaired -/- fetuses at (C) the labyrinth border or (D) the decidual border was significantly reduced (**P* = 0.04 and *P* = 0.02, respectively). L, placental labyrinth. [*Aborted, dead after Caesarean (C) section.] (E) Total VEGF165 levels in sera collected from pregnant mice were significantly reduced in -/- at E12.5 and returned to control levels at P10. (F) Urine protein/creatinine level ratio (U-P/U-Cr) was significantly elevated in -/- at E13.5 and E18.5 but back to control levels by P10. (G) BP measurements reveal that -/- mice exhibit elevated BP during the pregnancy time period associated with lack of pups (C-sectioned on E18.5); pre-, before implantation until E4.5; early-, E4.5 to E7.5; mid-, E12.5 to E15.5; late-, E17.5 to E18.5. MMP9^{+/-} and MMP9^{-/-} are pregnant females that had pups on E18.5 [*n* = 7 MMP9^{+/-} (7.2 pups plus 0.3 resorptions per female), *n* = 2 MMP9^{-/-} (five pups plus one resorption per female), and *n* = 9 MMP9^{-/-} plugged, no pups]. (H) Percentage of open glomerular capillaries in kidneys of females in G was significantly reduced, especially in the -/- plugged, no pups mice but also in -/- that had pups (**P* < 0.0001). Representative H&E glomeruli images are included (lower-magnification images in Fig. S3C).



Discussion

Our data answer a longstanding question regarding the role of MMP9 in trophoblast invasion and placental development. Moreover, we phenotypically connect MMP9 insufficiency to early manifestation of PE, characteristic of the more severe cases of this syndrome. Placental defects associated with MMP9 deficiency in mice were evident early, during EPC formation, and attributable to defective trophoblast differentiation. Similar to MMP9-null mice that exhibit impaired embryo development, early-onset forms of PE in women are almost always associated with IUGR and early manifestation of reduced placental volume. Moreover, MMP-null pregnant mice exhibit diagnostic criteria for PE as VEGF dysregulation and proteinuria, on the background of pre-existing elevated BP and signs of kidney pathology.

At E7.5, before the placenta had formed, all MMP9-null embryos exhibited delayed development linked to impaired endometrial decidualization and trophoblast invasion, resulting in poorly developed maternal–fetal vascular connections. The observed morphological and molecular alterations, which included delayed PED and Reichert membrane migration and expansion, likely contributed to the developmental delays of the MMP9 embryos and mirror defects in CTB invasion observed in PE (3).

These early effects of the absence of MMP9 probably contributed to the IUGR features of these runted and resorbing embryos detected at E10.5. Although all cell layers were present in the placentas of runted MMP9-null embryos, the TGC layer was significantly enlarged at the expense of the labyrinth and the ST layers. Because, at E10.5, MMP9 is mostly expressed in TGCs (18), its absence may correspond with this abnormal phenotype, although it can also be a compensatory effect for the loss of the labyrinth. The poorly developed labyrinth probably contributed to the runting of MMP9-null embryos by restricting nutrient and gas exchange.

Reciprocal embryo transfers indicated that, although embryonic MMP9 is more dominant, normal embryonic trophoblast invasion and differentiation also require maternal MMP9. These experiments were instrumental in differentiating the role of embryonic from maternal MMP9 because, in most heterozygous matings, in which maternal blood in the decidua contains MMP9, null pups are rescued as they appear in their expected Mendelian frequency. However, given the secreted, non–cell-autonomous nature of MMP9, it is also possible that MMP9 provided by the heterozygous and WT embryos in these heterozygous matings add to threshold levels of MMP9 that, in turn, enabled sufficient vascular development to help sustain the null embryos, resulting in this Mendelian frequency. The suggested threshold MMP9 levels needed to sustain normal litter size were further reinforced in C57BL/6J homozygous × heterozygous matings, which yielded 50% reduction in the size of litters, not all of which were the null fetuses (as analyzed at E20.5).

Our data also demonstrate that, during later stages of pregnancy, the lack of maternal and fetal MMP9 led to decreased serum VEGF, possibly as a result of diminished ECM degradation and failure to release stored VEGF (31). This phenomenon could also contribute to deficient trophoblast endovascular invasion. There is circumstantial evidence that antagonism of VEGF and placental growth factor may have a pathogenic role in the appearance of hypertension and proteinuria. The deficiency in total VEGF may therefore be reflected in an increase in sFlt1, thus leading to the observed proteinuria and hypertension (32). In fact, angiogenic factors are now used to predict PE (1). In addition to sFlt1 as a predictive marker for PE, other markers, which rely on VEGF levels, are being discovered, such as VEGF165b, an antiangiogenic splice variant of VEGF165 that may blunt some of the endothelial effects of VEGF165 (28). This could also explain why the concentration of free, biologically active VEGF

decreases in PE, but total VEGF in PE is still in a large excess compared with sFlt1.

We also suggest that MMP9-null mice are a model of PE with preexisting hypertension and kidney pathology. Often, women with a history of hypertension are more susceptible to developing PE upon pregnancy. There is evidence showing that preexisting hypertension can lead to complications of pregnancy such as placental abruption (33), IUGR, and perinatal death (34). Moreover, some MMP9-null mice exhibit kidney pathology and are also more prone to nephritis (35), possibly rendering their kidneys as more sensitive to a challenge like pregnancy.

In summary, as we show here, the cumulative decreases in MMP9 involving mostly the fetal but also the maternal contributions phenocopy aspects of PE accompanied by IUGR in the mouse, and suggest that a threshold level of MMP9 at the maternal–fetal interface is required for normal placentation. So far, no therapy exists that may cure defective trophoblast invasion in women with PE. The discovery of MMP9 as a regulator of this process may offer therapeutic insights to this condition. Even though the mouse and the human maternal–fetal interfaces are morphologically different, they are physiologically similar, hereby suggesting the MMP9-null mice as a model for delineating the yet unknown aspects of PE pathophysiology.

Materials and Methods

Mice Mating Schemes. All animal experiments were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Timed pregnancies. Plug day was set as E0.5. Homozygous matings were used to study the effect of MMP9 total elimination during early placentation using 129SVJ pure or mixed backgrounds, which putatively have a milder phenotype. The effect of MMP9 on the mother, which was more profound in the C57BL/6J background, was examined in MMP9-null females mated with heterozygous males compared with heterozygous females mated with WT males. Additional details are provided in *SI Materials and Methods*.

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Reciprocal embryo transfers. Blastocysts were harvested from pregnant MMP9-null or WT females and reciprocally transferred into day-2.5 pseudopregnant hosts, after which the implantation sites were analyzed, as described in *SI Materials and Methods*.

Proliferation and Apoptosis Assays. BrdU incorporation and TUNEL staining were performed by using kits from Zymed laboratories and Trevigen, respectively, as indicated in *SI Materials and Methods*.

Immunohistochemistry and Morphometric Analysis. Stainings were performed by using antibodies and kits from Zymed Laboratories, Pharmingen, Invitrogen, Molecular Probes, and DAKO, and quantified as indicated in *SI Materials and Methods*.

Serum VEGF and Urinary Protein Measurements. The total serum VEGF levels (Quantikine ELISA Mouse VEGF Immunoassay; MMV00; R&D Systems) and urine protein levels (colorimetric assay; Sigma) were measured as previously described (30).

Tail Cuff BP Measurement and Analysis. Mouse BP was measured by using a conventional tail-cuff method as indicated in *SI Materials and Methods*.

Statistics. Significance was determined at $P < 0.05$ by unpaired Student *t* test for datasets with two groups and by one-way ANOVA followed by Tukey multiple comparison test for datasets with three or more groups.

ACKNOWLEDGMENTS. We thank Dr. Susan Fisher for critical reading; Ying Yu for genotyping; Dr. Zoltan Laszik for kidney histology advice; and Jelena R. Linnemann, Eline C. Van Kappel, Jennifer Tai, and Charlotte D. Koopman for technical help. This work was supported by National Institutes of Health Grants CA057621 (to Z.W.), HD026732 (to Z.W.), CA075072 (to Z.W.), DK55001 (to R.K.), DK081976 (to R.K.), CA125550 (to R.K.), CA155370 (to R.K.), and CA151925 (to R.K.); American Cancer Society Fellowships (to J.R.); Machiah Foundation and Bikura/Israeli Science Foundation postdoctoral fellowships (to V.P.); a Weizmann Institute of Science–National Postdoctoral Award Program for Advancing Women in Science (to V.P.); and a foreign study grant from the Kanai Foundation for the Promotion of Medical Science in Japan (to K.K.).

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