

# Cellular Senescence Limits Regenerative Capacity and Allograft Survival

Heidi Braun,\* Bernhard M.W. Schmidt,<sup>†</sup> Mirja Raiss,\* Arpita Baisantry,\*<sup>†</sup>  
Dan Mircea-Constantin,\* Shijun Wang,<sup>‡</sup> Marie-Luise Gross,<sup>§</sup> Manuel Serrano,<sup>||</sup>  
Roland Schmitt,<sup>†</sup> and Anette Melk\*

\*Department of Kidney, Liver and Metabolic Diseases, Children's Hospital, Hannover Medical School, Hannover, Germany; <sup>†</sup>Department of Nephrology, Hannover Medical School, Hannover, Germany; <sup>‡</sup>Department of Cellular Pathology, German Cancer Research Centre, Heidelberg, Germany; <sup>§</sup>Department of Pathology, University of Heidelberg, Heidelberg, Germany; and <sup>||</sup>Tumor Suppression Group, Spanish National Cancer Research Center (CNIO), Madrid, Spain

## ABSTRACT

Long-term graft survival after kidney transplantation remains unsatisfactory and unpredictable. Interstitial fibrosis and tubular atrophy are major contributors to late graft loss; features of tubular cell senescence, such as increased p16<sup>INK4a</sup> expression, associate with these tubulointerstitial changes, but it is unknown whether the relationship is causal. Here, loss of the *INK4a* locus in mice, which allows escape from p16<sup>INK4a</sup>-dependent senescence, significantly reduced interstitial fibrosis and tubular atrophy and associated with improved renal function, conservation of nephron mass, and transplant survival. Compared with wild-type controls, kidneys from *INK4a*<sup>-/-</sup> mice developed significantly less interstitial fibrosis and tubular atrophy after ischemia-reperfusion injury. Consistently, mice that received kidney transplants from *INK4a*/*ARF*<sup>-/-</sup> donors had significantly better survival 21 days after life-supporting kidney transplantation and developed less tubulointerstitial changes. This correlated with higher proliferative rates of tubular cells and significantly fewer senescent cells. Taken together, these data suggest a pathogenic role of renal cellular senescence in the development of interstitial fibrosis and tubular atrophy and kidney graft deterioration by preventing the recovery from injury. Inhibiting premature senescence could have therapeutic benefit in kidney transplantation but has to be balanced against the risks of suspending antitumor defenses.

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Despite improvements in first-year graft survival, long-term failure of kidney transplants remains an important clinical problem.<sup>1</sup> Recent studies have indicated that most transplants fail due to specific causes such as antibody-mediated rejection and recurrent diseases.<sup>1,2</sup> Interstitial fibrosis and tubular atrophy occur in response to the injury caused by these processes and reflect the loss of functional nephron mass. Tubulointerstitial changes are a common feature in many chronic renal diseases and eventually are the cause for returning to dialysis after transplantation.<sup>3–5</sup> An explanation for the strong predictive value of donor age and transplantation-dependent tissue injury for the development of interstitial fibrosis and tubular atrophy<sup>6,7</sup> could be changes in cell fate programming, such as senescence,

that lead to the inability of tubular epithelial cells to repair and maintain nephron integrity.<sup>8–10</sup>

Cellular senescence is reached through two main pathways: telomere-dependent and telomere-independent senescence. Telomere-dependent

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H.B. and B.M.W.S. contributed equally to this work.

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**Correspondence:** Dr. Anette Melk, Department of Kidney, Liver and Metabolic Diseases, Children's Hospital, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany. Email: [melk.anette@mh-hannover.de](mailto:melk.anette@mh-hannover.de)

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senescence, also called replicative senescence, refers to telomere shortening and eventually cell cycle arrest with ongoing cell cycling.<sup>11–13</sup> Telomere-dependent senescence is circumvented in normal laboratory animals due to the expression of telomerase, an enzyme that maintains telomere length.<sup>14</sup> An alternate form of senescence caused by different cellular stresses, like DNA damage, oxidative stress, Ras induction, and epigenetic alterations, is called stress and aberrant signaling-induced senescence arrest or premature senescence.<sup>15</sup> The cell cycle regulator and tumor suppressor p16<sup>INK4a</sup> is associated with this telomere-independent growth arrest that is detectable in humans and rodents.<sup>16,17</sup>

Several studies have correlated senescence markers, mainly higher expression of p16<sup>INK4a</sup> and/or shorter telomeres, with the occurrence of interstitial fibrosis and tubular atrophy in CKD or transplant failure.<sup>18–22</sup> In experimental models, renal transplantation leads to a transient elevation in p21<sup>CIP1/WAF1</sup>, sustained increases of p16<sup>INK4a</sup>, induction of SA- $\beta$ -GAL expression, and substantial telomere shortening.<sup>23</sup> Importantly, old renal allografts show a more rapid emergence of epithelial deterioration that is accompanied by an increased expression of p16<sup>INK4a</sup> compared with young donor allografts.<sup>18</sup> In telomerase-deficient mice, critical telomere shortening in the kidney leads to increased senescence, thereby limiting regenerative capacity.<sup>24</sup> Consequently, it has been shown that senescence markers, in particular p16<sup>INK4a</sup> expression, in renal transplant implantation biopsies are useful to predict transplant outcome.<sup>25,26</sup>

Here, we evaluated whether loss of the *INK4a* locus could result in superior morphologic and functional outcome in mice undergoing either renal ischemia-reperfusion injury or receiving a fully MHC-mismatched, vascularized life-supporting kidney transplant.

## RESULTS

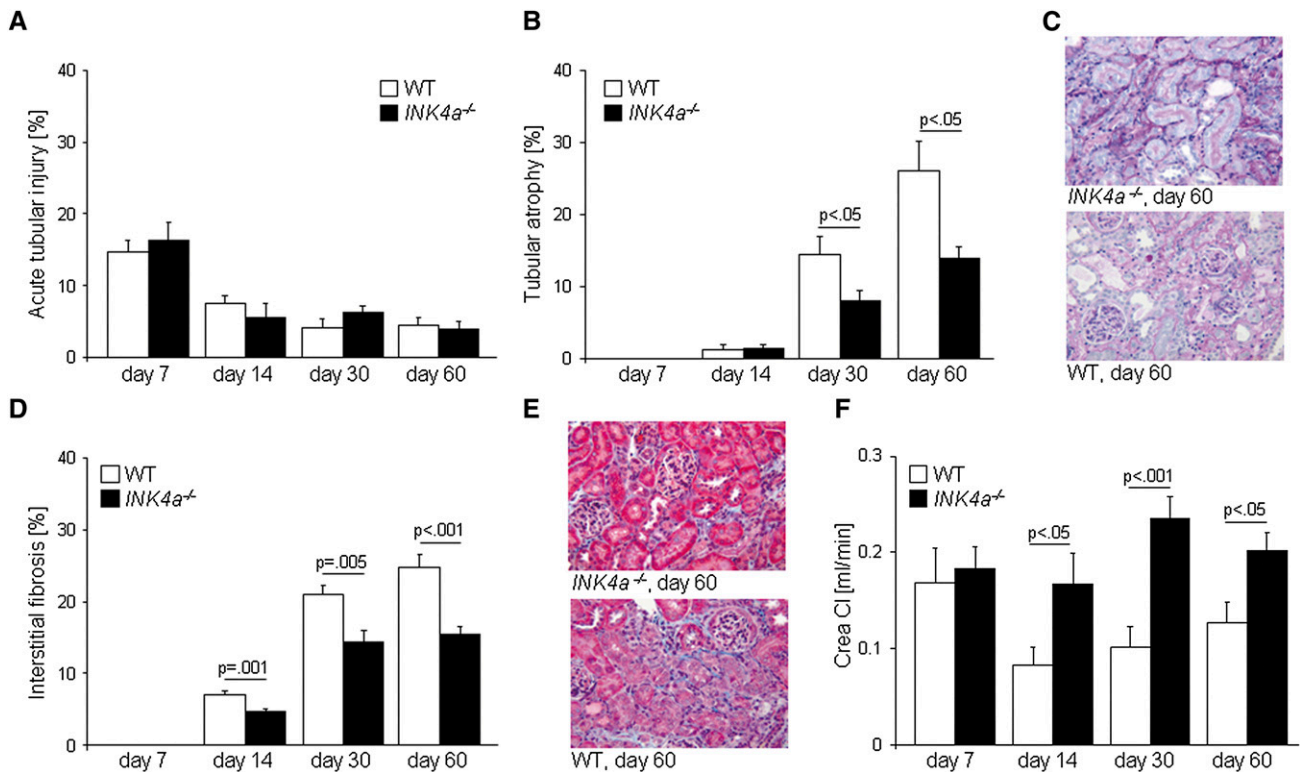
We subjected kidneys from wild-type or *INK4a*<sup>-/-</sup> mice to 30 minutes of warm ischemia. Acute tubular damage was similar in both groups, being highest on day 7 after ischemia with a decrease by day 14 (Figure 1A). Tubular atrophy, a marker of unsuccessful epithelial repair, was first observed at day 14 with a significant increase by days 30 and 60 in both groups (Figure 1, B and C). This increase was significantly higher in wild-type kidneys compared with kidneys from *INK4a*<sup>-/-</sup> mice. Similarly, interstitial fibrosis was significantly higher in wild-type kidneys (Figure 1, D and E). In wild-type kidneys, ischemia led to a significant increase of p16<sup>INK4a</sup> expression (Supplemental Figure 1A). Creatinine clearances, as a measure of renal function, showed no difference on day 7 between the two groups but were significantly lower in wild-type mice on days 14, 30, and 60 (Figure 1F).

We then sought to determine whether a higher proliferative capacity of renal tubular cells was responsible for the superior outcome of *INK4a*<sup>-/-</sup> mice after ischemia-reperfusion injury and whether this would result in fewer senescent tubular cells

in their kidneys. The expression of Ki67, a marker strictly associated with cell proliferation (labeling phases S, G<sub>2</sub>, and M of the cell cycle) was quantified. Proliferation rates were higher on day 7 compared with day 30. The number of proliferating tubular cells was significantly higher in renal sections from *INK4a*<sup>-/-</sup> mice on days 7 and 30 compared with wild-type mice (Figure 2, A and B). Senescent cells were detected by  $\gamma$ H2A.X/Ki67 co-staining on day 60. The number of senescent tubular cells, defined as Ki67 negative cells with high  $\gamma$ H2A.X foci density,<sup>27</sup> was significantly higher in wild-type kidneys (Figure 2C and Supplemental Figure 2). Apoptosis measured by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling was low overall in our experimental setting and no differences were seen between the two animal groups (data not shown). Taken together, these data suggest that p16<sup>INK4a</sup> expression leads to impaired renal repair by reducing the proliferative reserve of tubular epithelial cells. In turn, loss of p16<sup>INK4a</sup> resulted in a significantly lower amount of senescent cells.

Clinical studies have shown increased p16<sup>INK4a</sup> expression in transplants with interstitial fibrosis and tubular atrophy or allograft nephropathy, respectively.<sup>18,21</sup> To test our findings in the transplant setting, we performed transplants from *INK4a*/*ARF*<sup>-/-</sup> as well as wild-type mice, which are both on a C57BL/6 background, into fully MHC-mismatched wild-type mice on a C3H background. Importantly, the transplant model allowed us to exclude that a reduced immunologic response was the reason for the better outcome of kidneys from *INK4a*<sup>-/-</sup> mice after ischemia-reperfusion injury. To assess functional aspects, we used a life-supporting transplant model, in which both recipient kidneys are removed. We found that mice transplanted with *INK4a*/*ARF*<sup>-/-</sup> kidneys had a significantly better survival compared with mice that received a wild-type transplant (Figure 3A).

Human transplant biopsy studies have shown that peri- and post-transplantation stresses (such as ischemia-reperfusion injury,<sup>24,28,29</sup> acute rejection,<sup>30</sup> or hypertension<sup>31</sup>) lead to an increased p16<sup>INK4a</sup> expression. Our results were in agreement, and we found that experimental transplantation led to a significant increase in p16<sup>INK4a</sup> expression in wild-type kidneys (Supplemental Figure 1B). *INK4a*/*ARF*<sup>-/-</sup> transplants showed less chronic pathologic changes with a significant difference in the amount of interstitial fibrosis detected at day 21 (Figure 3B), which was comparable with what was seen after ischemia-reperfusion injury. The better functional integrity was not due to differences in the inflammatory response of the recipient: the amount of infiltrating cells as measured by the number of infiltrating CD3-positive lymphocytes and the typical lesions of tubulitis were similar in both groups (Supplemental Figure 3 and Supplemental Table 1). However, the proliferation rate of renal tubular cells was significantly greater in *INK4a*/*ARF*<sup>-/-</sup> kidneys before and after transplantation (Figure 3C). These observations provide evidence that loss of a major senescence pathway leads to increased allograft regenerative capacity and protects from the development of interstitial fibrosis and tubular atrophy.



**Figure 1.** Ischemia-reperfusion injury in *INK4a*<sup>-/-</sup> and wild-type mice. (A) Acute tubular injury (shown as percentage of affected area) is most pronounced 7 days after injury and decreases thereafter. There are no differences between the groups. (B) Tubular atrophy (percentage of affected area) is first detected 14 days after injury. Significant differences occur 30 and 60 days after injury, with *INK4a*<sup>-/-</sup> kidneys developing less tubular atrophy. (C) Representative sections from kidneys 60 days after injury. Tubular atrophy is visualized with the periodic acid–Schiff staining. (D) Interstitial fibrosis (percentage of blue-stained area) reveals a similar picture to tubular atrophy with significantly less interstitial fibrosis seen in *INK4a*<sup>-/-</sup> kidneys 14, 30, and 60 days after injury. (E) Representative Masson Trichrome staining of kidneys 60 days after injury. Interstitial fibrosis is visualized as blue-colored collagen fibers. (F) Creatinine clearances are significantly lower in wild-type mice 14, 30, and 60 days after injury. WT, wild-type; Crea Cl, creatine clearance. Original magnification, ×200.

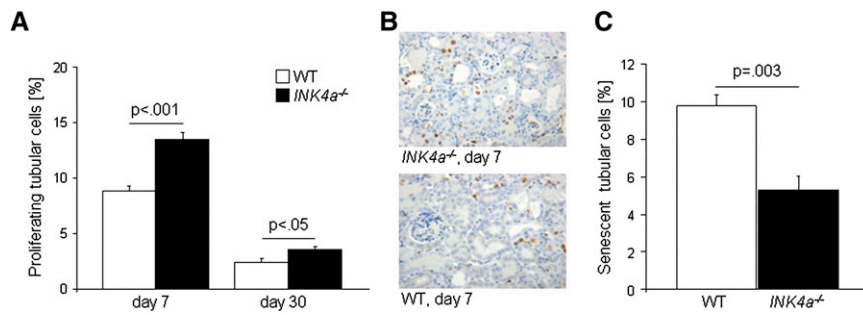
## DISCUSSION

Our data are consistent with the hypothesis that p16<sup>INK4a</sup> is crucially implicated in the mechanism of regenerative loss of renal aging. Irreversible parenchymal changes such as interstitial fibrosis and tubular atrophy were less common in either of the two transgenic mouse strains. *INK4a* deletion led to improved renal function and resulted in superior recipient survival in a life-supporting transplant model. We were able to confirm our results in two independent transgenic mouse strains. Obviously, these two mouse strains are not equivalent to each other because of the additional deletion of the *ARF* transcript in one of the strains and p19<sup>ARF</sup> being an additional potent cell cycle inhibitor. The confirmatory nature of both experimental settings strongly argues for the *INK4a* locus as the driving force for the observed phenotype, even though the contribution of the *ARF* transcript was not further explored.

The utilization of different models was also important in light of a recent renal study that showed an aggravating effect

of deleting *INK4a* in a model of obstructive uropathy.<sup>32</sup> The authors concluded from their results that p16<sup>INK4a</sup> limits inflammation and prevents fibrosis, whereas deletion of the gene increases tissue injury. Although it cannot be excluded that the apparent discrepancy to our study might be related to a difference in the strain of knockout mice, it seems likely that it reflects the heterogeneous effect of p16<sup>INK4a</sup> expression depending on the biologic context and on the affected cell type. While models of ischemic injury require a burst of epithelial cell proliferation as an instrumental part of their repair mechanism,<sup>33</sup> epithelial and/or interstitial cell proliferation might be detrimental in fibrosis models such as obstructive uropathy.<sup>34</sup>

Our data clearly show that the potentially promitogenic *INK4A* deletion does not necessarily induce a profibrogenic phenotype in renal fibroblasts. Instead, the primary problem in the postischemic kidney consists in the failure of epithelial repair, which is more successful in *INK4A* deleted mice with a better proliferative reserve. The development of interstitial fibrosis is a secondary process that results from defective



**Figure 2.** Proliferation and senescence of tubular cells from *INK4a*<sup>-/-</sup> and wild-type kidneys after ischemia-reperfusion injury. (A) Tubular cell proliferation (percentage of Ki67 positive tubular cells) measured 7 and 30 days after injury was significantly higher in kidneys from *INK4a*<sup>-/-</sup> mice. Proliferation rates declined from day 7 to day 30 in both groups ( $P < 0.05$ ). (B) Representative staining showing Ki67-positive cells in kidneys 7 days after injury. (C) The number of senescent tubular cells at day 60 after injury was significantly lower in kidneys from *INK4a*<sup>-/-</sup> mice. WT, wild-type. Original magnification,  $\times 200$ .

epithelial repair and can be regarded as a default mechanism of inadequate regeneration. In this context, our findings might extend previous data on the association of epithelial cell cycle arrest at G2/M and fibrotic outcome<sup>35</sup> by showing a similar phenotype if epithelial cells are arrested at the G1 state of cellular senescence. Tubular cells that are arrested in G2/M after acute injury secrete profibrogenic factors such as TGF- $\beta$ 1 and connective tissue growth factor.<sup>35</sup> On the basis of data from other cell types, it is likely that senescent renal tubular cells also secrete specific factors.<sup>36</sup> However, these might not overlap with the secretome of G2/M arrested cells. Further studies are needed to define effector molecules of senescent renal tubular cells in order to better understand the switch from defective epithelial repair to promotion of interstitial fibrosis.

Although our results suggest that inhibiting premature senescence could have therapeutic benefits, it is important to keep potential risks in mind. Not only is cellular senescence an integral part of the physiologic antitumor barrier<sup>37,38</sup> but it might also be tissue protective under specific circumstances senescence.

In summary, our results demonstrate that the development of premature senescence via p16<sup>INK4a</sup> expression is a critical regulator of cell repair and can contribute to postischemic interstitial fibrosis and tubular atrophy development. Interference with p16<sup>INK4a</sup> leads to increased regenerative capacity and may thus represent a novel strategy in improving the outcome of kidney transplantation. This will be of particular importance for aged donor kidneys that have to be increasingly utilized to overcome the shortage in available transplant organs.

## CONCISE METHODS

### Animals

All procedures performed on animals were done in accordance with institutional guidelines for animal research and were approved by the local government authorities.

### Mice

*INK4a*<sup>-/-</sup> mice on a 129/Ola-C57BL/6J background that carry a point mutation specifically affecting p16<sup>INK4a</sup> but not p19<sup>ARF</sup> expression<sup>39</sup> were provided by Dr. A. Berns (The Netherlands Cancer Institute, Amsterdam) and bred at the animal facility of the University of Heidelberg. *INK4a/ARF*<sup>-/-</sup> mice on a C57BL/6J background<sup>17</sup> used for the transplantation experiments came from the stock of Dr. M. Serrano.

### Ischemia-Reperfusion Injury

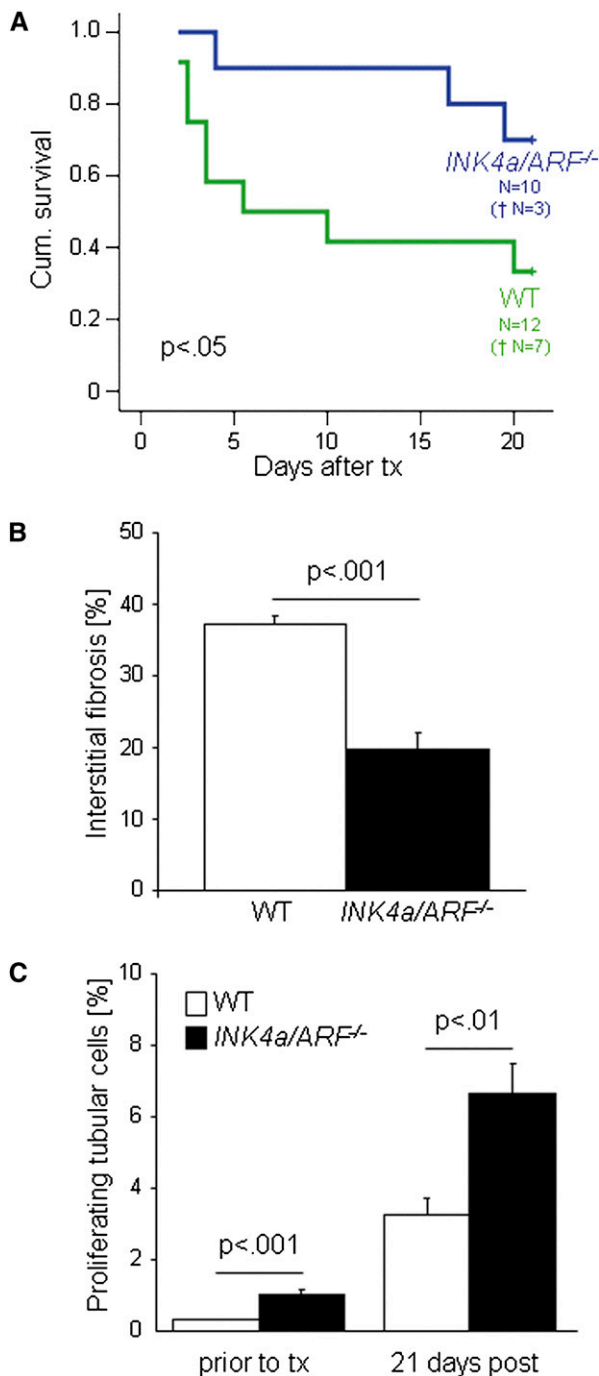
Ischemia-reperfusion surgery was performed according to current standard protocols in age-matched male *INK4a*<sup>-/-</sup> and wild-type mice that were 3–4 months old. Briefly, mice were anesthetized with inhalational isoflurane and were kept on a heating pad to maintain body temperature during surgery. A midline laparotomy was made. The bowel was gently placed aside by retractors. The renal pedicles were exposed and adjacent fat tissue was removed carefully. The left renal pedicle (artery and vein) was clamped for 30 minutes, using nontraumatic microsurgical vascular clips (Aesculap, Tuttlingen, Germany). Occlusion of blood flow was confirmed by visual inspection of the kidneys. The right kidney was left *in situ*. After removal of the clip, the kidneys were observed for approximately 5 minutes to ensure blood reflow, and then fascia and skin were sutured in two layers. All animals received the same volume of warm saline instilled in the peritoneal cavity during the surgical procedure and were allowed to recover with *ad libitum* access to food and water. Mice were sacrificed after 7 (wild-type,  $n=8$ ; *INK4a*<sup>-/-</sup>,  $n=8$ ), 14 (wild-type,  $n=7$ ; *INK4a*<sup>-/-</sup>,  $n=7$ ), 30 (wild-type,  $n=9$ ; *INK4a*<sup>-/-</sup>,  $n=9$ ), or 60 (wild-type,  $n=9$ ; *INK4a*<sup>-/-</sup>,  $n=9$ ) days, respectively.

After sacrifice, the kidneys were excised, adjacent tissue was carefully removed, kidneys were decapsulated, and weight was determined. Part of each kidney was immediately snap-frozen in liquid nitrogen for later RNA extraction; a second part was fixed in 4% buffered formalin and embedded in paraffin.

### Renal Transplantation

Donor mice (*INK4a/ARF*<sup>-/-</sup> mice on a C57BL/6J background) were anesthetized and the abdomen was opened through a midline incision. The right kidney was excised, flushed, and preserved in cold saline solution for 30 minutes. The host mice (C3H background purchased from Charles River Laboratories, Sulzfeld, Germany) were similarly anesthetized and both native kidneys were excised (life-supporting kidney transplantation). The donor kidney was anastomosed heterotopically to the inferior aorta, vena cava, and bladder. The mice were allowed to recover and were followed for 21 days. Mice that had not died within the observational period were killed by cervical dislocation at day 21. None of the transplanted hosts received immunosuppressive therapy. Mice with technical complications or pyelonephritis were removed from the study.

We studied 10 transplants from *INK4a/ARF*<sup>-/-</sup> mice and 12 transplants from wild-type mice.



**Figure 3.** Life-supporting allogeneic renal transplantation of *INK4a/ARF*<sup>-/-</sup> and wild-type kidneys. (A) Cumulative survival was significantly better in mice that received a transplant from a *INK4a/ARF*<sup>-/-</sup> mouse. (B) Interstitial fibrosis (percentage of blue-stained area) was significantly higher in wild-type transplants. (C) Tubular cell proliferation (percentage of Ki67-positive tubular cells) was significantly higher in *INK4a/ARF*<sup>-/-</sup> transplants, before and 21 days after transplantation. WT, wild-type; tx, transplant.

### Creatinine Clearance Determination

Mice (wild-type and *INK4a*<sup>-/-</sup>) were kept in metabolic cages for the last 24 hours before sacrifice. Twenty-four-hour urine was collected, urine volume was recorded, and aliquots were stored at -80°C for subsequent analysis. Blood was obtained by cardiac puncture under deep anesthesia right before sacrifice. Plasma and urinary creatinine were determined using an enzymatic method that has been validated in rodents.<sup>40</sup> Creatinine clearance (ml/min) was derived from the following formula: urinary creatinine × urine volume × 1440 min<sup>-1</sup> × plasma creatinine<sup>-1</sup>.

### Histopathology of Kidney

Tissue sections were cut at 3-μm sections with a Leica RM 2165 microtome (Leica Instruments, Nussloch, Germany) and stained with hematoxylin and eosin, periodic acid-Schiff, or Masson Trichrome, respectively. All analyses were performed using a Leica DM LB2 digitizing microscope and a Leica DFC 320 camera with QWin V3 software (all from Leica Microsystems GmbH, Wetzlar, Germany).

Acute and chronic tubular damage were quantified as the ratio of damaged tubules to total tubular area. Acute damage was mainly reflected by tubular necrosis, whereas chronic damage consisted of tubular deterioration including reduced tubular diameter, thickened tubular basement membrane, and loss of tubular nuclei. The degree of renal interstitial fibrosis was measured using Masson Trichrome stainings and a semiquantitative scoring system. The area of blue-stained interstitial fibrosis was detected using the QWin V3 software package and related to total tubulointerstitial area. Both analyses were based on the evaluation of 20 high-power fields (HPFs) (×200 magnification) for each animal.

### Real-Time RT-PCR

Total RNA was extracted from murine renal tissue samples using Trizol reagent (Invitrogen, Karlsruhe, Germany), RNA integrity verified by agarose gel electrophoresis, and cDNA obtained by reverse transcription of 1 μg total RNA using Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen, Karlsruhe, Germany). The following intron-spanning primers and probes were used for quantitative PCR of p16<sup>INK4a</sup>: forward, 5'GGGCACTGCTGGAAGCC 3'; reverse, 5'AACGTTGCCCATCATCATC 3'; and probe, 5'CCGAACCTCTTTCGGTCGTA 3'.

### Immunohistochemistry for Ki67 and CD3

Immunoperoxidase staining for Ki67 and CD3 was performed on paraffin-embedded tissue. Antigen retrieval was performed in citrate buffer, pH 6.0, using a pressurized heating chamber (Pascal Pressurized Heating Chamber; Dako, Hamburg, Germany). The sections were immersed in 3% H<sub>2</sub>O<sub>2</sub> in methanol, blocked with 1× Universal Blocking Reagent (BioGenex, San Ramon, CA), and then incubated at room temperature with the primary antibody for Ki67 (MIB-9, 190 mg/ml, at a dilution of 1:25; Dako) or for CD3 (CD3-12, 1 mg/ml, at a dilution of 1:50; Serotec, Martinsried, Germany) or with the appropriate isotype control antibodies, and rinsed with PBS. After 30 minutes of incubation with the Envision Monoclonal System (Dako) for Ki67 or with a biotinylated goat-anti-rat (Southern Biotech, Birmingham, AL), sections were washed again in PBS. In case of CD3 staining, 30 minutes of incubation with ABC reagent (Vectastain; Vector Laboratories, Burlingame, CA) was performed and rinsed with PBS. Visualization was



performed using the DAB substrate kit (Dako). The slides were counterstained with hematoxylin for Ki67 or methylene blue for CD3.

Analysis for Ki67 was done by counting 10 randomly photographed HPFs ( $\times 200$  magnification) within the cortex by a blinded observer. The percentage of positively stained tubular nuclei was related to the total number of nuclei. For Ki67, a subgroup of animals for days 7 (wild-type,  $n=8$ ; *INK4a*<sup>-/-</sup>,  $n=8$ ) and 30 (wild-type,  $n=9$ ; *INK4a*<sup>-/-</sup>,  $n=9$ ) were evaluated.

CD3-positive cells were assessed only in *INK4a/ARF*<sup>-/-</sup> transplants on 10 HPFs ( $\times 200$  magnification) by a blinded observer. Positive cells were attributed either to the interstitial, tubular, or glomerular compartment. In addition, the number of infiltrating CD3-positive cells per tubular cross-section was assessed.

### $\gamma$ H2A.X/Ki67 Co-Staining

Staining was performed on paraffin-embedded kidney sections. Antigen retrieval was performed in citrate buffer, pH 6.0, using a pressurized heating chamber (Pascal Pressurized Heating Chamber; Dako). The sections were incubated in blocking buffer (5% skim milk) for 60 minutes at room temperature. Primary antibodies for  $\gamma$ H2A.X (JBW301, 1:200; Millipore) and Ki67 (Clone SP6, 1:200; Thermo Scientific) were applied overnight at 4°C. Slides were then washed with PBS-Tween and incubated for 1 hour at room temperature with secondary antibodies for  $\gamma$ H2A.X (anti-mouse Alexa-555, A21422; Invitrogen) and for Ki67 (anti-rabbit Alexa-488, A21206; Invitrogen). Sections were counterstained with 4',6-diamidino-2-phenylindole and mounted with 1,4-diazabicyclo[2.2.2]octane antifade mounting medium.

Analysis was performed using 15 HPFs ( $\times 400$  magnification) within the cortex. The percentage of Ki67-negative and  $\gamma$ H2A.X-positive ( $\geq 4$  foci) tubular nuclei was related to total number of nuclei. For  $\gamma$ H2A.X/Ki67-co-staining, a subgroup of animals for day 60 (wild-type,  $n=5$ ; *INK4a*<sup>-/-</sup>,  $n=5$ ) was evaluated.

### Statistical Analyses

Means among different treatment groups were compared using ANOVA, and *t* tests with Bonferroni correction were applied for *post hoc* analysis. Data were evaluated using the SPSS 16.0 statistical software package (SPSS Inc, Chicago, IL). All data are shown as mean  $\pm$  SEM, unless otherwise indicated.

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### DISCLOSURES

None.

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